



Published in final edited form as:

Curr Protoc Hum Genet. ; 85: 13.11.1–13.11.9. doi:10.1002/0471142905.hg1311s85.

Neural stem cell-mediated delivery of oncolytic adenovirus

Julius W. Kim, J. Robert Kane, Jacob S. Young, Alan L. Chang, Deepak Kanojia, Shuo Qian, Drew A. Spencer, Atique U. Ahmed, and Maciej S. Lesniak

The Brain Tumor Center, The University of Chicago, Chicago, Illinois

Abstract

The use of stem cells (SCs) as carriers for therapeutic agents has by now progressed to early clinical trials. These clinical trials exploring SC-mediated delivery of oncolytic adenoviruses will commence in the near future, hopefully yielding meritorious results that could provoke further scientific inquiry. Preclinical animal studies have demonstrated that SCs can be successfully loaded with conditionally-replicative adenoviruses and, then, delivered to the tumor, upon which they may evoke pronounced therapeutic efficacy in the animal (Ahmed et al., 2011; Ahmed et al., 2012; Thaci et al., 2012; Tobias et al., 2013). Here in this protocol, we describe the maintenance of SCs, provide an analysis of optimal adenoviral titers for SC loading, and evaluate the optimized viral loading on SCs.

INTRODUCTION

As stem cell-mediated delivery of oncolytic adenoviruses has shown promising therapeutic efficacy, it is very necessary to standardize the protocol to enhance the efficacy and consistency of this approach. Only when healthy and minimally passaged SCs are loaded with an optimized number of virus particles/cell will these SCs migrate to its destined place(s) around the tumor area during the period of viral progeny production (48-72hr) and then release the virus progenies ($\sim 10^4$ particles/cell). We describe here how to properly culture and prepare the SCs for oncolytic virus loading. We further describe how to optimize a virus titer for loading on SCs to maximize the oncolytic virus progenies delivered to the target site.

BASIC PROTOCOL 1. Culture of neural stem cells

Through this protocol, we describe the means of culture of different type of stem cells for the loading of adenoviral vector.

Materials

Stem cells: Neural stem cells (HB1.F3-CD) or Mesenchymal stem cells (either bone marrow derived or adipose derived)

100X stock of Pen/Strep: Penicillin sodium (100 units/mL) and streptomycin (100 μ g/mL)

100X stock of Glutamine (200 mM, light sensitive): It is only needed when you purchase DMEM without Glutamine.

Fetal Bovine Serum (FBS)

1X PBS

Dulbecco's Modified Eagle's medium (DMEM, 500 mL)

Dimethyl sulfoxide (DMSO)

0.25% Trypsin-EDTA solution

T75 cell culture flask

15ml and 50ml Conical tubes

Microscope

37°C, 5% CO₂ humidified incubator

Note: All cell culture practices must be conducted under sterile conditions, including the reagents and workspace involved.

Before putting any materials in the sterile hood, 70% Et-OH has to be sprayed and let it completely dry up in the sterile hood. **Do not wipe out.** Sanitization can be only done with dehydration of Et-OH.

Initial culture from a frozen stock of SCs—This protocol is tailored to a thawing of a cryotube containing 2×10^6 cells in 1 mL.

1. Add 15 mL of cell growth media (10% FBS and 1X Pen/Step in DMEM) to a T75 flask.
2. Spread the cell growth media throughout the flask.
3. Thaw the frozen stock of SCs in 37°C water bath.
4. Transfer thawed cells in the cryotube into a sterile hood.
5. Slowly add the SCs to the growth media in the T75 flask.

Be cautious not to cause osmotic shock to the cells by slowly adding the SCs to the culture media.

6. Gently mix the cells in the media to evenly extend their presence on the surface of the T75 flask
7. Incubate the cells with growth media in a 37°C, 5% CO₂ humidified incubator.
8. On the following day, aspirate the media from the T75 flask and immediately add 15 mL of the fresh cell growth media.

In this step, the DMSO from the freezing media and any dead cells may be aspirated in removal.

Please be advised that it will take approximately 3-4 days for SCs to fully recover and propagate efficiently.

