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In vivo calpain knockdown using delivery of siRNA

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Abstract

Calpain is an intracellular Ca^{2+} -dependent non-lysosomal cysteine protease expressed ubiquitously in mammals. In endothelial cells, dysregulation of calpain has been shown to be involved in a wide variety of pathological conditions such as angiogenesis, vascular inflammation, and diabetes. Cell- or tissue-targeted *in vivo* delivery of small interfering RNA (siRNA) is a powerful research tool in analysis of protein function, and has been proposed as an attractive therapeutic modality that is applicable against a large number of human diseases including cancer. In this chapter we describe a method to knockdown calpain 1 in mouse pulmonary vascular endothelium using delivery of siRNA/cationic liposome complex. This technique results in a greater than 80% reduction in calpain 1 protein levels 48 hours after a single i.v. injection of calpain 1 siRNA (0.5 mg siRNA/kg)/cationic liposome complex. We also describe confocal imaging to verify the loss of calpain 1 expression in pulmonary microvessel endothelial cells and application of this technique in the mouse model of ventilator-induced lung injury.

Keywords

siRNA; calpain; knockdown; cationic liposome; endothelial cell; lung

1. Introduction

Calpain is an intracellular Ca^{2+} -dependent non-lysosomal cysteine protease. In humans, there are currently 15 known calpain isoform genes, which are defined by the presence of a protease domain. The best characterized, ubiquitous isoforms are calpain 1 (or μ -calpain) and calpain 2 (or m-calpain) which require micromolar (1 to 20 μM) and millimolar (0.25 to 0.75 mM) concentrations of intracellular Ca^{2+} for activation respectively. Calpains participate in a wide variety of physiological and pathological processes. Dysregulation of calpain expression and activity is related to myriad diseases including neurological disorders, cardiovascular diseases and cancers (1). Knocking down calpain isoforms *in vivo* and *in vitro* has identified important roles for calpains in the prevention and treatment of

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hyperhomocysteinemia, hyperglycemia (2), diabetes-induced renal injury (3), lung inflammation (4), atherosclerosis (5) and brain injury (6–7).

Gene silencing mediated by siRNAs, the 21- to 23-nt double-stranded RNA, is a sequence-specific RNA degradation mechanism. In contrast to the tangible and obvious effectiveness of RNA interference *in vitro*, the success of siRNA-mediated gene silencing *in vivo* has been limited due to multiscale barriers, including rapid blood clearance of molecules <10 nm in size, siRNA degradation in the bloodstream, nonspecific accumulation in tissues, poor cellular uptake and inefficient intracellular release (8). Delivering naked or liposome-complexed siRNA *in vivo* basically falls into two approaches: local and systemic administrations. Advantages of the local administration strategy for siRNA delivery *in vivo* include simple siRNA formulation, site-specific delivery for localized effects, and lower doses for efficacy with a lower risk of off-target effects. Systemic delivery by intravenous injection is the most common route of siRNA delivery and more widely applicable to clinical practice (9).

Liposomes are currently considered to be the most successful and effective drug-carrier system. The efficacy of liposomes depends on the physicochemical properties of their components and their size, surface charge, and lipid organization (10). Cationic liposomes have emerged as attractive siRNA carriers for systemic transfection *in vivo* due to their favorable characteristics such as biodegradability, minimal toxicity, nonimmunogenicity, relative ease of large-scale production, simplicity of use, and high *in vitro* transfection efficiency. Numerous laboratory studies (11–14) and clinical trials (15–17) have demonstrated the potential of cationic liposomes in gene therapy. Since cholesterol is one of the most promising siRNA conjugates that have shown efficient RNA interference *in vivo* (13, 18, 19), cholesterol has been utilized as an effective ‘helper’ lipid in dimethyl dioctadecyl ammonium bromide (DDAB) for preparation of the cationic liposome in our laboratory (4, 20, 21). Cationic liposomes, by virtue of their surface positive charge, bind selectively to endothelial cells via electrostatic interaction and have substantial endothelial uptake (18, 22, 23). Due to the large pulmonary vascular surface area and delivery of the entire cardiac output to pulmonary circuit, intravenous administration of siRNA-cationic liposome complex can be used to selectively deliver siRNA to pulmonary microvessel endothelial cells (24, 25–34).

