Published in final edited form as:
Methods Mol Biol. 2012 ; 797: 141–162. doi:10.1007/978-1-61779-340-0_11.

Oncolytic measles virus retargeting by ligand display

Pavlos Msaouel, MD, **Ianko D. Iankov, MD, PhD**, **Cory Allen**, **Stephen J. Russell, MD, PhD**, and **Evanthia Galanis, MD**
Departments of Oncology and Molecular Medicine, Mayo Clinic, Rochester, MN 55905

Abstract

Despite significant advances in recent years, treatment of metastatic malignancies remains a significant challenge. There is an urgent need for development of novel therapeutic approaches. Virotherapy approaches have considerable potential and among them measles virus (MV) vaccine strains have emerged as one of the most promising oncolytic platforms. Retargeted MV strains deriving from the Edmonston vaccine lineage (MV-Edm) have shown comparable antitumor efficacy to unmodified strains against receptor expressing tumor cells with improved therapeutic index. Here we describe the construction, rescue, amplification and titration of fully retargeted MV-Edm derivatives displaying tumor specific receptor binding ligands on the viral surface in combination with CD46 and SLAM entry H ablating mutations.

Keywords

oncolytic measles virus; measles retargeting; virotherapy; measles engineering

1. Introduction

Oncolytic measles virus has emerged as one of the most promising platforms for virus-based cancer therapeutics. In contrast to the wild-type measles virus which can cause potentially serious disease, the Edmonston measles virus (MV-Edm) is an attenuated vaccine strain that has an excellent safety profile. Our group and others have demonstrated the therapeutic potential of MV-Edm derivatives against a variety of solid tumors and hematologic malignancies including ovarian cancer (1, 2), glioblastoma multiforme (3), multiple myeloma (4, 5), lymphoma (6), breast cancer (7, 8), prostate cancer (9, 10) and hepatocellular carcinoma (11).

MV-Edm strains exhibit considerable local bystander oncolytic effect achieved via the massive cell-cell fusion of infected cells with neighboring uninfected cells. Of note attachment of measles virus to the target cells and fusion are mediated by two distinct envelope glycoproteins: the hemagglutinin (H) protein and the fusion (F) protein respectively. Measles virus enters the cells via interaction of the H glycoprotein with two known receptors: CD46 (12, 13) a regulator of complement activation that is found on all human nucleated cells but overexpressed in tumors and SLAM (14, 15); (16) the signaling lymphocyte activating molecule, which is expressed on activated T and B cells and macrophages. Receptor recognition by the H protein induces conformational changes of the F protein that result in fusion and viral entry (17). Thus, viral binding specificity is determined by the measles H protein (18). This allows retargeting strategies to focus on H protein modification without affecting the potent fusogenic capacity of the virus. Indeed, a

Corresponding Author: Evanthia Galanis, M.D. Mayo Clinic 200 First Street SW Rochester, MN 55905 Phone: (507) 284-3902 Fax: (507) 284-1803 galanis.evanthia@mayo.edu.

number of studies from our laboratory and others have confirmed that retargeted MV-Edm derivatives retain the potent oncolytic activity of the parent strain against cells or xenografts expressing the target receptors (19-25). A variety of ligands have been successfully displayed on H, including single chain antibodies against EGFRvIII(22), EGFR(20, 21) , CD38 (20), folate receptor alpha (FRalpha)(23), HER-2/neu(26), CD20(27), echistatin (an RGD containing peptide binding to integrins αvβ3 and α5β1)(28) and cytokines such as IL-13(19). An alternative MV-Edm targeting approach has also been developed utilizing protease-cleavable linkers that are inserted into the F protein in order to restrict proteolytic maturation of F at sites where the protease is abundantly secreted (29). It is thus now possible to reprogram MV-Edm specificity at different levels. Although ongoing clinical trials using engineered oncolytic MV-Edm strains have not demonstrated dose limiting toxicity to date (30), viral retargeting may address any future toxicity concerns associated with higher viral doses and use of potent therapeutic transgenes (where even tighter specificity might be desired), overcome variability in viral receptor expression by tumor cells, and increase the efficacy of targeted delivery following systemic administration.

In the following sections we will describe the methods employed in our laboratory for rescue and propagation of MV-Edm derivatives reprogrammed to specifically target cancer cells by displaying alternate receptor binding ligands on the viral surface. The first step to MV-Edm retargeting is the introduction of H protein mutations that ablate viral recognition and entry via the natural measles receptors CD46 and SLAM. Several H mutations that ablate entry through CD46 or SLAM have been identified (31, 32). We routinely use the combination of a single CD46 ablating mutation at position 481 (Y481A) and a single SLAM ablating mutation at position 533 (R533A) (20, 22, 31, 32) shown by Nakamura et al (32) to effectively ablate entry via both CD46 and SLAM with comparable fusogenicity to unablated strains. To facilitate viral rescue as well as allow *in vitro* and *in vivo* visualization of infection, the virus may also contain the enhanced green fluorescent protein (eGFP) gene at position 1 (**Fig. 1**). All retargeted viruses are derived from the Edmonston-Nse strain and are rescued using the pseudoreceptor STAR (Six-His Tagging And Retargeting) system (20). This system uses the 293-3-46 helper cells generated by Radecke et al. (33) to stably express the measles nucleocapsid (N) and phosphoprotein (P) proteins and T7 RNA polymerase for viral rescue (**Fig. 2**), and Vero-aHis cells for viral propagation. The protocol we describe here includes construction of the full length retargeted measles construct by cloning of the target receptor peptidic ligand at the C terminus of H, rescue and propagation of the virus as well as preparation, purification and titration of viral stocks. The targeting ligands introduced at the C-terminus of the H protein are coupled with a 6-histidine tag (**Fig. 1**) which allows propagation of retargeted MV-Edm derivatives using Vero-aHis cells. This cell line was generated by Nakamura et al. (20) to express a membrane-anchored single-chain antibody that binds to the 6-histidine tag allowing virus entry and propagation of the retargeted strain.

2. Materials

2.1 Construction of the full-length retargeted measles vector

2.1.1 Targeting ligand Amplification

1. Oligonucleotide primers for amplification of the targeting ligands. The primers should be designed to flank the ligand with SfiI/NotI digestion sites.
2. Standard polymerase chain reaction (PCR) reagents. We routinely use the Taq polymerase for PCR amplification which allows the use of the TA cloning system.
3. Standard reagents and apparatus for agarose gel electrophoresis.

2.1.2 Gel Fragment Purification

1. QIAEX Gel Extraction Kit (Qiagen, Almada, CA).
2. 10 mM Tris-HCl, pH 8.5 or H_2O with pH 7.0-8.5.

2.1.3 Cloning of the peptide ligand

1. Invitrogen TA Cloning Kit (Invitrogen, Carlsbad, CA).
2. Lysogeny broth (LB) plates containing 100 μg/ml ampicillin (store at 4°C).
3. LB medium containing 100 μg/ml of ampicillin (store at 4°C).

2.1.4 Plasmid Isolation and Analyzation

1. QIAprep Miniprep kit (Qiagen, Almada, CA).
2. 10 mM Tris-HCl, pH 8.5 or H_2O (pH 7.0-8.5).
3. Digestion enzymes and reagents used to verify DNA sequence were bought from New England Biolabs (Ipswich, MA).
4. DNA sequencer (Perkin-Elmer, Foster City, CA).