Subculture of SCs—SCs can grow rapidly until the passage limit reaches 25-30 (Aboody et al., 2008; Ahmed et al., 2012). During these passages, carefully check their growth every day. As the media becomes acidic (denoted by a yellow change of color), change the media.

9 Aspirate the existing cell growth media from the T75 flask.

10. Add 5ml of 1X PBS and gently spread to wash.

11. Add, and thoroughly spread, 1.5 mL of 0.25% Trypsin-EDTA solution to the T75 flask.

To accelerate cell detachment, the flask can be placed and incubated in a 37°C, 5% CO₂ humidified incubator.

12. When obvious cell detachment is observed, add 5 mL of the fresh cell growth media to the T75 flask.

13. Wash and homogenize the cells in the cell growth media.

Be especially gentle throughout this repeated pipetting. Strong agitation causes the cells to die.

One of confluent T75 flask of SCs can generate 3 of T75 flasks to be cultured.

14. Transfer the evenly divided volume of the cell-containing media (~2.3ml) to a new T75 flask.

15. Add 12mL of fresh cell growth media to the flask.

Preparation of Frozen Stocks of SCs—A minimum of $2-3 \times 10^6$ cells per 1 mL of freezing media in cryotube is required to restore the cells in a T75 flask. At high confluency, approximately 1.0×10^7 cells can grow in a T75 flask, therefore, 3 cryotubes of frozen stock can be made.

16. Follow the subculture directives from steps 9-13.

17. Transfer the cell-containing media (~6.5 mL) to a 50 mL tube.

18. Centrifuge the tube at 1,200rpm for 5 minutes.

19. While centrifuging, make an appropriate amount of the cell freezing media (10% DMEM in FBS: 300 μ L of DMSO + 2.7 mL of FBS).

20. Aspirate the supernatant media and add the cell freezing media.

21. Homogenize the cells well and transfer to 3 different cryotubes.
22. Store the cryotubes at -80°C overnight and transfer to -180°C the following day.

Basic Protocol 2. Optimizing the Loading Capability of Stem Cells

As the primary receptor, Coxsackievirus and adenovirus receptor (CAR) of the wild type human adenovirus serotype 5 (HAd5) is not expressed on the SCs, the Ad5 fiber domain which dictates the receptor binding, has been genetically modified to achieve the efficient, enhanced and/or directed specific infection in most of presently used adenoviral vectors. However, when this structural protein (fiber) is modified, it could in some cases lead to a lower number of virus progeny production (Kim et al., 2013; Tyler et al., 2009; Zheng et al., 2007).

Therefore, depending on the modification(s), the infectivity and/or production of viral progenies can be different in SCs. As such, Basic Protocol 2 is necessary to investigate *in vitro* the optimal virus titer that can keep the SCs alive until *in vivo* delivery and yet produce the maximum number of virus progenies. In the following protocol, we did not include the generation or production of adenoviral vectors since the defined protocol can be found in UNIT 12.4.

Materials

- 24-well plates
- 1.5 mL tubes
- Hemocytometer
- Trypan blue
- Adeno-X™ Rapid Titer Kit (Clontech)

Determine the viability of SCs post virus loading

1. Prepare one 24-well plate by adding 2×10^4 cells per well (Figure 1) and incubate overnight in a 37°C , 5% CO_2 humidified incubator.
2. On the following day, prepare a 100-fold dilution of virus stock by mixing 10 μL of original virus stock with 990 μL of 1X PBS). Keep this dilution readily available on ice.

For the most part (with some exception), adenovirus stocks are in 10~50% glycerol with a high number of virus particles (Unit 12.4).

The dissociation factor for adenovirus is very low (Shabram et al., 2002); therefore, it is highly recommended to make a 100-fold diluted virus stock first and use it for further virus infection to have better homogeneous mixture in the virus infection media.

3. By following Table 1, prepare a virus dilution: multiple of infection (MOI) of 0.1-1,000 vp/cell or pfu/cell with virus infection media (2% FBS and 1X Pen/Step in DMEM).

