BMI1 transduction of human airway epithelial cells for expansion of proliferation and differentiation

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Abstract

Air Liquid Interface (ALI) cultured cells are widely used as *in vitro* models of the human respiratory airway in studies of pulmonary physiology, disease, and therapies. However, the primary basal cells required to establish the ALI cultures generally lose their ability to differentiate by the second or third passage, requiring a fresh batch, which can be limiting, particularly from donors with rare genotypes or in studies where gene modification or editing is required. We have developed a method that preserves the ability to expand primary cells and maintain their capacity to differentiate by lentiviral transduction with *BMI1*. *BMI1* transduced basal airway cells are maintained in submerged culture in the same way as primary basal cells but can be passaged more than twenty times retaining their differentiation capacity in ALI cultures. *BMI1*-transduced basal cells can be frozen and stored long term in liquid nitrogen, enabling transfer of samples between research groups.

Key words

Air-liquid interface (ALI) culture, human airway epithelial cells, cystic fibrosis, primary ciliary dyskinesia, human lung model, primary cells.

1 Introduction

Primary human airway basal cells cultured in air-liquid interface (ALI) are particularly important in the study of pulmonary diseases [1] such as cystic fibrosis. Murine *cftr* knockouts do not develop characteristic features of CF respiratory disease although they do manifest intestinal symptoms [2]. Large animals such as pigs and ferrets [3], which have lungs with similar anatomical and physiological features to those of humans, do develop CF-like respiratory disease but are costly to maintain and limited in their availability. ALI cultures established from CF basal epithelial cells, for example, produce thick sticky mucus and display chloride transport defects while cells from donors with primary ciliary dyskinesia produce a ciliated epithelium with dysmotile cilia. Epithelial cells from healthy donors in ALI culture can be used for physiological studies as well as study of transmitted viral diseases such as SARS-CoV-2 [4]. However, the usefulness of these models is impacted by the limited proliferation capacity of primary basal cells which is particularly limited for the study of cells from donors with rare genetic mutations, necessitating repeated sampling from the donor.

Several approaches have been investigated to extend the proliferation capacity of basal epithelial cells including cultured by feeder layer culture where primary cells are cultured with Rho-associated protein associated kinase (ROCK) inhibitor on a layer of irradiated fibroblast cells, providing cell-driven growth factors [5,6]. However, the complexity of co-culture with feeder layer cells makes scale up difficult, and time-consuming and their differentiation capacity is limited to approximately 10 passages.

Human *BMI1* is a proto-oncogene that has been implicated as an anti-senescence gene and has an important role in self-renewal, cell-cycle, and DNA repair [7,8]. We have shown previously that both human non-CF and CF *BMI1*-transduced basal cells proliferate up to passage 23 [9], and, very importantly, maintained their potential to differentiate to ciliated epithelium on ALI. Dysmotile cilia were demonstrated in ALI cultures of *BMI1*-transduced basal cells from donors with primary ciliary dyskinesia (PCD) with DNAH5 mutations (manuscript in review) (Fig. 1). Furthermore, we have confirmed that *BMI1* transduced cells at ALI culture form tight junctions and display Na^+ and Cl^- ion channel properties (Fig. 2), and therefore, they also can be used for electrophysiological studies. Hence, *BMI1* transduction of human airway cells provides a useful model for pulmonary disease study.

2 Materials

2nd generation Lentivirus vectors:

VSV-G envelope expressing plasmid pMD2.G (Plasmid #: 12259 in Addgene).

2nd generation lentivirus packaging plasmid pCMVdR8.74.

Lentivirus expression vector pLVX-Puro.

Or 3rd generation Lentivirus vectors:

VSV-G envelope expressing plasmid pMD2.G (Plasmid #: 12259 in Addgene).

3rd generation lentivirus packaging plasmid pMDLg/pRRE (Plasmid #: 12251in Addgene).

3rd generation lentivirus packaging plasmid pRSV rev (Plasmid #: 12253 in Addgene).

3rd generation transfer plasmid.

HEK 293T cells.

Foetal calf serum (FCS).

Dulbecco’s Phosphate Buffered Saline (DPBS).

0.05% Trypsin/EDTA.

OptiMEM (Gibco, Life Technologies).

0.22 µm Stericup® with PES membrane (Millipore, Merks) or Millex-GP Syringe 0.22 µm filter Unit (Millipore, Merks).

