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# Lung endothelial cells strengthen, but brain endothelial cells weaken barrier properties of a human alveolar epithelium cell culture model

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## Abstract

The blood-air barrier in the lung consists of the alveolar epithelium, the underlying capillary endothelium, their basement membranes and the interstitial space between the cell layers. Little is known about the interactions between the alveolar and the blood compartment. The aim of the present study was to gain first insights into the possible interplay between these two neighboured cell layers. We established an *in vitro* Transwell model of the alveolar epithelium based on human cell line H441 and investigated the influence of conditioned medium obtained from human lung endothelial cell line HPMEC-ST1.6R on the barrier properties of the H441 layers. As control for tissue specificity H441 layers were exposed to conditioned medium from human brain endothelial cell line hCMEC/D3. Addition of dexamethasone was necessary to obtain stable H441 cell layers. Moreover, dexamethasone increased expression of cell type I markers (caveolin-1, RAGE) and cell type II marker SP-B, whereas decreased the transepithelial electrical resistance (TEER) in a concentration dependent manner. Soluble factors obtained from the lung endothelial cell line increased the barrier significantly proven by TEER values and fluorescein permeability on the functional level and by the differential expression of tight junctional proteins on the molecular level. In contrast to this, soluble factors derived from brain endothelial cells weakened the barrier significantly. In conclusion, soluble factors from lung endothelial cells can strengthen the alveolar epithelium barrier *in vitro*, which suggests communication between endothelial and epithelial cells regulating the integrity of the blood-air barrier.

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**Key words:** alveolar epithelium in vitro model, claudin-1, claudin-3, claudin-4, claudin-5

**Abbreviations:** ALI, acute lung injury; ARDS, acute respiratory distress syndrome, ATI, alveolar epithelial type I; ATII, alveolar epithelial type II; BBB, blood-brain barrier; SP-B, surfactant protein B; SP-C, surfactant protein C; TEER, transepithelial electrical resistance.

## 1. Introduction

The blood-air interface in the lung represents one of the vital barriers in the human body. The alveoli of the human lung - with an area of approximately 100–140 $m^2$ - are functionally the most important element of the lung (Gehr et al., 1978). The alveolar epithelial barrier plays a central role in lung diseases, like acute lung injury (ALI) and its most severe extreme form, the acute respiratory distress syndrome (ARDS) (Maniatis et al., 2008; Matthay, 1994). A key function of the epithelium is the formation of diffusion barriers that allow the generation and maintenance of compartments with different compositions, a fundamental requirement for the physiological organ function like in the lung (Gorin and Stewart, 1979; Balda and Matter, 1998). The lung alveolar epithelium *in vivo* consists of two epithelial cell types, the terminally differentiated squamous alveolar epithelial type I (ATI) cell, which constitutes approximately 93% of the alveolar epithelial surface area, and the surfactant-producing cuboidal alveolar epithelial type II (ATII) cell (Crapo et al., 1992). The major functions of ATII cells are surfactant synthesis, transepithelial ions and water movement as well as regeneration of alveolar epithelium after lung injury

(Uhal, 1997). The main functions of ATI cells are the control of peptide growth factors metabolism, transcellular ion transport, alveolar fluid regulation and immune modulation (Williams, 2003). It is believed that ATII cells are the sole progenitor for ATI cells (Fehrenbach, 2001). In ATI cells the caveolin-1 synthesis and the formation of caveolae are discussed as a characteristic feature of an ATI-like cell phenotype in the alveolar epithelium *in vivo* and *in vitro* (Campbell et al., 1999; Kasper et al., 1998; Kunzmann et al., 2011; Matthay, 1994). The caveolae membrane system is of interest because of its important transport function of molecules across the blood-air barrier in the lung (Gumbleton et al., 2000). As a second ATI marker the receptor for advanced glycation end-products (RAGE) was discussed, especially as a marker of ATI cell injury in ALI (Uchida et al., 2006). In comparison, surfactant proteins are restricted to the ATII phenotype; especially surfactant protein B (SP-B) and C (SP-C) are known to be synthesized with a high specificity by ATII cells (Phelps and Floros, 1991a; Weaver et al., 1988). In addition, glucocorticoids enhance fetal lung maturation by increasing production of SP-B in ATII cells (Phelps and Floros, 1991b; Ladenburger et al., 2010). Cell-cell contacts within the alveolar epithelium are sealed by tight junctions. Tight junctions, located at the most apical region of lateral membranes of epithelial cells, create a paracellular barrier in epithelial and endothelial cell layers, which protect the underlying tissue from the external environment (Forster, 2008; Rodriguez-Boulan and Nelson, 1989). Two different classes of integral membrane proteins constitute the tight junction strands in epithelial cells and endothelial cells, occludin and members of the claudin protein family, which are responsible for changes in the electrolyte and solute permeability in cells layers (Forster, 2008). The flux of fluid, ions, macromolecules, and inflammatory cells across airway epithelium depends in part upon the integrity of its apico-lateral tight junctions (Godfrey, 1997).

Because of the complexity of the pulmonary alveolar system, little is known about the molecular interactions between alveolar epithelial and endothelial cells. Up to now only few reports were published supporting the idea of a functional unit at the blood-air barrier consisting of alveolar epithelial and endothelial cells (Hermanns et al., 2004, 2009, 2010). Therefore, the establishment of *in vitro* models of the alveolar epithelial barrier, which could mimic different conditions in the alveolar region, would be useful to analyze the role of lung endothelial cells. Ideally, the *in vitro* model would form a tight barrier and consist of cells expressing ATI and ATII cell markers such as caveolin-1, RAGE and SP-B. NCI H441 cells have emerged as a well-established model system for distal pulmonary epithelial cells (Hermanns et al., 2004; Shlyonsky et al., 2005).

The aim of this study was to establish an alveolar cell culture model based on human cell line H441 and to use this model to study the influence of soluble factors derived from lung endothelial cells (HPMEC-ST1.6R) on the alveolar epithelium barrier. Human cell line HPMEC-ST1.6R was chosen as a model of the lung endothelium since it was reported that only HPMEC-ST1.6R exhibited the major constitutive and inducible endothelial cell characteristics and showed an angiogenic response on Matrigel similar to that of primary HPMEC in contrast to several other endothelial cell lines (Unger et al., 2002). Human brain endothelial cell line hCMEC/D3 represents an established model of the human blood-brain barrier (Weksler et al., 2005) and was used to check if possible effects of the lung endothelial cells on the alveolar epithelium model are tissue-specific.