In this chapter, we describe a method for preparation of the cationic liposome by mixing cholesterol and DDAB and then delivery of the calpain 1 siRNA/cationic liposome complex into mice through the tail vein to selectively deplete calpain 1 in the pulmonary vasculature. Efficiency of siRNA-mediated depletion of calpain 1 is confirmed by Western blot analysis of pulmonary endothelial and non-endothelial cell lysates and confocal immunofluorescence for direct visualization of protein expression in lung vascular endothelial cells. A mouse model of ventilator-induced lung injury is also described, to examine the effects of *in vivo* calpain knockdown on lung inflammation.

2. Materials

2.1. Preparation of siRNA/cationic liposome complex

1. Calpain 1 siRNAs: a pool of three target-specific 19–25 nt siRNAs.
2. Control siRNA: ON-TARGETplus Non-targeting control siRNA (Dharmacon).
3. Dimethyl dioctadecyl ammonium bromide (DDAB) stock solution: 400 mg DDAB in 20 mL chloroform.
4. Cholesterol stock solution: 400 mg cholesterol in 20 mL chloroform.
5. 5% (w/v) Glucose: 5 mg glucose in 100 mL deionized H₂O, sterilize the solution in an autoclave.
6. Sodium dodecyl sulfate.
7. Rotavapor.
8. Argon gas.
9. Sonicator.
10. Water bath.
11. Round-bottom flask.
12. 0.22 µm syringe filter.
13. Cell scrapers.

2.2 Experimental animals

1. Mice: House in microisolator cages under specific pathogen-free conditions, feed with autoclaved food, and use in experiments at 8 weeks of age (20–30 g).
2. Separate mice into two groups: calpain 1 siRNA/cationic liposome group and control siRNA/cationic liposome group.

2.3 Intravenous administration of siRNA/cationic liposome complex

1. Mouse injection restrainer.
2. 1 mL syringe.
3. Sterile 30 gauge needles.
4. Heating device.
5. Gauze sponges.

2.4 Isolation of lung endothelial cells

1. 1×DPBS: 9.55 g of Dulbecco's phosphate-buffered saline (DPBS) powder, 200 mg KCl, 200 mg K₂HPO₄, 8 g NaCl, 1.15 g NaH₂PO₄. Adjust pH to 7.4 and bring to 1L with deionized H₂O.
2. Collagenase / dispase: 1 mg/mL in DPBS.

3. Isolation buffer (0.1% BSA, 2 mM EDTA in DPBS, PH 7.4).
4. High glucose (4.5 g/L) Dulbecco's modified Eagle medium (DMEM).
5. Dynabeads magnetic beads: Sheep anti-rat IgG Dynabeads.
6. Anti-PECAM-1 antibody.
7. 40 μ m cell strainer.
8. 10 mL syringe.
9. MACS Separator.
10. MACS Column.

2.5 Western Blot

1. Modified RIPA lysis buffer: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate (Na_3VO_4), 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail (10 μ L/mL lysis buffer).
2. 50% (w/v) Bis-acrylamide (29:1) solution. Store at room temperature. Keep the solution in a dark glass bottle.
3. Separating gel buffer: 1 M Tris-HCl, pH 8.8. Store at 4 $^{\circ}\text{C}$.
4. 10% (w/v) sodium dodecyl sulfate (SDS). Store at room temperature.
5. Stacking gel buffer: 0.375 M Tris-HCl, pH 6.8. Store at 4 $^{\circ}\text{C}$.
6. 5% (w/v) ammonium persulfate (APS).
7. 8% separating gel solution: Total 12.54 mL of solution. 2 mL 50% Bis-acrylamide, 4.7 mL Tris-HCl pH 8.8, 125 μ L 10% SDS, 5.4 mL deionized H_2O , 312.5 μ L 5% APS, 3.125 μ L N, N, N', N'-tetramethylethane-1, 2-diamine (TEMED).
8. 4 $\times$ Laemmli sample buffer.
9. 4% stacking gel solution. Mix 6.315 mL of solution: 500 μ L 50% Bis-acrylamide, 2.1 mL Tris-HCl pH 6.8, 62.5 μ L 10% SDS, 3.15 mL deionized H_2O , 500 μ L 5% APS, 2.5 μ L TEMED.
10. 10 $\times$ Running buffer (1 L): 30.3 g (0.25 M) Tris Base, 144.0 g (1.92 M) glycine, and 10.0 g (1%, w/v) SDS. Bring to 1L with deionized H_2O . Store at room temperature.
11. 5 $\times$ Transfer buffer (1.6 L): 29.3 g glycine, 58.2 g Tris-Base, Bring to 1.6 L with deionized H_2O .
12. Tris-buffered saline with Tween (TBS-T) (1 L): 10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, and 1% (v/v) Tween-20. Bring to 1 L with deionized H_2O .
13. Blocking buffer: 5% (w/v) non-fat dry milk in TBS-T.

