BMI-1 extends proliferative potential of human bronchial epithelial cells whilst retaining their mucociliary differentiation capacity.

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ABBREVIATIONS LIST

ALI = Air-Liquid Interface
BEGM = Bronchial Epithelial Growth Media
CBF = Cilia Beat Frequency
CFBE = Cystic Fibrosis Bronchial Epithelial
CRCs = Conditionally Reprogrammed Cells
DMEM = Dulbecco's Modified Eagle Medium
GFP = Green Fluorescent Protein
HBE = Human Bronchial Epithelial
hESCs = human Embryonic Stem Cells
hTERT = human Telomerase Reverse Transcriptase
iPSCs = induced Pluripotent Stem Cells
I_sc = short circuit current
NHBE = Normal Human Bronchial Epithelial
ODA = Outer Dynein Arms
PBS = Phosphate Buffered Saline
PCD = Primary Ciliary Dyskinesia
ROCK = Rho-associated protein kinase
ABSTRACT

Air-liquid interface (ALI) culture of primary airway epithelial cells enables mucociliary differentiation providing an *in vitro* model of the human airway but their proliferative potential is limited. To extend proliferation, these cells were previously transduced with viral oncogenes or mouse *Bmi-1* + *hTERT* but the resultant cell lines did not undergo mucociliary differentiation. We hypothesised that use of human *BMI-1* alone would increase the proliferative potential of bronchial epithelial cells while retaining their mucociliary differentiation potential. CF and non-CF bronchial epithelial cells were transduced by lentivirus with *BMI-1* then their morphology, replication kinetics and karyotype were assessed. When differentiated at ALI, mucin production, ciliary function and transepithelial electrophysiology were measured. Finally, shRNA knockdown of DNAH5 in BMI-1 cells was used to model primary ciliary dyskinesia (PCD). BMI-1 transduced basal cells showed normal cell morphology, karyotype and doubling times despite extensive passaging. The cell lines underwent mucociliary differentiation when cultured at ALI with abundant ciliation and production of the gel-forming mucins MUC5AC and MUC5B evident. Cilia displayed a normal beat frequency and 9+2 ultrastructure. Electrophysiological characteristics of BMI-1 transduced cells were similar to un-transduced cells. shRNA knockdown of DNAH5 in BMI-1 cells produced immotile cilia and absence of DNAH5 in the ciliary axoneme as seen in cells from patients with PCD. BMI-1 delayed senescence in bronchial epithelial cells, increasing their proliferative potential but maintaining mucociliary differentiation at ALI. We have shown these cells are amenable to genetic manipulation and can be used to produce novel disease models for research and dissemination.
Key words: air-liquid interface, airway model, lung, mucociliary differentiation, primary ciliary dyskinesia
INTRODUCTION

The ciliated epithelium lining the airways provides the first line of defence to inhaled pathogens and particles and plays a crucial role in many respiratory diseases. It is possible to remove respiratory epithelial cells from the nose or upper airways of donors by brushing and culture them in the laboratory on collagen-coated, semi-permeable membranes. The progenitor basal epithelial cells from the brushings cultured at Air-Liquid Interface (ALI) differentiate into a fully ciliated, pseudostratified epithelium closely resembling that found in the airway (3).

If cells are obtained from a donor with a lung disease, e.g., cystic fibrosis, primary ciliary dyskinesia (PCD), asthma and chronic obstructive pulmonary disease, these ALI cultures provide a surrogate model of the diseased lung for research into pathogenic mechanisms and for the development of new therapeutics(9, 14, 16). However, basal epithelial cells can only be passaged 2-3 times before they lose their proliferation and differentiation potential (6, 18). Thus, to establish the wider use of basal cells in ALI epithelial culture models, methods are required that enable basal cells to be cultured for longer, genetically engineered, expanded and stored easily prior to differentiation on ALI cultures. Such cells would also overcome ethical issues related to repeated brushing of volunteers.

Recent approaches to extend the utility of primary, basal epithelial cells involved culturing them with rho-associated protein kinase (ROCK) inhibitors on a layer of irradiated feeder cells to provide cell-derived growth factors (18, 27). The requirement for irradiated feeder cells makes the maintenance of basal cell cultures complex and time-consuming, difficult to scale up and may limit the use of this approach to specialist laboratories. Alternatively, induced pluripotent stem cells (iPSCs) and embryonic stem cells (hESCs) were differentiated into mature respiratory epithelial cells and used to generate a pseudostratified epithelium expressing CFTR (30). However, the process takes several weeks and often the resulting cultures are not suitable for disease modelling as they are contaminated with endodermal
cell types (31) and often present with karyotypic anomalies which may confound drug screening efforts.