Dulbecco's Modified Eagle Medium (DMEM; Gibco, Life Technologies).

Penicillin/Streptomycin (10,000U/mL).

Polyethylenimine (PEI, Sigma).

TaqMan Universal PCR master mix (Thermo Fisher Scientific).

Primers for WPRE Forward: 5'-ACCACCTGTCAGCTCCTTTC-3' and Reverse: 5'-GAATTGTCAGTGCCCAACAG-3'.

Probe for WPRE: 5'-FAM-CTATTGCCACGGCGGAACT-TAMRA-3'.

TaqMan assay reagent for Actin B, Gene ID: Hs01060665_g1.

Plasmid coding one copy of WPRE and housekeeping gene (e.g., Actin B or Albumin) for titration used to generate the standard curve (Standard plasmid).

PureCol (Type I Bovine Collagen Solution at 3mg/mL, Advanced BioMatrix).

PneumaCult-Ex medium (STEMCELL technologies), supplemented according to the company's instruction.

PneumaCult-ALI medium (STEMCELL technologies), supplemented according to the company's instruction.

3 Protocols

3.1 The transfer vector cloning

Full-length human *BMI1* cDNA was cloned from pHR-EF1_-BMI1-IRES-GFP plasmid [10] by PCR with *Xho*I and *Bam*HI sites added and TOPO cloned [11] into pCR4-TOPO vector before being subcloned into pLVX-Puro vector digested with *Xho*I and *Bam*HI.

3.2 Lentivirus production (Table 1)

3 days before seeding cells on Day 1

- Seed $5x10^6$ HEK 293T cells per T175 flask in DMEM plus 10% heat-inactivated FCS and 1% penicillin/streptomycin

Day 1 – Seeding HEK 293T cells in T175 flasks

1. Remove the medium from each flask prepared 3 days prior and wash the cells with DPBS (10 – 15 mL).
2. Aspirate the DPBS and add 3 mL 0.05% Trypsin/EDTA to detach the cells from the plastic surface.
3. Incubate the cells in trypsin for 5 minutes at 37 ^{o}C in 5% CO_2.
4. Add 7 mL complete medium containing 10% foetal calf serum to stop trypsin activity. Pipette up and down and rinse the flask wall to remove all cells and to disrupt any cellular aggregates.
5. Transfer resuspended cells into a 50mL falcon tube.
6. To count cells, pipette 10 µL of the resuspended cells onto a haemocytometer and add 40 µL Trypan blue (dilute with trypan blue at 1 in 5). Count the number of cells.
7. Centrifuge the 50 ml tubes containing resuspended cells in at 300 x *g* for 5 minutes to pellet the cells.
8. Remove the supernatant of trypsin-containing medium and resuspend the cell pellet in 10 mL fresh complete medium.
9. Seed 2.1 – 2.5 $x10^7$ HEK 293T cells in 10 ml complete media in each T175 flask in preparation for transfection with lentiviral vector plasmids 24 hours later.

10. Seed 5x 10^6 cells in an additional T175 flask that will be used for lentiviral titration.

Day 2 -Transfection in HEK 293T cells

11. The HEK 293T cells should be 80% to 90% confluent in T175 flasks prior to transfection.
12. Prepare transfection mix for the required number of flasks (plus an excess of 1 extra flask)

Table 1. Reagents required for Lentiviral production

2nd generation	**1 flask**
pMD2.G	17.5 µg
pCMVdR8.74	32.5 µg
pLV-Puro-BMI1	25 µg
PEI	1 µL
OPTIMEM	10 mL

3rd generation	**1 flask**
pMD.G	17.5 µg
pMDLg/pRRE	32.5 µg
pRSV rev	25 µg
transfer plasmid	25 µg
PEI	1 µl
OPTIMEM	10 mL

13. DNA-OptiMEM mix: add the three plasmid DNAs for 2nd generation or four plasmids for the 3rd generation in 5 mL OptiMEM and mix by vortex. The DNA-OptiMEM mixture should be filtered using a 0.22 µm Stericup® (PES membrane) or filter. Pre-wet the filter membrane with 5 mL OptiMEM if Stericup® is used. Add 5 mL DNA-OptiMEM mix.
14. PEI-OptiMEM mix: add 11 µL PEI in 5 mL OptiMEM to the DNA solution and mix by vortexing briefly. Pre-wet the membrane of a 0.22 µm Stericup® (PES membrane) with 5 mL OptiMEM if Stericup® is used. Add 5 mL PEI-OptiMEM.
15. Add the PEI-OptiMEM (5 ml) mix to DNA-OptiMEM mix (5 ml). Mix gently and incubate at room temperature for 20 minutes.
16. Add 2 mL OptiMEM so that 12 mL transfection medium can be transferred each T175 flask.
17. Incubate the cells for 48 hours at 37 ^{o}C and 5% CO_2.
18. Replace the transfection media to 18 mL fresh complete medium 4 hour-post transfection.