# 2. Material and Methods

## 2.1. Cell culture

Human lung epithelial cell line H441 was purchased from ATCC. Cells were maintained in RPMI1640 (R8758, Sigma, Munich, Germany) supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mol/l Glutamax I, 1x ITS (100x ITS, 41400-045) (all from Gibco®, Life Technologies GmbH, Darmstadt, Germany) and 1 mol/l Na-pyruvate (L0473, Biochrom, Berlin, Germany) and were subcultured in a ratio 1:6 on gelatine (0.5%) coated cell culture tissue flasks (GreinerBioone, Frickenhausen, Germany) once a week. Human lung endothelial cell line HPMEC-ST1.6R were a kind gift from Prof. Kirkpatrick and Dr. Unger of the University Hospital Mainz in Germany (Krump-Konvalinkova et al., 2001). They were cultured on gelatine coated flasks in M199 supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mol/l Glutamax I (all from Gibco®, Life Technologies GmbH), 25 µg/ml sodium heparin (H3149, Sigma) and 25 µg/ml ECGS (E2759, Sigma) and were trypsinized in a ratio of 1:8 once a week. Human brain endothelial cell line hCMEC/D3 were obtained from Prof. Couraud (Weksler et al., 2005) and were maintained in EBM-2 (C-4147, Lonza, Cologne, Germany) medium supplemented according to the manufacturer's instruction with the components of the EGM®-2 bullet kit (C-3162, Lonza, FBS, VEGF, $R^3$-IGF-1, hEGF, hbFGF, heparin, ascorbic acid, gentamycine and amphotericin B) without hydrocortisone. hCMEC/D3 cells were splitted in a ratio of 1:3 onto gelatine coated cell culture flasks once a week. Growth media were renewed every 2-3 days. In order to obtain soluble factors secreted from HPMEC-ST1.6R and hCMEC/D3 cells growth medium supernatants were collected from non-confluent cell layers after 3-5 days after seeding on 75 $cm^2$

tissue flasks and sterile-filtered before usage. The terms ST1, ST1-cond., D3 and D3-cond. are used in the text for fresh or conditioned (cond.) growth medium of the corresponding cells (ST1=HPMEC-ST1.6R, D3=hCMEC/D3). H441 cells were used between passages 56-76, HPMEC-ST1.6R between passages 30-37 and hCMEC/D3 between passages 17-34.

### 2.2. Transwell model experiments

For Transwell insert experiments H441 cells were seeded at a cell density of 23,000 cells/cm$^2$ onto collagen-I (0.01%, C7661, Sigma) coated 6-well inserts (353090, BD, Heidelberg, Germany, pore size 0,4 µm, PVDF, transparent, apical volume 2 ml, basolateral volume 3 ml). Medium was renewed every 2-3 days. After five days of culture it was started to add dexamethasone (10, 100, 300 or 1000 nmol/l) to the apical side with every medium change in a ratio of 1:1000 of the appropriate ethanolic stock solution. In order to assess the development of the barrier properties the transepithelial electrical resistance (TEER) was determined using a WPI device with chopstick electrodes (World Presicion Instruments, Berlin, Germany) as previously published (Neuhaus et al., 2008). In addition to the TEER measurement, transport studies with the paracellular marker fluorescein were undertaken. 10 µmol/l fluorescein was added in the apical compartment to begin the transport studies. 300 µl samples were taken after 15, 45, 105, 165 and 225 minutes from the basolateral side and were replaced after each sampling step with 300 µl fresh, prewarmed H441 medium to maintain the same hydrostatic pressure conditions during the entire transport experiment. Fluorescence of samples, stock solution and supernatants of the apical compartment after the end of the experiment (90 µl/well in a black 96-well plate from GreinerBioone) were measured with a Tecan GeniosPro (excitation

wavelength: 485 nm; emission wavelength: 535 nm). Calculation of the permeability coefficients was accomplished according the clearance principle as previously published (Neuhaus et al., 2008) and described in the supplementary file in detail. In order to ease the comparison between different experiments, the permeability coefficient values of the control experiments were set to 100% and the other results within the same experiment were normalized to the 100% of the control experiment.

### 2.3. mRNA analysis

mRNA analysis is described in the supplementary file in detail. In brief. mRNA of cells grown on Transwell inserts was isolated with a Nucleospin-RNAII Kit (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions. RNA concentrations were determined by means of a Nanodrop ND 2000 spectrophotometer (FisherScientific, Schwerte, Germany). 1 µg total RNA per sample were reversely transcribed to 20 µl cDNA by means of the high capacity cDNA-kit (with random primer and RNAse inhibitor) from Applied Biosystems (Life Technologies GmbH, Darmstadt, Germany) according to the manufacturer's instruction. qPCR analysis were performed using either FAM-labelled probes for 18SrRNA (endogenous control, EUK18SrRNA, 4352930-0810022, Taqman®, Applied Biosystems), SP-B (Hs00167036_m1 Taqman®, Applied Biosystems), caveolin-1 (Hs00971716_m1; Taqman®, Applied Biosystems) and RAGE (Hs00153957_m1, Taqman®, Applied Biosystems) or primers from MWG Eurofins (Ebersberg, Germany) for claudin-1, claudin-3, claudin-4, claudin-5, occludin, ZO-1, and 18SrRNA. Primer sequences and detailed qPCR conditions are described in the supplementary file. qPCR analysis were conducted by means of a 7300 Real-Time PCR System (Applied Biosystems). Each sample was analyzed as triplicate. Relative

mRNA abundances to 18S rRNA were calculated by the dCt method using following formula: $2^{(\text{Ct of 18S rRNA-Ct of gene of interest})}$, where Ct is the threshold cycle value. For the qualitative proof of the presence of tight junctional proteins cDNA samples were amplified with a 2720 Thermal Cycler (Applied Biosystems) and amplification products were separated on a 2 % agarose gel in TAE-buffer with a peqGOLD 50 bp DNA-ladder (PEQLAB Biotechnologie GmbH, Erlangen, Germany) as marker at 90 V for half an hour. Bands were then visualised by UV-excited ethidium bromide using an ImageMaster VDS (Pharmacia Biotech).

### 2.4. Western blotting

Western blotting analysis is described in the supplementary file in detail. In brief, H441 cells cultured on 6-well inserts were scraped in RIPA buffer on ice. Sample's protein concentrations were determined by a Pierce BCA assay (FisherScientific). 20 µg protein per lane for claudin-1,-3,-4 and RAGE, 30 µg protein per lane for claudin-5, SP-B and caveolin-1 analysis were loaded onto 15% SDS-PAGE gels. After gel electrophoresis proteins were immunoblotted onto PVDF membranes by means of a semi-dry blotter. Membranes were blocked with 5% milk powder in PBS for 1 hour and primary antibodies were applied in 0.5% BSA/PBS solution at 4°C over night. Antibodies against claudin-1, claudin-3, claudin-5 (all 1:200), SP-B and caveolin-1 (1:100) were rabbit polyclonal (519000, 341700, 341600, Zymed®, Life technologies; sc-894, sc-13978, Santa Cruz Biotechnology, Heidelberg, Germany), antibodies against claudin-4 (1:200, 329400, Zymed®, Life technologies) and β-actin (1:15000, A5441, Sigma) were mouse monoclonal and antibody against RAGE (1:100, sc-8230, Santa Cruz Biotechnoloy) was goat polyclonal. Membranes were washed with 0.1% Tween 20/PBS for three times for 10 minutes and were blocked a further time with

5% milk powder/PBS for 25 minutes at RT. Afterwards membranes were incubated with secondary HRP-labelled antibodies diluted 1:1000-5000 in 1% BSA/PBS at RT for 1 hour and was then washed for three times with 0.1% Tween 20/PBS. To visualize the bands western blots were incubated with ECL-solutions for 2 minutes and were developed using a FluorChem FC2 Multiimager II (Alpha Innotech). Density values of the single tight junction protein bands were calculated with the software Alpha View and were related to the appropriate β-actin bands.