Extended proliferative potential of primary human bronchial epithelial (HBE) cells was described by transduction of basal cells with the mouse polycomb complex protein $Bmi-1$ and human telomerase reverse transcriptase ($hTERT$) (6). Unlike cells transformed with viral oncogenes, $Bmi-1+hTERT$ cell lines had no chromosomal abnormalities and produced a pseudostratified epithelium on ALI but gave only sparse ciliogenesis. This limited differentiation capacity may be explained by reports that $hTERT$, following long-term growth in culture, up-regulates expression of the potent mitogen c-Myc, so promoting entry into the cell cycle (21) thereby impeding ciliogenesis.

We hypothesised that BMI-1 transduction alone may overcome these issues observed with $Bmi-1+hTERT$, to produce basal cells with the potential for extended proliferation that retain their differentiation capacity on ALI. In this study, $BMI-1$ transduced primary basal epithelial cells from CF and healthy donors were investigated for their morphology, growth characteristics and karyotype. We also assessed the cells mucociliary differentiation potential at ALI along with their $\text{Na}^+$ and $\text{Cl}^-$ transport properties in Ussing chamber studies. We then demonstrate their use for the production of novel engineered disease models by shRNA knockdown of $DNAH5$, a gene associated with PCD, a ciliopathy with significant lung pathology resulting from abnormal mucociliary clearance. $BMI-1$ transduction offers a facile method to greatly extend the utility of basal epithelial cells for translational and basic research.
MATERIALS & METHODS

Materials

Primary antibodies used in this study can be found in Table 1. Secondary antibodies for immunofluorescence were anti-IgG antibodies conjugated with AlexaFluor dyes (Invitrogen, Life Technologies). Secondary antibodies for Western blots were horseradish peroxidase-conjugated (HRP-conjugated) anti-IgG antibodies (Dako, Agilent Technologies).

Collagen Coating

Tissue culture flasks and transwells were coated for 1 hour at room temperature with 1% (v/v) solution of a 3mg/mL bovine collagen solution (PureCol; Advanced Biomatrix) in phosphate buffered saline (PBS), then washed with distilled water and air-dried.

Cell Culture

HEK293T cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% (v/v) foetal bovine serum. Normal human bronchial epithelial (NHBE) cells, cystic fibrosis human bronchial epithelial cells (CFBE) cells were grown on collagen-coated plastic in bronchial epithelial growth media (BEGM; Lonza). All cells were grown at 37°C and 5% CO₂. NHBE and CFBE cells were purchased from Lonza and Epithelix SàRL.

Lentivirus Production and Transduction

Full-length human BMI-1 cDNA was PCR cloned from pHREF1α-BMI1-IRES-GFP plasmid(20) with Xhol and BamHl sites added and TOPO cloned into pCR4 TOPO vector before being subcloned into pLVX-Puro vector digested with Xhol and BamHl. Lentivirus was produced as previously described(20), concentrated by centrifugation
at 4,500 x g for 18 hours at 4°C, re-suspended in OptiMem and added to cell media to transduce NHBE and CFBE cells (Lonza) at passage 2.

**Doubling Time Analysis**

NHBE and NHBE BMI-1 cells at varying passage numbers were seeded at densities of 30,000 cells per well onto collagen-coated 12-well plates. Cells were detached using trypsin-EDTA following 1-4 days in culture and total cell numbers per well were counted using a haemocytometer. An online calculator was used to calculate the doubling time (Roth V. 2006 Doubling Time Computing, Available from: http://www.doubling-time.com/compute.php). Doubling times were calculated using the formula;

\[
doubling\ time = \frac{duration \times \log(2)}{\log(\text{final cell count}) - \log(\text{initial cell count})}
\]

Where cell count values were mean cell count of 3 independent wells.

**Western Blotting**

Cells were lysed with Cell Extraction Buffer (Life Technologies), boiled in the presence of NuPage LDS Sample Buffer (Life Technologies) and loaded onto NuPage Novex 4-12% Bis-Tris gels (Life Technologies). Electrophoresis and protein transfer onto Immobilon-P polyvinylidene fluoride membranes were performed using standard protocols. Antibodies against BMI-1, p16Ink4a and GAPDH and appropriate HRP-conjugated secondary antibodies were used for probing with bands visualised using Pierce ECL Western Blotting Substrate (Life Technologies, Paisley, UK) and a UVichemi chemiluminescence imaging system (UVItect).
Air-liquid Interface (ALI) Culture

Cells grown to ~80% confluence in T75 flasks were trypsinised, seeded at a density of 900,000 cells/cm² on Transwell inserts (Corning) and grown at an ALI as previously described(8). Cell were maintained at an ALI for 4 weeks before analyses were performed.

Quantitative Reverse Transcription PCR (qRT-PCR)

Unless indicated, all reagents for qRT-PCR were obtained from ThermoFisher. Total RNA was harvested from cells using RNeasy Mini Kit (Qiagen) and potential DNA impurities digested using DNase I enzyme (TURBO DNA-free kit). Purified RNA was reverse transcribed with 2.5U/µL murine leukaemia virus (MuLV) reverse transcriptase at 42°C for 1 hour in a reaction containing 1x GeneAmp PCR Gold Buffer, 1mM each dNTP, 5µM random hexamers, 5mM MgCl₂ and 1U/µL RNase inhibitor. The resulting cDNA was used in a qPCR reaction containing 1x Platinum Quantitative PCR SuperMix-UDG w/ROX and 1x TaqMan Gene Expression Assay primer/probe set (GAPDH primer/probe set Hs99999905_m1; DNAH5 primer/probe set Hs00292485_m1). The PCR reaction cycles used were 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Fluorescence data was collected at the end of each 60°C reaction and relative expression levels calculated using the delta-delta Ct (2⁻ΔΔCt) method(19).

Immunofluorescence Staining and Confocal Microscopy

Cells were fixed with 4% PFA for 10 minutes at room temperature, washed with PBS and permeabilised with PBS-Triton (PBS 0.1% (v/v) Triton-X100) for 10 minutes at room temperature before blocking, immunostaining and mounting on microscope slides as previously described(26). Images were obtained using an Inverted Zeiss
LSM 710 Confocal microscope with the appropriate excitation lasers selected for the dyes used.

**Fluorescence Microscopy**

Bright-field and fluorescence images were captured with a Nikon Digital Sight DS-QiMC video camera attached to a Nikon Eclipse Ti-U inverted microscope. Videos and images were processed using NIS Elements AR software (Nikon, v4.00.12).

**TEM for Cilia Ultrastructure**

Ciliated cells cultured at an ALI were scraped and cells washed off with 200µL warmed BEBM. Cells were fixed by addition of 2mL of 2.5% glutaraldehyde and stored at 4°C for at least 24 hours prior to further processing as previously described(24). Assessment of cilia ultrastructure was undertaken blinded by Dr Amelia Shoemark, a member of the PCD diagnostic service team at the Royal Brompton & Harefield NHS Foundation Trust, UK.

**High-Speed Video Microscopy**

High-speed video was recorded using a MotionPro X4 high-speed motion camera attached to a Nikon Eclipse Ti-U inverted microscope built with an environmental chamber. Videos were recorded at a frame rate of 500fps using Motion Studio software (IDT Vision, v2.11) with cells maintained at 37°C.

For cilia beat frequency (CBF) assessment, ALI cultures were washed twice with PBS to remove mucus that may have affected CBF. After washing, the cells were allowed to equilibrate at 37°C and 5% CO₂ for 20 minutes before video recording. At least four independent cultures per donor line were videoed with five areas recorded per culture, i.e., at least 20 videos were captured per donor line. To minimise bias
videos were recorded from the top, bottom, left, right and centre region of each
culture and cilia beat-frequency assessed using CiliaFA software (25).

Electrophysiology Studies

Cells were grown at ALI for 4 weeks on Snapwell membranes (Corning) to enable
mucociliary differentiation. Snapwells were then mounted on Ussing chambers and
short circuit current (Isc) was measured as previously described (32). Briefly,
monolayers were mounted in Ussing chambers in physiological salt solution
consisting of 117 mM NaCl, 25 mM NaHCO3, 4.7 mM KCl, 1.2 mM MgSO4, 1.2 mM
KH2PO4, 2.5 mM CaCl2 and 11 mM d-glucose. The solution was continuously
circulated throughout the course of the experiment and maintained at 37°C whilst
bubbled with 21% O2 + 5% CO2 premixed gas. Monolayers were first maintained
under open-circuit conditions until transepithelial potential difference (Vt) and
resistance stabilised. The cells were then short-circuited by clamping Vt at 0 mV
using a DVC-4000 voltage/current clamp, and Isc was measured and recorded using
a PowerLab computer interface. Every 30 seconds the preparations were returned to
open-circuit conditions for 3 seconds so that the spontaneous Vt could be measured
and trans-epithelial electrical resistance (TEER) calculated. Drugs were circulated in
physiological salt solution and added in the order of amiloride (10 μM, apical),
forskolin (25 μM, apical and basolateral) and GlyH-101 (10 μM, apical).
RESULTS