2.1.5 Subcloning of the peptide ligand into the pCG-H_{AA}-H6 vector

1. pCG-H_{AA}-H6 shuttle vector developed by Dr. T. Nakamura (20). Store at -20°C.
2. NEBuffer 2 10X (New England Biolabs, Ipswich, MA) [10X concentrated stock of 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), pH 7.9]. Store at -20°C.
3. BSA 100X (New England Biolabs, Ipswich, MA) [100X concentrated stock of 20mM KPO_4 (pH 7.0), 50 mM NaCl, 0.1 mM EDTA and 5% (w/v) glycerol]. Store at -20°C.
4. NotI and SfiI digestion enzymes (New England Biolabs, Ipswich, MA). Store at -20°C.
5. 10X ligation buffer (Invitrogen, Carlsbad, CA) [a 10X concentrated stock of 60 mM Tris-HCl, pH 7.5, 60 mM $MgCl_2$, 50 mM NaCl, 1 mg/ml bovine serum albumin, 70 mM β-mercaptoethanol, 1 mM ATP, 20 mM dithiothreitol (DTT), 10 mM spermidine]. Store at -20°C.
6. T4 DNA Ligase (4.0 Weiss units/μl) (Invitrogen, Carlsbad, CA). Store at -20°C.

2.1.6 Propagation and purification of transfection grade plasmid DNA

1. QIAprep Midiprep kit (Qiagen, Almada, CA).
2. LB medium containing 100 μg/ml of ampicillin. Store at 4°C.
3. 99.7% (v/v) pure isopropanol, 70% (v/v) ethanol solution.
4. H_2O (pH 7.0-8.5) or 10 mM Tris-HCl, pH 8.5.

2.1.7 Ligation into full-length plasmid

1. The p(+) MV-eGFP(-Spe) plasmid encodes the full-length infectious clone of the MV-NSe strain, deriving from MV-Edm. The green fluorescent protein gene (eGFP) has been inserted as an additional transcription unit upstream of the measles N gene (**Fig. 1**). The vector has been modified (34) to contain unique PacI

and SpeI digestion sites that facilitate cloning of the modified H cDNAs. Store at -20°C.

2. NEBuffer 4 10X (New England Biolabs, Ipswich, MA) [10X concentrated stock of 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate, 1 mM DTT, pH 7.9]. Store at -20°C.
3. BSA 100X (New England Biolabs, Ipswich, MA) [100X concentrated stock of 20mM KPO_4 (pH 7.0), 50 mM NaCl, 0.1 mM EDTA and 5% glycerol]. Store at -20°C.
4. PacI and SpeI digestion enzymes (New England Biolabs, Ipswich, MA). Store at -20°C.
5. 10X ligation buffer (Invitrogen, Carlsbad, CA) [10X concentrated stock of 60 mM Tris-HCl, pH 7.5, 60 mM $MgCl_2$, 50 mM NaCl, 1 mg/ml bovine serum albumin, 70 mM β-mercaptoethanol, 1 mM ATP, 20 mM dithiothreitol (DTT), 10 mM spermidine.] Store at -20°C.
6. T4 DNA Ligase (4.0 Weiss units/μl) (Invitrogen, Carlsbad, CA). Store at -20°C.

2.2 Rescue, propagation and titration of retargeted MV strains

1. 293-3-46 helper cell line generated by Radecke et al. (33) to stably express the T7 RNA polymerase as well as measles N and P proteins.
2. Vero-aHis cell line developed by Nakamura et al. (20) to stably express a membrane-anchored single-chain antibody recognizing the 6-histidine peptide (20, 35). Vero-aHis are derived from the Vero (African green monkey kidney) cell line and are grown in DMEM supplemented with 10% (v/v) FBS and 100 U/ml penicillin-streptomycin (*see* **Note 1**).
3. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum. The DMEM may also contain 100 U/ml penicillin-streptomycin and 1.2 mg/ml G418 when indicated. Store at 4°C.
4. Opti-MEM I Reduced Serum Medium (Invitrogen, Carlsbad, CA) supplemented with 100 U/ml penicillin-streptomycin as indicated. The Opti-MEM used in all protocols described herein does not contain fetal bovine serum (FBS). Store at 4°C.
5. Standard cell culture equipment and materials.

2.2.1. Transfection of 293-3-46 helper cells

1. ProFection Mammalian Transfection System–Calcium Phosphate kit (Promega, Madison, WI).
2. pEMC-La plasmid expressing the measles large (L) protein developed by Radecke et al.(33). Store at -20°C.
3. pSC6-N plasmid expressing the measles N protein developed by Radecke et al.(33). Store at -20°C.
4. p(+) MV-eGFP(-Spe) plasmid engineered to contain the chimeric fully retargeted measles H as described in subsection 3.1.7.

[1]Vero-aHis cells should typically be passaged on a subcultivation ratio of 1:8-1:10 every 3-4 days.

3. Methods

3.1 Construction of the full-length retargeted measles vector

The targeting ligand is subcloned into the C terminus of the measles H gene. To facilitate cloning, the pCG-H_{AA}-H6 plasmid contains SfiI/NotI restriction sites (**Fig. 1**) that are used to insert the ligand cDNA (*see* **Note 2**).

3.1.1 Peptide ligand Amplification

1. Begin by PCR amplifying the target receptor ligand. Any vector, tissue, cell line etc. that expresses the ligand of interest can be used for PCR amplification. Primers are designed to flank the ligand with SfiI/NotI digestion sites (*see* **Note 2**).
2. The PCR product is ran on a 1% agarose gel and visualized by ethidium bromide staining. The fragment is then excised and extracted from the agarose gel with a sterile scalpel and placed into a clean 1.5 ml microcentrifuge tube.

3.1.2 Gel Fragment Purification—We routinely use the QIAEX II Gel Extraction Kit for purification of ligands 500-3000 bp in size.

1. Prepare a water bath at 50°C.
2. In a clean 1.5 ml microcentrifuge tube mix 10 μl of the DNA adsorption resin (QIAEX II) with 650 μl of QX1 buffer. Then add the mix into the tube containing the gel fragment.
3. Incubate the tube at 50°C for 10-30 min. This will solubilize the agarose and adsorb the DNA to the resin which will appear as a pellet at the bottom of the tube.
4. Remove the supernatant (being careful not to disturb the pellet) with a pipette. Then wash the pellet with 750 μl of Buffer QX1, resuspend the pellet by vortexing and do a quick spin down for 30 sec. Decant the supernatant using a pipette without disturbing the pellet. All supernatant should be removed to clear any residual agarose contaminants.
5. Wash the pellet with 500 μl of wash buffer (buffer PE) and do a quick spin down for 30 sec. Again remove the supernatant using a pipette without disturbing the pellet. All supernatant should be removed to clear any residual salt contaminants.
6. Air-dry the pellet until it becomes white.
7. For DNA elution, add 20 μl of 10 mM Tris-HCl, pH 8.5 or H_2O with pH 7.0-8.5 and vortex until all the pellet is resuspended.
8. Incubate at room temperature for 10-15 min (up to 30 min)
9. Centrifuge for 1 min. The purified DNA will now be contained into the supernatant. Carefully place the supernatant into a sterile tube. It can now be used for cloning into the TA cloning vector or stored at -20°C until use.

3.1.3 Cloning of the ligand—These instructions assume the use of the Invitrogen TA cloning kit containing the pCR2.1 cloning vector but are easily adaptable to other cloning

[2]The following considerations apply in choosing the strategy for targeting ligand cloning: a. the primers used for amplification of the ligand should also include SfiI/NotI digestion sites necessary for subcloning the amplified fragment into the pCG-H_{AA}-H6 plasmid (**Fig. 1**); the presence of Sfi, NotI, PacI or SpeI restriction sites inside the ligand coding sequence itself may complicate cloning. b. For efficient viral transcription to occur, it is critical that the genome size of the engineered retargeted virus is a multitude of six nucleotides (rule of six) (33, 36).

systems. The TA cloning kit (Invitrogen, Carlsbad, CA) allows subcloning into the pCR2.1 vector without enzymatic digestion of the PCR product or vector.