Day 4- 1st virus harvest

1. Collect the medium from three T175 flasks per 50 mL tube (i.e., 3 flasks per tube) and centrifuge at 500 x *g* for 5 minutes to pellet any cells, retaining virus in the medium.
2. Add 18 mL fresh medium to the cells in the T175 flasks if the virus is to be harvested again (2nd virus harvest).
3. The supernatant from Day 4 step1 should be filtered using a 0.22 µm SteriCup® (PES membrane). Pre-wet the filter membrane with 5 mL OptiMEM and the add the supernatant.
4. Store the filtered supernatant at 4 ^{o}C.

Day 5- 2nd virus harvest (*see* **Note 1**)

1. Collect the medium from T175 flasks into 50 mL tubes (i.e., 3 flasks per tube) and centrifuge at 500 g for 5 minutes to remove cells.
2. The supernatant is filtered using a 0.22 µm SteriCup (PES membrane). Pre-wet the filter membrane with 5 mL OptiMEM and add the supernatant.
3. Mix the filtered 2^{nd} harvested virus with the 1^{st} harvest virus obtained the previous day.
4. Aliquot the filtered virus into 6 ultracentrifuge tubes. Start by adding 30 mL in each tube and balance each tube.
5. Place each bucket into the rotor inside the ultracentrifuge (matching the number on the bucket to the number on the rotor).
6. Concentrate the virus by ultracentrifugation at 100,000 x *g* for 2 hours at 4^{o}C.
7. Remove the tubes from the buckets using forceps and decant the supernatant to a waste container with Virkon (virus decontaminant). Using forceps, wipe the inside wall of the tubes with tissue paper wet with 70% Ethanol (70% IMS) to remove excess OptiMEM. Do not touch the pelleted virus on the bottom.
8. Add 110 µL OptiMEM (or medium of choice) to each tube and incubate the virus in OptiMEM in the tubes on ice for at least 30 minutes (no more than 2-3 hours).
9. Gently pipette the virus up and down in OptiMEM in the tubes to resuspend the virus. Set the Gilson pipette to lower volume (e.g., 50 µL) to avoid generating bubbles. Scrape the bottom with pipette tips.
10. Combine all tubes and prepare 25 µL aliquots in cryovials (*see* **Note 2**).
11. Snap-freeze the virus aliquots by placing the cryovials in dry ice and store at -80 ^{o}C.

3.3. Titration

Day 1 – Seed HEK 293T cells in a 24 well plate

Seed HEK 293T cells in a 24 well plate at $1x10^5$ cells per well

Day 2 – Transduction

1. Thaw the 21 µL aliquot of virus in ice.
2. Prepare 5-fold serial dilution of the virus in 1.5 mL tubes (Table 2).

Table 2. Titration serial dilution protocol

	OPTIMEM	VIRUS
TUBE 1	180 µl	20 µl from FROZEN VIAL
TUBE 2	240 µl	60 µl from TUBE 1
TUBE 3	240 µl	60 µl from TUBE 2
TUBE 4	240 µl	60 µl from TUBE 3
TUBE 5	240 µl	60 µl from TUBE 4
TUBE 6	240 µl	60 µl from TUBE 5
TUBE 7	300 µl	-

3. Count one or two wells to check the accurate cell number in the plate seeded yesterday.
4. Remove the medium from each well and add 200 µL DMEM (the complete medium for HEK 293T) to the wells.
5. Add 100 µL of virus-containing medium from tubes diluted in step 2 to the cells. One well should be transduced per dilution.
6. Add 700 µL complete medium to each well in the end of the day.

Transduced cells should be incubated at least three days and ideally were passaged once in 12-well plates.

Day 5 – check the titre using qRT-PCR (*see* **Note 3**).