Characterisation of BMI-1 transduced cells in submerged culture

Primary NHBE cells maintained in submerged cultures displayed a characteristic cobblestone appearance (Figure 1a) but by passage 3 cells became elongated in appearance (white arrow; Figure 1b) and squamous differentiation was evident (black arrow; Figure 1b). In contrast, BMI-1 transduced NHBE cells (NHBE-BMI-1) maintained their cobblestone appearance following extensive passaging, for example at passage 11 (Figure 1c) and passage 17 (Figure 1d). However, squamous cells became evident following 25 passages (Figure 1e) after which the cells senesced, with no observable cell division for ten days. The cells maintained a normal diploid karyotype even at passage 23 (Figure 2).

BMI-1 down-regulates expression of the pro-senescent protein p16Ink4A. NHBE cells transduced with BMI-1 had low levels of p16Ink4A protein and high levels of BMI-1 (Figure 3a). Levels of BMI-1 in untransduced NHBE cells declined with an increase in passaging whilst levels of p16Ink4A increased and were higher in senesced, untransduced NHBE cells at passage 6 while BMI-1 expression was not evident by Western blot (Figure 3a).

SV40 large T-antigen or ROCK inhibition extends the replication potential of basal cells but alters the proliferation rate of the cells (4, 7, 12) therefore we assessed the doubling times of BMI-1 transduced cells at different passages (Figure 3b). We determined that untransduced cells at passage 2 had a doubling time of 1.18 days similar to BMI-1 transduced cells at passages 12 and 15 (doubling times of 1.25 and 1.21 days respectively) although by passage 23 the doubling time had increased to 1.49 days, consistent with observations of senescence at passage 25.
Differentiation of NHBE-BMI-1 Cells

NHBE-BMI-1 basal cells were subsequently analysed for their differentiation potential when cultured at ALI. After 2 -3 weeks culture, both primary NHBE and NHBE-BMI-1 cells produced motile cilia (Video 1a and b respectively). NHBE-BMI-1 cells maintained the ability to differentiate and produce cilia even at passage 15.

To quantify cilia function, we assessed cilia beat frequency of both primary and BMI-1 transduced NHBE and CFBE cells. Beating cilia from CFBE cells could not be detected, most likely due to the build-up of viscous mucus hindering cilia beating, until cultures were washed. As such, CFBE and NHBE cultures were washed twice prior to video recording and CBF analysis as detailed in the methods section.

CBF analysis of both primary and BMI-1 transduced NHBE and CFBE cells showed mean values within the normal range for respiratory cilia of 9-17Hz(25) (Figure 4a and b). Primary NHBE and NHBE-BMI-1 cells had a CBF of 16.7±0.2Hz and 15.3±0.2Hz respectively (Figure 4a) and primary CFBE and CFBE-BMI-1 cells exhibited CBF values of 12.9±0.3Hz and 14.3±0.3Hz respectively.

Further evidence of differentiation was demonstrated by immuno-detection, in NHBE-BMI-1 cells, of the tight junction protein occludin (Figure3c) and the mucins MUC5AC and MUC5B (Figure 4d, e). In addition, basal cells were present and indicated by p63 staining (Figure 4f) and BMI-1 protein was present in all nuclei (Figure 4g). The ciliary protein acetylated α-tubulin was also detected by immunostaining and highlighted abundant ciliation (Figure 4h). Further analysis of the cilia in differentiated NHBE-BMI-1 cells by TEM showed that they had a normal 9+2 ultrastructure with both inner and outer dynein arms present (Figure 4i, Table 2 and Table 3).
**Electrophysiology studies**

Primary HBE cells grown on ALI develop a trans-epithelial electrical resistance (TEER) with ion transport properties that can be measured by mounting of cultured epithelia on Ussing chambers and addition of drugs that can activate or inhibit specific cell surface ion channels. Cultures of primary NHBE cells from two different donors showed baseline TEER values of $331.1 \pm 105.5 \Omega \cdot \text{cm}^2$ and $621.0 \pm 33.2 \Omega \cdot \text{cm}^2$ (Table 4) and primary CFBE cells developed TEER of $1307.9 \pm 36.6 \Omega \cdot \text{cm}^2$. Similarly, BMI-1 transduced NHBE and CFBE cells developed high TEER when grown at an ALI ($1268.4 \pm 78.4 \Omega \cdot \text{cm}^2$ and $917.6 \pm 165.3 \Omega \cdot \text{cm}^2$ respectively; Table 4) demonstrating the cells retained their ability to form an electrically resistive epithelium.