1. Ligation of the purified DNA into the pCR2.1 cloning vector: Prepare the 10 μl ligation reaction by mixing 1 μl of the purified DNA, with 1 μl of the pCR2.1 vector (25 ng/μl), 1 μl of the 10X Ligation Buffer, 1 μ of the T4 DNA ligase, and 6 μ of sterile water. This step should be executed on ice; the 10X ligation buffer is especially sensitive to temperature variations. Always do a quick spin of the ligation buffer before use.
2. Thaw one vial (50 μ) of frozen One Shot® Competent *E. coli*. Thawing should always be performed on ice.
3. The ligation reaction (ligated pCR2.1 vector) is then incubated at 14°C for at least 1 hour but preferably overnight; store at -20°C until ready for transformation.
4. Transformation of the ligand vector into competent *E. coli:* Our laboratory routinely uses the One Shot® TOP10 *E. coli* cells included into the TA cloning kit, but other competent strains may be used.
5. Immediately after thawing, add 2.5 μl of the ligated vector into the *E. coli* vial. Preferably do not pipette the mix because this may harm the bacteria.
6. Incubate the vial on ice for 30 minutes. The remaining ligated vector can be stored at -20°C.
7. The vial should then be placed at 42-50°C for 30 sec to heat shock the bacteria.
8. Immediately place the vial on ice for 2 min.
9. Add 250 μl of SOC medium (which has been thawed at room temperature) to the vial.
10. The vial should then be placed in a shaking incubator and shaken horizontally (225 rpm) at 37°C for 30-60 min.
11. Plate 50 μl of the vial on an LB agar plate containing 100 μg/ml ampicillin. Alternatively, one may spread 10 μl of the vial into an LB agar plate, 50 μl into a second plate and the rest of the transformation vial on a third plate. This ensures that at least one plate will have well-spaced colonies (*see* **Note 3**).
12. The LB plate/plates should then be incubated at 37°C overnight. The next day, bacterial colonies should be visible on at least one plate.
13. The transformed colonies may then be analyzed or the plates can be stored at 4°C for up to 2 weeks.
14. To analyze the presence of the insert, 6 colonies may be picked for plasmid isolation.
15. Each colony should be placed in a tube with 1.5 ml LB medium containing 100 μg/ml of ampicillin and then shaken (225 rpm) at 37°C for 4-5 hours.
16. Centrifuge at 13000 rpm (~17900 × g) in a conventional, table-top microcentrifuge. The bacterial cells will appear as a pellet at the bottom of the tube. Remove the supernatant by inverting the tube. The bacteria can now be used for plasmid isolation or stored at -20°C.

[3]The pCR2.1 vector also encodes a lacZα fragment that allows for blue-white screening of bacterial colonies by supplementation of X-Gal into the LB agar plate. Due to the high efficiency of the cloning system in our experience, we do not typically use blue-white screening when selecting bacterial colonies. One may nevertheless use this feature when needed.

3.1.4 Plasmid Isolation and Analysis

1. Our laboratory routinely employs the QIAprep Miniprep kit for plasmid DNA purification, but this can be exchanged for any other appropriate plasmid purification method. Before starting, it is important to check Buffers P2 and N3 for salt precipitation. Any such salts should be redissolved by warming to 37°C. The P2 buffer should not be vigorously shaken. Also ensure that RNAse A and Lyseblue have been added to the P1 buffer. The pelleted bacteria in each of the 6 tubes should first be resuspended in 250 μl of resuspension buffer (buffer P1) and vortexed until no pellet or bacterial clumps are visible.
2. Add to the tubes 250 μl of lysis buffer (buffer P2). The bacterial suspension should then turn blue due to the dissolving effect of buffer P2 on the Lyseblue reagent contained in the P1 buffer. Gently shake the tube until the solution is homogenously colored blue and let it rest at room temperature for no more than 5 minutes.
3. Add to the tubes 350 μl of buffer N3 or 4.2 M Gu-HCl, 0.9 M potassium acetate, pH 4.8 and mix by inverting the tube until the suspension becomes completely colorless. Let it rest at room temperature for 5-10 min.
4. Transfer the tubes to a microcentrifuge and centrifuge at 13,000 rpm (~17,900 × g) for 1.5 min. The supernatant will contain the plasmid.
5. Apply the supernatant of each tube to a spin column and wait for ~10 min.
6. Centrifuge the columns at ~2500 rpm for 1.5 min and just before the end increase to 13000 rpm. The flow-through should then be discarded.
7. Add 500 μl of buffer PB to each spin column and centrifuge at 13000 rpm for 1 min. Again the flow-through should be discarded.
8. Add 500 μl wash buffer (buffer PE) to each spin column and centrifuge at 13000 rpm for 1 min. The flow-through should always be discarded before proceeding to the next step.
9. Centrifuge at 13000 rpm for 2 min to clear any residual wash buffer.
10. Each spin column should be placed in a sterile 1.5 ml microcentrifuge tube. The plasmid DNA can be eluted by applying 50 μl of 10 mM Tris-HCl, pH 8.5 or H_2O with pH 7.0-8.5 to the center of the column. Let it rest for 5 min at room temperature and then centrifuge at ~5000 rpm for 2 min.
11. Quick spin at 13000 rpm. The tube will now contain the eluted plasmid DNA which may be stored at -20°C.
12. The sequence of the ligated plasmid should now be verified. We usually perform analytical digestions of all 6 isolated plasmids and then perform full sequencing of at least one of the plasmids that appear to have the correct sequence according to the analytical digestion. After confirming the correct sequence of the ligated plasmid we proceed with subcloning into the pCG-H_{AA}-H6 vector.

3.1.5 Subcloning of the targeting ligand into the pCG-H_{AA}-H6 vector—The pCG-H_{AA}-H6 shuttle vector (provided by Dr. T. Nakamura) (20) encodes a modified measles H sequence that incorporates both a CD46 (Y481A) and SLAM (R533A) ablating mutation. As previously noted it also includes unique SfiI and NotI restriction sites (**Fig. 1**) that can be used to insert the ligand cDNA.

1. Prepare a 20 μl digestion reaction for each of the two plasmids (pCR2.1 containing the peptide ligand cDNA and pCG-H_{AA}-H6) by mixing 0.5-1.0 μg of the corresponding plasmid DNA with 2 μl NEBuffer 2 10X, 0.5 μl of BSA 100X, 0.5 μl of NotI and sterile water as needed to reach a volume of 20 μl. Each reaction should be incubated at 37°C for ~2 hours, then 0.5 μl SfiI should be added and the temperature raised to 50°C for 40 min.
2. The digestion product from the pCR2.1-ligand reaction can be ran on a 1% agarose gel and visualized by ethidium bromide staining. After confirming the correct size of the fragment, it can be excised and gel purified as described in subsection 3.1.2. The digested pCG-H_{AA}-H6 vector is similarly visualized and purified.
3. The gel purified fragment is then ligated to the SfiI/NotI-digested pCG-H_{AA}-H6 vector. For this purpose we typically use the T4 DNA Ligase (Invitrogen) in our laboratory, but this may be substituted for any other effective ligation procedure. A 20 μl ligation reaction is prepared by mixing 2 μl of the T4 reaction buffer 10 X, 2 μl of the T4 ligase, 5 μl of the gel purified open pCG-H_{AA}-H6 vector, 5 μl of the gel purified SfiI/NotI-digested fragment and 6 μl of sterile water. Incubate overnight at 14°C.
4. The ligated pCG-H_{AA}-H6 plasmid containing the targeting ligand can now be transformed into competent *E. coli*. and propagated as described in subsection 3.1.3. Plasmid isolation and analysis is performed as described in subsection 3.1.4.

3.1.6 Propagation and purification of transfection grade pCG-H_{AA}-H_6 plasmid DNA—Before proceeding to constructing the full-length plasmid, it is important that transfection grade preparations of the ligated pCG-H_{AA}-H6 plasmid are produced. Occasionally, ligation of the reengineered H cDNA into the full-length plasmid may fail and we have observed that plasmid quality can be a critical factor in resolving such issues. After confirming the correct sequence of the ligated pCG-H_{AA}-H6 by analytical digestions and DNA sequencing the plasmid should be transformed into competent *E. coli*. and plated as described in subsection 3.1.3. For plasmid DNA purification we have found the QIAprep Midiprep kit to yield excellent high-quality transfection grade DNA. Numerous competitive systems are available from other commercial sources.