4. Aspirate the media and add a virus-containing media with proper titers.
5. After incubation for 2 hours in a 37°C, 5% CO₂ humidified incubator, aspirate the virus-containing media and wash with 0.5 mL of 1 × PBS.
6. After washing, add 0.5 mL of cell growth media and incubate for 72 hours in a 37°C, 5% CO₂ humidified incubator.
7. After 72 hours, transfer the media to 1.5 mL tubes.
8. Add 150 µL of 0.25% Trypsin-EDTA and incubate until the cells detach.
9. Add 300 µL of 1X PBS and transfer to the corresponding tubes from step 7.

In the time between 48 and 72 hours, the initially added viruses will propagate and start to induce the detachment of the cells. To analyze the cell viability, both adhered and detached cells have to be collected.

10. Homogenize the cells in the tube.
11. Mix 20 µL of the cell suspension and 20 µL of trypan blue.
12. Quantify the live and dead cells with hemocytometer.

Only dead cells will uptake trypan blue which is visibly dark coloured microscopically. The ideal percentage of viable cells should be at least 80-90%. If the percentage of live cells is less than this, the virus titer should be reduced.

Analysis of viral progeny production in SC

13. Follow the steps from 1 – 9 from the above SC Cell viability post virus loading protocol.
14. Centrifuge at 1,200 rpm for 5 minutes.
15. Aspirate the supernatant media and resuspend in 50 µL of PBS.
16. Freeze the cell-containing tubes at –80°C, thaw the cell containing tubes at 37°C, and vortex well.
17. Repeat step 16, three times.

Adenovirus particles are relatively stable during the ‘freezing and thawing’ steps. These steps will, however, rupture the cell membrane and release the virus progenies into the supernatant.

18. Check the virus titer with Adeno-X™ Rapid Titer Kit (Clontech).

Around 10⁴ virus particles can be produced from one cell. Therefore with an optimized viral titer (between 10-100 infectious units) loaded 1.0 × 10⁶ SCs, it is expected to produce and release approximately 1.0 × 10⁸ to 10¹⁰ of virus particles with 72hr.

Basic Protocol 3. Loading of adenovirus into Stem Cells

Through the above analysis, optimal adenovirus titers that can achieve maximum neural stem cell viability and viral progeny production should be determined and these parameters used in the final loading of adenovirus (Ahmed et al., 2013; Bolontrade et al., 2012; Yong et al., 2009).

1. Follow steps 9-13 from the subculture of SC (Basic protocol 1).
2. Centrifuge at 1,200 rpm for 5 minutes.
3. Aspirate the supernatant media and add 5 mL of 1X PBS.
4. Quantify the cell number using a hemocytometer.
5. Transfer approximately 1.0×10^6 cells into a new 15 mL tube and add 5 mL of 1X PBS for the second wash.
6. Centrifuge at 1,200 rpm for 5 minutes.
7. While centrifuging, prepare optimal virus titers, as determined in Basic Protocol 2, in a solution of 100 μ L of 1X PBS.
8. Aspirate the cell supernatant and add 100 μ L of optimal virus titer containing solution.
9. After gently mixing, incubate for 2 hours at room temperature.
10. After incubation, add 0.5 mL of 1X PBS and centrifuge at 1,200 rpm for 5 minutes.
11. Remove the supernatant and add 0.5 mL of 1X PBS.
12. Repeat steps 10-11 three times.
13. After washing, resuspend the cells in a desired amount of 1XPBS (Ahmed et al., 2013; Bolontrade et al., 2012; Yong et al., 2009).

The amount of 1X PBS needed will vary depending on the application. For intracranial injection, 5 μ L/injection would be the maximum volume so it should be less than 5 μ L but in other cases, it could be 100 μ L.

14. Keep the virus-loaded SCs cool on ice until their use.

It is recommended to use the virus-loaded SCs as soon as possible but they can be stored on ice for up to an hour.