Short circuit current ($I_{sc}$) analysis in Ussing chambers of NHBE and CFBE cells revealed that both primary NHBE cells and passage 13 NHBE-BM-1 cells cultured at ALI also had similar electrophysiology. Amiloride (10$\mu$M), an inhibitor of the epithelial Na$^+$ channel ENaC reduced $I_{sc}$ in all cultures, although the amiloride-sensitive $I_{sc}$ was variable. Subsequent elevation of cellular cAMP with forskolin (25$\mu$M) increased $I_{sc}$ and this elevation was inhibited by the CFTR inhibitor Gly-H101 (10$\mu$M) (Figure 5 a, b). Thus, ENaC and CFTR-mediated ion transport was retained in NHBE-BMI-1 cells. Primary CFBE cells and passage 17 CFBE BMI-1 cells cultured at ALI also exhibited amiloride-inhibitable $I_{sc}$ but no response to either forskolin or GlyH-101 was observed, as expected due to the lack of CFTR in these cells (Figure 5 c, d). Thus, CFBE-BMI-1 cells, like NHBE-BMI-1, also maintain the Na$^+$ and Cl$^-$ ion transport characteristics of non-transduced primary CF cells.

**Use of BMI-1 transduced cells to generate PCD cell models**
We next explored the potential use of the BMI-1 transduced NHBE cells to generate an in vitro model of PCD. The outer dynein arm protein DNAH5 is the most commonly mutated gene but even so this is a rare disease and cells are often not readily available. Cells with DNAH5 mutations lack the DNAH5 protein in the ciliary axoneme and have missing outer dynein arms (ODAs) (13). NHBE cells transduced with BMI-1 were additionally transduced with a DNAH5 shRNA lentiviral construct that also expresses green fluorescent protein (GFP).

DNAH5 expression in shRNA-transduced cells was silenced by approximately 75% relative to untransduced cells (Figure 6a) while scrambled shRNA had no effect on DNAH5 expression indicating silencing specificity.

NHBE-BMI-1 cells transduced with the two shRNAs were subsequently cultured at ALI to promote differentiation and ciliation. Following mucociliary differentiation, NHBE-BMI-1 GFP-positive cells, transduced with scrambled shRNA had motile cilia, (Video 2a) whereas GFP-positive DNAH5 shRNA silenced cells had immotile cilia (Video 2b). However, in GFP negative cells (and by extension also DNAH5 shRNA negative) motile cilia were still observed (Video 2c).

In untransduced NHBE BMI-1 cells and those GFP-positive cells transduced with the scrambled shRNA, DNAH5 was localised to the ciliary axoneme in all ciliated cells assessed as shown by co-localisation with acetylated α-tubulin expression. In contrast, in DNAH5 shRNA transduced GFP-positive cells, only 2.9% (5/173) of ciliated cells had DNAH5 in the ciliary axoneme (Figure 6b and Table 5).
DISCUSSION

Airway diseases are a significant cause of morbidity and mortality. Mucociliary differentiation of primary airway epithelial cells using ALI culture methods provides an *in vitro* model that faithfully recapitulates the *in vivo* airway epithelium for the study of disease pathology and therapies. However, these cells can only be cultured for 2-3 passages before they lose their ability to differentiate(5). This has important practical, ethical and cost implications for research in the field. Traditional cell transformation methods, using viral oncogenes that promote entry into the cell cycle, produce immortal cell lines incapable of mucociliary differentiation most likely due to their inability to suspend cell division and allow cilia production and differentiation.

We have shown that prevention of cellular senescence by expression of *BMI-1* allows extended passaging of HBE cells from CF and non-CF donors. Western blot analysis highlighted that senescent primary NHBE cells had accumulated high levels of the pro-senescent protein p16<sup>ink4a</sup> in agreement with other studies (1, 6, 20). *BMI-1* transduced cells, however, showed low levels of p16<sup>ink4a</sup> thereby delaying cell senescence as reported previously(15).