### 2.5. Immunofluorescence microscopy

Immunofluorescence microscopy was carried out as published recently (Neuhaus et al., 2008) and is described in the supplementary file in detail.

### 2.6. Data analysis

Data are presented as means±SD. The statistical significance between culture conditions was determined by a t-test utilizing SigmaStat3.5 software. In case of test failure on normality or equality of variance of the used data set, a Mann-Whitney Rank Sum Test was carried out. A probability value less than 0.05 was considered statistically significant.

## 3. Results

### 3.1. Dexamethasone influences tightness of the barrier in a concentration dependent manner

After five days of culture of H441 cells onto 6-well Transwell inserts, the surface was approximately 80% confluent and dexamethasone was added apically in different

concentrations. Figure 1A shows the time course of TEER values of H441 cell layers treated with dexamethasone. H441 cell layers grown in medium without dexamethasone started to form holes on days 8-10 until cells completely detached on days 13-15. TEER values were partly still measurable in these time frames, but light microscopic control revealed holes and detachment of the cells indicating that dexamethasone was necessary to stabilize the confluent layer onto the inserts after reaching confluence. Highest TEER values of confluent cell layers were detected on days 14-15. Addition of 10 nmol/l dexamethasone increased TEER from 122 ± 33 Ohm*cm$^2$ to 438 ± 9 Ohm*cm$^2$ on day 15, whereas 100 nmol/l elevated TEER only to 322 ± 17 Ohm*cm$^2$ ($p<0.05$, mean ± SD, n=3). This concentration dependent negative tendency was confirmed including data of 30 nmol/l (400 ± 16 Ohm*cm$^2$), 300 nmol/l (245 ± 13 Ohm*cm$^2$) and 1 µmol/l (207 ± 11 Ohm*cm$^2$) dexamethasone.

### 3.2. Tight junction proteins claudin-1,-3,-4,-5, occludin and ZO-1 are expressed in the alveolar epithelium *in vitro* model

In order to investigate the presence of some main tight junctional proteins in our model, mRNA analysis of claudin-1, claudin-3, claudin-4, claudin-5, occludin and ZO-1 of H441 samples of day 15 treated with 100 nmol/l dexamethasone (apical addition) was accomplished. PCR products separated on an agarose gel confirmed the presence of all analyzed tight junctional molecules, but the band of claudin-5 was very weak indicating low expression (figure 1B). Thus, qPCR with GAPDH as endogenous control was carried out in order to rank the abundances of the tight junctional proteins which were then related to claudin-1. Claudin-4 was the most abundant (7-fold) tight junctional protein, followed by occludin (4-fold), ZO-1 (1.24-fold), claudin-1 (1-fold) and claudin-3 (0.46-fold). Confirming the agarose gel result

claudin-5 exhibited the weakest, but detectable mRNA expression (0.001-fold). In order to confirm the presence of tight junction proteins on the protein level, immunofluorescence images of H441 cells (day 15) treated with 100 nmol/l dexamethasone were generated. As shown in figure 1C, pictures demonstrate the presence of claudin-1, claudin-3, claudin-4, claudin-5, occludin and ZO-1 in H441 cells grown on Transwell inserts for 15 days also on the protein level. In concordance with the low mRNA expression of claudin-5, only for claudin-5 it was not able to present a continuous tight junction network over the entire H441 cell layer.

### 3.3. Dexamethasone modulates expression of ATI markers caveolin-1 and RAGE as well as of ATII marker SP-B

One major aim was to generate a model consisting of cells expressing ATI as well as ATII markers in order to mimic both types of alveolar epithelial cells in one model. Therefore, the expression of mRNA for the ATI markers, caveolin-1 and RAGE, as well as the ATII marker, SP-B, was examined in the presence and absence of dexamethasone by qPCR. For this, H441 cells were grown on Transwell inserts as before for the TEER-experiments and were lysed for mRNA analysis on days 7 and 15. ATI markers caveolin-1 and RAGE were regulated in a similar manner. As shown in figure 2A, mRNA expression of caveolin-1 as well as RAGE did not change significantly on day 7 after apical addition of 10 or 100 nmol/l dexamethasone on day 5, but was significantly decreased to 0.37-fold (caveolin-1, 10 nmol/l dexamethasone, $p<0.05$) or to 0.64-fold (RAGE, 10 nmol/l dexamethasone, $p<0.05$) on day 15 in comparison to the non-treated control of day 7. Interestingly, the treatment of H441 cells with 100 nmol/l dexamethasone revealed a statistically significant increase of caveolin-1 ($p<0.05$) and RAGE ($p<0.05$) mRNA expression compared to the 10

nmol/l dexamethasone treated cells on day 15. Untreated H441 samples of day 15 were not obtainable since the cells started to detach at days 8-10 as mentioned above. In case of cell type 2 marker SP-B, dexamethasone induced SP-B expression very significantly in a concentration dependent manner. For example, 10 nmol/l dexamethasone increased SP-B expression on day 7 7.9-fold ($p<0.05$). which was further increased by 100 nmol/l to 107.8-fold ($p<0.05$). On day 15 SP-B mRNA expression of dexamethasone treated samples was still higher than on day 7 without dexamethasone, but decreased in comparison to day 7 dexamethasone treated samples. In detail, H441-cells treated with 10 nmol/l dexamethasone revealed a 2.3-fold SP-B expression and 100 nmol/l dexamethasone application still increased SP-B expression 16-fold in comparison to the 7 days old untreated cell samples ($p<0.05$). In order to confirm the presence of type 1 and type 2 cell markers caveolin-1, RAGE and SP-B on the protein level immunofluorescence images of H441 cells (day 15) treated with 100 nmol/l dexamethasone were generated. As shown in figure 2B, pictures demonstrate the presence of caveolin-1, RAGE and SP-B in H441 cells grown on Transwell inserts for 15 days also on the protein level. Caveolin-1 was found at the cell boarders, whereas RAGE and SP-B were recognizable in the cytosol.

### 3.4. Soluble factors derived from lung endothelial cells enhance, but from brain endothelial cells decrease the tightness of the alveolar epithelial barrier

In order to study the effects of soluble factors derived from the lung endothelial cell line HPMEC-ST1.6R, H441 cells were seeded on the apical side of the Transwell inserts. In the apical compartment H441 cells were fed with H441 growth medium as before, whereas different growth media were added in the basolateral side during the entire cultivation. The effects of ST1-medium, conditioned ST1-medium, D3-medium

and conditioned D3-medium were studied in comparison to the original H441 medium. Conditioned ST1-medium was obtained from lung endothelial cell line HPMEC-ST1.6R, conditioned D3-medium from human brain microvascular cell line hCMEM/D3, whereas ST1 and D3-medium represent the fresh, unconsumed growth media. It was decided to add 100 nmol/l dexamethasone to the apical side beginning on day 5 in order to obtain cell layers with a distinct barrier (ATI cell feature), but also a maximum of SP-B expression (ATII cell feature). Highest tightness of H441 cell layers was achieved after 15 days of culture (figure 1A), thus data comparison focused on this time point.

Figure 3 depicts the influence of the different growth media on TEER and permeability of the paracellular marker fluorescein across H441 cell layers. In comparison to the standard H441 medium (set to 100%), basolateral addition of conditioned ST1 medium increased TEER of H441 cell layers significantly to 127.1 ± 6.4 % ($p<0.05$). In contrast to ST1 media, basolaterally added conditioned D3 medium derived from the human blood-brain barrier cell line hCMEC/D3 decreased TEER to 44.1 ± 9.7 %, which was significantly different from both H441 and fresh D3 medium (both $p<0.01$).

In order to support TEER data, additional permeability experiments with the paracellular marker fluorescein were carried out. Calculated permeability coefficients were between 0.22-3.35 µm/min. To facilitate data comparability, permeability coefficients obtained across cell layers grown in H441 medium in both compartments were set to 100 % and the others were related to them. Basolaterally added ST1 medium decreased fluorescein permeability to 85.3 ± 4.7 %, which was further decreased by conditioned ST1 medium to 61.1 ± 1.5 %. These differences were statistically significant (ST1 vs. H441: $p<0.05$, ST1 cond. vs. H441: $p<0.01$, ST1 vs.

ST1 cond.: $p<0.01$) and confirmed TEER data that soluble factors secreted from lung endothelial cells maintain or even enhance the barrier in our alveolar epithelium *in vitro* model. In concordance to the TEER data, application of conditioned D3 medium increased the permeability of fluorescein to 279.2 ± 4.6 % in comparison to basolateral H441 medium. Basolateral fresh D3 medium did not change the permeability of fluorescein significantly.

### 3.5. Alterations of the expression patterns of claudins accord with tightness changes of the alveolar barrier

In order to investigate the functional changes of the tightness on the molecular level the expression of claudin-1, -3, -4 and -5 were analyzed. The changes of mRNA expression of claudin-1 and -3 matched well to the observed TEER values (Figure 4). mRNA expression of claudin-4 was interestingly increased by both conditioned media (ST-1 cond.: 1.28 ± 0.19 –fold; D3 cond.: 1.48 ± 0.11-fold) and were statistically significant in comparison to their corresponding fresh media. In the case of claudin-5 no significant changes were detected.

In order to confirm mRNA data and to include possible non-genomic effects on the tight junction molecules, the expression of the proteins was also analyzed by western blotting. The influence of growth media on the protein expression of claudin-1 and claudin-3 was similar to the effects onto the mRNA expression as well as on TEER. However, only the differences of samples treated with conditioned D3-medium (claudin-1: 46.7 ± 0.05 %; claudin-3: 68.9 ± 0.06 %) exhibited a statistically significant reduction in comparison to the fresh D3 medium (claudin-1: 91.1 ± 0.08 %; claudin-3: 144 ± 29.9 %) as well as to the H441-medium ($p<0.05$, mean ± SD, n=3). In the case of claudin-4 no significant difference was detected on the protein

level. In contrast to mRNA data of claudin-5, analysis of protein amounts of claudin-5 revealed significant differences. ST1 medium increased claudin-5 expression to 192.1 ± 69.1 % ($p<0.05$, mean ± SD, n=3), which was further elevated to 225.6 % by conditioned ST1 medium, whereas conditioned medium of brain endothelial cells (D3 cond.) decreased claudin-5 expression to 48.8 ± 15.7 % ($p<0.05$, mean ± SD, n=3)

### 3.6. Soluble factors of lung and brain endothelial cells modulate the expression of ATI markers caveolin-1 and RAGE as well as of ATII marker SP-B

In order to assess whether the changes of the H441 barrier by soluble factors of lung and brain endothelial cells correlated with alterations of ATI and ATII markers, mRNA and protein expressions of caveolin-1, RAGE and SP-B were analyzed. On the mRNA level caveolin-1 was significantly upregulated 2.28-fold ($p<0.05$) by conditioned D3-medium, which was concordant with a 2.75-fold increase of the protein expression (not significant). In contrast to caveolin-1, mRNA of the second ATI marker RAGE was significantly downregulated by the conditioned ST1 medium (0.49-fold, $p<0.05$) as well as by the conditioned D3 medium (0.67-fold, $p<0.05$). This was also confirmed by the according western blot data (ST1 cond.: 0.78-fold, n.s.; D3 cond.: 0.82-fold, $p<0.05$). Similar to caveolin-1, ATII marker SP-B was significantly upregulated 2.51-fold by conditioned D3 medium on the mRNA level, which was consistent with the 2.51 fold increase of SP-B protein expression. In summary, the two ATI markers caveolin-1 and RAGE were differentially regulated by soluble factors of lung or brain endothelial cells. Furthermore, no direct correlation between the expression of ATI marker expression and barrier functionality was found.

# 4. Discussion

The alveolar epithelial barrier function is involved in different pulmonary physiology processes, such as fluid homeostasis, defence mechanisms and gas exchange. Also in pulmonary disease the alveolar epithelial barrier has been shown to play a central role (Godfrey, 1997; Matthay, 1994). Clinical studies have highlighted the importance of this intact barrier function to clinical outcomes (Maniatis et al., 2008; Ware and Matthay, 2000, 2001). Since the microenvironment (neighbouring cells, basal matrix) of biological barriers in mammalian organisms can regulate the barrier's functionality in a significant manner, we aimed at investigating the influence of the lung endothelium on the alveolar epithelium in an *in vitro* model.

Up to now, only few *in vitro* models based on stable cell lines of the alveolar epithelium are established, which consist of cells expressing either type I or type II markers and form a tight barrier (Hermanns et al., 2004, 2009, 2010). Currently, only two promising cell lines are available to model the alveolar epithelium, namely A549 and NCI H441. Several reports showed that cell line A549 is useful as a type II cell model. However, attempts to transdifferentiate them into type I cells and build up a sufficient barrier have failed until now (Hermanns et al., 2004). In case of H441 cells, recent publications showed that treatment of H441 cells with dexamethasone leads to the upregulation of the type-2 marker, SP-B, to dome formation, to a tight barrier and an increase of the expression of tight junctional molecule claudin-4 (Hermanns et al., 2004; Ladenburger et al., 2010; Shlyonsky et al., 2005). Consequently, we decided to establish an alveolar cell culture model based on human cell line H441 and to use this model to study the influence of soluble factors derived from lung as well as from brain endothelial cells.

First, in order to validate this cell culture model the influence of dexamethasone on the barrier (TEER) and on the expression of caveolin-1, RAGE (type-1 markers) and SP-B (type-2 marker) was investigated. It was known from other studies with primary alveolar type II cells as well as cell lines that addition of dexamethasone or hydrocortisone was necessary to form a barrier (Daugherty et al., 2004; Hermanns et al., 2004; Hermanns et al., 2009; Steimer et al., 2007). To our best knowledge, we are the first to present data showing that the tightness of the H441 barrier is dependent on the dexamethasone concentration. This seems to be important considering the fact that reseachers applied dexamethasone in a rather wide concentration range (50 nmol/l – 1 µmol/l) for their H441 studies (Hermanns et al., 2010; Shlyonsky et al., 2005). One major aim of the establishment of the H441-model was to generate tight cell layers which at the same time possess type I and type II markers. Previous studies showed that H441 layers treated with dexamethasone were able to exhibit several properties of the alveolar epithelium such as apical villi, laminar as well as multivesicular bodies, type-2 marker TTF-1 as well as epithelial marker E-cadherin (Hermanns et al., 2004, 2010). Thus, we have decided to concentrate on the analysis of the markers caveolin-1, RAGE and SP-B in order to present relevant, but also novel data of H441 cells concerning the presence of both cell types within the H441 Transwell model. Ladenburger et al. (2010) showed recently that dexamethasone was able to increase SP-B in H441 cells significantly. This was concordant with our results where dexamethasone increased SP-B in a concentration dependent manner, whereas caveolin-1 and RAGE expression was not altered compared to the untreated samples on day 7. Interestingly, immunofluoresence microscopy of double-stainings against caveolin-1 and SP-B showed cells expressing caveolin-1 but not SP-B at the same time confirming the presence of two differentially differentiated cell types in the

H441-layers representing type 1 and type 2 cells (see supplementary file, figure 2). So we were able to show that the established H441 model consists of cells expressing both type 1 (caveolin-1, RAGE) and type 2 (SP-B) markers on the mRNA as well as on the protein level and that the marker expression can be regulated by dexamethasone.

With regard to the tightness of the established H441-Transwell model, TEER values indicated that the chosen culture conditions resulted in a distinct barrier which is associated on the molecular level with the presence of tight junctional proteins. For the alveolar epithelium the presence of claudin-1,-3,-4,-5,-7 and 18 were reported (Daugherty et al., 2004; Kaarteenaho et al., 2010; Koval et al., 2010; Wang et al., 2003). Claudins are important for a stable barrier of the alveolar epithelium in health as well as in disease (Capaldo and Nusrat, 2009; Coyne et al., 2002; Crosby and Waters, 2010; Daugherty et al., 2004; Godfrey et al., 1993; Lecuona et al., 1999; Soini, 2011; Wray et al., 2009). We have analyzed the presence of ZO-1, occludin, claudin-1,-3,-4 and -5 on the mRNA as well as on the protein level in our established H441-model. In case of ZO-1 and occludin, their presence was already shown for H441 layers (Hermanns et al., 2004, 2010; Shlyonsky et al., 2005), whereas the analysis of claudin expression was carried out for the first time. In comparison to claudin-1,-3 and -4, the expression of claudin-5 was very weak in our model. Claudin-1 is relative specific for type I cells (Daugherty et al., 2004), whereas claudin-3 and claudin-4 were found mainly in type II pneumocytes (Kaarteenaho-Wiik and Soini, 2009). In human lung development claudin-5 was only weakly expressed in the lung epithelium during the pseudoglandular and the canalicular phase observed by immunofluorescence microscopy. In the later phases (saccular and alveolar) claudin-5 was not longer detected and only found in the surrounding lung microvasculature

(Kaarteenaho et al., 2010), whereas claudin-3 and claudin-4 were found in the alveoli during all developmental stages. These facts supported our data and demonstrate that our model mimics several properties of the alveolar epithelium including both type I and type II cells of the alveolar epithelium.

After establishment and characterisation of our H441-model the influence of conditioned endothelial growth media on its barrier properties was investigated. Recently, it was shown that co-cultures of primary type II cells as well as of H441-layers with either primary lung endothelial cells or the endothelial cell line ISO-HAS-I resulted in significantly increased tightness indicated by an increase of TEER and ZO-1 localisation (Hermanns et al., 2009, 2010). In our case, application of conditioned medium of HPMEC-ST1.6R cells to the basolateral compartment increased the tightness of the H441-layers shown by TEER values and fluorescein permeability. On the contrary, conditioned medium of human brain endothelial cells hCMEC/D3 decreased the tightness of H441-layers significantly confirming tissue-specific effects. To link these observations on the functional level to the molecular level mRNA as well as protein expression of claudin-1,-3,-4 and -5 and the alveolar cell type markers (caveolin-1, RAGE, SP-B) were quantified by qPCR and western blotting. Interestingly, almost the same results to ours were found by Fernandez et al. (2007), who analyzed the claudin expression patterns of rat lungs after chronic alcohol ingestion. Claudin-1 and -3 were reduced in lung tissue, whereas claudin-4 was unchanged. Also concordant to our results, they found no changes of claudin-5 mRNA, but reported changes of claudin-5 on the protein level. This confirmed our assumption that claudin-5 is regulated on the posttranscriptional level. Up-regulation of claudin-5 was reported in alveolar epithelial cell layers with increased tightness (Wang et al., 2003). Since the basal expression of claudin-5 in our H441-cells is quite

low in comparison to claudin-1,-3 and -4, a significant regulation of claudin-5 is maybe connected with a significant change in barrier functionality. In this context, we were able to observe a distinct up-regulation of the claudin-5 network of H441-layers fed basolaterally with fresh ST1-medium by immunofluorescence microscopy (see supplementary file, figure 1). With regard to the ATI and ATII markers, no direct correlation was found between changes of the barrier tightness and the expression of caveolin-1, RAGE or SP-B. Notably, caveolin-1 and SP-B were regulated in a similar manner, whereas RAGE expression decreased due to addition of conditioned endothelial medium independent from the tissue origin. These data showed that the two ATI markers caveolin-1 and RAGE reacted differently on the endothelial factors in our model suggesting differences in their usability as ATI markers. This was concordant to the classification of ATI markers by McElroy and Kaspar (2004). They added caveolin-1 to the group of ATI selective proteins, but RAGE only to the ATI selective/associated markers, since mRNA of RAGE was also detected in ATII cells (McElroy and Kaspar, 2004).

In summary, after validation of the H441-cell culture as an alveolar epithelium model consisting of cells expressing either ATI or ATII markers, it was shown for the first time that soluble factors of lung endothelial cells (HPMEC-ST1.6R) enhance the barrier of the alveolar epithelium. Furthermore, this functional improvement of the barrier was linked with the quantitative expression of claudins on the mRNA as well as on the protein level. Moreover, soluble factors of brain endothelial cells (hCMEC/D3) disrupted the barrier's integrity proving tissue-specific effects of endothelial cells. With regard to the clinical context, influencing the alveolar permeability by manipulation of the claudin expression may be a future target in the treatment of different lung diseases (Soini, 2011).

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## References

Balda, M.S., Matter, K., 1998. Tight junctions. J Cell Sci. 111, 541-547.

Campbell, L., Hollins, A.J., Al-Eid, A., Newman, G.R., von Ruhland, C., Gumbleton, M., 1999. Caveolin-1 expression and caveolae biogenesis during cell transdifferentiation in lung alveolar epithelial primary cultures. Biochemical and Biophysical Research Communications 262, 744-751.

Capaldo, C.T., Nusrat, A., 2009. Cytokine regulation of tight junctions. Biochimica et Biophysica Acta 1788, 864-871.

Coyne, C.B., Vanhook, M.K., Gambling, T.M., Carson, J.L., Boucher, R.C., Johnson, L.G., 2002. Regulation of airway tight junctions by proinflammatory cytokines. Molecular Biology of the Cell 13, 3218-3234.

Crapo, J.D., Barry, B.E., Gehr, P., Bachofen, M., Weibel, E.R., 1982. Cell number and cell characteristics of the normal human lung. American Review of Respiratory Diseases 126, 332-337.

Crosby, L.M., Waters, C.M., 2010. Epithelial Repair Mechanisms in the Lung. American Journal of Physiology. Lung Cellular and Molecular Physiology 298, L715-731.

Daugherty, B.L., Mateescu, M., Patel, A.S., Wade, K., Kimura, S., Gonzales, L.W., Guttentag, S., Ballard, P.L., Koval, M., 2004. Developmental regulation of claudin localization by fetal alveolar epithelial cells. American Journal of Physiology. Lung Cellular and Molecular Physiology 287, L1266-1273.

Fehrenbach, H., 2001. Alveolar epithelial type II cell: defender of the alveolus revisited. Respiratory Research 2, 33-46.

Fernandez, A.L., Koval, M., Fan, X., Guidot, D.M., 2007. Chronic alcohol ingestion alters claudin expression in the alveolar epithelium of rats. Alcohol 41, 371-379.

Forster, C., 2008. Tight junctions and the modulation of barrier function in disease. Histochemistry and Cell Biology 130, 55-70.

Gehr, P., Bachofen, M., Weibel, E.R., 1978. The normal human lung: ultrastructure and morphometric estimation of diffusion capacity. Respiration Physiology 32, 121-140.

Godfrey, R.W., Severs, N.J., Jeffery, P.K., 1993. Structural alterations of airway epithelial tight junctions in cystic fibrosis: comparison of transplant and postmortem tissue. American Journal of Respiratory Cell and Moleluar Biology 9, 148-156.

Godfrey, R.W., 1997. Human airway epithelial tight junctions. Microscopy Research and Technique 38, 488-499.

Gorin, A.B., Stewart, P.A., 1979. Differential permeability of endothelial and epithelial barriers to albumin flux. Journal of Applied Physiology 47, 1315-1324.

Gumbleton, M., Abulrob, A.G., Campbell, L., 2000. Caveolae: an alternative membrane transport compartment. Pharmaceutical Research 17, 1035-1048.

Hermanns, M.I., Fuchs, S., Bock, M., Wenzel, K., Mayer, E., Kehe, K., Bittinger, F., Kirkpatrick, C.J., 2009. Primary human coculture model of alveolo-capillary unit to study mechanisms of injury to peripheral lung. Cell and Tissue Research 336, 91-105.

Hermanns, M.I., Kasper, J., Dubruel, P., Pohl, C., Uboldi, C., Vermeersch, V., Fuchs, S., Unger, R.E., Kirkpatrick, C.J., 2010. An impaired alveolar-capillary barrier in vitro: effect of proinflammatory cytokines and consequences on nanocarrier interaction. Journal of the Royal Society, Interface 7 Suppl 1, S41-54.

Hermanns, M.I., Unger, R.E., Kehe, K., Peters, K., Kirkpatrick, C.J., 2004. Lung epithelial cell lines in coculture with human pulmonary microvascular endothelial cells: development of an alveolo-capillary barrier in vitro. Laboratory Investigation 84, 736-752.

Kaarteenaho, R., Merikallio, H., Lehtonen, S., Harju, T., Soini, Y., 2010. Divergent expression of claudin -1, -3, -4, -5 and -7 in developing human lung. Respiratory Research 11, 59.

Kaarteenaho-Wiik, R., Soini, Y., 2009. Claudin-1, -2, -3, -4, -5, and -7 in usual interstitial pneumonia and sarcoidosis. The Journal of Histochemistry and Cytochemistry 57, 187-195.

Kasper, M., Reimann, T., Hempel, U., Wenzel, K.W., Bierhaus, A., Schuh, D., Dimmer, V., Haroske, G., Muller, M., 1998. Loss of caveolin expression in type I

pneumocytes as an indicator of subcellular alterations during lung fibrogenesis. Histochemistry and Cell Biology 109, 41-48.

Koval, M., Ward, C., Findley, M.K., Roser-Page, S., Helms, M.N., Roman, J., 2010. Extracellular matrix influences alveolar epithelial claudin expression and barrier function. American Journal of Respiratory Cell and Molecular Biology 42, 172-180.

Krump-Konvalinkova, V., Bittinger, F., Unger, R.E., Peters, K., Lehr, H.A., Kirkpatrick, C.J., 2001. Generation of human pulmonary microvascular endothelial cell lines. Laboratory Investigation 81, 1717-1727.

Kunzmann, S., Collins, J.J., Yang, Y., Uhlig, S., Kallapur, S., Speer, C.P., Jobe, A.H., Kramer, B.W., 2011. Antenatal Inflammation Reduces Cav-1 Expression and Influences Multiple Signaling Pathways in Preterm Fetal Lungs. American Journal of Respiratory Cell and Molecular Biology 45, 969-976.

Ladenburger, A., Seehase, M., Kramer, B.W., Thomas, W., Wirbelauer, J., Speer, C.P., Kunzmann, S., 2010. Glucocorticoids potentiate IL-6-induced SP-B expression in H441 cells by enhancing the JAK-STAT signaling pathway. American Journal of Physiology. Lung Cellular and Molecular Physiology 299, L578-584.

Lecuona, E., Saldias, F., Comellas, A., Ridge, K., Guerrero, C., Sznajder, J.I., 1999. Ventilator-associated lung injury decreases lung ability to clear edema in rats. American Journal of Respiratory and Critical Care Medicine 159, 603-609.

Maniatis, N.A., Kotanidou, A., Catravas, J.D., Orfanos, S.E., 2008. Endothelial pathomechanisms in acute lung injury. Vascular Pharmacology 49, 119-133.

Matthay, M.A., 1994. Function of the alveolar epithelial barrier under pathologic conditions. Chest 105, 67S-74S.

McElroy, M.C., Kaspar,.M., 2004. The use of alveolar epithelial type I cell-selective markers to investigate lung injury and repair. European Respiratory Journal 24, 664–673.

Neuhaus, W., Plattner, V.E., Wirth, M., Germann, B., Lachmann, B., Gabor, F., Noe, C.R., 2008. Validation of in vitro cell culture models of the blood-brain barrier: tightness characterization of two promising cell lines. Journal of Pharmaceutical Sciences 97, 5158-5175.

Phelps, D.S., Floros, J., 1991a. Localization of pulmonary surfactant proteins using immunohistochemistry and tissue in situ hybridization. Experimental Lung Research 17, 985-995.

Phelps, D.S., Floros, J., 1991b. Dexamethasone in vivo raises surfactant protein B mRNA in alveolar and bronchiolar epithelium. American Journal of Physiology 260, L146-152.

Rodriguez-Boulan, E., Nelson, W.J., 1989. Morphogenesis of the polarized epithelial cell phenotype. Science 245, 718-725.

Shlyonsky, V., Goolaerts, A., Van Beneden, R., Sariban-Sohraby, S., 2005. Differentiation of epithelial Na+ channel function. An in vitro model. The Journal of Biological Chemistry 280, 24181-24187.

Soini, Y., 2011. Claudins in lung diseases. Respiratory Research 12, 70.

Steimer, A., Franke, H., Haltner-Ukomado, E., Laue, M., Ehrhardt, C., Lehr, C.M., 2007. Monolayers of porcine alveolar epithelial cells in primary culture as an in vitro model for drug absorption studies. European Journal of Pharmaceutics and Biopharmaceutics 66, 372-382.

Uchida, T., Shirasawa, M., Ware, L.B., Kojima, K., Hata, Y., Makita, K., Mednick, G., Matthay, Z.A., Matthay, M.A., 2006. Receptor for Advanced Glycation End-

Products Is a Marker of Type I Cell Injury in Acute Lung Injury. American Journal of Respiratory and Critical Care Medicine 173, 1008-1015.

Uhal, B.D., 1997. Cell cycle kinetics in the alveolar epithelium. American Journal of Physiology 272, L1031-1045.

Unger, R.E., Krump-Konvalinkova, V., Peters, K., Kirkpatrick, C.J., 2002. In vitro expression of the endothelial phenotype: comparative study of primary isolated cells and cell lines, including the novel cell line HPMEC-ST1.6R. Microvascular Research. 64, 384-397.

Wang, F., Daugherty, B., Keise, L.L., Wei, Z., Foley, J.P., Savani, R.C., Koval, M., 2003. Heterogeneity of claudin expression by alveolar epithelial cells. American Journal of Respiratory Cell and Molecular Biolology 29, 62-70.

Ware, L.B., Matthay, M.A., 2001. Alveolar fluid clearance is impaired in the majority of patients with acute lung injury and the acute respiratory distress syndrome. American Journal of Respiratory and Critical Care Medicine, 163, 1376-1383.

Ware, L.B., Matthay, M.A., 2000. The acute respiratory distress syndrome. New England Journal of Medicine 342, 1334-1349.

Weaver, T.E., Sarin, V.K., Sawtell, N., Hull, W.M., Whitsett, J.A., 1988.: Identification of surfactant proteolipid SP-B in human surfactant and fetal lung. Journal of Applied Physiology 65, 982-987.

Weksler, B.B., Subileau, E.A., Perrière, N., Charneau, P., Holloway, K., Leveque, M., Tricoire-Leignel, H., Nicotra, A., Bourdoulous, S., Turowski, P., Male, D.K., Roux, F., Greenwood, J., Romero, I.A., Couraud, P.O., 2005. Blood-brain barrier-specific properties of a human adult brain endothelial cell line. The FASEB Journal 19, 1872-1884.

Williams, M.C., 2003. Alveolar type I cells: molecular phenotype and development. Annual Review of Physiology 65, 669-695.

Wray, C., Mao, Y., Pan, J., Chandrasena, A., Piasta, F., Frank, J.A., 2009. Claudin-4 augments alveolar epithelial barrier function and is induced in acute lung injury. American Journal of Physiology. Lung Cellular and Molecular Physiology 297, L219-227.

# Figure Captions

**Fig. 1.** Concentration dependent influence of dexamethasone on the time courses of TEER development of H441-cell layers (A). Presence of tight junction molecules claudin-1 (233 bp), claudin-3 (247 bp), claudin-4 (187 bp), claudin-5 (239 bp), occludin (170 bp) and ZO-1 (224 bp) is proven on mRNA level of H441-layers treated with 100 nmol/l dexamethasone (apical) and lysed on day 15, relative abundances were determined by qPCR presented in the table below the agarose gel image (B). Immunofluorescence microscopic images of H441-layers (day 15) treated with 100 nmol/l dexamethason (apical) prove the presence on the protein level and localisation at the plasma membrane of claudin-1, -3,-4 and -5, occludin and ZO-1 (green=tight junction protein, red=propidium iodide stained nuclei) (C). In the case of claudin-5, low predicted abundance by mRNA analysis was confirmed and visualized by immunofluorescence microscopy.

**Fig. 2.** Concentration dependent influence of dexamethasone on the mRNA expression of cell type-I markers caveolin-1 and RAGE and cell type-II marker SP-B in H441-cell layers on day 7 and day 15 determined by qPCR (A). Immunofluorescence microscopic images of H441-layers (day 15) treated with 100 nmol/l dexamethason (apical) prove the presence on the protein level and the expected localisation of caveolin-1, RAGE and SP-B. (green=marker protein, red=propidium iodide stained nuclei) (B). Data are presented as means ± SD (n=3). Statistically significant differences are marked by * ($p<0.05$, t-test) in comparison to the samples of non-treated cells and by # ($p<0.05$, t-test) in comparison to 10 nmol/l dexamthasone treated cells on day 15.

**Fig. 3.** Influence of soluble factors from lung and brain endothelial cells on the barrier of the H441 model. H441 cells were fed basolaterally with fresh or conditioned growth media from human lung endothelial cell line HPMEC-ST1.6R (ST1) or from human brain endothelial cell line hCMEC/D3 (D3) for 15 days, 100 nmol/l dexamethasone was added in the apical side with every medium exchange from day 5 on. Changes of the barrier functionality of H441 layers were measured by TEER (A) and by the permeability of paracellular marker fluorescein (B). H441 means conventional H441 growth medium, ST1 and D3 are the fresh growth media for HPMEC-ST1.6R and hCMEC/D3 cells, whereas ST1-cond. and D3-cond. means the conditioned growth media obtained from HPMEC-ST1.6R and hCMEC/D3 cells. Data are normalized to data obtained from H441 cell layers grown in H441-medium which were set to 100% and are presented as means ± SD (n=3). Statistically significant differences are marked by # ($p<0.05$, t-test) in comparison to H441-growth medium and by * ($p<0.05$, t-test) in comparison to the according fresh medium ST1 or D3.

