Identification of AAV serotypes for lung gene therapy in human embryonic stem cell derived lung organoids.

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Short Report

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Abstract

Gene therapy is being investigated for a range of serious lung diseases, such as cystic fibrosis and emphysema. Recombinant adeno-associated virus (rAAV) is a well-established, safe, viral-vector for gene delivery with multiple natural and artificial serotypes available displaying alternate cell, tissue and species-specific tropisms. Efficient AAV serotypes for the transduction of the conducting airways have been identified for several species; however, efficient serotypes for human lung parenchyma have not yet been identified. Here, we screened the ability of multiple AAV serotypes to transduce lung bud organoids (LBOs) - a model of human lung parenchyma generated from human embryonic stem cells. Microinjection of LBOs allowed us to model transduction from the luminal surface, similar to dosing via vector inhalation. We identified the natural rAAV2 and rAAV6 serotypes, along with synthetic rAAV6 variants, as having tropism for the human lung parenchyma. Positive staining of LBOs for surfactant proteins B and C confirmed distal lung identity and suggested the suitability of these vectors for the transduction of alveolar type II cells. Our findings establish LBOs as a new model for pulmonary gene therapy and stress the relevance of LBOs as a viral infection model of the lung parenchyma as relevant in SARS-CoV-2 research.

Background

Recombinant adeno-associated virus (rAAV) is a well-established vector for gene delivery, currently in use clinically for gene therapy, with multiple, naturally occurring serotypes and artificial variants facilitating species-specific cell and tissue tropisms [1]. Engineering of new AAV capsids has been the focus of extensive research, but capsids selected in animal models and cancer cell lines often translate poorly to large animal models and humans. Clinical trials of gene therapy for Cystic Fibrosis lung disease using rAAV serotype 2 failed to show efficacy [2] and of the many potential reasons the lack of serotype screening for airway tropism and animal studies not translating to the human airways is an important factor [3].

Identification of AAV serotypes for gene delivery to the human lung has focused mainly on transduction of the human airway epithelium [4, 5]. The lung parenchyma, however, is the target for treating genetic diseases such as surfactant deficiencies; in particular the surfactant producing Alveolar Type II (ATII) pneumocytes, which express proteins crucial for surfactant function. In addition, ATII cells are an important cell target for several respiratory viral infections including MERS-CoV and SARS-CoV−1 and 2 [6].

In this study, we aimed to identify AAV serotypes that permit efficient gene delivery to the human lung parenchyma. We hypothesised that an ideal model for capsid selection should be of human origin and should also offer a polarised cell layer that mimics the tissue surface available to viral vectors, including the distribution of viral entry receptors. We chose a human 3D cell culture model of the lung for serotype
screening—lung bud organoids (LBOs) [7]. LBOs are generated from human embryonic stem cells (hESC) and exhibit a strong bias towards the generation of lung parenchyma cell types, especially alveolar type II (ATII) pneumocytes [7]. *It provides a reproducible, in vitro model in which to study human / viral vector interactions, that is substantially more similar to the native tissue environment than traditional, immortalised, submerged cell culture models. The* polarised 3D structure of LBOs allows for vector transduction from the luminal surface, mimicking vector administration by inhalation, and thus provides an attractive translational model for diseases of the human parenchyma.

**Results**

To generate LBOs, the hESC cell line AND-2 was sequentially differentiated via endoderm and branching induction according to the timeline shown in Figure 1 [7]. After 59 or 79 days of differentiation in culture, LBOs were microinjected with multiple rAAV vectors, or a negative control buffer, to mimic vector delivery to the apical/luminal surface of the lung. Injection of rAAV vectors expressing Enhanced Green Fluorescent Protein (EGFP) from the CMV promoter resulted in EGFP-dependent fluorescence in LBOs as early as day 3 post-injection. On day 5 after injection (Figure 2), high levels of EGFP expression were observed following transduction with rAAV2, rAAV6 and variants rAAV6.2 and rAAV6.2FF ([4, 8], Figure 2g, b, c, and d respectively). EGFP-derived fluorescence was much lower in cultures injected with rAAV1 and rAAV8 (Figure 2f and i), while fluorescence with rAAV serotypes 5 and 9 (Figure 2h and j) was indistinguishable from mock injection (Figure 2a). For rAAV6.2, EGFP expression from the CMV promoter was considerably more robust than that achieved with the hCEFI promoter [9], which yielded only low levels of fluorescence (Figure 2c and e).

The LBOs were sectioned and stained for ATII cell markers Surfactant Protein C (SP-C) and Surfactant Protein B (SP-B) to confirm distal lung maturity (Figure 3a & b). Native EGFP-dependent fluorescence was observed alongside positive SP-B immunostaining in LBOs transduced with AAV6.2-CMV-eGFP (Figure 3c) compared with non-transduced organoids (Figure 3b) indicating the suitability of this vector to transduce the human lung parenchyma. To further understand the basis for rAAV transduction, which can depend on both primary glycan receptors and protein co-receptors [1], we also stained the LBOs for the universal AAV co-receptor (AAVR or KIAA0319L, Figure 3d), α-2,3-linked sialic acid (Figure 3e & f), and heparan sulphate (Figure 3g & h). Scattered cells staining positive for α-2,3-linked sialic acid were observed (Figure 3e & f), along with staining of subcellular structures characteristic of AAVR ([10], Figure 3d) and the ‘spotted’ staining pattern commonly observed for heparan sulphate ([11] Figure 3g & h). The observations confirmed the presence of all three rAAV receptor molecules in the LBO cultures.

**Discussion**

The development of models of the human lung is important for the investigation of new treatments, but is often challenging when human lung tissue is scarce. Moreover, isolated adult alveolar stem cells quickly de-differentiate in culture. The generation of LBOs from hESC provides a 3D model of human lung parenchyma, which has been shown to model aspects of embryonic development, RSV infection and
genetic diseases such as Hermansky-Pudlak syndrome [7, 12]. We chose to use this model to identify rAAV serotypes with tropism for human lung parenchyma - a crucial step in developing novel viral gene therapies for diseases of the lung.

We showed that reporter EGFP fluorescence was greatest following transduction with rAAV serotypes 2, 6 and variants of serotype 6 in d59 organoids, with similar transduction patterns for organoids injected at d79 (data not shown). The AAV5 vector was negative for eGFP expression in this human model (Figure 2h) although serotype 5 was previously identified as suitable for transduction of murine lung parenchyma [4]. This highlights the variation in vector tropism observed between model species, a well-known challenge in the field of viral in gene therapy, and also more generally a problem for viral infection studies. Staining of the LBOs for the lung parenchymal markers SP-B and SP-C confirmed distal lung identity and suggests the suitability of these rAAV serotypes 2, 6 and variants of 6 for transduction of ATII cells. Serotypes 6.2 (AAV6 capsid + F129L) and 6.2FF (AAV6 + F129L + Y445F + Y731F) were designed for improved lung transduction [4, 8], but in this qualitative study no differences were observed. The hCEFI promoter was investigated to avoid promoter silencing and to enable long-term therapeutic transgene expression, however, expression levels in the LBOs suggests that other promoter options should be explored for the human lung parenchyma. Furthermore, staining revealed the presence of the universal AAVR co-receptor, and also α−2,3-linked sialic acid and heparan sulphate, which have been previously observed in resected adult, human lung tissue [13, 14], although the location of these receptors and their subtypes within the human lung is not fully understood [11].

The highly efficient transduction of LBOs with rAAV serotypes 2 and 6 is consistent with the observed strong positive staining for heparan sulphate (Figure 3g & h), a key entry receptor for these serotypes [1]. These findings indicate that LBO cultures may be a useful model for screening vectors targeting the human parenchyma, particularly in the early (neonatal) stages of lung development, as required in, for example, treatment of congenital surfactant deficiencies. LBOs might also be suitable for the generation of new capsids targeting the human parenchyma via directed evolution and screening of AAV capsids libraries. The LBOs also have potential to model aspects of viral infection in the human parenchyma, including infection of ATII cells with SARS-CoV–1 and 2.

In future studies, we anticipate that LBOs can be generated from genome-edited hESCs, potentially giving rise to concomitant morphological and functional phenotypes, that could serve as refined human disease models to facilitate the investigation of gene therapy vectors for disease treatment. In summary, we have established human LBOs as a model to screen for vector transduction, identifying serotypes suitable for transduction of the human lung parenchyma.

**Methods**

The hESC line AND–2 was sequentially differentiated to LBOs (for details see supplementary methods, figure 1 and references [7, 15, 16]). Needles with long continuous taper were pulled for LBO microinjection, which was deemed successful when the organoid visibly pulsated during injection (aiming
for 3–4 successful injections per site and, depending on the LBO size and number of buds, 2–3 locations; see supplementary methods for detailed microinjection protocol). This corresponded to $3.5E8–1E9$ rAAV genome copies (GC) of vector per LBO. Recombinant AAV vectors were produced by triple transfection in HEK293T cells and purified via iodixanol density gradient centrifugation [17]. Vector purity was tested via SDS-PAGE and titres were determined using quantitative PCR. For immunohistochemistry analysis, LBOs were processed in fixed-frozen sections. Cellular proteins and glycan receptors were stained using primary antibodies to α-surfactant protein B (#sc–133143, Santa Cruz Biotech), α-prosurfactant protein C (#ab3785, Merck), α-KIAA0319L (AAVR, #PA5–67257, Invitrogen) and α-heparan sulphate, clone F58–10E4 (#370255, Amsbio), as well as Maackia Amurensis Lectin II to detect α–2,3-linked sialic acid (#B–1265, Vectorlabs). As a negative control for staining, glycan receptors were digested using heparinase III or sialidase A and sections processed in parallel.

**Abbreviations**

AAV, Adeno-associated virus; AAVR, AAV receptor; ATII: Alveolar type II cell; BMP: Bone morphogenetic protein; CMV, cytomegalovirus; EGFP, Enhanced Green Fluorescent Protein; FGF, Fibroblast growth factor; GC, genome copies; hCEFI, human CMV enhancer coupled to EF1 promoter; hESC: Human embryonic stem cell; KGF: Keratinocyte growth factor; LBO: Lung bud organoid; rAAV, recombinant AAV; RSV, respiratory syncytial virus; SARS-CoV, Severe Acute Respiratory Syndrome coronavirus; SP: Surfactant protein.

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Declarations

Ethics approval and consent to participate
The use of the hESC line AND–2 and the experimental procedures of this study were approved by the ISCIII Ethics Committee and the National Committee of Guarantees for the Use and Donation of human Cells and Tissues (ref. no. 436351 1).

Availability of data and materials
Please contact the corresponding author to request datasets used and analysed in this study are available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests.

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Authors’ contributions
HMB performed the experiments and analysed and interpreted data. LZY generated organoids and assisted microinjections. MP was instrumental in the development of the organoid microinjection protocol. AZ supervised organoid generation, microinjections and discussed study design. HMB, SH and DG designed the study and interpreted the results. HMB, AZ, SH and DG wrote the manuscript in consultation with all authors. All authors read and approved the final manuscript.

Figures
Figure 1

Key stages of Lung Bud Organoid generation. Representative images of the key stages in lung bud organoid generation are shown together with the key respective differences in culture, plate type/coating and cellular factors. 

d0: hESC are routinely cultured in adherent mode on Matrigel coated plates. 

- d0-d4: Transition to suspension culture allows differentiation (via embryoid bodies) to definite endoderm.
- d4-d6: Transition to adherent culture on fibronectin coated plates and a two-step treatment with cellular factors induces anterior foregut endoderm.
- d6-d28: Transition to suspension culture allows generation of nascent organoids.
- d28-d45+: mature LBOs are generated after nascent organoids are placed in a Matrigel sandwich, with 'buds' starting to emerge after a few days as highlighted by the arrow. Mature LBOs are typically used for experiments from d45 onwards (in this study d59 and d79). Scale bars 200 µm.
Figure 2

Native EGFP fluorescence in lung bud organoids on day 5 after rAAV transduction. Lung bud organoids (aged d59) were microinjected with 3.5E8 to 1E9 GC of rAAV serotypes expressing EGFP from either the CMV promoter or hCEFI promoter as indicated. Mock injection with buffer served as a negative control. Five days after injection, LBOs were imaged en face for EGFP fluorescence. One representative image from n=3-4 injected organoids is shown; for serotypes rAAV6.2, rAAV5, and rAAV9 the image is representative of two independent (n=3-4) experiments. Scale bar 200 µm.
Figure 3

Immunohistochemistry for markers of alveolar type II cells and AAV entry receptors. Representative images are shown of fixed-frozen sections of LBOs, with nuclei stained with DAPI (blue) and various markers (red), including: ATII cell marker SP-C (a), ATII cell marker SP-B (b, c), universal AAV co-receptor AAVR (d), glycan receptor α-2,3-linked sialic acid (e, f) and glycan receptor heparan sulphate (g, h). Native fluorescence is observed following microinjection of AAV6.2-CMV-eGFP (c) compared with negative control LBOs (b). Sections digested with sialidase A (e) and heparinase III (g) to remove glycans are included as negative staining controls. Scale bar 50 µm.

Supplementary Files

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