In addition to exhibiting delayed senescence, *BMI-1* transduced cells retained their cell phenotype, karyotype, ion transport characteristics and mucociliary differentiation potential with abundant ciliation observed when cultured at ALI. Ussing chamber studies revealed that, like primary HBE cells, *BMI-1* transduced NHBE and CFBE cells formed electrically resistive cultures and the direction of change in I<sub>sc</sub> was as expected upon addition of amiloride, forskolin and the CFTR inhibitor Gly-H101. We note that baseline TEER values varied between HBE donors as did the magnitude of change in I<sub>sc</sub> upon addition of amiloride, forskolin and the CFTR.
inhibitor Gly-H101. Such variation has also been observed by Tosoni et al. (29) who recently demonstrated baseline TEER values ranged from 309 to 2963Ω.cm² in ALI cultures generated from the cells of 18 healthy donors.

In agreement with our findings, Torr et al (28) recently demonstrated that transduction of basal cells, from different two donors, with human BMI-1 alone extends the proliferative potential of NHBE cells whilst retaining their differentiation potential as demonstrated by immunostaining and scanning electron microscopy. Our study extends on these findings demonstrating that passaging capacity of diseased cells (CFBE) can also be extended using this method. Taken together this would suggest BMI-1 transduction of bronchial epithelial cells permits extended passaging and mucociliary differentiation independent of donor and/or disease status although further studies are needed to confirm this.

BMI-1 transduction did not immortalise the HBE cells in contrast to viral antigens such as the SV40 large T-antigen used to produce the 16HBE14o- cell line(5). However, BMI transduced cells could still be differentiated at 20-25 passages representing a significant advantage of this method over use of viral antigens. Using the ALI culture protocol outlined in the current study one can routinely obtain from 6-8 functional epithelial transwells in a 24-well ALI culture format per passage enabling the generation of a minimum of ~90-100 transwells from a single donor. This is significantly higher than the 10-15 epithelial transwells that can be generated with ~1x10⁶ primary bronchial epithelial cells (typical quantity obtained from commercial providers) or brushing of the nasal turbinate of a single donor(29). Furthermore, sub-culturing of BMI-1 transformed cells, as opposed to seeding ALI cultures, would enable banking of early passage cells and the potential to generate exponentially more functional epithelia at each passage.
Tosoni et al. (29) recently demonstrated that ALI cultures generated from different healthy donors can yield epithelia with vastly different physiological properties and drug responses. The BMI-1 transduction protocol enables the generation of a large number of epithelia generated from donors with similar genetic backgrounds, or indeed from a single donor, allowing the study of disease pathophysiology in a manner that avoids the influence of genetic variability in cells from different donors. This highlights the potential for the development of personalised treatments using BMI-1 transduced cells.

In addition, an extended passaging capacity affords the opportunity for modification of HBE cells to create new models, to better understand disease and find novel treatments. As a proof of concept, we transduced NHBE BMI-1 cells with shRNA targeted against DNAH5 in an attempt to create a model of PCD. The shRNA construct contained a GFP reporter to allow for selection of cells in which the DNAH5 shRNA was expressed. Focussing on cells expressing GFP, we demonstrated loss of ciliary motility and absence of DNAH5 in the ciliary axoneme of cells transduced with the DNAH5 targeted shRNA so mimicking the phenotype seen in patient cells (13). shRNA-mediated knockdown has been previously used to model PCD in otherwise healthy primary HBE cells (10, 11, 17) but these cells were not long lived so could not be used for further study to assess, for example, protein interactions or novel treatments. Gene addition, shRNA knockdown, or genome editing of BMI-1 transduced HBE cells could therefore provide a more useful tool for the study of a number of airway diseases.

Recently the use of pharmacological Rho-kinase inhibition along with co-culture of HBE cells with irradiated feeder-layer fibroblasts has been described to allow indefinite passage of HBE cells whilst retaining the cells differentiation capacity when
placed at ALI (18, 27). However, studies where the mucociliary differentiation potential of CRCs have been assessed have not reported successful mucociliary differentiation beyond passage 11(2, 22, 27). Furthermore, CRC morphology and doubling times differ significantly to their parent cells with CRC cells being smaller and growing in colonies as well as showing faster proliferation rates(18, 27). Following viral transduction, *BMI-1* expressing NHBE and CFBE cells are cultured exactly as non-transformed primary cells, without the need for a feeder layer, a factor that is likely to aid in the rapid uptake of this method of transformation and dissemination of the resulting cell models between laboratories and in the maintenance of cells in biobanks.