1. Before starting, it is important to check the P2 Buffer for SDS precipitation and to pre-chill the P3 buffer at 4°C. Also ensure that RNAse A and Lyseblue have been added to the P1 buffer. A single colony from a fresh plate should be inoculated into 3 ml of 3 ml LB medium containing 100 μg/ml ampicillin to prepare a starter culture.
2. Place the tube in a shaking incubator (225 rpm) at 37°C for approximately 9 hours. If the LB medium is still clear after 9 hours (due to slow bacterial growth) then lower the temperature to 30°C and leave the tube overnight in the shaking incubator.
3. Inoculate 25-50 μl of the starter culture into 50 ml of LB medium (100 μg/ml ampicillin), shake at 37°C for approximately 5 hours and then change the temperature to 30°C for approximately 5 hours.
4. Add ampicillin to a total concentration of 200 μg/ml and continue incubation at 30°C overnight.
5. Centrifuge at 6000 rpm for 15 min at 4°C.

6. The bacterial cells will be pelleted at the bottom of the tube. Discard the supernatant by tube inversion. The bacteria may then be used for plasmid isolation or stored at -20°C.
7. Resuspend the bacteria in 4 ml of resuspension buffer (buffer P1) and vortex until no pellet or bacterial clumps are visible.
8. Add 4 ml of lysis buffer (buffer P2). The bacterial suspension should then turn blue due to the dissolving effect of buffer P2 on the Lyseblue reagent contained in the P1 buffer. Gently shake the tube until the solution is homogenously colored blue and let it stay at room temperature for no more than 5 minutes.
9. Add 4 ml of pre-chilled buffer P3 and mix by inverting the tube until the suspension becomes completely colorless. Let it stay at room temperature for 10 min.
10. During this time, equilibrate a QIAGEN-tip 100 column by applying 4 ml of equilibration buffer (Buffer QBT). The column should be allowed to empty by gravity flow and the flow-through should be discarded.
11. Pour the bacterial suspension into the barrel of the QIAfilter syringe (QIAfilter Midi Cartridge) and let it stay at room temperature for another 10 min. The outer nozzle of the syringe should be capped.
12. Remove the cap from the syringe; insert the syringe plunger (provided in the kit) and press it to filter the bacterial lysate into the equilibrated QIAGEN-tip.
13. Allow the lysate to pass through the column (may take a few minutes) and discard the flow-through (approximately 10 ml volume).
14. Wash the column twice with 10 ml of wash buffer (Buffer QC). Discard the flow-through each time.
15. Place a clear 50 ml tube below the column and elute the plasmid DNA by applying 5 ml of elution buffer (buffer QF).
16. Precipitate the eluted DNA by adding 3.5-4 ml of isopropanol mix gently (do not vortex) and immediately centrifuge at ≥15000 × g for 30 min at 4°C. Alternatively, DNA precipitation can be accomplished by centrifuging at 10000-11000 × g for 1 hour at 4°C.
17. Carefully remove the supernatant and wash with 25 ml of 70% ethanol.
18. Centrifuge at 8000 × g for 5 min at 4°C.
19. Carefully remove all of the supernatant without disturbing the DNA pellet.
20. Allow the pellet to air-dry for 5-10 min and redissolve the DNA with 100 μl of 10 mM Tris-HCl, pH 8.5 or H_2O with pH 7.0-8.5. To recover all DNA, vortexing may be performed to resuspend the DNA from the tube walls.
21. Determine DNA yield by UV spectrophotometry at 260 nm (*see* **Note 4**).

3.1.7 Subcloning of the retargeted H into of the full-length MV-Edm infectious clone—The ablated H protein displaying the targeting ligand is subsequently subcloned

[4]Before proceeding to the more complicated and time-consuming process of rescuing the reengineered virus, the chimeric pCG-H_{AA}-H6 containing the targeting ligand may now be used for co-transfection along with plasmid expressing the MV-Edm F protein to confirm that the chimeric ablated H is expressed into the cell surface at a proper conformation that allows fusion of cells expressing the target receptor as well as of Vero-aHis cells.

into the plasmid p(+) MV-eGFP (-Spe) that encodes the full-length infectious clone of the MV-Edm NSe strain and also contains the eGFP gene at position 1. This vector has been modified to contain a unique SpeI restriction site (34). Since both the pCG-H_{AA}-H6 and the full-length p(+) MV-eGFP (-Spe) plasmids contain unique PacI and SpeI digestion sites, these can be used to subclone the ablated chimeric H cDNA into the full-length plasmid.

1. Prepare a 20 μl digestion reaction for each of the two plasmids [pCG-H_{AA}-H6 containing the targeting ligand cDNA and p(+) MV-eGFP (-Spe)] by mixing 0.5-1.0 μg of each plasmid DNA with 2 μl NEBuffer 4 10X, 0.5 μl of BSA 100X, 0.5-1 μl of PacI, 0.5-1 μl of SpeI and sterile water as needed to reach a volume of 20 μl. Each reaction should be incubated at 37°C for ~3-4 hours.
2. The product from the chimeric pCG-H_{AA}-H6 digestion can be ran on a 1% agarose gel and visualized by ethidium bromide staining. After confirming the correct size of the fragment it can be excised and gel purified as described in subsection 3.1.2.
3. The gel purified fragment is subsequently ligated to the PacI/SpeI-digested p(+) MV-eGFP (-Spe) vector, as described in subsection 3.1.5. A 20 μl ligation reaction is prepared by mixing 2 μl of the T4 reaction buffer 10 X, 2 μl of the T4 ligase, 2 μl of the gel purified Pacl/Spel digested p(+) MV-eGFP (-Spe) vector, 8 μl of the gel purified PacI/SpeI -digested fragment and 6 μl of sterile water. Incubate overnight at 14°C.
4. The ligated full-length p(+) MV-eGFP (-Spe) plasmid containing the chimeric ablated H can now be transformed into competent *E. coli*. and propagated as described in subsection 3.1.3. Due to the vector size this transformation reaction may be less efficient but at least a few colonies should be visible on the agar plate. Plasmid isolation and analysis is then performed as described in subsection 3.1.4. After confirming the correct sequence of the full-length infectious clone, the plasmid is propagated and purified in transfection grade quality as described in subsection 3.1.6.

3.2 Rescue, propagation and titration of the retargeted virus

To rescue the retargeted MV-Edm derivative, a modified version of the rescue system developed by Radecke et al. (33) is utilized. The helper cells 293-3-46 are derived from the human embryonic kidney 293 cell line and engineered to stably express the measles N and P proteins as well as the T7 RNA polymerase (33). To rescue the virus (**Fig. 2**), the 293-3-46 cells are co-transfected with a plasmid expressing the measles large (L) protein (pEMC-La), a plasmid expressing the measles N protein (pSC6-N) and the full-length p(+) MV-eGFP (-Spe) plasmid containing the chimeric ablated H (*see* **Note 5**). Rescue of MV-Edm strains capable of interacting with the natural measles CD46 receptor on the cell surface of 293-3-46 cells can easily be visualized because of the formation of giant mononuclear cell aggregates (syncytia) due to cell to cell fusion. On the other hand, fully retargeted strains are incapable of fusing the 293-3-46 cells, since these cells in most cases are not expressing the receptor recognized by the engineered H. Thus the expression of the eGFP gene at position 1 of the retargeted strains is an effective way to visualize initial virus propagation in the helper cells. As the next step, overlaying the infected 293-3-46 cells onto Vero-aHis cells (**Fig. 2**) will result in syncytia formation via interaction of the anti-His single chain antibody, displayed on the surface of the Vero-aHis cells, with the 6-histidine tag at the C-terminus of

[5]The original rescue system by Radecke et al.(33) required the transfection of only two plasmids (pEMC-La and the full-length vector) into the 293-3-46 cells. The additional co-transfection of the N expressing plasmid during rescue is a modification used at the Galanis laboratory, which in our experience has significantly increased the rescue efficacy for both retargeted and non-retargeted cloned MV-Edm strains (19).

the retargeted H. Unless otherwise specified, all following steps should be performed in a tissue culture hood.