Commentary

Background Information

Initially isolated from human adenoids as infectious particles in the mid-20th century, adenoviruses may be now engineered for use as delivery vehicles for gene therapy and for

their oncolytic activity against cancer (Nandi and Lesniak, 2009). More specifically, adenovirus-mediated cancer therapy has steeped particular interest in treating glioma (Nandi and Lesniak, 2009). This is due to the fact that these viruses merit particularly well as delivery vehicles, offering specific tropism to the engineered site so desired (Kim et al., 2013; Tyler et al., 2009; Zheng et al., 2007). However, despite many advantages of adenoviral vectors, there have been major challenges for their use such as pre-existing anti-adenoviral immunity within the population, and minimal penetration and spreading of viruses in the tumor mass (Hendrickx et al., 2014). The use of the stem cell carrier can overcome these challenges and enhance the oncolytic activity of adenovirus.

Since the discovery of stem cells (SCs), their biological classification, function, and potential for therapeutic use has been investigated in a litany of preclinical and translational studies. The self-renewal and regenerative capabilities of SCs make them attractive agents of treatment for neurodegenerative disorders. However, it is their tropism to brain tumors that motivated the study of their potential as a therapeutic delivery vehicles (Aboody et al., 2008). This remarkably intrinsic tumor tropism of neural stem cells allows them to specifically deliver a therapeutic agent, such as an oncolytic adenovirus, to the tumor, drastically reducing the off-target side effects all too commonly experienced. Using SCs to deliver oncolytic viruses can enhance the viral distribution throughout the tumor bed while simultaneously shielding the viruses from the immune system (Ahmed et al., 2010). Furthermore, SCs can deliver oncolytic viruses across the blood brain barrier to the tumor, making multiple, less invasive intravenous doses of therapy possible (Aboody et al., 2000).

The groundbreaking use of SCs as a carrier for therapeutic agents has already progressed to early clinical trials, and hopefully clinical trials exploring stem cell-mediated delivery of oncolytic adenoviruses will commence in the near future. Preclinical studies have demonstrated that SCs can successfully be loaded with conditionally replicative viruses, deliver them to tumor, and provide a therapeutic benefit in animal models (Ahmed et al., 2011; Ahmed et al., 2012; Thaci et al., 2012; Tobias et al., 2013). The innate limitations and obstacles facing oncolytic virotherapy can be overcome by delivering them with stem cell carriers like NSCs and MSCs, and the future is bright for this promising combination of molecular agents against currently devastating malignancies.

Critical Parameters and Troubleshooting

Several steps are critical for the efficient loading of adenoviruses on SCs. The initial recovery of cells from the freezing stock takes about 3 days. At this point, SCs grow slowly. However, SCs grow exponentially after 2-3 passages. Therefore during this initial passage step, extra careful observation is absolutely required.

SCs can be cultured for 25-30 passages. After this time point, SCs start to grow extremely slow; therefore, it is highly recommended to make frozen stocks at the earliest time point. It is not recommended to culture and use the cells for any experiments after 30 passages.

In addition, SCs are highly sensitive to pH change. When the SCs are confluent and left to themselves for too long, the pH of the media become acidified which causes the death of SCs. Observe the SCs on a daily basis and change the media if any media acidification is

observed. Optimization of virus titer to maximize virus progeny production and minimize cytopathic effects on SCs is the most important step for a stem cell-mediated adenovirus delivery approach. Depending on how well it is optimized, it will completely change the therapeutic effect from the SC-mediated adenovirus delivery approach. As mentioned above, within 72hrs the viral progeny production should reach the maximum without killing the SCs. At least 80-90 % of virus loaded SCs should be healthy but detached from the bottom of the well which will generate approximately 10^4 virus particles per cell.

For optimized virus titer, it is recommended to dilute an original virus stock to 100-fold in 1XPBS and use it for infection. Keep in mind that the dissociation factor of adenovirus particles is low and the sample must be thoroughly mixed to achieve homogeneity of virus particles in the solution, minimizing any variation on each experiment.

Anticipated Results

It takes approximately 48 to 72 hours for adenovirus to produce the maximum number of virus progenies (10^4 virus particles) in a host cell and to be released from the host cell. When healthy and minimally passaged, SCs are loaded with optimized virus particles/cell and these SCs will migrate to its destined place(s) around the tumor area during this progeny production period and then release the virus progenies. When adenoviruses with an optimized titer (between 10-100 infectious units) loaded 1.0×10^6 SCs are used, it is expected to produce and release approximately 1.0×10^8 to 10^{10} of virus particles in their destination.