1. Wash the transduced cells with DPBS and aspirate the DPBS.

2. Add 100 µL Trypsin/EDTA and incubate the cells in an incubator.
3. Add 400 µL DPBS to stop Trypsin activity and centrifuge the cells at 300g for 5 minutes.
4. Extract DNA according to the instructions of the DNA extraction kit (GeneJet genomic DNA purification kit, Thermo Fisher Scientific).
5. Elute DNA in 50 µL DNase free water and measure the concertation of DNA in each sample.
6. Mix TaqMan Universal PCR mater mix (Applied Biosystems, Thermo Fisher Scientific), TaqMan assay of β Actin or mixture of two primers WPRE Forward: ACCACCTGTCAGCTCCTTTC, Reverse: GAATTGTCAGTGCCCAACAG and Probe: CTATTGCCACGGCGGAACT at 900 nM, 300 nM and 100 nM final concentration respectively and 100 ng DNA. Total reaction volume is adjusted to 20 µL with water (*see* **Note 4**).
7. Add the standard plasmid serial diluted in water to 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 WPRE/house-keeping gene copies into TaqMan Universal PCR mater mix, TaqMan assay of β Actin or mixture of WPRE primers and probe in 20 µL per well (in triplicate).
8. Run the samples in a qRT-PCR machine following the company's instruction of the qPCR master mix.
9. Obtain a standard curve of WPRE and β Actin (housekeeping gene) from the Ct values (average of the triplicate) of the standard samples.
10. Calculate the average of the Ct values of the triplicate and choose one point from the serial dilutions that is on the standard curve range. i.e., between the Ct values of 10^7 and 10^2 of the standard.
11. Calculate the WPRE copy number and β Actin copy number from the Ct values using the standard curve.
12. Calculate BMI-1 copy number from the formula:

    BMI1 copy number = 2x WPRE copy number/Albumin copy number.

Calculate titre (TU/mL) from the formula:

TU/mL (Titre) = BMI1 copy number x the number of seeded cells (i.e., 1 x10^5) / the volume of virus added into the cells (Table 3)

Table 3. Volumes of virus for *BMI1* copy number determination

TUBE 1	TUBE 2	TUBE 3	TUBE 4	TUBE 5	TUBE 6
10 µl	2 µl	0.4 µl	0.08 µl	0.016 µl	0.0032 µl

3.4 Transduction with BMI-1 lentivirus in primary airway epithelial cells

3.4.1 Expanding primary cells in a collagen coated flask

1. Collagen coating: Dilute PureCol (Advanced BioMatrix) with PBS at 1 in 100 and add 2 to 5 mL collagen in DPBS into flasks then incubate the flasks at room temperature for 30 minutes.
2. Alternatively dilute PureCol with DPBS at 1 in 10 and incubate for 5 minutes at room temperature.
3. Remove the PureCol/PBS solution from wells and air-dry the flasks.
4. Thaw a primary cell vial from liquid nitrogen in water bath (*see* Note 5).
5. Add the cells into 10 mL complete medium (e.g., PneumaCult-Ex medium) and transfer to a collagen coated flask. A T25 flask is normally enough size to obtain plenty of cells required for the transduction.
6. Incubate cells in an incubator with 5% CO2 at 37 ^{o}C.
7. Change the medium next day to remove DMSO.
8. Change the medium every 2 to 3 days until cells are confluent.

Day 1 – seeding primary cells in collagen coated 6-well plates

1. Collagen coating: Dilute PureCol (Advanced BioMatrix) with PBS at 1 in 100 and incubate the plate with collagen at room temperature for 30 minutes.
2. Remove the PureCol/PBS solution from wells and air-dry the plates.
3. Seed Primary cells (up to passage 3) at $1x10^5$ cells a day before transduction in collagen coated 6 well plates and the cells will be 30 to 40% next day.

Day 2 – Transduction in primary cells with BMI-1 by lentivirus:

1. Dilute *BMI1* lentivirus in 200 µL OptiMEM and add 800 µL PneumaCult-Ex Medium (STEMCELL technology) supplemented according to the company's instructions. For example, if the titre is $2x10^5$ TU/mL and MOI-1 is desired, mix 0.5 µL of the BMI-1 lentivirus and 200 µL OptiMEM. Mix 800 µL complete medium for the primary cells.
2. Add the virus in 1 mL per well of 6 well plates

Day 3 - Top up 1 mL complete medium (PneumaCult-Ex Medium) next day of the transduction. Do not remove the virus-containing medium.

