CLINICALLY SAFE THERAPEUTIC DELIVERY USING HUMAN MESENCHYMAL STEM/STROMAL CELL-DERIVED EXOSOMAL HYBRIDS

by

Joseph D. Takacs

B.A. (Hendrix College) 2016

MOCK GRANT PROPOSAL

Submitted in partial satisfaction of the requirements

for the degree of

MASTER OF SCIENCE

in

BIOMEDICAL SCIENCE

in the

GRADUATE SCHOOL

of

HOOD COLLEGE

January 2019

Accepted:

_____	_____
Ricky R. Hirschhorn, Ph.D. Committee Member	Ann L. Boyd, Ph.D. Director, Biomedical Science Program
_____	
Ann L. Boyd, Ph.D. Committee Member	
_____	_____
Joseph E. Candiello, Ph.D. Project Adviser	April Boulton, Ph.D. Dean of the Graduate School

STATEMENT OF USE AND COPYRIGHT WAIVER

I do authorize Hood College to lend this mock grant proposal, or reproductions of it, in total or in part, at the request of other institutions or individuals for the purpose of scholarly research.

DEDICATION

I would like to dedicate this work to my family, friends, and loved ones. The continued love and support has allowed me to reach this point in life knowing that they would always be there to help and support.

ACKNOWLEDGEMENTS

I would like to thank my committee for the continued support throughout my academic career at Hood College. You have allowed me to grow as a student, a researcher, and, most importantly, an individual. Dr. Boyd, I would like to thank you for your continued support throughout the entirety of my time at Hood. Your continued support always made me strive to become a better student, as well as expanding my knowledge base. Dr. Hirschhorn, I would like to thank you for continually challenging me throughout my time at Hood college and continually engaging students. Your teaching methods always inspired me to look more in depth in every topic discussed in class. Dr. Candiello, I would like to personally thank you for the continual guidance and hands-on approach in the generation of this document.

Mock Grant Application

Modeled after Department of Health and Human Services
Public Health Services
(based on Form PHS 398)

LEAVE BLANK—FOR OFFICIAL USE ONLY.

Type	Activity	Version – HCBMS.011712
Review Group		Formerly
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT *(Do not exceed 81 characters, including spaces and punctuation.)*
Clinically safe therapeutic delivery using human mesenchymal stem cell-derived exosomal hybrids.

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☒ NO ☐ YES
(If "Yes," state number and title)
Number: Title:

3. PROGRAM DIRECTOR/PRINCIPAL INVESTIGATOR — **New Investigator** ☐ **No** ☒ **Yes**

3a. NAME (Last, first, middle)	3b. DEGREE(S)	3h. eRA Commons User Name
Takacs, Joseph, D	B.A.	N/A

3c. POSITION TITLE
Graduate Student

3d. MAILING ADDRESS *(Street, city, state, zip code)*
401 Rosemont Ave
Frederick, MD 21701

3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
Department of Biology

3f. MAJOR SUBDIVISION
Biomedical Science Program

3g. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: N/A FAX: N/A

E-MAIL ADDRESS:
jtakacs012@gmail.com

4. HUMAN SUBJECTS RESEARCH ☒ No ☐ Yes

4a. Research Exempt: N/A

4b. Federal-Wide Assurance No.: N/A

4c. Clinical Trial ☒ No ☐ Yes

4d. NIH-defined Phase III Clinical Trial ☒ No ☐ Yes

5. VERTEBRATE ANIMALS ☒ No ☐ Yes

5a. Animal Welfare Assurance No. N/A

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
01/01/2019	12/31/2019				

9. APPLICANT ORGANIZATION
Name: Joseph D. Takacs
Address: Department of Biology
Hood College
401 Rosemont Ave
Frederick, MD 21701

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☐ State ☐ Local
Private: → ☒ Private Nonprofit
For-profit: → ☐ General ☐ Small Business
☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER
DUNS NO. N/A Cong. District N/A

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name: Joseph D. Takacs
Title: Graduate Student
Address: Hood College
401 Rosemont Ave
Frederick, MD 21701
Tel: N/A FAX: N/A
E-Mail: jtakacs012@gmail.com

13. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: Joseph D. Takacs
Title: Graduate Student
Address: Hood College
401 Rosemont Ave
Frederick, MD 21701
Tel: N/A FAX: N/A
E-Mail: jtakacs012@gmail.com

14. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 13. *(In ink. "Per" signature not acceptable.)*
Joseph Takacs

DATE
09/29/2018

PROJECT SUMMARY (See instructions):

As gene therapy techniques become more aggressive with site-specific therapeutics aimed at treating diseases such as hereditary, infectious, and neoplastic diseases at the DNA level, the parallel development of standardized, clinically safe delivery methods is paramount. Completion of this study will result in a clinically safe method to deliver large therapeutic payloads *in vitro*, while avoiding randomly integrated sites within the genome, incomplete therapeutic effects, and presentation of other unwanted activity typically attributed to other common viral-based delivery methods. The proposed exosome-liposome hybrid will serve as a baseline for the systems' translational potential as further modifications to the liposomal construct must be explored before clinical application.

RELEVANCE (See instructions):

Therapeutic genome editing technologies and research are plagued by troubleshooting issues, most notably inefficient and clinically unsafe delivery methods *in vivo* and *ex vivo*. Within CRISPR-Cas9 systems, the most common delivery methods (i.e. AAV) are associated with adverse side effects and immunogenicity which further halt the advancement of the therapeutic gene editing field. In response to the need of a clinically "safe" and effective intracellular delivery system, we propose that a natural carrier, such as cell-derived exosomes, function as a novel and clinically "safe" delivery system. As such, exosomes loaded with Cas9 and sgRNA-expressing plasmids would elicit a targeted response and would serve as a native delivery system that can be derived from a multitude of cell types.

PROJECT/PERFORMANCE SITE(S) (if additional space is needed, use Project/Performance Site Format Page)

Project/Performance Site Primary Location			
Organizational Name: Department of Biology, Hood College			
DUNS:			
Street 1: 401 Rosemont Ave		Street 2:	
City: Frederick	County: Frederick		State: MD
Province:	Country: United States		Zip/Postal Code: 21702
Project/Performance Site Congressional Districts: 6th Congressional District			

Additional Project/Performance Site Location			
Organizational Name:			
DUNS:			
Street 1:		Street 2:	
City:	County:		State:
Province:	Country:		Zip/Postal Code:
Project/Performance Site Congressional Districts:			

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Joseph D. Takacs	Graduate Student
eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Hendrix College Conway, AR	B.A.	2016	Biochemistry and Molecular Biology
Hood College Frederick, MD	M.S.	2019 (anticipated)	Biomedical Science

A. Positions and Honors

2016 – Present: Full time Graduate Student
2016 – Present: Research Associate, RoosterBio

B. Selected peer-reviewed publications (in chronological order)

Takacs JD, Forrest TJ, Basura GJ.2017. Noise exposure alters long-term neural firing rates and synchrony in primary auditory and rostral belt cortices following bimodal stimulation. Hear Res.; 356:1-15.

C. Research Support

N/A

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the project/performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. If research involving Select Agent(s) will occur at any performance site(s), the biocontainment resources available at each site should be described. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:

Hood College facilities will be utilized to perform this project. All necessary equipment and laboratory space needed to perform this project will be available for use including but not limited to: pipettes, centrifuges, biological safety cabinets, liquid nitrogen dewars, refrigerators, and freezers.

Clinical:

N/A

Animal:

N/A

Computer:

Hood College is fully-equipped with computational equipment for the analysis of data and execution of this project.

Office:

Common materials will be available within Hood College for use during execution of this project.

Other:

N/A

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

The following major equipment is available on site:

- Centrifuges
- Incubators
- Microscopes
- Biological Safety Cabinets (BSCs)
- Liquid Nitrogen Dewars
- Limited Cell Culture Materials

The following consumables will be purchased with grant funding:

- Human Mesenchymal Stem Cell Working Cell Banks
- Extensive Cell Culture Materials
- NanoSight (Malvern Panalytical)
- Neon™ transfection system (ThermoFischer)

SPECIFIC AIMS

Aiuti *et al.*2013 and Shim *et al.* 2017 describe persistent, unwanted symptoms such as randomly integrated sites and incomplete therapeutic effects caused by viral-based therapeutic delivery methods. This study's objective is to bypass these bottlenecking features by proposing a feasible, biologically native therapeutic delivery method with a strong focus on clinical "safety" and a quantitative comparison to current delivery methods. The specific aims are as follows:

1. Size determination of exosome-liposome hybrids to assess loading capacity.
2. Optimization and standardization of exosome loading procedures.
3. Quantitative analysis of current and proposed delivery methods *in vitro*.

BACKGROUND AND SIGNIFICANCE

Research field advancements in the past quarter of a century resulted in an exponential growth to the human understanding of genetics within animal models and humans themselves. The Human Genome Project, beginning in 1990, had the largest impact to the ever-growing field of genetics (Shapiro 1993). As a part of this project, researchers combined efforts to process millions of nucleotide sequences correlated to diseases, disorders, monogenic traits, and haplotypes resulting in the identification of the underlying genetic components. The observations, analyses, mappings, and identifications within the human genome allow for streamlining proposals for technological advancements aimed to combat or overturn experienced symptoms presented by targeted diseases (Shapiro 1993).

Understanding the human genome as it relates to individual specificity opened broad windows for advancement in screening and therapeutic techniques. As this field of sequencing expands, parallel gene therapy investigations are underway with 18 current site-specific gene-editing-based therapeutics in clinical trials which are aimed to treat hereditary, infectious, and neoplastic diseases (Shim *et al*. 2017). Typically exemplified by viral-based therapeutic delivery methods *in vivo* and *ex vivo*, these transient expression-modulating gene therapies ultimately remained unsuccessful in clinical trials due to reoccurring randomly integrated sites within the genome, incomplete therapeutic effects, and presentation of other unwanted activity (Aiuti *et al.* 2013; Shim *et al.* 2017). As both clinical and model systems studies progress, these past contributions broadened our scope and understanding of gene therapy without providing a consistent, permanent, and safe gene editing-based therapeutic delivery method (Aiuti *et al.* 2013).

Gene-Editing Nucleases

New technologies within the gene editing field incorporate nuclease engineering as a mode for accurate, programable gene modification within a variety of systems (Cox *et al.* 2015; Maeder and Gersbach 2016). These nucleases, such as Zinc Finger Nucleases (ZFN), Transcription Activator-Like Effector Nuclease (TALEN), and Clustered Regularly Interspaced Short Palindromic Repeats-Associated Nuclease Cas9 (CRISPR-Cas9), are restriction enzymes with engineered DNA-binding domains that cleave targets to produce double-stranded breaks (DSB) in DNA (Urnov *et al.* 2010; Joung and Sander 2013; Sander and Joung 2014). Once strand breaks and the corresponding DNA-damage signaling cascade occur, two native eukaryotic cellular pathways, non-homologous end joining (NHEJ) and homology-dependent repair (HDR), can then mediate the repair of the double-stranded breaks and, simultaneously, be utilized to eliminate target regions by joining ends or to eliminate target regions by inserting donor DNA, respectively (Shim *et al.* 2017).

While these efforts resulted in promising candidates for therapeutic gene editing, experimental results using the CRISPR-Cas9 system were found to be superior (Table 1) (Hsu *et al.* 2014). Based on the prokaryotic innate microbial defense system, the customizable RNA-guided site specific DNA binding domain within the CRISPR-Cas9 system allows for the simplification of specific DNA targeting and avoids many of the apparent limitations presented by the protein-DNA interactions within the DNA-binding domains of ZFN and TALEN (Hsu *et al.* 2014). Predictable target designation based upon CRISPR-Cas9's endogenous ability to target any 20 base-pair sequence flanked by a 2-6 base pair DNA sequence (typically 5'-NGG-3'; "N" representative of nonspecific nucleobase followed by two guanines, "G") known as a protospacer adjacent motif, or

PAM. The simplification of designing CRISPR-Cas9 guide RNA and multiplexing availability allows for the gene editing system to further accelerate the therapeutic gene-editing field in the years to come, despite multiple challenges remaining such as target specificity and off-target effects presented by the nuclease systems. (Cong *et al.* 2013).

Table 1. Comparison of common gene-editing nucleases (Shim et al. 2017).

	ZFN	TALEN	CRISPR/Cas9
DNA binding	Zinc finger protein	TALE protein	Guide RNA
DNA cleavage DNA recognition range	FokI 18–36 bp (3 bp/Zinc finger module)	FokI 30–40 bp (1 bp/TALE module)	Cas9 22 bp (DNA-RNA base pairing)
Recognition sequence	Sequence containing G base as follows: 5'-GNNGNNGNN-3'	Sequence starting from 5'-T and ending with A-3'	Sequence immediately followed by an adjacent protospacer motif 5'-NGG-3'
Advantages	Sequence-based module engineering Small protein size (<1 kb)	High specificity Accurate recognition by 1 bp Relatively easy selection of target region	Free selection of target region Simple synthesis of guide RNA Multiplexing ability
Limitations	Difficult sequence selection and zinc finger engineering Expensive and time-consuming	Not applicable to methyl cytosine Expensive and time-consuming Large protein size (>3 kb)	Large protein size (>4 kb)

Current Delivery Systems of CRISPR-Cas9

Although the therapeutic significance of CRISPR-Cas9 systems is widely recognized within the research and clinical community, there are still significant limitations, such as identifying a clinically "safe" delivery method. Currently, there are two main modes of therapeutic gene editing delivery: *in vivo* and *ex vivo* (Shim *et al.* 2017). *In vivo* delivery entails the gene-editing nuclease being administered directly to the patient, while e*x vivo* delivery first requires the isolation of the target cells from the patient and then transfection before being re-administered to the patient (Shim *et al.* 2017).

Current clinical models of *in vivo* delivery systems include adeno-associated virus (AAV), cationic liposomes, Polyethyleneimine (PEI), and cell-penetrating peptides (CPP) conjugation (Wang *et al.* 2016). Additionally, some studies further show success *in vitro* using physical means such as electroporation and microinjection (Wang *et al.* 2016). Unfortunately, despite the historical delivery methods showing preliminary success in feasibility studies, these methods are not optimized and can potentially illicit unwanted immune responses. In many cases, the immune response is against the viral components further limiting the repeatable use of the delivery vector which is necessary for a significant therapeutic response (Shim *et al.* 2017). AAV, currently the most common clinical delivery vector for CRISPR-Cas9, was previously shown to induce antibody and T-cell responses, impurities in viral vector preparations, and propensity to influence the immunogenicity of recombinant viral vectors encoding nucleases (Mingozzi and High 2011; Hareendran *et al.* 2013; Mingozzi and High 2013). Furthermore, the prolonged expressions of the viral capsid proteins could also elicit antibody and T-cell immune

responses (Shim *et al.* 2017). As the therapeutic gene editing field expands, it is necessary to determine a systemized mode of delivery regarding clinical safety.

Exosomes for Therapeutic Delivery

Exosomes are nanosized particles that are released from the lipid bilayer into the extracellular environment through the endosomal sorting complexes required for transport (ESCRT) to natively facilitate intercellular communication (Figure 1; Figure 2). Originating from multivesicular bodies (MVBs), these nanoparticles are drawing widespread attention as a possible nanoscale drug delivery system for various chemical and biomolecular drugs (Table 2) (Ibrahim and Marbán 2016; Liu *et al.* 2016). Initial isolation and discovery of exosomes relied upon size determination when differentiating exosomes from other (similar) types of particles. Although, more recent advancements resulted in exosome identification primarily based upon the biological pathway of origin (biogenesis) and composition of the membrane, consisting of an abundance of lipids, proteins, and other molecules (Figure 3; Figure 4) (Tan *et al.* 2013; Lin *et al.* 2018). The native membrane composition of the exosomes results in favorable functional traits such as facilitating escape from phagocytosis, passing through biological barriers, antigen presentation, and signal transduction, but also contribute to high biocompatibility and low immunogenicity properties *in vivo* (Ibrahim and Marbán 2016; Min Kim *et al.* 2017). Previous studies (Table 2) have already identified a multitude of therapeutic applications for engineering exosomes as nanoparticle delivery platforms. Despite preliminary data remaining highly effective, the overall feasibility of widespread application of exosomes as a standardized drug delivery method for increasing clinical safety remains limited due to the overall size and carrying capacity of exosomes (Table 2) (Tan *et al.* 2013).

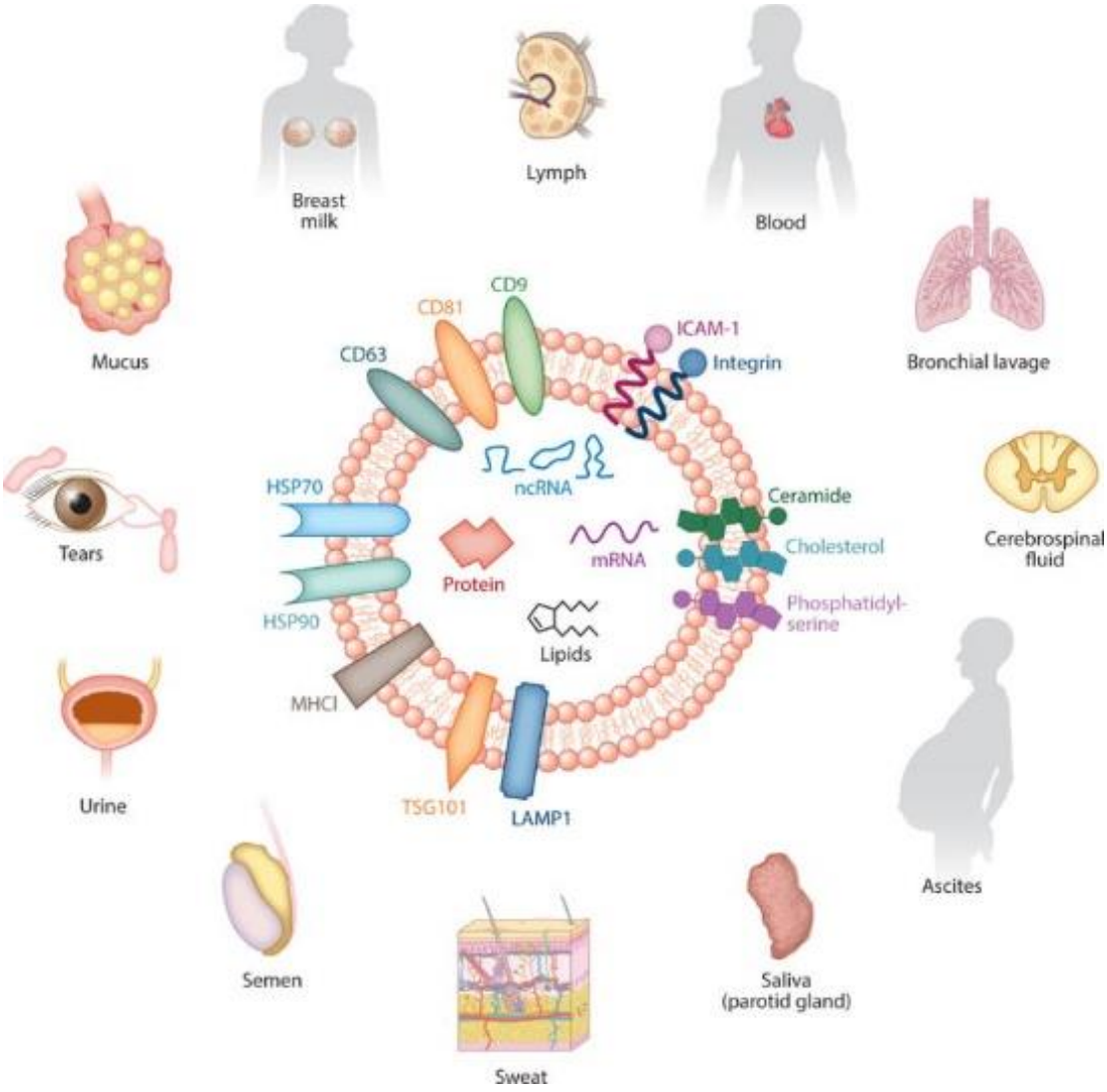

Figure 1. Membrane composition of exosomes enriched in cholesterol, ceramide, and phosphatidylserine. Isolate determination includes tetraspanins as targets (CD9, CD63, and CD81). (Ibrahim and Marbán 2016).

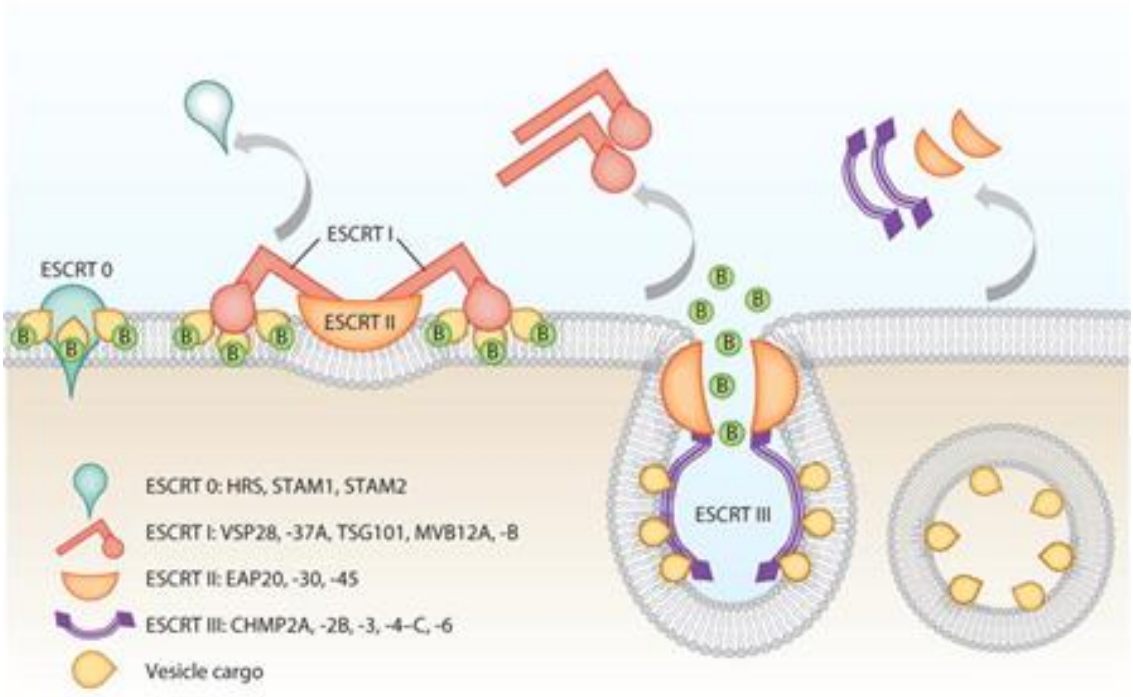

Figure 2. ESCRT cellular pathway utilized for loading constructs. Furthermore, ESCRT pathway components can be utilized to target exosomes within heterogenous populations of nanoparticles within biological assays (Ibrahim and Marbán 2016)**.**

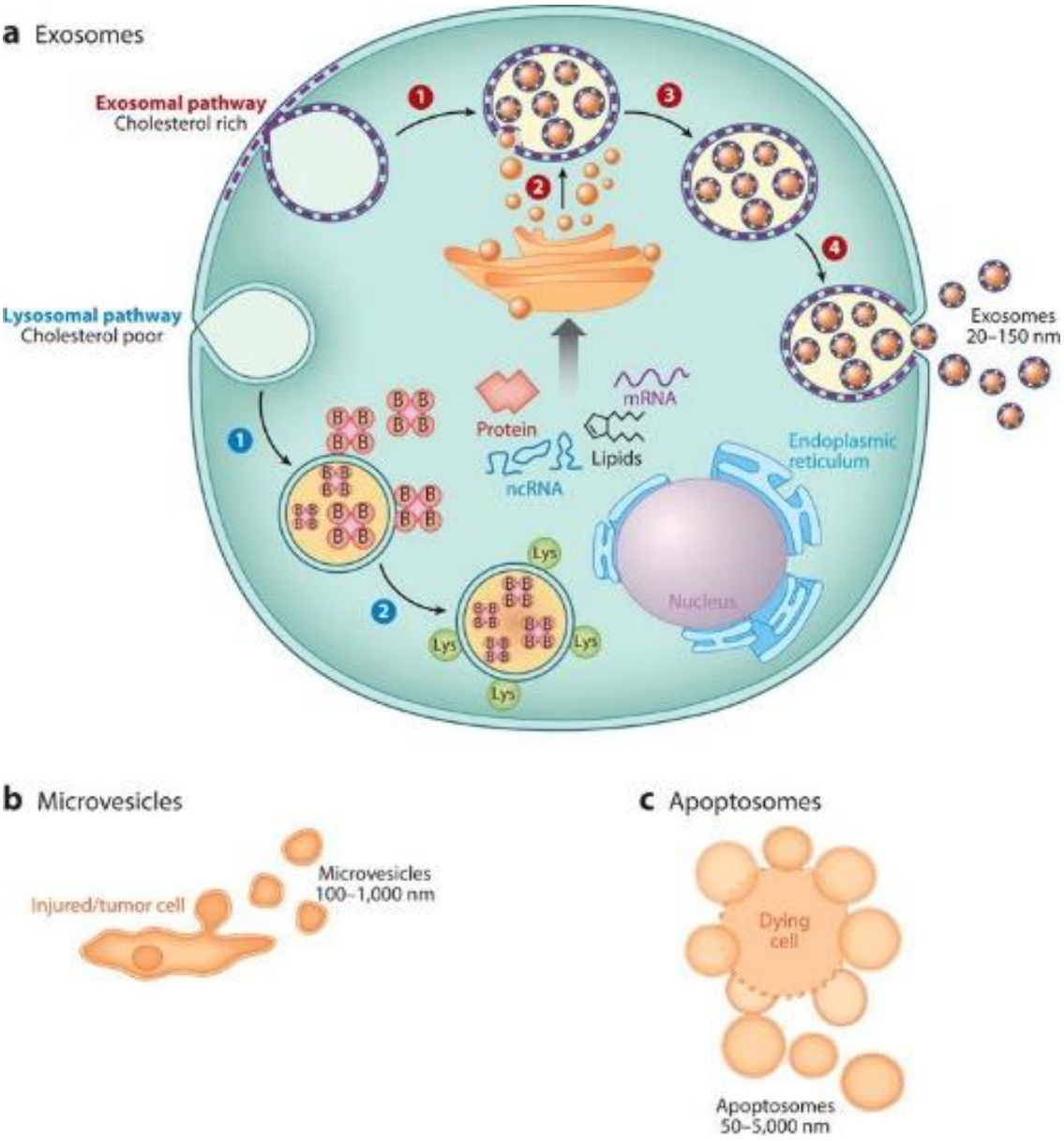

Figure 3. Exosome biogenesis. Determination of release or degradation cellular pathways based upon cholesterol concentration of multivesicular bodies. (Ibrahim and Marbán 2016)

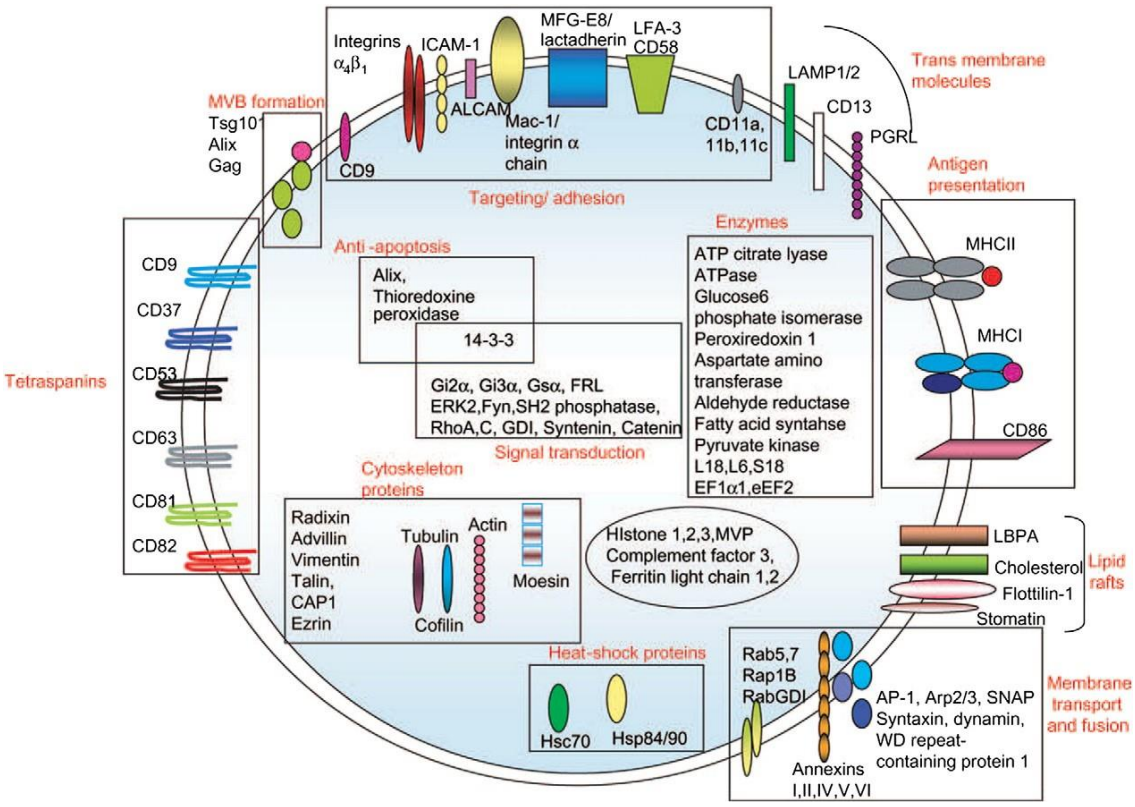

Figure 4. Variable expression of surface molecules facilitates exosomal functionality. Furthermore, exosomal membrane composition is hypothesized to play large role in cellular interaction mechanisms when engineered as a therapeutic delivery platform (Tan *et al.* 2013)**.**

Table 2. Literature summary of previous studies utilizing engineered exosomes for therapeutic delivery. Extensive studies utilized miRNA and siRNA; small load capacity therapeutics (Tan *et al.* 2013)**.**

Author	Exosome source	Exosome payload
Akao 2011	Human monocytic leukemia THP-1 cells	Chemically-modified miR-143 RNA
Eldh 2010	MC/9 mouse blast cells	Various types of RNA strands, dependent on exposure to oxidative stress
Keller 2010	Human amniotic fluid, saliva, urine	Exosome shuttle RNA (esRNA)
Kogure 2011	Human hepatocellular carcinoma	miRNA
Kosaka 2010	HEK293 human embryonic kidney cell line COS-7 African green monkey kidney fibroblast-like cell line	siRNA
Lasser 2010	Human nasal lavage fluid	mRNA miRNA
Lasser 2011	Human saliva, plasma, breast milk	RNA mRNA
Lee 2011	B16F1 murine melanoma cell line	Class II transactivator gene (CIITA)
Luo 2009	Human placenta	Placenta-specific miRNAs
Meckes 2010	EBV-positive nasopharyngeal carcinoma	Viral mRNA
Michael 2010	Human saliva	miRNA
Mittelbrunn 2011	Lymphoblastoid B cell line	miRNA
Muller 2011	Rat adipocytes	miRNA
Nazarenko 2010	Rat adenocarcinoma	mRNA
Pegtel 2010	EBV-positive lymphoblastoid B cells	miRNA
Taylor 2008	Human ovarian cancer cells	miRNA
Valadi 2007	Mouse MC/9 mast cell line Human HMC-1 mast cell line Mouse primary bone marrow-derived mast cell	mRNA miRNA
Yang 2011	Macrophages from HEK-293 T cells	miRNA
Alvarez-Erviti 2011	Mouse dendritic cells	siRNA

As the therapeutic gene editing field expands, the focus remains on establishing a standardized, clinically safe, delivery method. In this study, we will examine the feasibility of bypassing limitations on load capacity by encapsulating and delivering large nucleic acids *in vitro* using a novel exosome-liposome hybrid construct identified by Lin *et al.* 2018. Utilizing CRISPR-dCas9 machinery targeting KRAS genes, exosome-liposome constructs will carry and deliver large plasmid payloads to human mesenchymal stem/stromal (hMSC) cultures for green fluorescent protein (GFP) expression. After successful engineering the exosome delivery platform, the loading efficiency, delivery efficiency, and bio-distribution will be quantitatively compared with other leading therapeutic delivery methods to further assess the proposed methodology.

RESEACH DESIGN / METHODS

Aim 1: Size determination of exosome-liposome hybrids to assess loading compacity.

Methods

Commercially available Human Mesenchymal Stem/Stromal Cells (hMSCs) and bioprocessing media will be purchased in a cryopreserved state from RoosterBio Inc (Frederick, MD). Mesenchymal Stem Cells will be cultured in NUNC tissue culture-treated flasks using proprietary medium for up to seven days or relevant cell confluence is observed (>80%) following standardized cell culture techniques. The proprietary media supporting proliferation will then be discarded, and the remaining cell monolayers will be washed with Dulbecco's phosphate-buffered saline solution (DPBS; Gibco™) to remove any remaining media. Post expansion, exosomes will be collected using DMEM+ 10% exosome depleted FBS; (Gibco) for 48 hours. After incubation, the media supernatant will be collected for processing.

Cell debris will be removed from solution by high-rate centrifugation, 500xg for thirty min and 12,000xg for another thirty minutes. Exosomes will then be isolated from the purified media using the Total Exosome Isolation Kit (ThermoFisher Scientific), which forces exosomes out of the media solution when coupled with centrifugation, 10,000xg for one hour. The resulting pellet will then be resuspended in (exosome-depleted) DMEM and analyzed for size determination and stock concentration based upon Brownian motion light scattering using the NanoSight (Malvern Panalytical) (Lin *et al.* 2018).

Liposomal reagent (Lipofectamine 2000; Life Technologies, or Liposome Kit; Sigma) will be prepared and resuspended in DMEM. The stocks of liposomes and exosomes will be mixed and incubated at 37°C + 5% CO_2 for 12-hours to facilitate

exosome-liposome fusion (Lin *et al*. 2018). Following incubation, the resulting suspension will be sampled and analyzed for size determination and stock concentration based upon Brownian motion light scattering using the NanoSight (Malvern Panalytical).

Expected Outcomes

Nanotracking analysis will allow for the mean size determination and concentration of the hMSC isolated exosomes (historically ranging 30 to 100-nm) and the liposome-exosome hybrid constructs. Completion of Aim 1 will result in the generation of preliminary data for future objectives. We expect to optimize and standardize the exosome-liposome fusion regarding process parameters to increase yield and efficiency for potential commercial scalability.

Potential Pitfalls and Alternative Strategies

Lin *et al.* 2018 proposes that the fusion protocol is facilitated by correlated surface constituents shared between exosomes and liposomes, although the exact mechanism is still unknown. This risk factor may attribute to additional experiments to commence during this stage of the study. To gain additional confidence, we will monitor native protein expression typically expressed by exosomes using enzyme-linked immunosorbent assays (ELISA) targeting ALIX or CD63. Furthermore, the unclarity attributed to the mechanism of fusion may lead us to assess multiple different vendors for liposomal constructs to determine which method/protocol best suits our needs.

Aim 2: Optimization and standardization of exosome loading procedures.

Methods

Following completion of AIM 1, a standardized method of generating exosome-liposome hybrid constructs will be implemented. The resulting products will then be

assessed for loading capacity. The hybrid constructs will be loaded with a large plasmid (encoding CRISPR-Cas9) for eventual application in AIM 3.

Assembly of a complete vector system will be purchased through Sigma-Aldrich and utilized as a stable, ready-to-use reagent. The CRISPR/Cas9-GFP by Sigma-Aldrich includes a single vector system U6-gRNA cassette allowing for the co-expression of Cas9 and GFP from the same mRNA (Figure 5), despite common methods typically expressing gRNA and Cas9 being expressed from two separate vectors. A site specific Kras-U6gRNA-Cas9-GFP will then be incorporated using sgRNA (developed using the Synthego interface; Figure 6). The sgRNA, Sequence: 5'-UCCCUUCUCAGGAUUCCU AC-3', will target a sequence (downstream of PAM sequence) in the KRAS gene allowing for the dCas9 to bind the sense strand located on exon 3 of chromosome 12.

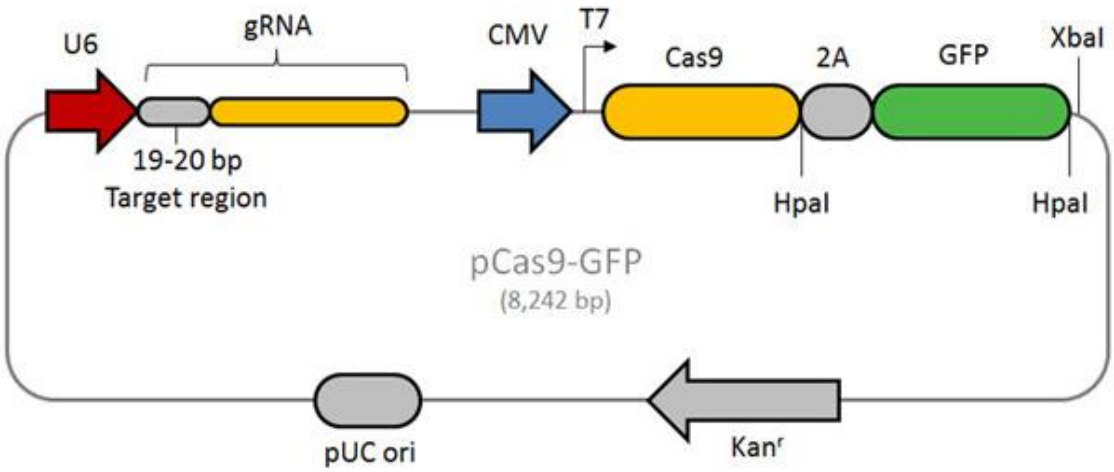

Figure 5. Schematic of CRISPR/Cas9-GFP single vector system by Sigma-Aldrich.

Figure 6. Schematic representation of proposed sgRNA by Synthego. PAM sequence represented in blue.

The commercially available single vector system will be preliminarily mixed and incubated with liposome solution in DMEM. The liposome-plasmid complex will then be added to an exosome solution and incubated according to previously optimized methods. The incubation procedure should allow the exosomes to fuse with the liposomes, furthermore encapsulating the plasmid (Lin *et al.* 2018). To ensure encapsulation of the large payload, samples would be assessed through 4',6-diamidino-2-phenylindole (DAPI) staining and size determination using nanotracking analysis (Malvern Analytical).

Expected Outcomes

DAPI staining and size determination allow us to determine the loading efficiency of the proposed methods. It is expected that the methods and associated protocols would be tailored towards scalability for industry standards and clinical relevance.

Potential Pitfalls and Alternative Strategies

Although the single expression cassette is favorable to other common expression cassettes, it may deem to be an unachievable goal dependent upon the results and size

determination observed in AIM 1. In this case, subsequent experiments will follow to optimize new strategies of plasmid encapsulation based upon historical methods (Table 3). This includes incorporation of a two plasmid technique previously shown to be successful within Lin *et al.* 2018.

Table 3. Previous Successful Exosome Loading Techniques within Literature (Luan *et al.* 2017).

		Advantages	Disadvantages
I) Passive loading	a) Incubation of exosomes and free drugs	Simple Do not compromise membrane integrity	Low drug loading efficiency
	b) Incubation of the donor cells with free drugs	Simple Do not compromise membrane integrity	Low drug loading efficiency Drugs may cause cytotoxicity to the donor cells
II) Active loading	a) Sonication	High drug loading efficiency	Compromise membrane integrity
	b) Extrusion	High drug loading efficiency	Compromise membrane integrity
	c) Freeze/thaw	Medium drug loading efficiency	Aggregations
	d) Electroporation	Liposome-exosome fusion	Aggregations
	e) Incubation with saponin	Loading with large molecules such as siRNA, miRNA Enhanced drug loading	Toxicity
	f) Click chemistry	Quick and efficient	
	g) Antibody binding	Better control over the conjugation site Specific and easy to operate	

Aim 3: Quantitative analysis of current and proposed delivery methods *in vitro*.

Methods

The finalized plasmid construct will be applied into current industry and clinical leading techniques for delivery: viral (AAV) and non-viral (electroporation). Human mesenchymal stem cells will be cultured until approximately 80% confluent according to previous culture techniques. Once confluent, cell monolayers will be washed with DPBS and replaced with exosome-depleted DMEM. The previously engineered exosome-liposome plasmid containing hybrids will be added to the hMSC cell cultures. Concurrently, the plasmid will also be cloned into an AAV expression vector by Vigene Biosciences, as well as applied directly to cultures using the Neon™ transfection system (while in suspension) (ThermoFisher). The three experimental sets of hMSC cell cultures will then be incubated at 37°C + 5% CO_2 and will be observed over the course of multiple days for a qualitative analysis of GFP expression using simple microscopy.

Following specific applications of the separate delivery systems to hMSC cell cultures, the hMSC cultures will be washed multiple time to remove any excess components. The cells will then be dissociated from the tissue culture flasks using Tryple-Select (GIBCO). The solutions then will be centrifuged and resuspended in Cryostor5 (5% DMSO; BioLife Solutions) for cryopreservation. The cells will then be sent to an outside collaborator for quantitative flow cytometry based on GFP expression.

Table 4. Optimized hMSC Neon™ transfection system parameters as supplied by ThermoFisher.

Electroporation parameters

Pulse voltage (v)	Pulse width (ms)	Pulse number	Cell density (cells/ml)	Transfection efficiency	Viability	Tip type
990	40	1	5×10^6	54%	90%	10 µl

Expected Outcomes

These results will allow for the determination of transfection efficiency between the various delivery methods. Furthermore, we will assess the validity and toxicity (if any) of the clinically safe therapeutic delivery method in direct comparison to other leading methods.

Potential Pitfalls and Alternative Strategies

Human mesenchymal stem cells are notoriously hard to transfect and replicatively senescent, as are many primary cell strains. If experiments result in less than expected expression of GFP, it potentially may not be attributed to the exosome-liposome hybrid. Therefore, it is understood that simple delivery of the exosomes to culture may not be sufficient, and that more development of optimal delivery parameters may be necessary, possibly even coupling some passive and active delivery techniques.

SUMMARY

Just as the Human Genome Project resulted in rapid advances within the sequencing field, the completion of this study should result in a feasible, clinically safe therapeutic delivery platform that will allow for additional streamlined proposals for future site-specific therapeutics without the hesitation caused by viral-based delivery platforms. Furthermore, repetitive analysis of these proposed experiments will delve deeper into assessing the exosome-liposome hybrid delivery methods regarding clinical safety by looking further into off-target genotoxicity, immunogenicity, pharmacokinetics, biodistribution, and genome-edited hMSC cell characterization in accordance to ISCT guidelines proposed by Dominici *et al.* 2006.

REFERENCES

A CRISPR/Cas-GFP Vector for Rapid Expression Verification and Enrichment of Genome Edited Cells. Sigma-Aldrich. [accessed 2018 Apr 24]. https://www.sigmaaldrich.com/technical-documents/articles/biology/crispr-cas-gfp-vector.html.

Aiuti A, Biasco L, Scaramuzza S, Ferrua F, Cicalese MP, Baricordi C, Dionisio F, Calabria A, Giannelli S, Castiello MC, *et al.* 2013. Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. Science 341:1233151. doi:10.1126/science.1233151.

Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, *et al.* 2013. Multiplex genome engineering using CRISPR/Cas systems. Science 339:819–823. doi:10.1126/science.1231143.

Cox DBT, Platt RJ, Zhang F. 2015. Therapeutic genome editing: prospects and challenges. Nat. Med. 21:121–131. doi:10.1038/nm.3793.

Donley Greer, Hull Sara Chandros, Berkman Benjamin E. 2012. Prenatal whole genome sequencing. Hastings Center Report 42:28–40. doi:10.1002/hast.50.

Hareendran S, Balakrishnan B, Sen D, Kumar S, Srivastava A, Jayandharan GR. 2013. Adeno-associated virus (AAV) vectors in gene therapy: immune challenges and strategies to circumvent them. Rev. Med. Virol. 23:399–413. doi:10.1002/rmv.1762.

Hsu PD, Lander ES, Zhang F. 2014. Development and applications of CRISPR-Cas9 for genome engineering. Cell 157:1262–1278. doi:10.1016/j.cell.2014.05.010.

Ibrahim A, Marbán E. 2016. Exosomes: fundamental biology and roles in cardiovascular physiology. Annu Rev Physiol. 78:67–83. doi:10.1146/annurev-physiol-021115-104929.

Joung JK, Sander JD. 2013. TALENs: a widely applicable technology for targeted genome editing. Nat. Rev. Mol. Cell Biol. 14:49–55. doi:10.1038/nrm3486.

Lamichhane TN, Raiker RS, Jay SM. 2015. Exogenous DNA loading into extracellular vesicles via electroporation is size-dependent and enables limited gene delivery. Mol Pharm 12:3650–3657. doi:10.1021/acs.molpharmaceut.5b00364.

Lin Y, Wu J, Gu W, Huang Y, Tong Z, Huang L, Tan J. 2018. Exosome–Liposome hybrid nanoparticles deliver CRISPR/Cas9 system in MSCs. Advanced Science 0:1700611. doi:10.1002/advs.201700611.

Liu D, Yang F, Xiong F, Gu N. 2016. The smart drug delivery system and its clinical potential. Theranostics 6:1306–1323. doi:10.7150/thno.14858.

Luan X, Sansanaphongpricha K, Myers I, Chen H, Yuan H, Sun D. 2017. Engineering exosomes as refined biological nanoplatforms for drug delivery. Acta Pharmacologica Sinica 38:754–763. doi:10.1038/aps.2017.12.

Maeder ML, Gersbach CA. 2016. Genome-editing Technologies for Gene and Cell Therapy. Mol. Ther. 24:430–446. doi:10.1038/mt.2016.10.

Min Kim S, Yang Y, Ja Oh S, Hong Y, Jang M. 2017. Cancer-derived exosomes as a delivery platform of CRISPR/Cas9 confer cancer cell tropism-dependent targeting. Journal of Controlled Release 266. doi:10.1016/j.jconrel.2017.09.013.

Mingozzi F, High KA. 2011. Immune responses to AAV in clinical trials. Curr Gene Ther 11:321–330.

Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. - PubMed - NCBI. [accessed 2018 Nov 16]. https://www.ncbi.nlm.nih.gov/pubmed/16923606.

RoosterBio Inc. [accessed 2018 Apr 24]. https://www.roosterbio.com/.

Sander JD, Joung JK. 2014. CRISPR-Cas systems for editing, regulating and targeting genomes. Nat. Biotechnol. 32:347–355. doi:10.1038/nbt.2842.

Sercombe L, Veerati T, Moheimani F, Wu SY, Sood AK, Hua S. 2015. Advances and challenges of liposome assisted drug delivery. Front Pharmacol 6. doi:10.3389/fphar.2015.00286. [accessed 2018 Apr 11]. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4664963/.

Shapiro LJ. 1993. Human genome project. West J Med 158:181.
Shim G, Kim D, Park GT, Jin H, Suh S-K, Oh Y-K. 2017. Therapeutic gene editing: delivery and regulatory perspectives. Acta Pharmacol Sin 38:738–753. doi:10.1038/aps.2017.2.

Stable transfection of MSCs by electroporation | Gene Therapy. [accessed 2018 Apr 25]. https://www.nature.com/articles/3302163.

Synthego - World-Class Synthetic Guide RNA for CRISPR Genome Editing. Synthetic Guide RNA for CRISPR Genome Editing | Synthego. [accessed 2018 Apr 24]. http://www.synthego.com/.

Tan A, Rajadas J, Seifalian AM. 2013. Exosomes as nano-theranostic delivery platforms for gene therapy. Adv. Drug Deliv. Rev. 65:357–367. doi:10.1016/j.addr.2012.06.014.

Total Exosome Isolation Reagent (from cell culture media) - Thermo Fisher Scientific. [accessed 2018 Apr 24]. https://www.thermofisher.com/order/catalog/product/4478359.

Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD. 2010. Genome editing with engineered zinc finger nucleases. Nat. Rev. Genet. 11:636–646. doi:10.1038/nrg2842.

Wang L, Li F, Dang L, Liang C, Wang C, He B, Liu J, Li D, Wu X, Xu X, *et al.* 2016. *In vivo* delivery systems for therapeutic genome editing. Int J Mol Sci 17. doi:10.3390/ijms17050626. [accessed 2018 Apr 10]. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4881452/.

Yu B, Zhang X, Li X. 2014. Exosomes derived from mesenchymal stem cells. Int J Mol Sci 15:4142–4157. doi:10.3390/ijms15034142.