In summary, here we have shown that *BMI-1* transduction delays senescence in HBE cells from healthy and CF donors whilst maintaining the cells mucociliary differentiation potential. We have undertaken extensive characterisation of the differentiated cells showing normal ciliary beat frequency and ciliary ultrastructure. Ussing chamber studies with BMI-1 transformed NHBE and CFBE cells showed that these cells exhibit similar Na+ and Cl- ion transport characteristics to their respective primary cells, validating their use as models of CF. Furthermore, we have demonstrated how BMI-1- transduced cells can be engineered by further transduction with DNAH5 shRNA to recapitulate an in vitro disease model of primary ciliary dyskinesia, a valuable feature when studying rare diseases such as PCD where patient samples are difficult to obtain.
REFERENCES


AUTHOR CONTRIBUTIONS

M.M.M., A.S., R.A.H., J.M.D. and D.L.B. contributed to data collection. All authors contributed to study design, data analysis, interpretation of the data and critical revision of the final manuscript. All authors approved the final version of the manuscript.

GRANTS

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DISCLOSURES

The authors declare no competing financial interests.
Supplementary videos available.
FIGURE LEGENDS

Figure 1. BMI-1 maintains healthy cell morphology in 2D culture.

The morphology of (a) NHBE cells at passage 1 and (b) passage 3 was observed under light microscopy and compared to NHBE BMI-1 cells after passages (c) 11, (d) 17 and (e) 25. White arrows highlight elongated cells and black arrows highlight squamous cells. Scale bars are 100µm.

Figure 2. Karyotype analysis of NHBE-BMI-1 cells.

Karyotype of passage 23 NHBE-BMI-1 cells was undertaken by The Doctors Laboratory, London.

Figure 3. Elevated p16Ink4a precedes senescence and BMI-1 functions by inhibiting p16Ink4a and retains a normal cell doubling time.

(a) Western blot was used to assess levels of BMI-1 and p16Ink4a in serially passaged NHBE cells and BMI-1 transduced cells and (b) cell counting was used to determine the replication kinetics of NHBE and NHBE BMI-1 cells at varying passages. Growth curves are presented as percent of mean of day 1 cell count. Data are mean ± S.E.M. For each data point n=3 biological replicates.

Figure 4. BMI-1 cells retain their mucociliary differentiation capacity.

Extensively passaged BMI-1 transduced cells (passage 15) were differentiated on ALI and cilia beat frequency of (a) NHBE and (b) CFBE cells was determined using ciliaFA plugin(25) for ImageJ. Data are mean ± S.E.M; n= 4 independent ALI cultures, 5 fields videoed per culture. Immunostaining of NHBE-BMI-1 cells was used to show tight junction formation (occludin; c), mucin production (MUC5AC and MUC5B; d and e respectively), the presence of basal cells (p63+; f), widespread BMI-1 expression (BMI-1; g), and extensive ciliation (acetylated α-tubulin; h). TEM was used to determine cilia ultrastructure (i). Images are representative of 4 independent ALI cultures per marker. Scale bars for c-h are 50µm and 100nm for i.

Figure 5. BMI-1 cells form ALI cultures suitable for Ussing chamber studies.

Representative Ussing chamber traces and changes in short-circuit current (Isc) in response to administration of amiloride (apical), forskolin (apical and basolateral) and GlyH-101 (apical) in primary and BMI-1 transduced (a and b) NHBE and (c and d) CFBE cells are shown. Data are mean ± S.E.M; n= at least 3 independent ALI cultures (see Table 4 for exact values).

Figure 6. DNAH5 knockdown recapitulates PCD phenotype.

(a) qRT-PCR was used to assess DNAH5 mRNA expression in NHBE-BMI-1 cells and NHBE BMI-1-transduced with lentivirus expressing either a scrambled or
DNAH5-targeting shRNA and grown in submerged 2D culture. **P<0.01; one-way ANOVA with Bonferroni’s post-test used to assess significance. Data are mean ± S.E.M. (b) Immunostaining for DNAH5 and acetylated α-tubulin was used to assess the presence or absence of DNAH5 in the ciliary axoneme of shRNA transduced and untransduced NHBE BMI-1 cells differentiated at ALI. Presence of GFP fluorescence denotes cells transduced with the GFP-shRNA construct and so expressing the shRNA. Scale bars are 20µm. Images are representative of 4 independent ALI cultures per condition.
### Table 1. Primary antibodies used in this study.