14. Primary antibody: rabbit anti-VE-Cadherin, mouse anti-calpain 1, and rabbit anti-GAPDH.
15. Secondary antibody: donkey anti-rabbit and donkey anti-mouse IgG conjugated to horseradish peroxidase.
16. Odyssey Fc Imager (LI-COR Biosciences).
17. BCA protein assay kit.
18. Nitrocellulose Membrane.
19. Super Signal West Pico Chemiluminescent substrate (Thermo Fisher Scientific).
20. Image J software (NIH).

2.6 Confocal Immunofluorescence Microscopy

1. 4% (v/v) paraformaldehyde: Dilute 16% (v/v) paraformaldehyde to 4% (v/v) with 1× DPBS.
2. Wash solution: 0.1% (v/v) Triton X-100 in DPBS.
3. Blocking buffer: 5% (v/v) donkey serum and 1% (w/v) BSA in wash solution.
4. Antibody dilution buffer: 1% (v/v) donkey serum and 1% (w/v) BSA in wash solution.
5. Primary antibody: rabbit anti-PECAM-1 and mouse anti-calpain 1 antibodies.
6. Secondary antibody: donkey anti-rabbit Alexa Fluor 594, and donkey anti-mouse Alexa Fluor 488.
7. Cryostat with metal grids
8. Inert support medium: optimal cutting temperature (OCT) compound.
9. Confocal Microscopy.
10. 12-mm microscope cover glass.
11. Mounting medium: Antifade mounting medium with DAPI.

2.7 Mouse model of ventilator-induced lung injury

1. Animal: Mice should be housed in microisolator cages, fed with autoclaved food, and used in experiments at 8–12 wk of age (25–30 g).
2. Anesthetics: ketamine (75 mg/kg), 1% isoflurane.
3. Ventilator: small animal ventilator for rodents.
4. Grouping: Mice transfected with control (CON) and calpain 1 (Calpn1) siRNA should be divided into three groups: control group, CON siRNA-treated mice ventilated with normal tidal volume; mechanical ventilation (MV) group, CON siRNA-treated mice ventilated with high tidal volume; and MV plus Calpn 1 group, Calpn 1 siRNA-treated mice ventilated with high tidal volume.

2.8 Polymorphonuclear neutrophil (PMN) counts in bronchoalveolar lavage (BAL) fluid

1. 1 mL sterile syringe.
2. Cytocentrifuge.
3. Diff-Quick dye.
4. Hemocytometer.
5. Light microscope.
6. Slides.

3. Methods

3.1 Preparation of cationic liposome

1. Add 315 μ L of DDAB stock solution (i.e., 6.3 mg DDAB), 200 μ L of cholesterol stock solution (i.e., 4 mg cholesterol) and 9.5 mL of chloroform to a sterilized round-bottom flask, and gently swirl until they are completely mixed.
2. Connect the round-bottom flask to the Rotavapor and evaporate the chloroform under vacuum at 40 °C, 100 rpm for approximately 20 minutes (see Fig. 1). All of the chloroform should be removed by rotary evaporation yielding a thin lipid film on the sides of a round bottom flask (see Note 1). For small volumes (< 1 mL), the chloroform may be evaporated using a dry nitrogen or argon stream in a fume hood.
3. Add 2 mL of 5% glucose to resuspend the lipid. Scratch the flask bottom thoroughly using the cell scrapers to dissolve all of the lipid.
4. Add a pinch of SDS powder to the water of the bath sonicator, sonicate the lipid for at least 20 minutes using the flask holding stand and just barely touch the bottom of the round-bottom flask to the water in the vat (see Note 2).
5. Transfer the sonicated liposome from the flask to an Eppendorf tube, extrude and sterilize the liposome with a 0.22 μ m syringe filter (see Note 3).

