Research Article

Human Alveolar Epithelial Cells Expressing Tight Junctions to Model the Air-Blood Barrier

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Summary

This paper describes a new human alveolar epithelial cell line (hAELVi – human Alveolar Epithelial Lentivirus immortalized) with type I-like characteristics and functional tight junctions, suitable to model the air-blood barrier of the peripheral lung. Primary human alveolar epithelial cells were immortalized by a novel regimen, grown as monolayers on permeable filter supports and characterized morphologically, biochemically and biophysically. hAELVi cells maintain the capacity to form tight intercellular junctions, with high trans-epithelial electrical resistance (> 1000 Ω*cm²). The cells could be kept in culture over several days, up to passage 75, under liquid-liquid as well as air-liquid conditions. Ultrastructural analysis and real-time PCR revealed type I-like cell properties, such as the presence of caveolae, expression of caveolin-1, and absence of surfactant protein C. Accounting for the barrier properties, inter-digitations sealed with tight junctions and desmosomes were also observed. Low permeability of the hydrophilic marker sodium fluorescein confirmed the suitability of hAELVi cells for *in vitro* transport studies across the alveolar epithelium. These results suggest that hAELVi cells reflect the essential features of the air-blood barrier, as needed for an alternative to animal testing to study absorption and toxicity of inhaled drugs, chemicals and nanomaterials.

Keywords: lentivirus immortalization, ATI cells, pulmonary drug delivery, inhalation toxicology

1 Introduction

The alveolar epithelium of the peripheral lung is mainly comprised of two cell types, alveolar type I (ATI) and alveolar type II (ATII) cells, both forming the air-blood barrier, which is responsible for gas exchange. While the cuboidal ATII cells produce lung surfactant proteins to reduce the alveolar surface tension, the squamous ATI cells essentially represent the barrier between blood and air space of the lung; they form a tight epithelium sealed by intercellular connections, i.e., tight junction complexes (Crandall and Matthay, 2001). ATI cells cover about 95% of the alveolar surface (Crapo et al., 1982) and shield the organism from outer influences, like inhaled toxins, particles or microorganisms. While paracellular transport is thus restricted, transport of solutes mainly occurs via transcellular pathways, either by passive diffusion or by active transport via various transporter proteins (Endter et al., 2009).

Isolated human ATI-like cells in primary culture (hAEpC) are to date the best model to reflect the *in vivo* situation of the air-blood barrier due to their ability to form tight junctions and hence to exhibit high trans-epithelial electrical resistance (TEER). The latter is a widely accepted parameter for tightness of an epithelium and its barrier function. hAEpC is the most reliable alveolar cell model reflecting the *in vivo* situation of the air-blood barrier (ABB), especially regarding the study of absorption and transport of xenobiotics (Bur et al., 2006; Endter et al., 2009; Forbes and Ehrhardt, 2005). The primary cell model is also used in the context of infection research, e.g., Chan et al. investigated the

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pro-inflammatory response induced by influenza viruses (Chan et al., 2005). Furthermore, hAEpC cells are applied in advanced co-culture models for various purposes, e.g., to investigate the interaction of epithelial cells and immune cells, like human blood monocyte-derived macrophages and dendritic cells (Blank et al., 2011; Lehmann et al., 2011). Nevertheless, primary cells are limited by certain factors, including availability, short life span, inter-individual differences, and health status of the donor, which may negatively affect reproducibility and also preclude their use in high-throughput screening of new drug candidates (Daum et al., 2012; Elbert et al., 1999; Fuchs et al., 2003).

A number of human (Ehrhardt et al., 2003; Forbes, 2003; Foster et al., 1998; Grainger et al., 2006) and non-human (Horálková et al., 2009; Rosenberger et al., 2014) lung epithelial cell lines are commercially available; however, in terms of mimicking the tight barrier formed by ATI cells, they have limited application. The human alveolar cell line A549, derived from an adenocarcinoma and commonly used in toxicity studies (Roggen et al., 2006), does not exhibit high TEER values and is therefore not well suited for drug absorption studies (Foster et al., 1998). A new human ATI cell line (TT1), recently described by Tetley et al. (van den Bogaard et al., 2009), was obtained through the immortalization of primary ATII and used as a model for inflammatory response studies and nanoparticle uptake. However, TT1 cells do not appear to develop tight intercellular junctions, and therefore still lack important barrier properties (van den Bogaard et al., 2009; Kemp et al., 2008). Other widely used lung cell lines are 16HBE14o- and Calu-3, but they are both of bronchial origin and therefore – although they build TEER of 300-600 Ω*cm^2 – are not suitable as a model of the alveolar epithelium (Ehrhardt et al., 2003; Forbes, 2003; Grainger et al., 2006).

Thus, there is a need for cell lines reflecting the specific properties of alveolar cells required for investigating biological aspects of the respiratory tract, such as infection pathways in the context of aerosol transmitted infectious diseases. To minimize the use of animal tests for the safety assessment of inhaled drugs, chemicals and (nano-) materials according to the 3R principle (replace, reduce, refine), new *in vitro* models of the air-blood barrier are of utmost importance. Given the lack of a suitable cell line, a novel immortalization regimen was applied in this study, leading to the generation of a new human alveolar immortalized cell line, namely human alveolar epithelial lentivirus immortalized (hAELVi) cells, through viral transduction of a defined set of immortalizing genes. Two clones of this cell line were obtained (hAELVi.A and hAELVi.B) and characterized with respect to their morphological and functional properties and their ability to serve as a model of the air-blood barrier as needed for drug transport studies.

2 Materials and methods

2.1 Isolation of human primary alveolar epithelial cells (hAEpC)

Primary alveolar epithelial cells (hAEpC) were isolated according to the established protocol by Daum et al. (2012). Briefly, the tissue was obtained from the healthy lung areas removed from patients undergoing lung tumor resection surgery. Tissues were used with ethical approval from the *Ärztekammer des Saarlandes* and patients' informed consent. The isolated ATII cells were seeded on fibronectin/collagen-coated six-well cell culture plates and maintained in Small Airway Growth Medium (SAGM) from Lonza, supplemented with 10% (v/v) fetal calf serum (FCS) and 10% (v/v) penicillin/streptomycin (P/S).

2.2 Transfection via lentiviral vectors

The hAEpC cells seeded on 6-well cell culture plates were infected on day 5 of culture with self-inactivating lentiviral vectors. For immortalization a lentiviral gene library composed of 33 different expansion genes was employed (CI-SCREEN gene library; InSCREENeX GmbH; International patent application PCT/EP2011/005528 "Methods and Vectors for Cell Immortalisation"). The expression of these recombinant genes is driven by the SV40 promoter. For infection, the lentiviral vectors were incubated with the primary cells overnight in the presence of 8 µg/ml polybrene (hexadimethrine bromide, Sigma Aldrich) The following day infection medium was aspirated and the cells were further cultivated with the abovementioned culture medium for five weeks until colonies of proliferating cells became apparent. These cells were pooled and further cultivated as polyclonal cell lines.

2.3 Cell culture/growth curves

All cell types, primary as well as transfected, were cultured in SAGM on fibronectin/collagen-coated cell culture plastic or Transwell® filter devices (3470, Corning). The cells were maintained in SAGM, supplemented with 10% fetal calf serum (FCS) and 10% penicillin/streptomycin (P/S). The transfected cells were passaged 1:1 in the first months of culturing when they reached confluence, and afterwards cultivated in a 1:3 ratio every 14 days. The medium was changed every two to three days and the cells incubated at 37°C, 5% CO_2 and 95%. To obtain the growth curves, hAELVi cells, passage 18 (1x10^4 cells per well) were seeded on 24-well plates; thereafter the cells were trypsinized and counted 24 h after seeding and then every 48 h with the CASY® Cell Counter (Roche) (n = 3).

To cultivate the cells under liquid-liquid conditions (LLC) or air-liquid interface (ALI), hAELVi cells were seeded on Transwell® filters (either 1.12 cm² (3460) or 0.33 cm² (3470), Corning). The cells were cultivated for 2 days under LLC (with cell culture medium feeding from both, apical and basolateral sides). The cells were then divided into two groups: some wells continued LLC cultivation, while others were transferred to the air-liquid interface (ALI) condition, in which the cell culture medium is supplied only from the basolateral compartment. Finally, the cells were grown for 14 days under the respective culture conditions at 37°C, 5% CO_2 and 95% humidity. Microplasma controls were performed on a regular schedule and never showed infection.

2.4 TEER measurement

The barrier property of primary cells or cell lines was determined by measuring the trans-epithelial electrical resistance (TEER) as previously described (Daum et al., 2012; Srinivasan et al., 2015). In summary, the cells were placed on a heating plate at 37°C to

avoid temperature shock-related TEER fluctuation while handling the cells under the sterile hood. TEER was measured with a Chopstick electrode and an epithelial voltohmmeter (EVOM) (World Precision Instruments, Sarasota, USA). For measuring TEER of ALI cultures the cells were set up to LLC conditions 2 h prior to measurement by adding SAGM culture medium. ALI culture condition requires no culture medium in the apical compartment and less culture medium in the basolateral compartment compared to LLC conditions. To avoid detachment of the cell monolayer and liquid pressure-related stress, medium was added first to the apical and then to the basolateral compartment. After the measurement the medium was removed, first from the basolateral, then from the apical compartment; hence, the cells were returned to the ALI condition.

2.5 Morphology and ultrastructure

For morphological and ultrastructural characterization hAELVi cells were grown on Transwell® membranes with a pore size of 0.4 µm and a growth area of 1.12 cm^2 (3460, Corning), cultivated under LLC and ALI conditions, and evaluated by cross sections, confocal laser scanning microscopy (CLSM), transmission electron microscopy (TEM) or scanning electron microscopy (SEM).

Histology

hAELVi cells previously grown on Transwell® filters were fixed on days 7 and 14 with 3% paraformaldehyde (PFA) for 30 min at room temperature (RT). Afterwards, the samples were dehydrated with an ethanol dehydration row (35-50-70-95-95-100% for 10 min each), followed by treatment with Histoclear II (Histological Clearing Agent-Fa. National diagnostics) for 10 min. Subsequently the samples were embedded in paraffin (Histowax Embedding Medium-Leica Microsystems) for 1 h, stored at 4°C overnight, and were cut the next day into 4 µm slices using a Microtom-Reichert Jung 2040 Autocut. The sections were stained with hematoxylin/eosin and analyzed with a Zeiss light microscope (Zeiss Imager M1m, Zeiss, Germany) using a 100x objective.

Confocal Laser Scanning Microscopy (CLSM)

Immortalized cells were grown on Transwell® membranes with a pore size of 0.4 µm and growth area of 1.12 cm^2 (3460, Corning) for 7 and 14 days, under LLC and ALI conditions, as described previously. Samples were then fixed with 3% PFA in phosphate buffered saline (PBS) for 30 min at RT. Afterwards, the samples were quenched with 50 mM NH_4Cl/PBS for 10 min and subsequently blocked and permeabilized using a mixture of 0.5% bovine serum albumin (BSA)/0.025% saponin/PBS for 30 min at RT. The primary antibodies against ZO-1 (rabbit anti-ZO-1, Catalog No 61-7300, Invitrogen) and occludin (mouse anti-occludin, Catalog No 33-1500, Invitrogen) were diluted 1:200 in 0.5% BSA/0.025% saponin/PBS and incubated at 4°C overnight. The secondary antibodies for occludin (polyclonal Alexa-Fluor 488 conjugated rabbit anti-mouse, Catalog No. A11059, Invitrogen) and ZO-1 (polyclonal Alexa-Fluor 633, conjugated goat anti-rabbit, Catalog No. A21070, Invitrogen) were diluted in PBS (1:400) and incubated for 1 h at 37°C. The samples were washed with PBS and counterstained with DAPI (1:50,000). Transwell® membranes were then mounted in DAKO medium (Product No. S302380-2, DAKO), as previously described by de Souza Carvalho et al. (2011), and analyzed by confocal laser scanning microscopy (Zeiss LSM710, Zeiss, Germany). Microscopic images of fixed samples were acquired at 1024 x 1024 resolution using a 63X water immersion objective and z-stacks of around 6 µm. Confocal images were analyzed using Zen 2012 software (Carl Zeiss Microscopy GmbH) and Fiji Software (Fiji is a distribution of ImageJ available at http://fiji.sc).

Transmission electron microscopy (TEM)

hAELVi cells (passage 36) were grown on Transwell® membranes, under LLC and ALI conditions, for 12 days. Thereafter, the samples were processed as described by Susewind et al. (2016), with minor modifications. Briefly, the membranes were fixed with a solution containing SAGM plus 1% glutaraldehyde for five minutes at 37°C, followed by incubation with 1% glutaraldehyde in 200 mM HEPES buffer, pH 7.4, overnight at 4°C. For epon embedding the samples were postfixed with 2% OsO_4 (EMA, PA, USA) solution containing 1.5% potassium ferricyanide for one hour on ice, and stained *en bloc* with 1.5% aqueous uranyl acetate (EMS, PA, USA) for 30 min. Cells were then dehydrated at RT using a graded ethanol series (70-80-90-96-(4x)100% for 10 min each) and progressively infiltrated with epoxy resin (50-75-100%) (Sigma-Aldrich). Transwell® membranes with cells were flat embedded and blocks were polymerized overnight at 70°C. Ultrathin sections of 70-80 nm, perpendicular to the filter plane, were cut with a Leica ultramicrotome Ultracut EM UCT (Leica Microsystems, Austria) using an ultra-diamond knife (Diatome, Switzerland) and examined with a CM100 transmission electron microscope (FEI, The Netherlands). The images were recorded digitally with a Quemesa TEM CCD camera (Olympus Soft Imaging Solutions, Germany) and iTEM software (Olympus Soft Imaging Solutions, Germany).

Scanning electron microscopy (SEM)

SEM images were taken with a Zeiss SEM EVO® HD15 (Zeiss, Germany) under high pressure conditions with a secondary electron detector and using 10 kV acceleration voltage. hAELVi cells (passage 41) on Transwell® filters were fixed after 8 days in culture, in either ALI or LLC, in 200 mM HEPES buffer containing 1% glutaraldehyde at 4°C, overnight. The following day, cells were washed twice for 10 min in HEPES buffer and dehydrated with gradual ethanol concentrations (50-60-70-80-90-96-99-100% for 20 min each). The filters were then sputtered with gold and examined by SEM.

2.6 Transport studies

To evaluate the transport of sodium fluorescein (FluNa) across the monolayers, hAELVi.A cells ($1x10^5$ cells/cm^2) were seeded on fibronectin/collagen-coated Transwell® membranes with a pore size of 0.4 µm and a growth area of 1.12 cm^2 (3460, Corning). The cells were cultured under LLC and ALI, respectively, and TEER measurements were performed every other day for

14 days. Transport experiments were then performed according to Elbert et al. (1999) with minor modifications. Briefly, before the cells were washed twice with pre-warmed Krebs-Ringer Buffer (KRB; NaCl 142.03 mM, KCl 2.95 mM, K_2HPO_4*$3H_2O$ 1.49 mM, HEPES 10.07 mM, D-glucose 4.00 mM, $MgCl_2$*$6H_2O$ 1.18 mM, $CaCl_2$*$2H_2O$ 4.22 mM; pH 7.4) and incubated in KRB for 45 min. After measuring the TEER, the medium was aspirated and 520 µl FluNa (10 µg/ml in KRB) ± 16mM EDTA were added to the apical compartment (donor) and 1.7 ml KRB were added to the basolateral compartment (acceptor). Directly after adding the solutions, samples were taken from the donor (20 µl) and the acceptor (200 µl), respectively, and transferred into a 96-well plate to measure the start concentrations. Afterwards, the plates were placed on a MTS orbital shaker (150 rpm; IKA, Germany) in the incubator and 200 µl samples were taken every 30 min, from the basolateral compartment only, for 3 h. Taken volumes were refilled with 200 µl KRB. At the end of the experiment the TEER was measured again and the samples in the 96-well plate were measured with a Tecan® plate reader using wavelengths of 488 nm (em) and 530 nm (ex).

2.7 RNA isolation and real-time PCR

The total RNA from immortalized cell lines, freshly isolated ATII cells, and ATI-like hAEpCs, previously grown on Trans-well® filters, was used to check the expression of Cav-1, SP-C and AQP-5 (Tab. 1). The total RNA was collected from immortalized cell lines on day 14 of cell culture on six-well plates, from freshly isolated ATII cells and from ATI-like hAEpCs on day 8 of cell culture on Transwell® filters. mRNA was isolated with the RNeasy mini kit from Qiagen (Cat. No. 74106). Semi-quantitative real-time PCR was performed with a Bio-Rad CFX96 real-time PCR machine. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as the internal control to correct for variations in the cDNA content among the samples. The data were normalized to the GADPH expression levels and were presented as the average of three independents experiments. The relative gene expression levels were calculated using the comparative Ct (ΔΔCt) method (Livak and Schmittgen, 2001). Sequences of primers and probes are presented in Table 1. The primers were constructed and ordered from Eurofins. Reverse transcription was performed with QuantiTect reverse transcription kit (Qiagen, Cat.No. 205314) and the real-time PCR reaction with QuantiTect Sybr Green PCR kit (Qiagen, Cat.No. 204143). Run information: 7 min 95°C; 40 cycles of 10 sec 95°C, 30 sec 65°C, 30 sec 72°C; and 1 min 65°C. Data analysis was performed with the Bio-Rad CFX Manager software.

Tab. 1: Primers for lung cell-specific marker rtPCR

Marker	Nucleotide sequence (5'→3')/ product length in bp
AQP5 forward	CCTACCATCCTGCAGATCGCGC/210
AQP5 reverse	TGCCACACCGTAGAGGATGCCA/210
SP-C forward	AAGCCCGCAGTGCCTACGTCTA/273
SP-C reverse	TGGATGACCCCGCTTCAGTGGA/273
CAV-1 forward	ACAGTTTTCATCCAGCCACGGGC/202
CAV-1 reverse	GGTGTTTAGGGTCGCGGTTGACC/202
GAPDH forward	GGAGAAGGCTGGGGCTCATTTGC/364
GAPDH reverse	CCCGTTCAGCTCAGGGATGACCT/364

2.8 Chromosome microarray analysis

Genomic DNA samples were extracted from $1x10^6$ cells using NaCl/chloroform and proteinase K treatment. Genomic hybridization was performed with the CytoScan™ HD array (Affymetrix, USA) according to the manufacturer's instructions. The Cytoscan HD array is characterized by 743,000 genotypeable SNP probes and > 1,953,000 non-polymorphism probes. Results were visualized and analyzed with the Chromosome Analysis Suite (ChAS) software package (Affymetrix) based on the GRCh37/hg19 assembly.

2.9 Statistical analysis

Data are representative of 2-3 experiments and shown as mean ± SEM. Two-way ANOVA with Bonferroni's posttest was performed using GraphPad Prism 5 software (GraphPad).

3 Results

hAEpC cells were isolated from human lung as previously described (Daum et al., 2012), cultivated on tissue culture plates and transduced with a small lentiviral gene library composed of 33 different genes. (CI-SCREEN gene library; InSCREENeX GmbH, for details see Section 2). Due to proprietary proceedings, no further information on the implemented genes can be provided at this time.

After transduction, the cells were expanded to establish immortalized cell lines. PCR analysis of the genomic DNA of the immortalized cell lines confirmed integration of the genes Id2, Id3, E7, Bcl2, Core, Myc and Nanog (Fig. S1A[1]). From more than 50 transfections, seven cell lines showed a prolonged life span and two clones, named hAELVi.A and hAELVi.B, developed high TEER values after 15 days in culture (690 Ω*cm^2 and 2400 Ω*cm^2, respectively) (Fig. S1B). These clones were subsequently propagated and used in all further experiments.

Both hAELVi cell lines showed a sigmoidal growth curve, with a characteristic lag phase after seeding, developing into an exponential growth phase and ending in a stationary phase (Fig. 1A), resulting in formation of a monolayer (Fig. 1B).

TEER was determined every day or every other day during 25 days (Fig. 1C). The hAEpC developed a maximum TEER of 2000 Ω*cm^2 at approximately day six to eight, followed by a subsequent decline of TEER value, demonstrating a short life span of around 15 days, a typical behavior for primary cells

[1] Supplementary figures S1-S4 at http://dx.doi.org/10.14573/ altex.1511131s

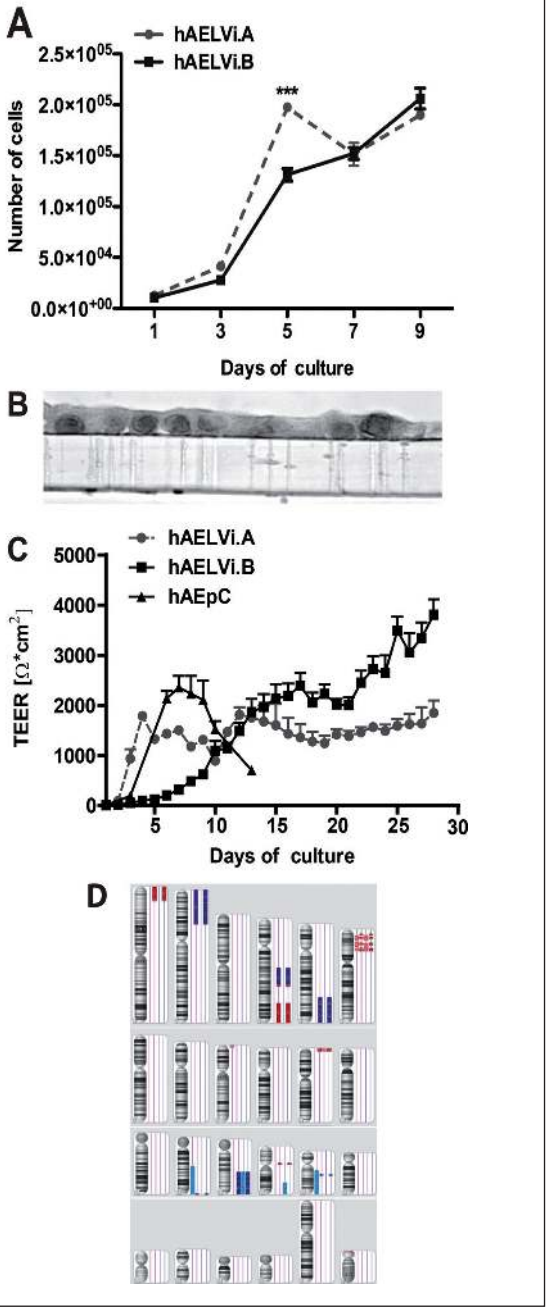

Fig. 1: hAELVi cell line characterization
(A) Growth curve of immortalized cell lines (hAELVi.A and hAELVi.B), grown in 24-well cell culture plates, passage 18, for ten days. (B) Histological cross-sections of hAELVi.A after 7 days in culture under liquid-liquid conditions (100x). (C) Comparative TEER curve of immortalized cells, both in passage 36, and primary ATI-like cells (hAEpC), growing on Transwell® membranes. (D) Chromosome profiling was performed with Affymetrix Cytoscan HD array. For each immortalized cell line an overview of the karyotype at cell culture passage 19 is presented: hAELVi.A (pink) and hAELVi.B (purple). Loss of a chromosome region is indicated as a red sign and duplication of a chromosome region is indicated as a blue sign. Data shown as mean ± SEM (n = 3); ***p < 0.001.

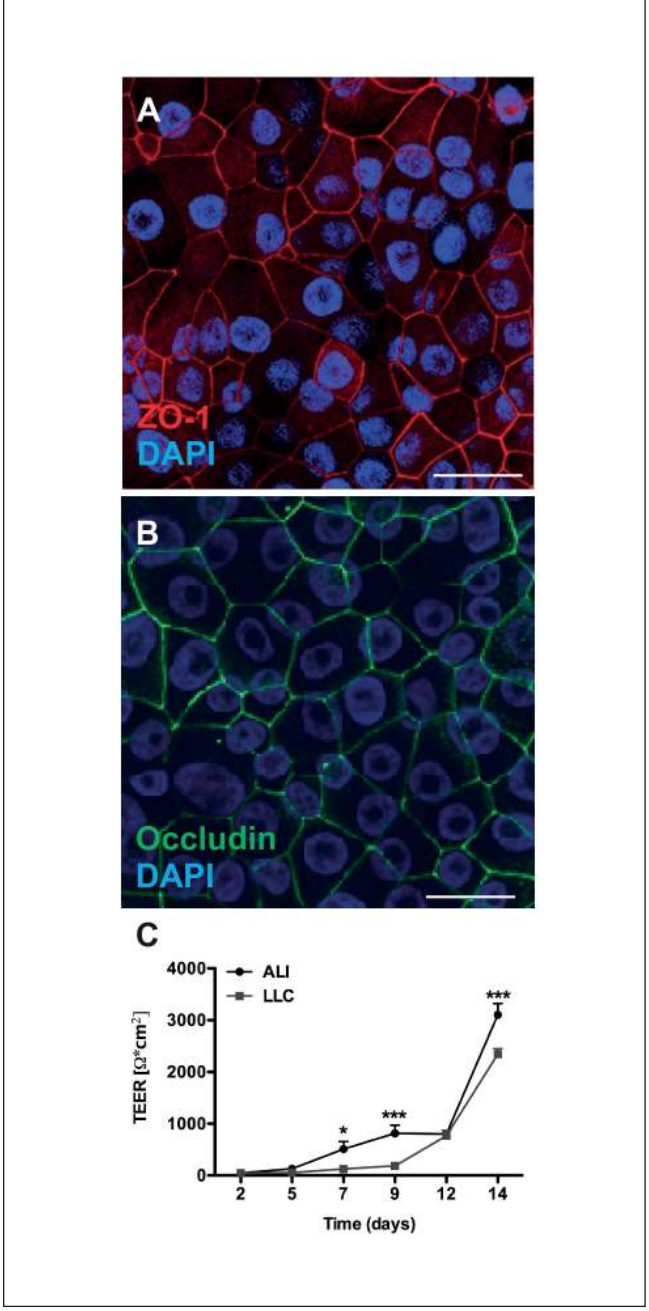

Fig. 2: Alveolar type I cell characteristics in hAELVi cells
(A,B) Z-stacks of images from confocal laser scanning microscopy of hAELVi.B growing under liquid-liquid conditions (LLC) for 14 days, immunolabeled with anti-ZO-1 (red) antibody (A) or anti-occludin (green) antibody (B). Nuclei stained with DAPI (blue). Bar: 20 µM. (C) TEER measurement of hAELVi.B growing up to 14 days under LLC and air-liquid interface (ALI).

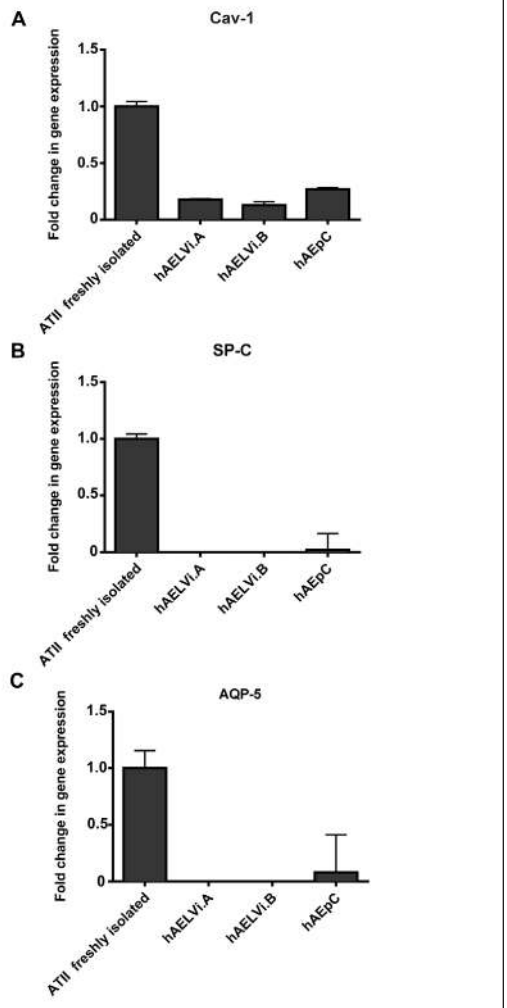

Fig. 3: rtPCR of lung markers
(A) Caveolin-1 (CAV), (B) surfactant protein C (SPC) and (C) aquaporin-5 (AQP). The relative quantification of gene expression in ATII freshly isolated cells was taken as value 1 and correlated to the gene expression in hAELVi.A, hAELVi.B and hAEpC. Data shown as mean ± SEM (n = 3)

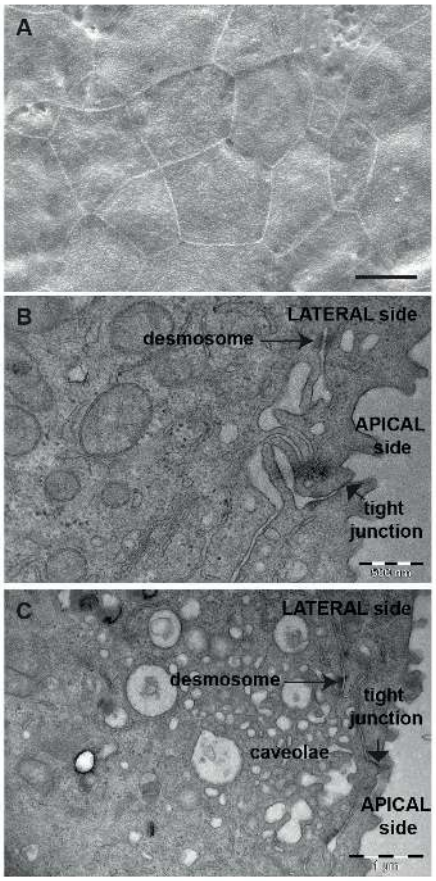

Fig. 4: Ultrastructure of hAELVi cells grown under LLC
(A) Scanning electron microscopy and (B and C) transmission electron microscopy of hAELVi.B on Transwell® after 12 days. Desmosomes (arrow), tight junctions (arrowhead) and caveolae are indicated in the TEM images. Bars (A: 5 µM; B: 500 nm; C: 1 µM).

(Daum et al., 2012; Fuchs et al., 2003). hAELVi cells, depicted in the same graph, reached a TEER of 2000 Ω*cm^2 six to ten days later that was conserved for up to 25 days. Higher TEER (~3000 Ω*cm^2) was also observed in hAELVi cells cultivated up to passage 75 (Fig. S2A) and for different passages over 14 days in culture (Fig. S2B). Genomic DNA from hAELVi cells was investigated for ploidy variation (Fig. 1D, Fig. S2C). In brief, 13 chromosomes revealed no alterations in copy number, in neither an earlier (Fig. 1D) nor a late passage (Fig. S2C). The remaining chromosomes revealed several deleted or duplicated chromosome regions.

To confirm the formation of tight junctions we investigated the expression of characteristic proteins – zona occludens (ZO-1) and occludin in hAELVi cells (Fig. 2A,B) grown under liquid-liquid conditions (LLC). A densely packed monolayer with clearly labeled tight junction complexes appeared as a continuous thin line between adjacent cells (Fig. 2A,B). Under air-liquid interface (ALI) conditions hAELVi.B showed higher TEER up to 500 Ω*cm^2 after seven days compared to 123 Ω*cm^2 observed with LCC, in the same period of time (Fig. 2C). After 12 days in culture the cells reached even higher TEER values (up to 2000 Ω*cm^2) in both conditions. The cells culti-

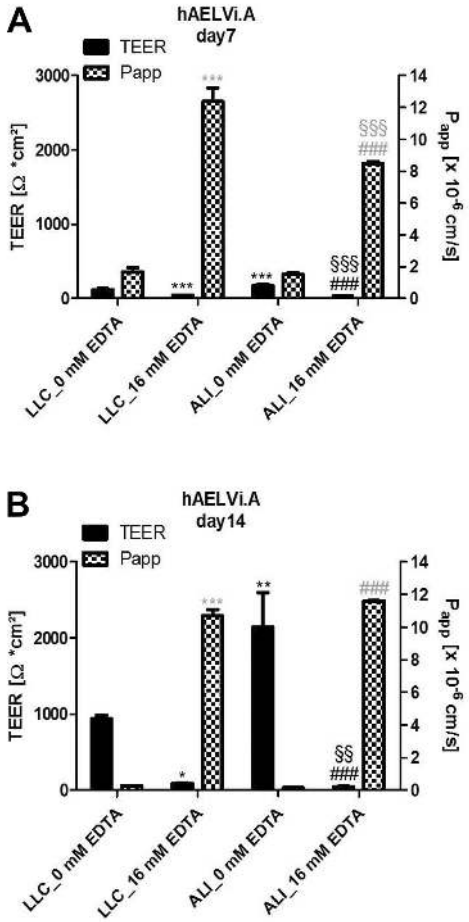

Fig. 5: Permeability assay in hAELVi cells

Transport of sodium fluorescein (FluNa) across a monolayer of hAELVi.A after (A) 7 days or (B) 14 days, cultivated under LCC and ALI, respectively. Both graphs show the relation of TEER and Papp. Data shown as mean ± SEM (n = 3); *p < 0.05; **p < 0.01; ***p < 0.001 vs. LLC_0 mM EDTA; ###p < 0.001 vs. ALI_0mM EDTA; §§p < 0.01; §§§p < 0.001 vs. LLC_16 mM EDTA.

vated under ALI conditions for seven days (Fig. S3A) showed no differences in the monolayer compared to those grown for 14 days under the same conditions (Fig. S3B); also, no difference was observed in the tight junction formation, as shown by ZO-1 immunolabeling after 7 or 14 days (Fig. S3C and S3D, respectively).

Real-time PCR (rtPCR) showed the expression of alveolar epithelial cell-specific markers in hAELVi cells compared to the hAEpC (Fig. 3). The markers caveolin (CAV-1), surfactant protein C (SP-C) and aquaporin (AQP-5) were chosen based on their known cell type-specific expression. We compared the expression level of these three proteins relative to the housekeeping gene GAPDH (Tab. 1); the results are shown (as a percentage of their expression levels) relative to freshly isolated human ATII cells. The expression of CAV-1 for both hAELVi cell lines was at a similar level as for primary culture type I like hAEpC (Fig. 3A). The type II cell marker SP-C however, could not be detected in either hAELVi cell line and was only marginally expressed in hAEpC (Fig. 3B). Conversely, AQP-5 was expressed at a low level in primary hAEpC, but could not be detected in the immortalized hAELVi cells (Fig. 3C).

As expected from CLSM results (Fig. 2 and Fig. S3), no difference was observed in the ultrastructure of cells cultivated under both conditions. SEM images showed the monolayer with clear cell-cell contacts (Fig. 4A and Fig. S4A) and TEM pictures showed inter-digitations between the cells, sealed with tight junction complexes and desmosomes (Fig. 4B and Fig. S4B). Other typical ATI cell structures observed close to the apical cell membrane were the caveolae (Newman et al., 1999) (Fig. 4C and Fig. S4C). These results, together with the observations regarding the tight junction complexes and TEER values corroborate the ATI-like character of the new cell line hAELVi.

The potential use of hAELVi cells as a model to predict drug absorption kinetics was also evaluated in cultures under LLC and ALI conditions, for 7 (Fig. 5A) or 14 days (Fig. 5B), by measuring the permeability of the hydrophilic molecule sodium fluorescein (FluNa), typically used as a paracellular transport maker. Higher TEER is always accompanied with lower paracellular transport, and this relationship could be observed at day 7 and even more prominently at day 14, when the cells displayed a TEER of more than 1000 Ω*cm^2. In the presence of EDTA as a modulator of the tight junctions, the TEER dropped to almost zero and the Papp value of FluNa reached essentially the same maximal level, indicating complete opening of the tight junctions under such conditions, with more FluNa transported to the basolateral compartment.

4 Discussion

Here, we report the generation of a new immortalized type I cell line from human alveolar epithelium that exhibits functional tight junctions and hence high TEER values.

Following a novel approach to reduce the possible de-differentiation of cells caused by transformation, the cells were transduced with a set of 33 genes, which are able to immortalize various cell types, producing cell lines with conserved physiological functionality. This is different from carcinoma-derived cell lines, which are beneficial for studying cancer development, e.g., a blastoma-derived cell line (Camerlingo et al., 2011) or an *in vitro* carcinogenesis model, where ATII cells are exposed to tobacco-specific carcinogens (Mennecier et al., 2014) but hardly represent normal epithelial cells. The widely used A549 cell line, derived from an adenocarcinoma, which is commonly employed as a model of the lung epithelium, has a rather ATII-like phenotype, including lamellar bodies (Shapiro et al., 1978). However, it has been used in drug transport studies (Wang and Zhang, 2004) despite its lack of barrier formation ability. A pre-

viously reported immortalization approach with a thermo-sensitive mutant of the SV40 Large T-Antigen led to the development of an immortalized human lung cell line that is well suited for many applications, e.g., nanoparticle-uptake (Kemp et al., 2008) or inflammatory response (van den Bogaard et al., 2009). However, these cells also lack the capacity to form functional tight junctions, which are needed to build a formidable diffusion barrier also to smaller hydrophilic molecules. Consequently, these cell lines are of limited value for conducting transport studies across the notoriously tight alveolar epithelium. Human lung cell lines that do form tight junctions are available, such as Calu-3 (Daum et al., 2009; Grainger et al., 2006) and 16HBE14o- (Forbes, 2003); however, these are derived from the upper airways, show columnar rather than squamous shape, and feature cilia as well as mucus. Due to lack of better alternatives, these cell lines have been used to assess pulmonary drug transport *in vitro*, either alone or in co-culture with other primary cells or cell lines, e.g., A549 together with primary human pulmonary microvascular endothelial cells to form a model of the alveolo-capillary unit (Hermanns et al., 2004); or to develop a model to study the interaction of xenobiotics with the alveolar epithelium by implementing not only epithelial cells, such as A549 or 16HBE14o-, but also human blood monocyte-derived macrophages and dendritic cells (Lehmann et al., 2011). The relevance of such studies to predict transport across the alveolar epithelium, however, must be interpreted very cautiously.

The new cell line hAELVi could offer significant advantages over other cell lines, either as monoculture or in co-culture systems with other cell types for the purpose of drug transport studies. As demonstrated by (ultra)structural and functional studies, hAELVi cells form tight intercellular junctions. Besides serving as a model of the tight alveolar air-blood barrier for drug transport and metabolism studies, they may also help to understand the role of tight junctions in disease development, since it is known that the alteration of tight junction integrity may be important for the course of infections, asthma and cystic fibrosis (Godfrey, 1997; Vermeer et al., 2009). Our results imply that hAELVi cells can be used in LLC and ALI conditions, for which the latter also allows deposition of aerosol particles or dry powder formulations in an existing set-up, the Pharmaceutical Aerosol Deposition Device on Cell Cultures (PADDOCC) (Hein et al., 2011). For the two clones obtained, only minor differences were observed in the time by which the cells start to build functional tight junctions, reflected by higher TEER values: hAELVi.A reached higher TEER values earlier than hAELVi.B, suggesting a faster differentiation. However, no morphological or ultrastructural differences were observed between these cells, and the final TEER as well as its time course during culture was consistently maintained also in higher passages, up to number 75.

At the molecular level the expression of the alveolar epithelial markers Cav-1, SPC and AQP-5 was assessed by rtPCR. CAV-1 is a protein of the caveolae membrane system that plays a role in the signaling platform and in the transport of macromolecules through the cell (Gumbleton, 2001); its expression increases during the transition from the ATII phenotype to the ATI phenotype (Fuchs et al., 2003). SP-C is highly abundant in ATII cells, which are the surfactant-producing cells of the lung epithelium (Phelps and Floros, 1991). AQP-5 is a water channel protein expressed on the apical side of ATI and bronchial epithelium cells (McElroy and Kasper, 2004; Nielsen et al., 1997) and seems to regulate the volume of the ATI cell (King and Agre, 1996).

The presence of caveolae at the plasma membrane, which can be clearly seen in the TEM images as well as the CAV-1 transcripts detected via rtPCR studies, together with the absence of multilamellar bodies and SP-C, indicates that the new immortal cell lines resemble an ATI-like rather than an ATII-like phenotype (Campbell et al., 1999; Fuchs et al., 2003; Gumbleton, 2001; Newman et al., 1999). Small amounts of the ATII cell marker SP-C in ATI-like hAEpC, as detected by rtPCR, could be a result of not yet complete differentiation of the originally isolated ATII cells into the ATI phenotype. The water channel protein AQP-5, often used as an ATI marker, could not be detected in the immortalized hAELVi cell lines. The reasons for this result and its implication for epithelial transport studies deserve further investigation, like immunofluorescence studies, because the marker has been found in cells using labeled antibodies even though it could not be detected at the transcriptional level before (Hermanns et al., 2009).

Another interesting marker, which could provide more information about the differentiation status of hAELVi cells, is the receptor for advanced glycation endproducts (RAGE). This highly selective differentiation marker was shown to be expressed upon the trans-differentiation of ATII into ATI-like cells *in vitro* and to promote the spreading of ATI cells, contributing to achieve their squamous phenotype (Demling et al., 2006). The detection of RAGE at the transcriptional and translational level has to be addressed in future work to further classify the hAELVi cell line.

Nevertheless, transport studies with FluNa showed that the hAELVi cells maintain their barrier properties and thus could be useful for drug permeability studies. Active transporters of peptides, e.g., PEPT-2 (Groneberg et al., 2001) or organic cations OCT (Salomon and Ehrhardt, 2012) as well as efflux systems, such as MDR1/P-gp (Cordon-Cardo et al., 1990) or BCRP (Ejendal and Hrycyna, 2002), should be examined in further experiments to enable the comparison with the primary cells and other cell lines.

In summary, the new cell line hAELVi displays morphological as well as physiological similarities with ATI cells. Most importantly, these cells develop tight intercellular junctions and high TEER (> 1000 Ω*cm^2) resulting in a formidable diffusion barrier to a hydrophilic marker molecule, which can be modulated by EDTA. We expect that the hAELVi cells described here have great potential to become an alternative to animal testing, both in the context of pulmonary drug delivery as well as inhalation toxicology.

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Conflict of interest

Tobias May filed a patent for the immortalization of mammalian cells which covers the immortalization regimen (CI-SCREEN gene library; InSCREENeX GmbH; International patent application PCT/EP2011/005528 "Methods and Vectors for Cell Immortalisation"). Tobias May is shareholder of InSCREENeX GmbH which commercializes immortalized cell lines.

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