Day 4 - Change the medium at Day 4. Change media every 2 or 3 days

- Transfer to T75 flasks (collagen coated) when the cells are confluent. The passage number will be p2,1 in the first T75 flask if the primary cells were transduced at passage 2, and the next passage number will be p2,2.

3.5 Cell maintenance

3.5.1 Freezing BMI-1 cells

1. Freeze cells in cryovials when they are approximately 80 to 90 % confluent.
2. Rinse BMI-1 cells with 1 mL 0.05 % Trypsin/EDTA then aspirate the Trypsin/EDTA.
3. Add 1 mL 0.05 % Trypsin/EDTA to detach cells and incubate them at 37 ^oC and 5% CO_2 for 5 minutes.

4. Add 4 mL PneumaCult-Ex medium to stop trypsin activity and transfer the cells into a 5 mL tube.
5. Centrifuge at 300 x *g* for 5 minutes.
6. Aspirate the supernatant and add 4 mL 10% DMSO (v/v) in fresh PneumaCult-Ex medium.
7. Aliquot 1 mL per cryovial and put the vials in dry ice (snap freezing).
8. Optional. Transfer approximately $5x10^5$ to $7x10^5$ cells into a new T75 flask and expand if necessary.

3.5.2 Thawing BMI-1 cells

1. Put cryovials in a water bath at 37 ^{o}C until the ice is melted.
2. Transfer the cells in 15 mL tubes and add 4 mL fresh PneumaCult-Ex medium
3. Centrifuge at 300 x *g* for 5 minutes.
4. Aspirate the supernatant and transfer the cells in 10 to 13 mL of fresh media to collagen-coated T75 flasks.
5. Change medium every 2 or 3 days (*see* **Note 6**).

The morphology of human airway cells transduced with BMI-1 is round or rectangle shape (Fig. 3 a,b). Passage BMI-1 transduced cells if cells are elongated and are growing close to each other (Fig. 3c,d) otherwise they stop growing. The cells normally stop growing at 70-80 % and never reach 100% confluence.

3.6 Air Liquid Interface Culture

1. Collagen-coat the apical sides of ALI plates. 12-trans well or 6-snapwell plates.
2. Trypsinise BMI-1 cells as described above.
3. Add 4 mL media to stop Trypsin activity.
4. Transfer cell suspension to 15 mL tubes.
5. Count the number of cells while tubes are centrifuged at 300 x *g* for 5 minutes.

6. Seed $3.5x10^5$ cells in 250 µL per well ($1.12 cm^2$) on transwell or snapwell membrane.
7. Add 0.5 to 1 mL media in the basolateral sides.
8. Incubate and grow cells in submerged culture in the transwells for approximately 5 days until cells are confluent. Change the media every 2 or 3 days.
9. Remove the media from the basolateral sides and add 0.5 to 1 mL of PneumaCult-ALI medium supplemented according to the company's instruction.
10. Remove the media from the apical sides.
11. Change ALI media on the basolateral sides every 2 or 3 days.
12. Grow the cells at ALI culture for 4 weeks to allow differentiation (*see* **Note 7**).

4 Notes

1 2nd round can harvest 5- to 7-fold fewer virus that the first one. The first harvested virus might be enough for your application of the virus, and the second harvesting step is, therefore, optional.

2 Save an aliquot (at least 20 µL required) for onwards titration

3 This step requires a primer set and probe (TaqMan assay) for the HIV element WPRE and a housekeeping gene such as β actin or albumin, and a standard plasmid coding WPRE and a house keeping gene of your choice.

4 Three technical repeats from each sample should be run. The total reaction number will be: (7 samples of DNA from transduced cells + 6 points of standards + water negative control) x3 = 42

5 Do not leave primary cells in water bath after the cells are thawed.

6 BMI-1 transduced cells can be passaged more than 22 times.

7 Human airway cells transduced with BMI-1 secrete excessive mucus when they grow in PneumaCult-ALI medium. Rinse mucus at least once a week. Incubate the cells in DPBS or HBSS for 10-15 minutes in an incubator at 37 ºC and 5% CO_2 and repeat 2 or 3 times. If necessary, rinse mucus with 30 µM N-Acetylcysteine in DPBS. For

transfections, wash off the mucus two- or three-days in the week before transfections and wash again just before transfections.

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