3.2 Preparation of the mixture of siRNA/cationic liposome

1. Prepare the siRNA/liposome complex by addition of 0.25 or 0.5 mg/kg calpain 1 siRNA (stock concentration, 10 μ M) into 200 μ L (i.e., 20 g mice, 10 μ L/g) (3, 18–19) of liposome suspensions. A scrambled siRNA should be employed as a control (see Note 4).

1. When the round-bottom flask is rotated, the water bath temperature should be kept at 40°C strictly. The optimal lipid film is thin and well-distributed. The bottom of the round-bottom flask should just barely touch the water.

2. The ultrasound should not be stopped until the lipid changes from large precipitates to small particles. The lipid suspension should begin to clarify to yield a slightly hazy transparent solution.

3. To obtain liposomes with a narrow size distribution, liposome is extruded by 0.22 μ m syringe filter. The diameter of the liposomes synthesized here is < 220 nm. To ensure size homogeneity, multiple extrusions (10 times) are needed.

4. An optimal efficient concentration of siRNA and the appropriate ratio of siRNA/cationic liposome are important for higher gene silencing efficiency of the siRNA.

2. Mix immediately and gently by shaking and incubate at room temperature for 15–20 minutes at room temperature (see Note 5).

3.3 Delivering siRNA/cationic liposome complex into mice by intravenous injection.

1. Hold the mice in a standard mouse restrainer.
2. Stimulate dilation of the tail veins in mice by placing the tail in warm (30–35 °C) water.
3. Grasp the tail at the distal end. Place the index and middle fingers of the non-dominant hand around the tail above where the needle will be inserted. Hold the lower part of the tail between the thumb and ring fingers below the injection site.
4. Slightly oppose pressure with both sets of fingers to straighten and stabilize the tail. Release pressure immediately before injection.
5. Insert the needle into the vein towards the direction of the head with the dominant hand. Hold the needle and syringe parallel to the tail with bevel side up.
6. Gently pull blood into the syringe to confirm proper placement in the vessel before injecting. Alternately, verify proper placement by advancing the needle smoothly into the vein without any resistance.
7. Slowly inject the total siRNA/liposome mixture (see Note 6).
8. Remove the needle and apply gentle compression until bleeding has stopped.
9. Return mice to their cage and monitor for an addition 5–10 minutes to ensure that bleeding has not resumed.

3.4 Determination of efficiency of calpain 1 knockdown

3.4.1. Western blot analysis

1. Anesthetize mice by injection of ketamine/xylazine (100 mg/kg/10 mg/kg, i.p.) at 48 hours post-transfection.
2. Sacrifice mice, harvest the lung after perfusing with DPBS (0.6 mM EDTA in DPBS, pH 7.4), followed by perfusing the lung intratracheally with collagenase/dispase solution, and then place the lung into ice-cold DMEM high glucose (4.5 g/L) medium.
3. Collect the lung tissues in collagenase/dispase solution after being minced, and then incubate at 37°C for 35 min with shaking.

5. The siRNA and liposome should be mixed immediately by pipetting or vortexing to avoid unequal mixing of the lipid and siRNA components that can cause formation of precipitates. The siRNA/cationic liposome complex should be freshly prepared just prior to injection using DNAase, RNAase and Pyrogen free tubes and tips. Keep sterile operation to the greatest extent.

6. A small volume of siRNA/liposome complex is injected gently in order to determine whether the needle is in the lumen of the vein. The entire volume of siRNA/liposome complex is then injected slowly. There should be little resistance if the mixture of siRNA/liposome is being injected into the tail vein. If there is significant resistance to injection, the needle may be not in the lumen of the vein. Keep sterile operation to the greatest extent.

4. After digestion, filter cell suspension with a 40 μ m cell strainer, and centrifuge at 400g for 10 minutes.
5. Resuspend the cells in an isolation buffer, and then incubate with the Dynabeads magnetic beads pretreated with PECAM-1 antibody for 20 min at 4°C with gentle rotation (see Note 7).
6. Isolate lung endothelial cells and separate them from the non-endothelial cell fraction by a MACS Column, which is placed in the magnetic field of a MACS Separator.
7. Wash the isolated cells with ice-cold DPBS.
8. Add the cold RIPA buffer to each tube.
9. Homogenize the cells with sonicator, and then lyse the cells on ice for 30 minutes.
10. Centrifuge at $16,000 \times g$, 4°C for 15 minutes.
11. Transfer supernatants to new Eppendorf tubes.
12. Measure protein concentration with a BCA assay kit.
13. Mix 25 μ g protein from each sample with 4 $\times$ Laemmli sample buffer in Eppendorf tubes. Boil the mixture at 95–100°C for 5 min.
14. Load prepared samples into wells of 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 4% stacking gel and make sure not to overflow.
15. Transfer the protein blots that have been separated by SDS-PAGE onto nitrocellulose membrane at 170 mA for 90 minutes.
16. Place the membranes into the blocking buffer for 1 hour at room temperature on a rocking platform.
17. Incubate the membranes with primary antibodies overnight at 4°C.
The dilutions of primary antibodies are as follows:
Anti-rabbit VE-cadherin antibody: 1:1,000
Anti-mouse calpain 1 antibody: 1: 300
Anti-rabbit GAPDH antibody: 1:2,000
18. Remove the primary antibody and wash the membranes for three times with TBS-T on a rocking platform.
19. Incubate the membranes with secondary antibodies (1:2,000) at room temperature for 1 hour.

7. Dynabeads (100 μ L/mouse) must be pre-coated with anti-PECAM-1 antibody (6 μ g/mouse) by co-incubating for at least 30 minutes at 4 °C with gentle tilting and rotation. To improve the purity of lung endothelial cells, the Dynabeads to cell suspension ratio must be optimized.

20. Discard the secondary antibodies and wash the membrane for three times with TBS-T on a rocking platform.
21. Immerse the membranes with SuperSignal West Pico Chemiluminescent Substrate for a while.
22. Detect protein bands with Odyssey Fc Imager.
23. Quantify the intensity of immunoblot bands by Image J software (NIH) (see Fig. 2).

3.4.2. Confocal immunofluorescence.

1. Freeze a fresh, unfixed lung tissue sample, up to 2.0 cm in diameter, in OCT in a suitable tissue mold. Freeze the OCT containing the tissue onto the specialized metal grids that fit onto the cryostat.
2. Cut lung sections 5 μ m thick in the cryostat at -20°C .
3. Immediately transfer the lung section to microscope slide by touching the slide to the tissue at room temperature. Thaw the slides mounted with cryosections (5 μ m thick) of mouse lung tissue for 20~30 minutes at room temperature, if the slides are stored in -80°C freezer (see Note 8).
4. Wash the slides in DPBS to remove the OCT compound.
5. Fix slides with 4% paraformaldehyde for 8 minutes at room temperature.
6. Discard paraformaldehyde and wash the slides three times with DPBS for 6 minutes each at room temperature on the rocking platform.
7. Place the slides in a blocking buffer for 60 minutes at room temperature.
8. Incubate the slides with primary antibodies overnight at 4°C .

The dilutions of primary antibodies are as follows:

Anti-rabbit PECAM-1 antibody: 1:200

Anti-mouse calpain 1 antibody: 1:40

9. Remove the primary antibody and wash the slides three times for 6 minute each with washing solution.
10. Pre-incubate the slides with a blocking buffer again for 30 minutes at room temperature.
11. Incubate the slides with the secondary antibody donkey anti-rabbit Alexa Fluor 594 (1:750) and donkey anti-mouse Alexa Fluor 488 (1:750) for 1 hour at room temperature. And from this step, keep the slides be in dark.
12. Wash the slides five times for 6 minutes each with washing solution.

8. To prevent the lung tissues from curling-up or detaching from the surface of the slide, frozen slides are warmed to room temperature for 20~30 minutes.

13. Mount the slides to glass microscope slides properly with mounting medium and avoid trapping air bubbles on the slide.
14. Dry the slides at room temperature for several hours in dark. Store the slides at -20°C in dark for up to several months, if necessary.
15. Acquire images by a laser-scanning confocal microscope (see Fig. 3).

3.5. Ventilator-induced lung injury

1. Intravenously inject control siRNA or calpain 1 siRNA into mice.
2. Anesthetize mice with ketamine (75 mg/kg) at 48 hours after injection.
3. Perform tracheostomy and the intubation cannula into the mouse trachea.
4. Ventilate mice for 2 hours with 1% isoflurane in room air to maintain anesthesia, using either high (28 mL/kg) or normal tidal volume (7 mL/kg) (4).

3.4.5. Determination of PMN counts in BAL fluid

1. Perform BAL by intratracheal injection of 1 mL of PBS followed by gentle aspiration.
2. Centrifuge the pooled BAL fluid at 400 g for 5 min.
3. Suspend cell pellets in PBS at a final concentration of 1×10^5 cells/mL.
4. Cytospin 200 μL volume of resuspended cells onto slides at 300 rpm for 5 minutes.
5. Stain the slides with Diff-Quick dye.
6. Examine the slides at a magnification of $\times 20$ and $\times 40$ by light microscopy.
7. Calculate the percentage of PMNs after counting 300 cells in randomly selected fields.
8. Manually measure the total cell count in BAL fluid using a hemocytometer (see Fig. 4).

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5. References

1. Ono Y, Saido TC, Sorimachi H. (2016) Calpain research for drug discovery: challenges and potential. *Nat Rev Drug Discov* 15, 854–876. [PubMed: 27833121]
2. Cheng Z, Jiang X, Pansuria M, Fang P, Mai J, Mallilankaraman K, Gandhirajan RK, Eguchi S, Scalia R, Madesh M, Yang X, Wang H. (2015) Hyperhomocysteinemia and hyperglycemia induce and potentiate endothelial dysfunction via μ -calpain activation. *Diabetes* 64, 947–59. [PubMed: 25352635]
3. Covington MD, Schnellmann RG. (2012) Chronic high glucose downregulates mitochondrial calpain 10 and contributes to renal cell death and diabetes-induced renal injury. *Kidney Int* 81, 391–400. [PubMed: 22012129]

4. Liu D, Yan Z, Minshall RD, Schwartz DE, Chen Y, Hu G. (2012) Activation of calpain s mediates early lung neutrophilic inflammation in ventilator-induced lung injury. *Am J Physiol Lung Cell Mol Physiol* 302, L370–9. [PubMed: 22140070]
5. Miyazaki T, Taketomi Y, Takimoto M, Lei XF, Arita S, Kim Kaneyama JR, Arata S, Ohata H, Ota H, Murakami M, Miyazaki A. (2011) m-Calpain induction in vascular endothelial cells on human and mouse atheromas and its roles in VE-cadherin disorganization and atherosclerosis. *Circulation* 124, 2522–32. [PubMed: 22064597]
6. Bevers MB, Ingleton LP, Che D, Cole JT, Li L, Da T, Kopil CM, Cohen AS, Neumar RW. (2010) RNAi targeting micro-calpain increases neuron survival and preserves hippocampal function after global brain ischemia. *Exp Neurol* 224, 170–7. [PubMed: 20298691]
7. Sha D, Jin Y, Wu H, Wei J, Lin CH, Lee YH, Buddhala C, Kuchay S, Chishti AH, Wu JY. (2008) Role of mu-calpain in proteolytic cleavage of brain L-glutamic acid decarboxylase. *Brain Res* 1207, 9–18. [PubMed: 18377878]
8. Shim MS, Kwon YJ. (2010) Efficient and targeted delivery of siRNA in vivo. *FEBS J* 277, 4814–27. [PubMed: 21078116]
9. Akhtar S, Benter IF. (2007) Nonviral delivery of synthetic siRNAs in vivo. *J Clin Invest* 117, 3623–32. [PubMed: 18060020]
10. Bozzuto G, Molinari A. (2015) Liposomes as nanomedical devices. *Int J Nanomedicine* 10, 975–99. [PubMed: 25678787]
11. Zimmermann TS, Lee AC, Akinc A, Bramlage B, Bumcrot D, Fedoruk MN, Harborth J, Heyes JA, Jeffs LB, John M et al. (2006) RNAi-mediated gene silencing in non-human primates. *Nature* 441, 111–114. [PubMed: 16565705]
12. Mikhaylova M, Stasinopoulos I, Kato Y, Artemov D, Bhujwalla ZM. (2009) Imaging of cationic multifunctional liposome-mediated delivery of COX-2 siRNA. *Cancer Gene Ther* 16, 217–26. [PubMed: 18927599]
13. Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M, Elbashir S, Geick A, Hadwiger P, Harborth J et al. (2004) Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* 432, 173–178. [PubMed: 15538359]
14. Wolfrum C, Shi S, Jayaprakash KN, Jayaraman M, Wang G, Pandey RK, Rajeev KG, Nakayama T, Charrise K, Ndungo EM et al. (2007) Mechanisms and optimization of in vivo delivery of lipophilic siRNAs. *Nat Biotechnol* 25, 1149–1157. [PubMed: 17873866]
15. Caplen NJ, Alton EW, Middleton PG, Dorin JR, Stevenson BJ, Gao X, et al. (1995) Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. *Nat. Med* 1, 39–46. [PubMed: 7584951]
16. McLachlan G, Ho LP, Davidson-Smith H, Samways J, Davidson H, Stevenson BJ, et al. (1996) Laboratory and clinical studies in support of cystic fibrosis gene therapy using pCMV-CFTR-DOTAP. *Gene Ther* 3, 1113–1123. [PubMed: 8986438]
17. Gill DR, Southern KW, Mofford KA, Seddon T, Huang L, Sorgi F, et al. (1997) A placebo-controlled study of liposome-mediated gene transfer to the nasal epithelium of patients with cystic fibrosis. *Gene Ther* 4, 199–209. [PubMed: 9135733]
18. Predescu DN 1, Bardita Cristina, Predescu Dan, Predescu Sanda. (2013) Long-term Silencing of Intersectin-1s in Mouse Lungs by Repeated Delivery of a Specific siRNA via Cationic Liposomes. Evaluation of Knockdown Effects by Electron Microscopy. *J Vis Exp* 76, 50316.
19. Neamu R, Bardita C, Wang M, Predescu SA. (2012) Impaired caveolae function and upregulation of alternative endocytic pathways induced by experimental modulation of intersectin-1s expression in mouse lung endothelium. *Biochem Res Int* 2012, 672705. [PubMed: 22506115]
20. Hu G, Vogel SM, Schwartz DE, Malik AB, Minshall RD. (2008) Intercellular adhesion molecule-1 dependent neutrophil adhesion to endothelial cells induces caveolae-mediated pulmonary vascular hyperpermeability. *Circ. Res* 102, e120–e131. [PubMed: 18511851]
21. Wang YL, Malik AB, Sun Y, Hu S, Reynolds AB, Minshall RD, Hu G. (2011) Innate Immune Function of the adherens junction protein p120-catenin in endothelial response to endotoxin. *J Immunol* 186, 3180–3187. [PubMed: 21278343]
22. Bai L, Andersson HA, McConnell KI, Chan DL, Hernandez M, Gonzalez J, Liu X, La Francesca S, Sakamoto JH, Serda RE. (2015) Silencing of Tumor Necrosis Factor Receptor-1 in Human Lung