Time Considerations

Initial cell recovery time from a frozen stock can vary depending on the condition of the cell stock and personal skills however, sub-culturing of SCs should be done on a basis of less than 72 hours to keep them healthy. When the SCs do not grow well at the initial recovery or during the subculture period, aspirate and discard the old media and replace with a fresh cell growth media. All of the procedures, including the preparation of SCs and adenovirus infection (loading) until *in vivo* delivery, should be done in 3-4 hours to maximize the therapeutic efficacy of SC-mediated adenovirus delivery.

Acknowledgement

This work was supported by NIH/NINDS grant U01NS069997 to MSL.

Literature Cited

- Aboody KS, Brown A, Rainov NG, Bower KA, Liu S, Yang W, Small JE, Herrlinger U, Ourednik V, Black PM, Breakefield XO, Snyder EY. Neural stem cells display extensive tropism for pathology in adult brain: evidence from intracranial gliomas. *Proc Natl Acad Sci U S A*. 2000; 97:12846–12851. [PubMed: 11070094]
- Aboody KS, Najbauer J, Danks MK. Stem and progenitor cell-mediated tumor selective gene therapy. *Gene Ther*. 2008; 15:739–752. [PubMed: 18369324]
- Ahmed AU, Alexiades NG, Lesniak MS. The use of neural stem cells in cancer gene therapy: predicting the path to the clinic. *Curr Opin Mol Ther*. 2010; 12:546–552. [PubMed: 20886386]
- Ahmed AU, Thaci B, Alexiades NG, Han Y, Qian S, Liu F, Balyasnikova IV, Ulasov IY, Aboody KS, Lesniak MS. Neural stem cell-based cell carriers enhance therapeutic efficacy of an oncolytic

- adenovirus in an orthotopic mouse model of human glioblastoma. *Mol Ther.* 2011; 19:1714–1726. [PubMed: 21629227]
- Ahmed AU, Thaci B, Tobias AL, Auffinger B, Zhang L, Cheng Y, Kim CK, Yunis C, Han Y, Alexiades NG, Fan X, Aboody KS, Lesniak MS. A preclinical evaluation of neural stem cell-based cell carrier for targeted antiglioma oncolytic virotherapy. *J Natl Cancer Inst.* 2013; 105:968–977. [PubMed: 23821758]
- Ahmed AU, Ulasov IV, Mercer RW, Lesniak MS. Maintaining and loading neural stem cells for delivery of oncolytic adenovirus to brain tumors. *Methods Mol Biol.* 2012; 797:97–109. [PubMed: 21948472]
- Bolton MF, Sganga L, Piaggio E, Viale DL, Sorrentino MA, Robinson A, Sevlever G, Garcia MG, Mazzolini G, Podhajcer OL. A specific subpopulation of mesenchymal stromal cell carriers overrides melanoma resistance to an oncolytic adenovirus. *Stem Cells Dev.* 2012; 21:2689–2702. [PubMed: 22462538]
- Hendrickx R, Stichling N, Koelen J, Kuryk L, Lipiec A, Greber UF. Innate immunity to adenovirus. *Human gene therapy.* 2014; 25:265–284. [PubMed: 24512150]
- Kim JW, Glasgow JN, Nakayama M, Ak F, Ugai H, Curiel DT. An adenovirus vector incorporating carbohydrate binding domains utilizes glycans for gene transfer. *PLoS One.* 2013; 8:e55533. [PubMed: 23383334]
- Nandi S, Lesniak MS. Adenoviral virotherapy for malignant brain tumors. *Expert Opin Biol Ther.* 2009; 9:737–747. [PubMed: 19456208]
- Shabram P, Vellekamp G, Scandella C. Purification of Adenovirus Elsevier. 2002
- Thaci B, Ahmed AU, Ulasov IV, Tobias AL, Han Y, Aboody KS, Lesniak MS. Pharmacokinetic study of neural stem cell-based cell carrier for oncolytic virotherapy: targeted delivery of the therapeutic payload in an orthotopic brain tumor model. *Cancer Gene Ther.* 2012; 19:431–442. [PubMed: 22555507]
- Tobias AL, Thaci B, Auffinger B, Rincon E, Balyasnikova IV, Kim CK, Han Y, Zhang L, Aboody KS, Ahmed AU, Lesniak MS. The timing of neural stem cell-based virotherapy is critical for optimal therapeutic efficacy when applied with radiation and chemotherapy for the treatment of glioblastoma. *Stem Cells Transl Med.* 2013; 2:655–666. [PubMed: 23926209]
- Tyler MA, Ulasov IV, Sonabend AM, Nandi S, Han Y, Marler S, Roth J, Lesniak MS. Neural stem cells target intracranial glioma to deliver an oncolytic adenovirus in vivo. *Gene Ther.* 2009; 16:262–278. [PubMed: 19078993]
- Yong RL, Shinojima N, Fueyo J, Gumin J, Vecil GG, Marini FC, Bogler O, Andreeff M, Lang FF. Human bone marrow-derived mesenchymal stem cells for intravascular delivery of oncolytic adenovirus Delta24-RGD to human gliomas. *Cancer Res.* 2009; 69:8932–8940. [PubMed: 19920199]
- Zheng S, Ulasov IV, Han Y, Tyler MA, Zhu ZB, Lesniak MS. Fiber-knob modifications enhance adenoviral tropism and gene transfer in malignant glioma. *J Gene Med.* 2007; 9:151–160. [PubMed: 17351980]