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<tr>
<th>Name</th>
<th>Supplier</th>
<th>Dilution WB/IF</th>
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<td>Anit-MUC5AC</td>
<td>Life Technologies</td>
<td>NA/1:100</td>
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<tr>
<td>Anti-Acetylated α-tubulin</td>
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<td>Anti-BMI-1</td>
<td>Life Technologies</td>
<td>1:200/1:100</td>
</tr>
<tr>
<td>Anti-GAPDH</td>
<td>Life Technologies</td>
<td>1:1000/1:500</td>
</tr>
<tr>
<td>Anti-MUC5B</td>
<td>Kind gift from Professor Dallas Swallow(23)</td>
<td>NA/neat</td>
</tr>
<tr>
<td>Anti-Occludin</td>
<td>Invitrogen, Life Technologies</td>
<td>NA/1:100</td>
</tr>
<tr>
<td>Anti-p16INK4</td>
<td>Pharmingen, BD Biosciences</td>
<td>1:200/NA</td>
</tr>
<tr>
<td>Anti-p63</td>
<td>Invitrogen, Life Technologies</td>
<td>NA/1:100</td>
</tr>
</tbody>
</table>

### Table 2. Microtubule organisation of motile cilia.

<table>
<thead>
<tr>
<th>Microtubule Organisation</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal 9+2</td>
<td>92.05</td>
</tr>
<tr>
<td>Central Pair Defect</td>
<td>0.66</td>
</tr>
<tr>
<td>Disarranged</td>
<td>3.31</td>
</tr>
<tr>
<td>Other Defect</td>
<td>3.97</td>
</tr>
</tbody>
</table>

### Table 3. Dynein arm presence in motile cilia.

<table>
<thead>
<tr>
<th>Dynein Arms</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ODA and IDA Present</td>
<td>100.00</td>
</tr>
<tr>
<td>ODA Only</td>
<td>0.00</td>
</tr>
<tr>
<td>IDA Only</td>
<td>0.00</td>
</tr>
<tr>
<td>ODA and IDA Absent</td>
<td>0.00</td>
</tr>
</tbody>
</table>
Table 4. Trans-epithelial electrical resistance (TEER) measurements.

<table>
<thead>
<tr>
<th>Name</th>
<th>Passage</th>
<th>TEER (Ω.cm² ±S.E.M)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHBE (AB053901)</td>
<td>P1</td>
<td>621.0 ± 33.2</td>
<td>5</td>
</tr>
<tr>
<td>NHBE (AB037501)</td>
<td>P1</td>
<td>331.1 ± 105.5</td>
<td>3</td>
</tr>
<tr>
<td>NHBE BMI-1</td>
<td>P13</td>
<td>1268.4 ± 78.4</td>
<td>4</td>
</tr>
<tr>
<td>CFBE</td>
<td>P2</td>
<td>1307.9 ± 36.6</td>
<td>5</td>
</tr>
<tr>
<td>CFBE BMI-1</td>
<td>P17</td>
<td>917.6 ± 165.3</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 5. DNAH5 localisation.

<table>
<thead>
<tr>
<th>shRNA Target</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untransduced</td>
<td>157</td>
<td>0</td>
</tr>
<tr>
<td>Scrambled</td>
<td>147</td>
<td>0</td>
</tr>
<tr>
<td>DNAH5</td>
<td>5</td>
<td>173</td>
</tr>
</tbody>
</table>
**a**

BMI-1 transduced

<table>
<thead>
<tr>
<th>P3</th>
<th>P4</th>
<th>P5</th>
<th>P6</th>
<th>P15</th>
<th>P16</th>
</tr>
</thead>
</table>

BMI-1 ~ 38kDa

p16Ink4A ~ 16kDa

GAPDH ~ 38kDa

---

**b**

![Graph showing cell count over days in culture](image)

Cell count (% of Day 1 count) vs. Days in Culture

- NHBE P2
- NHBE BMI-1 P12
- NHBE BMI-1 P15
- NHBE BMI-1 P23
a

b

NHBE BM-1 P2.14
NHBE P2 (AD201)

CFBE BM-1 P2.13
CFBE P2 (CFAD507)

C.F. [Hz]

0 5 10 15 20

C.F. [Hz]

0 5 10 15 20

(c, d, e, f, g, h, i)

Scale bar = 100 nm