3.2.1 293-3-46 cell culture and plating (*see* Note 6)

1. The 293-3-46 helper cells are grown as monolayers in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum along with 100 U/ml penicillin-streptomycin and 1.2 mg/ml G418; cells are grown on T75 flasks and passaged when 70-80% confluent, using an 1:4-1:8 split rate.
2. The helper cells should be plated in a 6-well plate 24 hours before transfection. After splitting a T75 flask, prepare 1:20, 1:40 and 1:80 cell dilutions of the aspirated cells and plate each dilution in 2 wells of a 6-well plate (use a total of 2 ml per well). Incubate the plate at a 37°C CO_2 incubator to allow the cells to attach and grow. It is important that on the day of transfection the monolayer consists of isolated cells (there should be as little cell clumping as possible) evenly spread throughout the well at 50-60% confluency. Plating 3 different dilutions in 2 wells each ensures optimal cell density at the time of transfection.

3.2.2. Transfection of 293-3-46 helper cells (Note 7)

1. At least 4 hours prior to transfection the growth medium is removed and replaced with DMEM, with 10% FBS. The medium should be prewarmed in a water bath at 37°C prior to being added to the cells.
2. In our experience, calcium phosphate-mediated transfection has shown excellent results for viral rescue. Our laboratory typically uses the ProFection Mammalian Transfection System–Calcium Phosphate kit (Promega, Madison, WI). For a one-well transfection 5 μg of the full-length p(+) MV-eGFP (-Spe), 50 μg of the pEMC-La plasmid and 250 μg of the pSC6-N will provide optimum efficiency. The plasmids are solubilized in a sterile 15 ml tube containing 175 μl of nuclease-free water.
3. Add 25 μl of 2M $CaCl_2$ to the tube and mix well. Let it rest at room temperature for 2-5 minutes.
4. In a separate sterile 15 ml tube add 200 μl of 2X HBS.
5. Gently vortex the second tube containing the 2X HBS. While continuing vortexing slowly add the content of the first tube dropwise to the second tube. Incubate the tube at room temperature for 30 minutes.
6. Add the transfection solution to the well containing the helper cells dropwise and at various locations around the well. Gently swirl the 6-well plate to evenly distribute the transfection precipitate over the helper cells.
7. Incubate the plate in a 37°C CO_2 incubator for 16-20 hours.
8. Replace growth medium with DMEM 10% FBS containing penicillin-streptomycin (G418 is no longer required) to avoid any unintentional contamination during the next steps.
9. Heat shock the plate in a water bath at 44°C for 2 hours.

---

[6]To increase the likelihood of a successful rescue, it is important that a healthy culture of 293-3-46 helper cells is maintained. Furthermore, plating before transfection must yield well-spaced, well-attached helper cells to optimize plasmid uptake.

[7]We recommend that at least one additional well is used to simultaneously rescue a non-ablated unmodified MV-Edm strain expressing eGFP (MV-eGFP) as a positive control confirming that the helper cells are in good condition.

10. Return the plate to a 37°C CO_2 incubator.

3.2.3. Vero-aHis cell overlay—Approximately 3 days after the heat-shock, if the rescue is successful, isolated green 293-3-46 cells can be visualized under the fluorescent microscope due to eGFP expression (*see* **Note 8**). Even if no green fluorescent cells are visible, the helper cells should be overlaid onto Vero-aHis cells at this time.

1. Plate one or two 10 cm tissue culture plates with 50-60% confluent Vero-aHis cells (in 5-10 ml of DMEM, 10% FBS, 100 U/ml penicillin-streptomycin).
2. Allow >3 hours for the Vero-aHis to attach to the plate.
3. Scrape the 293-3-46 helper cells from the surface of the transfected well into the overlying medium using a sterile tissue culture cell scraper.
4. Slowly add the helper cell suspension dropwise and at various locations to the Vero-aHis plate.
5. Return the plates to a 37°C CO_2 incubator.
6. Examine the plates daily under the microscope to identify the formation of syncytia which indicate the rescue of infectious viral particles (*see* **Note 9**).

3.2.4. Viral clone harvesting

1. As soon as one or more syncytia are formed, harvesting of each syncytium (corresponding to a different viral clone) should commence. Plate a 6-well plate with 50-60% confluent Vero-aHis cells in 1 ml Opti-MEM containing 100 U/ml penicillin-streptomycin. Prepare one well for each visible syncytium.
2. Allow >3 hours for the Vero-aHis to attach to the well surface.
3. Use a marker pen to circle the location of each syncytium on the undersurface of the 10 cm tissue culture plate.
4. Remove the growth medium from the tissue culture plate.
5. Use a standard micropipette with a 10 μl tip to take 5 μl of Opti-MEM and repeatedly pipette the Opti-MEM to the location of the syncytium. The goal is to detach the syncytium and resuspend it into the Opti-MEM. Collect the Opti-MEM and add it to a Vero-aHis well on the 6-well plate and place on a 37°C CO_2 incubator for 2-3 hours. Repeat this step for every visible syncytium which should be harvested and placed into a separate well corresponding to a distinct viral clone.
6. 2-3 hours later, add 1 ml of DMEM 10%FBS, 100 U/ml penicillin-streptomycin to each well and return the plate to a 37°C CO_2 incubator.
7. Two to three days later, wells are covered with syncytia.
8. Plate two 10 cm tissue culture plate with 50-60% confluent Vero-aHis cells for each well.
9. Allow >3 hours for the Vero-aHis to attach to the plate surface.

[8]While this is a good indicator that viral rescue is progressing smoothly, not all of these green cells will be able to assemble infectious virus particles.

[9]Syncytia may appear at different time points up to 10 days after transfection of the L93-3-46 helper cells. Syncytia formation beyond 10 days significantly increases the likelihood that the rescued virus is a mutated strain. Sequencing and other characterization techniques, including Western immunoblotting for detection of the correct size viral H protein (19), should be performed to verify that one or more of the rescued viral clones represents the correct retargeted strain.

10. Remove the growth medium from each 10 cm plate and add 5 ml of Opti-MEM, 100 U/ml penicillin-streptomycin.
11. Remove the growth medium from each well and add 1 ml of Opti-MEM.
12. Scrape the Vero-aHis from each well into the overlying Opti-MEM using a sterile tissue culture cell scraper.
13. Place the suspension of scraped cells/syncytia into a cryovial and vortex for 1 sec.
14. Snap-freeze the cryovial by submersion in liquid N_2.
15. Once completely frozen, thaw the cryovial using a water bath set to 37°C. When the suspension is half-thawed, vortex for 1 sec and then return the cryovial to the water bath.
16. Following complete thawing, vortex for 1 sec and then repeat the cycle of snap-freezing/thawing (steps 14-15). This will release the infectious viral particles into the supernatant.
17. Centrifuge at ~4000 rpm, for 5 minutes at 4°C to pellet the cell debris. Collect the cell lysate (supernatant) containing the virus. It may now be stored at a -80°C or used to proceed immediately to the additional purification steps.
18. Slowly add the cell lysate of each well drop wise and at various locations around two 10 cm Vero-aHis dishes.
19. Incubate the dishes to a 37°C CO_2 incubator for 2-3 hours.
20. Add 5 ml of DMEM 10% FBS, 100 U/ml penicillin-streptomycin to each plate.
21. Return the plates to a 37°C CO_2 incubator.
22. Approximately 2-3 days later each plate should be covered with syncytia.
23. Plate two-three 15 cm tissue culture plates with 50-60% confluent Vero-aHis cells corresponding to each infected 10 cm tissue culture plate.
24. Allow >3 hours for the Vero-aHis to attach to the plate surface.
25. Remove the growth medium from each 15 cm plate and add 10 ml of Opti-MEM.
26. Remove the growth medium from each 10 cm plate and add 5-6 ml of Opti-MEM.
27. Scrape the Vero-aHis from each 10 cm plate into the overlying medium using a sterile tissue culture cell scraper.
28. Place the suspension of scraped cells/syncytia into a 50 ml tube and vortex for 1 sec. Perform the two cycles of freeze-thawing as described in steps 14-17 to collect a cleared cell lysate containing the virus.
29. Slowly add the cell lysate of each 10 cm plate dropwise and at various locations around two-three 15 cm Vero-aHis plates.
30. Incubate the 15 cm plates to a 37°C CO_2 incubator for 2-3 hours.
31. Add 10 ml of DMEM 10% FBS, 100 U/ml penicillin-streptomycin to each plate.
32. Return the 15 cm plates to a 37°C CO_2 incubator.
33. Two to three days later, wells are covered with syncytia.
34. Remove the growth medium from each 15 cm plate and add 8-10 ml of Opti.

35. Scrape the Vero-aHis from each 15 cm plate into the overlying medium using a sterile tissue culture cell scraper.

36. Place the suspension of scraped cells/syncytia into a 50 ml tube and vortex for 1 sec. Perform the two cycles of freeze-thawing as described in steps 14-17 to collect a cleared cell lysate containing the virus.

37. The virus preparation may now be aliquoted into cryovials, stored at -80°C and titered. One of the aliquots can be marked to be used later for titration (*see* **Note 10**).

3.2.5. Measles virus titration

1. The following titration method is used to calculate the 50% tissue culture infective dose ($TCID_{50}$) of a viral aliquot. First prepare a 96 well plate by plating each well of the first 8 columns with 100 μl of 10^4 Vero-aHis (DMEM 10% FBS, 100 U/ml penicillin-streptomycin). One or more additional columns of the 96-well plate may also be plated and used as control wells (*see* **Note 11**).

2. Incubate the plate in a 37°C CO_2 incubator.

3. The next day, add 900 μl of Opti-MEM (100 U/ml penicillin-streptomycin) in each of eight 1.5 ml microcentrifuge tubes. These tubes will be used to prepare serial dilutions (10^{-1} to 10^{-8}) of the viral aliquot. Place the tubes in a rack immersed in ice and label them -1 to -8 from left to right.

4. Thaw the cryovial marked for virus titration and add 100 μl of the virus preparation into the first 1.5 ml tube (labelled -1). Mix well by pipetting and inverting the tube. This tube will contain the first (1:10) dilution of the viral preparation.

5. Replace the pipette or tip and add 100 μl of the 1:10 (10^{-1}) dilution to the second tube (labelled -2) and mix well by pipetting and inversion. This second tube will correspond to the second (1:100) dilution of the viral preparation. Repeat this process to prepare all dilutions. Remember to always use a fresh pipette or tip before transferring 100 μl of previous virus dilution to next dilution.

6. The serial dilutions should now be inoculated (50 μl/well) to the Vero-aHis in the 96-well plate. Each dilution should be inoculated to all wells of one corresponding column (total of 8 columns) starting from the highest (10^{-8}) to the lowest (10^{-1}) dilution.

7. Place the 96 well plate to a 37°C CO_2 incubator.

8. Three to four days later when a fully confluent monolayer of Vero-aHis cells is seen in control wells, all wells (inoculated with different virus dilutions) are examined under microscope for the presence of syncytia. An inoculated well is considered positive if the presence of even a single syncytium is detected (*see* **Note 12**).

---

[10] Each freeze/thaw cycle will likely decrease the viral titer. To determine the correct titer of the stored viral preparation, this should be aliquoted before transferring to -80°C. One aliquot can be thawed and titrated at a later time point, as described in subsection 3.2.5. The calculated titer will correspond to that of each of the remaining aliquots when thawed for the first time.

[11] When preparing viral stocks we typically prefer to titrate twice (in two 96-well plates inoculated simultaneously) and use the mean $TCID_{50}$ calculated by these two titrations.

[12] The cytopathic effect (syncytia formation) of MV-Edm derivatives is relatively easy to recognize under light microscopy even in cases when only a single syncytium is present. The expression of eGFP by infected cells further facilitates detection. By definition, no syncytia should be detectable on the non-inoculated control wells. The presence of cytopathic effect in these wells may indicate contamination during titration or, worse, of the prime Vero-aHis culture.

9. Mark the syncytia-positive inoculated wells on the plate. The $TCID_{50}$ will be equal to 10^X where X=absolute value of the exponent corresponding to the highest dilution where all wells in the column are positive + 1.8 + the fraction of positive wells past this dilution (*see* **Note 13**).

Acknowledgments

Grant Support:

NIH Grants CA 136547, CA 136393, CA 108961, CA 123831, and CA 103276.

References

1. Peng KW, TenEyck CJ, Galanis E, Kalli KR, Hartmann LC, Russell SJ. Intraperitoneal therapy of ovarian cancer using an engineered measles virus. Cancer research. 2002; 62:4656–4662. [PubMed: 12183422]
2. Hasegawa K, Pham L, O'Connor MK, Federspiel MJ, Russell SJ, Peng KW. Dual therapy of ovarian cancer using measles viruses expressing carcinoembryonic antigen and sodium iodide symporter. Clin Cancer Res. 2006; 12:1868–1875. [PubMed: 16551872]
3. Phuong LK, Allen C, Peng KW, Giannini C, Greiner S, TenEyck CJ, Mishra PK, Macura SI, Russell SJ, Galanis EC. Use of a vaccine strain of measles virus genetically engineered to produce carcinoembryonic antigen as a novel therapeutic agent against glioblastoma multiforme. Cancer research. 2003; 63:2462–2469. [PubMed: 12750267]
4. Peng KW, Ahmann GJ, Pham L, Greipp PR, Cattaneo R, Russell SJ. Systemic therapy of myeloma xenografts by an attenuated measles virus. Blood. 2001; 98:2002–2007. [PubMed: 11567982]
5. Dingli D, Peng KW, Harvey ME, Greipp PR, O'Connor MK, Cattaneo R, Morris JC, Russell SJ. Image-guided radiovirotherapy for multiple myeloma using a recombinant measles virus expressing the thyroidal sodium iodide symporter. Blood. 2004; 103:1641–1646. [PubMed: 14604966]
6. Grote D, Russell SJ, Cornu TI, Cattaneo R, Vile R, Poland GA, Fielding AK. Live attenuated measles virus induces regression of human lymphoma xenografts in immunodeficient mice. Blood. 2001; 97:3746–3754. [PubMed: 11389012]
7. McDonald CJ, Erlichman C, Ingle JN, Rosales GA, Allen C, Greiner SM, Harvey ME, Zollman PJ, Russell SJ, Galanis E. A measles virus vaccine strain derivative as a novel oncolytic agent against breast cancer. Breast cancer research and treatment. 2006; 99:177–184. [PubMed: 16642271]
8. Iankov ID, Msaouel P, Allen C, Federspiel MJ, Bulur PA, Dietz AB, Gastineau D, Ikeda Y, Ingle JN, Russell SJ, Galanis E. Demonstration of anti-tumor activity of oncolytic measles virus strains in a malignant pleural effusion breast cancer model. Breast cancer research and treatment. 2009
9. Msaouel P, Iankov ID, Allen C, Morris JC, von Messling V, Cattaneo R, Koutsilieris M, Russell SJ, Galanis E. Engineered measles virus as a novel oncolytic therapy against prostate cancer. The Prostate. 2009; 69:82–91. [PubMed: 18973133]
10. Msaouel P, Iankov ID, Allen C, Aderca I, Federspiel MJ, Tindall DJ, Morris JC, Koutsilieris M, Russell SJ, Galanis E. Noninvasive imaging and radiovirotherapy of prostate cancer using an oncolytic measles virus expressing the sodium iodide symporter. Mol Ther. 2009; 17:2041–2048. [PubMed: 19773744]
11. Blechacz B, Splinter PL, Greiner S, Myers R, Peng KW, Federspiel MJ, Russell SJ, LaRusso NF. Engineered measles virus as a novel oncolytic viral therapy system for hepatocellular carcinoma. Hepatology (Baltimore, Md. 2006; 44:1465–1477.
12. Dorig RE, Marcil A, Chopra A, Richardson CD. The human CD46 molecule is a receptor for measles virus (Edmonston strain). Cell. 1993; 75:295–305. [PubMed: 8402913]

[13]For example, if all wells up to and including the 10^{-5} dilution are syncytia-positive, 3 wells in the 10^{-6} are positive, 1 well in the 10^{-7} dilution is positive and all wells in the 10^{-8} dilution are negative then the TCID50 will be equal to $10^{5+1.8+3/8+1/8+0/8} = 10^{7.3}$ which is approximately equal to 2×10^7 $TCID_{50}$.

13. Naniche D, Varior-Krishnan G, Cervoni F, Wild TF, Rossi B, Rabourdin-Combe C, Gerlier D. Human membrane cofactor protein (CD46) acts as a cellular receptor for measles virus. Journal of virology. 1993; 67:6025–6032. [PubMed: 8371352]

14. Tatsuo H, Ono N, Tanaka K, Yanagi Y. SLAM (CDw150) is a cellular receptor for measles virus. Nature. 2000; 406:893–897. [PubMed: 10972291]

15. Hahm B, Arbour N, Naniche D, Homann D, Manchester M, Oldstone MB. Measles virus infects and suppresses proliferation of T lymphocytes from transgenic mice bearing human signaling lymphocytic activation molecule. Journal of virology. 2003; 77:3505–3515. [PubMed: 12610126]

16. Schneider-Schaulies S, Bieback K, Avota E, Klagge I, ter Meulen V. Regulation of gene expression in lymphocytes and antigen-presenting cells by measles virus: consequences for immunomodulation. Journal of molecular medicine (Berlin, Germany). 2002; 80:73–85.

17. Yanagi Y, Takeda M, Ohno S. Measles virus: cellular receptors, tropism and pathogenesis. The Journal of general virology. 2006; 87:2767–2779. [PubMed: 16963735]

18. Nussbaum O, Broder CC, Moss B, Stern LB, Rozenblatt S, Berger EA. Functional and structural interactions between measles virus hemagglutinin and CD46. Journal of virology. 1995; 69:3341–3349. [PubMed: 7745681]

19. Allen C, Paraskevakou G, Iankov I, Giannini C, Schroeder M, Sarkaria J, Schroeder M, Puri RK, Russell SJ, Galanis E. Interleukin-13 displaying retargeted oncolytic measles virus strains have significant activity against gliomas with improved specificity. Mol Ther. 2008; 16:1556–1564. [PubMed: 18665158]

20. Nakamura T, Peng KW, Harvey M, Greiner S, Lorimer IA, James CD, Russell SJ. Rescue and propagation of fully retargeted oncolytic measles viruses. Nature biotechnology. 2005; 23:209–214.

21. Paraskevakou G, Allen C, Nakamura T, Zollman P, James CD, Peng KW, Schroeder M, Russell SJ, Galanis E. Epidermal growth factor receptor (EGFR)-retargeted measles virus strains effectively target EGFR- or EGFRvIII expressing gliomas. Mol Ther. 2007; 15:677–686. [PubMed: 17299404]

22. Allen C, Vongpunsawad S, Nakamura T, James CD, Schroeder M, Cattaneo R, Giannini C, Krempski J, Peng KW, Goble JM, Uhm JH, Russell SJ, Galanis E. Retargeted oncolytic measles strains entering via the EGFRvIII receptor maintain significant antitumor activity against gliomas with increased tumor specificity. Cancer research. 2006; 66:11840–11850. [PubMed: 17178881]

23. Hasegawa K, Nakamura T, Harvey M, Ikeda Y, Oberg A, Figini M, Canevari S, Hartmann LC, Peng KW. The use of a tropism-modified measles virus in folate receptor-targeted virotherapy of ovarian cancer. Clin Cancer Res. 2006; 12:6170–6178. [PubMed: 17062694]

24. Jing Y, Tong C, Zhang J, Nakamura T, Iankov I, Russell SJ, Merchan JR. Tumor and vascular targeting of a novel oncolytic measles virus retargeted against the urokinase receptor. Cancer research. 2009; 69:1459–1468. [PubMed: 19208845]

25. Hummel HD, Kuntz G, Russell SJ, Nakamura T, Greiner A, Einsele H, Topp MS. Genetically engineered attenuated measles virus specifically infects and kills primary multiple myeloma cells. The Journal of general virology. 2009; 90:693–701. [PubMed: 19218216]

26. Hasegawa K, Hu C, Nakamura T, Marks JD, Russell SJ, Peng KW. Affinity thresholds for membrane fusion triggering by viral glycoproteins. Journal of virology. 2007; 81:13149–13157. [PubMed: 17804513]

27. Ungerechts G, Springfeld C, Frenzke ME, Lampe J, Johnston PB, Parker WB, Sorscher EJ, Cattaneo R. Lymphoma chemovirotherapy: CD20-targeted and convertase-armed measles virus can synergize with fludarabine. Cancer research. 2007; 67:10939–10947. [PubMed: 18006839]

28. Hallak LK, Merchan JR, Storgard CM, Loftus JC, Russell SJ. Targeted measles virus vector displaying echistatin infects endothelial cells via alpha(v)beta3 and leads to tumor regression. Cancer research. 2005; 65:5292–5300. [PubMed: 15958576]

29. Springfeld C, von Messling V, Frenzke M, Ungerechts G, Buchholz CJ, Cattaneo R. Oncolytic efficacy and enhanced safety of measles virus activated by tumor-secreted matrix metalloproteinases. Cancer research. 2006; 66:7694–7700. [PubMed: 16885371]

30. Msaouel P, Dispenzieri A, Galanis E. Clinical testing of engineered oncolytic measles virus strains in the treatment of cancer: an overview. Current opinion in molecular therapeutics. 2009; 11:43–53. [PubMed: 19169959]

31. Vongpunsawad S, Oezgun N, Braun W, Cattaneo R. Selectively receptor-blind measles viruses: Identification of residues necessary for SLAM- or CD46-induced fusion and their localization on a new hemagglutinin structural model. Journal of virology. 2004; 78:302–313. [PubMed: 14671112]

32. Nakamura T, Peng KW, Vongpunsawad S, Harvey M, Mizuguchi H, Hayakawa T, Cattaneo R, Russell SJ. Antibody-targeted cell fusion. Nature biotechnology. 2004; 22:331–336.

33. Radecke F, Spielhofer P, Schneider H, Kaelin K, Huber M, Dotsch C, Christiansen G, Billeter MA. Rescue of measles viruses from cloned DNA. The EMBO journal. 1995; 14:5773–5784. [PubMed: 8846771]

34. Hadac EM, Peng KW, Nakamura T, Russell SJ. Reengineering paramyxovirus tropism. Virology. 2004; 329:217–225. [PubMed: 15518802]

35. Douglas JT, Miller CR, Kim M, Dmitriev I, Mikheeva G, Krasnykh V, Curiel DT. A system for the propagation of adenoviral vectors with genetically modified receptor specificities. Nature biotechnology. 1999; 17:470–475.