- Microvascular Endothelial Cells Using Particle Platforms for siRNA Delivery. *Curr Drug Targets* 16, 1531–9. [PubMed: 26201489]
23. Kowalski PS, Zwiers PJ, Morselt HW, Kuldo JM, Leus NG, Ruiters MH, Molema G, Kamps JA. (2013) Anti-VCAM-1 SAINT-O-Somes enable endothelial-specific delivery of siRNA and downregulation of inflammatory genes in activated endothelium in vivo. *J Control Release* 176, 64–75.
24. Zhou MY, Lo SK, Bergenfeldt M, Tiruppathi C, Jaffe A, Xu N, Malik AB. (1998) In vivo expression of neutrophil inhibitory factor via gene transfer prevents lipopolysaccharide-induced lung neutrophil infiltration and injury by a beta2 integrin-dependent mechanism. *J Clin Invest* 101, 2427–37. [PubMed: 9616214]
25. Zhu N, Liggitt D, Liu Y, and Debs R (1993) Systemic gene expression after intravenous DNA delivery into adult mice. *Science* 261, 209–211. [PubMed: 7687073]
26. Thierry AR, Lunardi-Iskandar Y, Bryant JL, Rabinovich P, Gallo RC, and Mahan LC (1995) Systemic gene therapy: biodistribution and long-term expression of a transgene in mice. *Proc. Natl. Acad. Sci. USA* 92, 9742–9746. [PubMed: 7568209]
27. Li S and Huang L (1997) In vivo gene transfer via intravenous administration of cationic lipid-protamine-DNA (LPD) complexes. *Gene Ther* 4, 891–900. [PubMed: 9349425]
28. Liu F, Qi H, Huang L, and Liu D (1997) Factors controlling the efficiency of cationic lipid-mediated transfection in vivo via intravenous administration. *Gene Ther* 4, 517–523. [PubMed: 9231067]
29. Liu Y, Mounkes LC, Liggitt HD, Brown CS, Solodin I, Heath TD, and Debs RJ (1997) Factors influencing the efficiency of cationic liposome-mediated intravenous gene delivery. *Nat. Biotechnol* 15, 167–173. [PubMed: 9035144]
30. Song YK, Liu F, Chu S, and Liu D (1997) Characterization of cationic liposome-mediated gene transfer in vivo by intravenous administration. *Hum. Gene Ther* 8, 1585–1594. [PubMed: 9322091]
31. Templeton NS, Lasic DD, Frederik PM, Strey HH, Roberts DD, and Pavlakis GN (1997) Improved DNA: liposome complexes for increased systemic delivery and gene expression. *Nat. Biotechnol* 15, 647–652. [PubMed: 9219267]
32. Barron LG, Meyer KB, and Szoka FC Jr. (1998) Effects of complement depletion on the pharmacokinetics and gene delivery mediated by cationic lipid-DNA complexes. *Hum. Gene Ther* 9, 315–323. [PubMed: 9508049]
33. McLean JW, Fox EA, Baluk P, Bolton PB, Haskell A, Pearlman R, Thurston G, Umemoto EY, McDonald DM (1997) Organ-specific endothelial cell uptake of cationic liposome-DNA complexes in mice. *Am. J. Physiol* 273, H387–404. [PubMed: 9249514]
34. Liu D, Knapp EJ, and Song YK (1999) Mechanisms of cationic liposome mediated transfection of the lung endothelium, in *Nonviral Vectors for Gene Therapy* (Huang L, Wagner E, and Hung MC, eds.), Academic Press, San Diego, CA, pp. 313–335.

Figure 1. Scheme of a rotary evaporator.

The liposome is placed in the evaporation flask (right) which is rotated (100 rpm) by a motor unit. The flask is lowered into the water bath (40°C). Chloroform is removed under vacuum and trapped by a condenser.

Figure 2. Expression of calpain 1 protein in calpain-depleted mice.

Mice were transfected with control (CON) and calpain 1 siRNA. At 48 hours posttransfection, pulmonary endothelial cells (ECs) were isolated as described in *Methods*. CON siRNA-treated mice were used as the control group. A, The purity of isolated ECs was confirmed by the expression of VE-cadherin. NECs = non-endothelial cells. B, Effective knockdown of calpain 1 expression was confirmed by Western blotting of pulmonary ECs. Results are representative of 3 independent experiments.

Figure 3. Confocal images showing downregulation of calpain 1 in pulmonary vasculature with a specific calpain 1 siRNA.

All images were acquired from lung tissues treated with control and calpain 1 siRNA (0.5 mg/kg) for 48 hours. After fixation and permeabilization, the tissues were stained with anti-PECAM-1 (red) and anti-calpain 1 (green) antibodies followed by Alexa 594-conjugated secondary antibody and Alexa 488-conjugated secondary antibody, respectively. Endothelial calpain 1 expression was ascertained in PECAM-1 positive structure. Scale bars = 20 μ m. Shown are representative results of 3 independent experiments.

Figure 4. Decreased lung inflammation by mechanical ventilation in calpain-depleted mice. Mice were transfected with Control (CON) and calpain 1 (Calpn 1) siRNA (si). After 48 h, mice (n=6/group) were mechanically ventilated with high tidal volume (28 ml/kg, MV) for 2 h. Control siRNA-treated mice ventilated with normal tidal volume were used as the control groups. PMN counts in BAL fluid were calculated. *P<0.05 vs. control groups. †P<0.05 vs. CON siRNA+MV groups (see ref. 4).