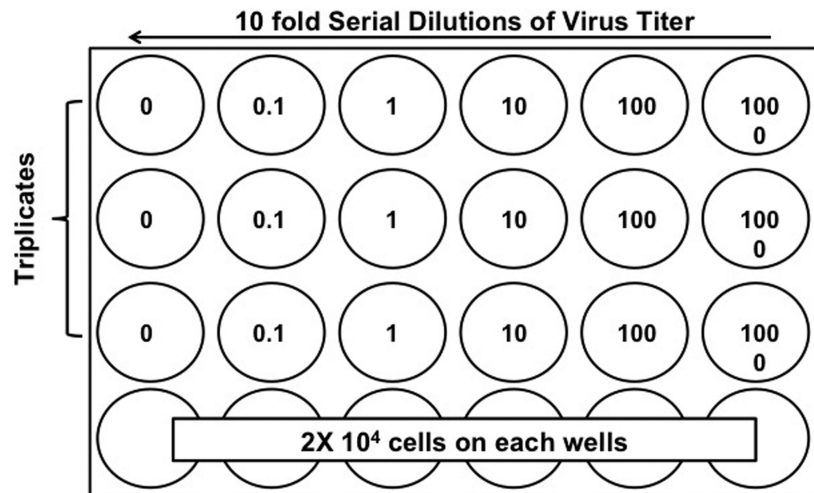


Figure 1. NSC plating and virus dilution for cell viability and viral progeny production assay. To assess the optimal titer of adenovirus for NSC infection, NSCs can be seeded in the 24 well plate with 2×10^4 cells in each well followed by different virus titers (from 0.1 to 1,000). Each virus titer should be evaluated in triplicates to minimize experimental errors.

Table 1

Formula for viral infection

Name	Vp/ml or pfu/ml	Cell/wells	MOI	Wells	Amount (mL)	Virus titer	100 dil. titer	Add amount(μ L) in E_ml
	virus unit	The number of cells in one well	Multiple of infection : how many viruses per cells	Number of total wells to use	Amount of the total media needed	Virus titer to be used	100 fold diluted virus titer	Required Amount of 100 fold diluted virus
	A	B	C	D	E	F = B \times C \times D	G = A /100	H = F / G \times 1000
Ex. You want to use 1.0^{12} virus to infect 3 well of 2×10^4 cells with 1000 MOI								
JK	1.0×10^{12}	2.0×10^4	1000	4wells (3 wells + 1 extra)	2mL (500 μ L X4well)	8.0×10^7	1.0×10^{10}	8 μ L
→ You can add 8μL of 100 fold diluted stock into 2ml of infection media. Then add 500μL to each well to achieve 1000 MOI virus infection.								

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript