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研究課題名（和文）CRISPR Cas9 protein delivery via virus-like particles for in vivo treatment of Duchenne muscular dystrophy

研究課題名（英文）CRISPR Cas9 protein delivery via virus-like particles for in vivo treatment of Duchenne muscular dystrophy

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研究成果の概要（和文）：We developed a novel protein delivery system based on virus-like particles (NanoMEDIC) which can be used to deliver proteins hard to transfect cells and in vivo in mice. Genome editing technology such as CRISPR-Cas9 could be delivered into a variety of cells and achieved high editing efficiency.

研究成果の学術的意義や社会的意義
Transient delivery of CRISPR-Cas9 RNP will allow for safer use of genome editing technology in vivo and may be applicable future clinical work. We also developed a serum-free culture system using flow electroporation to potentially apply the production of our NanoMEDIC system for industrial use.

研究成果の概要（英文）：We developed a novel protein delivery system based on virus-like particles that we term NanoMEDIC (nanomembrane-derived extracellular vesicles for the delivery of macromolecular cargo) which can be used to deliver proteins of interest in a variety of cell types including human iPS cells, monocytes, T cells, cancer cell lines, and also in vivo. In particular, genome editing technology such as CRISPR-Cas9 could be delivered into a variety of cells and achieved high editing efficiency. The system utilizes the FKBP12 and FRB dimerization domains fused with HIV-Gag and SpCas9, respectively. We optimized the FRB-SpCas9 fusion protein by testing the incorporation of SpCas9 when FRB was fused either to the N-, C- or N- and C- terminal ends of the protein. Furthermore, mutation of the FRB domain abrogated the dimerization activity.

研究分野：Gene Therapy

キーワード：CRISPR　DMD　Genome editing　Delivery

１．研究開始当初の背景

Clustered regularly interspaced short palindromic repeat (CRISPR) associated protein (Cas9) mediated DNA editing has enabled efficient editing of human cells in culture and is being touted for its potential as a therapeutic tool for treating human diseases. On the other hand, *in vivo* delivery of CRISPR-Cas9 is needed for targeting tissues of interest, such as liver, neurons or skeletal muscle, depending on the target disease. Duchenne muscular dystrophy (DMD) is a severe muscle degenerative disease that affects 1 in 3,500 boys and is caused by mutations in the X-linked gene, dystrophin. The absence of dystrophin protein in skeletal and cardiac muscle cells leads to a loss in muscle stability and results in muscle wasting.

Adeno-associated viruses (AAV) have been the leading tool for *in vivo* gene delivery, and have been utilized to treat DMD mouse and dog models by delivering the CRISPR-Cas9 system as an episomal DNA vector. However, there are several limitations and concerns regarding its potential use in humans including a limited viral genomic DNA packaging size of less than 5 kb, pre-existing immunity against AAV capsids, and immunogenicity to its capsid or delivered protein. Moreover, prolonged expression of Cas9 transgenes for up to several years could result in unwanted off-target mutagenesis.

To this end, ribonucleoprotein (RNP) delivery of CRISPR-Cas9 offers several advantages over DNA delivery by facilitating potent on-target cleavage while reducing unwanted off-target effects, as the RNP complex has been reported to be rapidly degraded in cultured cells compared with DNA plasmid mediated expression vectors. However, in order to deliver CRISPR RNP complexes into hard-to-transduce tissues, including muscle tissues, a suitable delivery system which can efficiently package, protect and deliver cargo of interest into target tissues is needed.

Viruses are natural carriers of proteins and nucleic acids for delivery into cells. By expressing viral envelope and/or structural proteins, extracellular vesicles (EVs) which lack a viral nucleic acid genome can be harnessed into protein and RNA delivery vehicles, and have been utilized for clinical trials, mainly in the vaccination field. The structural polyprotein from retroviruses, Gag, is an ideal candidate to package cargo into EVs as it is well studied and can induce active release of EVs from cells, which have been estimated to contain up to 5,000 Gag molecules per virus particle. Previously, we and other groups have fused HIV and MLV Gag with proteins of interest in order to deliver them into cells, which can be liberated by a viral protease from Pol protein.

While several EV-mediated CRISPR-Cas9 RNP delivery methods have been reported utilizing fusion of SpCas9 with retroviral Gag, such as Cas9P LV, VEsiCas, Gesicle, and NanoBlades systems, they all rely on stochastic or passive incorporation of sgRNA. Furthermore, direct fusion of SpCas9 with Gag requires the supplementation of wildtype Gag-Pol molecule in order to liberate Cas9 from Gag via protease-mediated cleavage, which competes for space within the EV and reduces the number of SpCas9 molecules packaged. Inclusion of protease in Pol also runs the risk of protease-mediated degradation at cryptic sites in the target protein, inadvertently reducing the number of functional proteins to be delivered. Thus, there is a need for active incorporation machinery for Cas9 protein and sgRNA, which does not involve direct fusion of Cas9 protein with Gag.

２．研究の目的

The purpose of this research project is to develop and apply a novel and transient CRISPR-Cas9 delivery platform for in vitro and in vivo delivery in a pre-clinical setting to treat Duchenne muscular dystrophy mice. By doing so, we hope to reduce off-target and immunological risks associated with long term expression of the CRISPR Cas9 system by viral vector systems.

Adeno-associated viruses have been used extensively for in vivo delivery of CRISPR-Cas9 in mouse models, however there are two main concerns with this approach. For one, prolonged expression of CRISPR-Cas9 and sgRNA significantly increases the off-target risk. For adeno-associated viruses, expression of these components could potentially be extremely long term, for years. Thus, there is a significant increased risk of off-target mutagenesis. Previous reports have indicated that transient delivery of SpCas9 is important for reducing these risks. Another concern with viral vector delivery is that immunogenicity to the viral vector or to the SpCas9 protein itself could cause elimination of the cells that are edited because SpCas9 could become an antigen.

３．研究の方法

The research method involves the production of virus-like particles containing CRISPR-Cas9 protein via chemical induced heterodimerization system to package the genome editing protein. The VLPs are then inoculated onto cells in culture or in vivo in a mouse. Genome editing was initially quantified using a GFP reporter system by single strand annealing assay. T7E1 analysis and TIDE analysis were also used to assess the mutation frequency. HEK293T cells were maintained in DMEM high glucose (Nacalai Tesque) supplemented with 10% FBS (BioSera) and penicillin/streptomycin (Nacalai Tesque). Hu5 KD3 cells (a kind gift from Dr. Naohiro Hashimoto at National Center for Geriatrics and Gerontology) were maintained in DMEM, 20% FBS, 2% Ultroser G (PALL) and penicillin/streptomycin. U937 cells (RIKEN) and Jurkat cells (a kind gift from Hirohide Saito, Kyoto University) were maintained in RPMI (Gibco), 10% FBS, and penicillin/streptomycin. C2C12 cells were a kind gift from Hidetoshi Sakurai and maintained in DMEM, 15% FBS, 0.1 mM nonessential amino acids, 100 mM sodium pyruvate (Gibco), 100 mM 2-mercaptoethanol (Gibco), and penicillin/streptomycin. For differentiation, C2C12 cells were seeded at a density of 15,000 cells on a 12-well plate coated with collagen type I (Nippy). Differentiation media (DMEM, 5% horse serum, 100 mM sodium pyruvate, 100 mM 2-mercaptoethanol, and penicillin and streptomycin) was added and the cells were differentiated for 4 days. Healthy donor iPS cells (404C2 and 1383D2, and FF13096NOR) were a kind gift from Dr. Keisuke Okita (Kyoto University) and Dr. Hidetoshi Sakurai (Kyoto University). DMD patient iPS cells lacking exon44 (FFDMD111) or exon 45-46 (FF12020) were also kindly provided by Dr. Hidetoshi Sakurai. iPSCs were maintained in StemFit AK03N (Ajinomoto).

HEK293T cells were seeded in 10 cm plates at a density of 2-3 × 106 cells per plate. The next day, plasmids encoding VSV-G, FKBP12-Gag, FRB-SpCas9, and sgRNA were transfected by lipofectamine 2000 (ThermoFisher Scientific). The following day, the transfection media was replaced with 10 mL of fresh DMEM media containing 10 % FCS and 300 nM of AP21967 (Takara Clontech). Supernatant was harvested 36-48 hours after transfection, filtered through a 0.45 um syringe filter (Sartorius), and then centrifuged at 8,800×g's overnight in an Avanti JXN-30 centrifuge (Beckman Coulter). The pelleted EVs were resuspended in 100 ul of HBSS or PBS (ThermoFisher Scientific) and then aliquoted into 1.5 mL centrifuged tubes and stored at −80C.

For NanoMEDIC inoculation, HEK293T, C2C12, and Hu5 EGxxFP reporter cells, 25,000 or 50,000 cells were seeded in a 48-well plate. The following day, the reporter cells were inoculated with approximately 10 µl of 80-fold concentrated NanoMEDIC particles and then analyzed by flow cytometry 3 days post inoculation using an BD LSRFortessa Flow Cytometer. For iPSC inoculation, 1×10^5 cells were inoculated with NanoMEDIC in a 24-well plate.

For TIDE analysis, Sanger sequencing of the amplified genomic DNA region of interest was carried out and then the resulting trace files were used to

calculate the % indels by the TIDE webtool.

４．研究成果

We developed a novel ribonucleoprotein delivery system utilizing distinct homing mechanisms. First, we used chemical induced dimerization to recruit Cas9 protein into extracellular nanovesicles based on the FKBP12 and FRB dimerization system. Second, we were able to efficiently recruit sgRNA into nanovesicles. We term our fully engineered delivery system NanoMEDIC (nanomembrane-derived extracellular vesicles for the delivery of macromolecular cargo) and demonstrate efficient genome editing in various hard-to-transfect cell types, including human iPS cells, myoblasts, lymphocytes, and cortical-derived neurons. We were able to target 5 endogenous genes (SAMHD1, EMX1, DMD, VEGFA, CCR5) at 7 loci and observed high mutation frequencies above 50%.

Using previously reported promiscuous sgRNAs at the EMX1 and VEGFA loci, we found that NanoMEDIC had low to undetectable off-target activity compared with DNA plasmid delivery of SpCas9 and sgRNA with over 50-fold on to off target selectivity. Furthermore, NanoMEDIC production is scalable for industrial production as a xeno-free suspension culture system. Toxicity was also not observed with NanoMEDIC even after high editing efficiency was observed.

The dimerization system was optimized by constructing various plasmids expressing FRB fused to the N-, C- or N- and C- terminal domains of FKBP12. We found the N-terminal FRB-SpCas9 fused construct had the best incorporation potential. Furthermore, we made an FRB mutant to show that the incorporation of SpCas9 into NanoMEDIC was induced by chemical induced dimerization.

Interestingly, we also discovered that, compared with other traditional methods to fuse Gag with the protein of interest, SpCas9, our dimerization system showed improved delivery of the protein of interest because it does not rely on the viral protease to cleave the protein of interest from Gag. We found the viral protease itself actually cleaved the protein of interest, thereby reducing the amount of available protein delivered into target cells. When we introduced a protease inhibitor during NanoMEDIC production, the protein of interest was not cleaved. We also compared NanoMEDIC to a commercially available system from Clontech and we found that NanoMEDIC performed significantly better in our GFP reporter system.

As a disease model, therapeutic exon skipping in the dystrophin gene locus was targeted and resulted in over 90% exon skipping efficiencies in skeletal muscle cells derived from Duchenne muscular dystrophy patient iPS cells. Simultaneous targeting of splicing acceptor and splicing donor sites of an an exon showed a synergistic effect in exon skipping as opposed to using the sgRNAs individually. Dystrophin protein expression was also restored. Finally, we generated novel luciferase-based reporter mice to demonstrate that NanoMEDIC could induce exon skipping and sustain skipping activity for over 160 days, even though NanoMEDIC itself was rapidly degraded within 3 days, indicating its utility for transient in vivo genome editing therapy of DMD and beyond.

In addition to CRISPR-Cas9, other proteins of interest could be incorporated into NanoMEDIC. We were able to show luciferase protein packaged and then delivered in vivo into mice. In the future, other therapeutic proteins could potentially be packaged. Alternatively, other orthologues of SpCas9 could be included when PAM is limiting and the more recently reported base editors fused with nickase Cas9 could be used to edit either C to T or A to G transitions, as the majority of diseases are due to single base pair mutations.

５．主な発表論文等

Currently, no publications.

〔雑誌論文〕（計　　0 件）
Currently, no publications.

〔学会発表〕（計　　1 件）
Title: Development of a CRISPR-Cas9 RNP delivery system using virus-like particles
Presenter: Peter Gee
Conference: The 3rd Annual Meeting of the Japanese Society for Genome Editing in Hiroshima
Year: 2018

〔産業財産権〕
○出願状況（計　　1 件）

名称：ウイルスタンパク質を使用した CRISPR ヌクレアーゼおよび RNA の送達方法
発明者：Peter Gee and Akitsu Hotta
権利者：Peter Gee and Akitsu Hotta
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６．研究組織

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