36. Kolakofsky D, Pelet T, Garcin D, Hausmann S, Curran J, Roux L. Paramyxovirus RNA synthesis and the requirement for hexamer genome length: the rule of six revisited. Journal of virology. 1998; 72:891–899. [PubMed: 9444980]

37. Peng KW, Donovan KA, Schneider U, Cattaneo R, Lust JA, Russell SJ. Oncolytic measles viruses displaying a single-chain antibody against CD38, a myeloma cell marker. Blood. 2003; 101:2557–2562. [PubMed: 12433686]

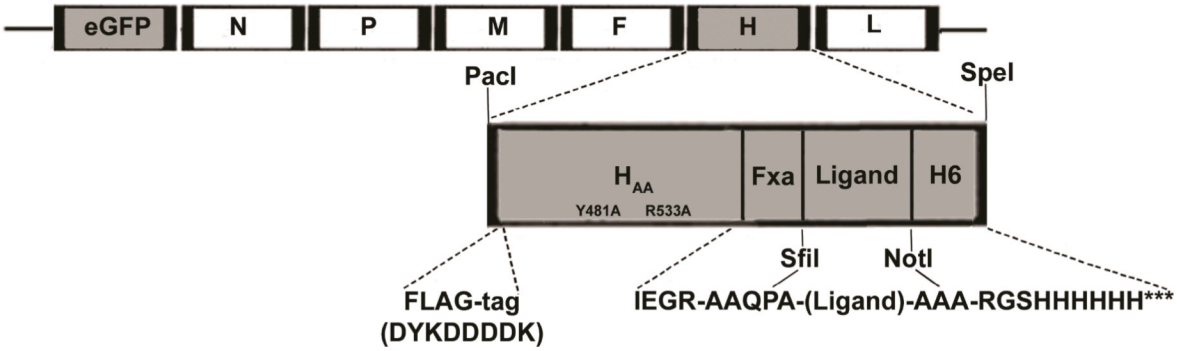

Fig. 1.

Schematic representation of the retargeted His-tagged MV-GFP-H_{AA} strain. The Y481A and R533A mutations in the hemagglutinin (H) gene ablate CD46 and SLAM interaction respectively (H_{AA}). The target receptor-binding ligand is displayed on the C-terminus of the ablated H_{AA} protein. The ligand is flanked by SfiI (encoding AAQPA amino acid sequence) and NotI (encoding AAA amino acid sequence) restriction sites. A six histidine peptide (H6) is attached to the C-terminus displayed ligand via a short linker sequence (AAARGS). H6 facilitates the "pseudoreceptor" interaction which allows propagation of the retargeted strain using Vero cells (Vero-aHis) generated to stably express a membrane-anchored single-chain antibody that binds to the H6 tag allowing virus entry and propagation. The FLAG-tag inserted after the start codon at the N-terminus of H_{AA} is used to facilitate detection by Western immunoblotting. Fxa is a factor Xa protease cleavage sequence (IEGR) that links the H_{AA} glycoprotein with the target receptor ligand peptide. It may be used to determine the recombinant nature of the chimeric H_{AA} (37). Thus, factor Xa protease cleavage allows removal of the peptide ligand resulting in an H_{AA} protein with similar molecular weight as that of unmodified virus. PacI/SpeI restriction enzymes are used to subclone the chimeric H_{AA} into the full-length infectious clone of the MV-NSe strain, deriving from MV-Edm. The virus also contains the gene encoding green fluorescent protein (eGFP) in position 1. (N, nucleoprotein gene; P, phosphoprotein gene; M, matrix protein gene; F, fusion protein gene; L, large protein gene; *, stop codons; standard one-letter abbreviations are used to denote amino acid residues). (modified from Nakamura T et al, Nature Biotechnology 23(2): 209-14, 2005).

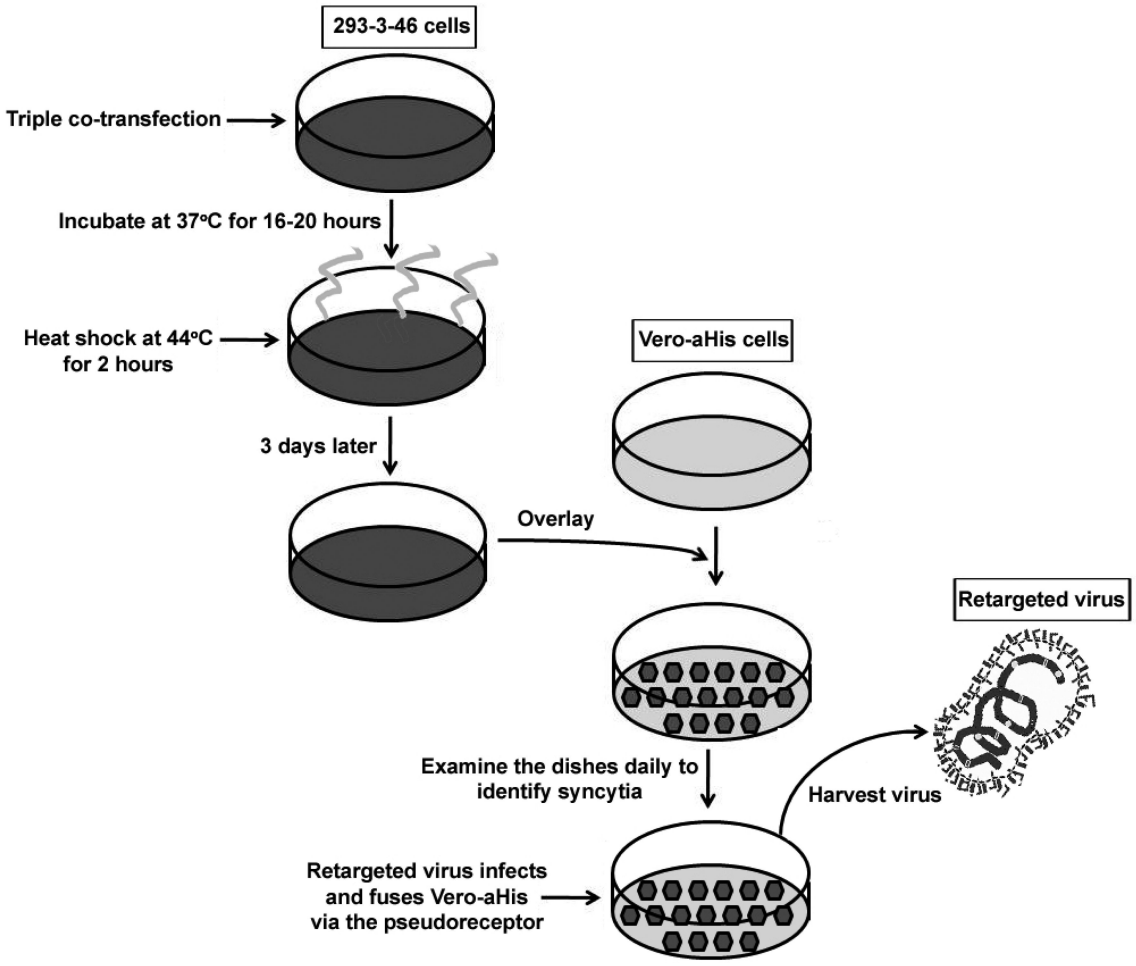

Fig. 2.

Rescue procedure for retargeted measles strains. The helper 293-3-46 cells are transfected with the full-length infectious clone expressing the retargeted ablated at as well as the pEMC-La and pSC6-N plasmids expressing measles L and N proteins respectively. The cells are incubated at 37°C CO_2 for 16-20 hours and then heat-shocked at 44°C for 2 hours. The plate is returned to the 37°C CO_2 incubator for approximately 3 days and then overlain on Vero-aHis cells. Binding of the single chain antibody expressed on the cell surface of Vero-aHis (pseudoreceptor) with the 6-histidine tag on the C-terminus of the recombinant H protein allows entry of the retargeted virus and propagation in Vero-aHis cells. Plates are examined for the characteristic cytopathic effect (syncytia formation), and the infectious virus clone is subsequently harvested (modified from Nakamura T et al, Nature Biotechnology 23(2):209-14, 2005)).