**Fig. 4.** Influence of soluble factors from lung and brain endothelial cells on the mRNA expression of claudins of H441 layers. H441 cells were fed basolaterally with fresh or conditioned growth media from human lung endothelial cell line HPMEC-ST1.6R (ST1) or from human brain endothelial cell line hCMEC/D3 (D3) for 15 days, 100 nmol/l dexamethasone was added in the apical side with every medium exchange from day 5 on. Changes of the mRNA expression of claudin-1, claudin-3, claudin-4 and claudin-5 of H441 cell layers were measured by qPCR. H441 means conventional H441 growth medium, ST1 and D3 are the fresh growth media for HPMEC-ST1.6R and hCMEC/D3 cells, whereas ST1-cond. and D3-cond. means the conditioned growth media obtained from HPMEC-ST1.6R and hCMEC/D3 cells. Data are

normalized to data obtained from H441 cell layers grown in H441-medium which were set to 1 and are presented as means ± SD (n=3). Statistically significant differences are marked by * ($p<0.05$, t-test) in comparison to the according fresh medium ST1 or D3.

**Fig. 5.** Influence of soluble factors from lung and brain endothelial cells on the protein expression of claudins of H441 layers. H441 cells were fed basolaterally with fresh or conditioned growth media from human lung endothelial cell line HPMEC-ST1.6R (ST1) or from human brain endothelial cell line hCMEC/D3 (D3) for 15 days, 100 nmol/l dexamethasone was added in the apical side with every medium exchange from day 5 on. Changes of the protein expression of claudin-1, claudin-3, claudin-4 and claudin-5 of H441 cell layers were measured by western blotting normalized to β-actin. H441 means conventional H441 growth medium, ST1 and D3 are the fresh growth media for HPMEC-ST1.6R and hCMEC/D3 cells, whereas ST1-cond. and D3-cond. means the conditioned growth media obtained from HPMEC-ST1.6R and hCMEC/D3 cells. One representative western blot out of three with similar results is shown. Data are normalized to data obtained from H441 cell layers grown in H441-medium which were set to 1 and are presented as means ± SD (n=3 per condition). Statistically significant differences are marked by # ($p<0.05$, t-test) in comparison to H441-growth medium and by * ($p<0.05$, t-test) in comparison to the according fresh medium ST1 or D3.

**Fig. 6**. Influence of soluble factors from lung and brain endothelial cells on the mRNA as well as protein expression of cell type-I markers caveolin-1 and RAGE and cell type-II marker SP-B of H441 layers. H441 cells were fed basolaterally with fresh or conditioned growth media from human lung endothelial cell line HPMEC-ST1.6R

(ST1) or from human brain endothelial cell line hCMEC/D3 (D3) for 15 days, 100 nmol/l dexamethasone was added in the apical side with every medium exchange from day 5 on. Changes of the expression of caveolin-1, RAGE and SP-B of H441 cell layers were measured by qPCR (mRNA) or western blotting normalized to β-actin (protein). H441 means conventional H441 growth medium, ST1 and D3 are the fresh growth media for HPMEC-ST1.6R and hCMEC/D3 cells, whereas ST1-cond. and D3-cond. means the conditioned growth media obtained from HPMEC-ST1.6R and hCMEC/D3 cells. One representative western blot out of three with similar results is shown. Data are normalized to data obtained from H441 cell layers grown in H441-medium which were set to 1 and are presented as means ± SD (n=3 per condition). Statistically significant differences are marked by # ($p<0.05$, t-test) in comparison to H441-growth medium and by * ($p<0.05$, t-test) in comparison to the according fresh medium ST1 or D3.

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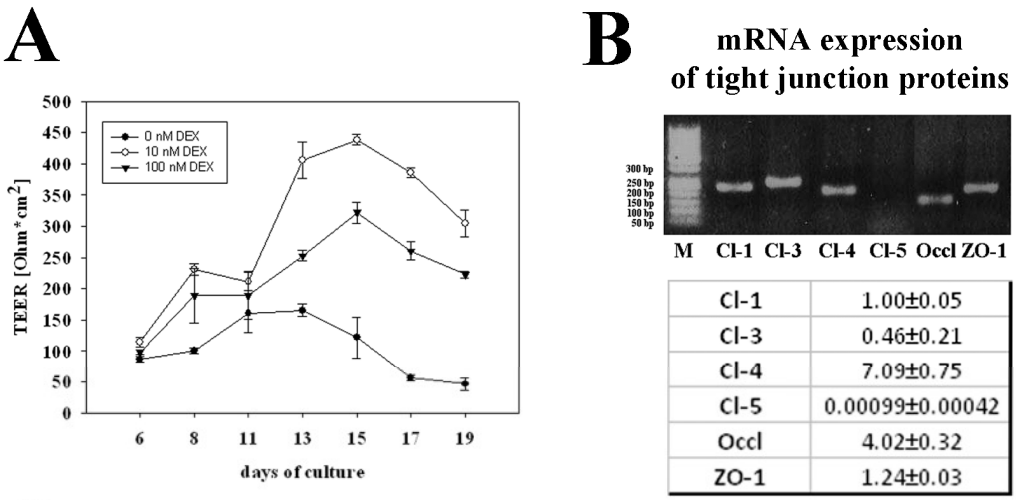
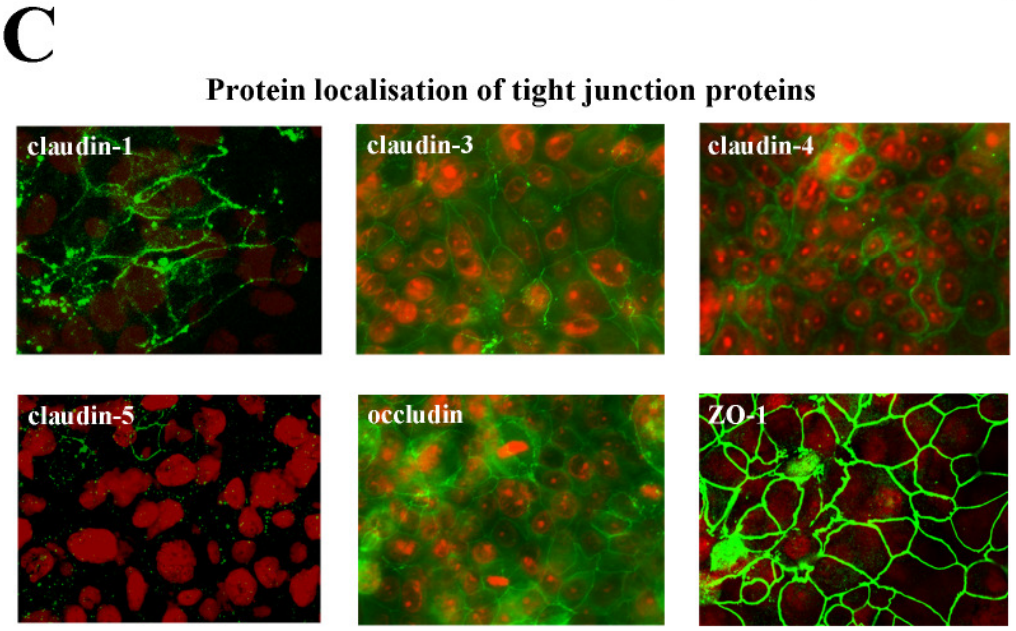
**Figure 1**



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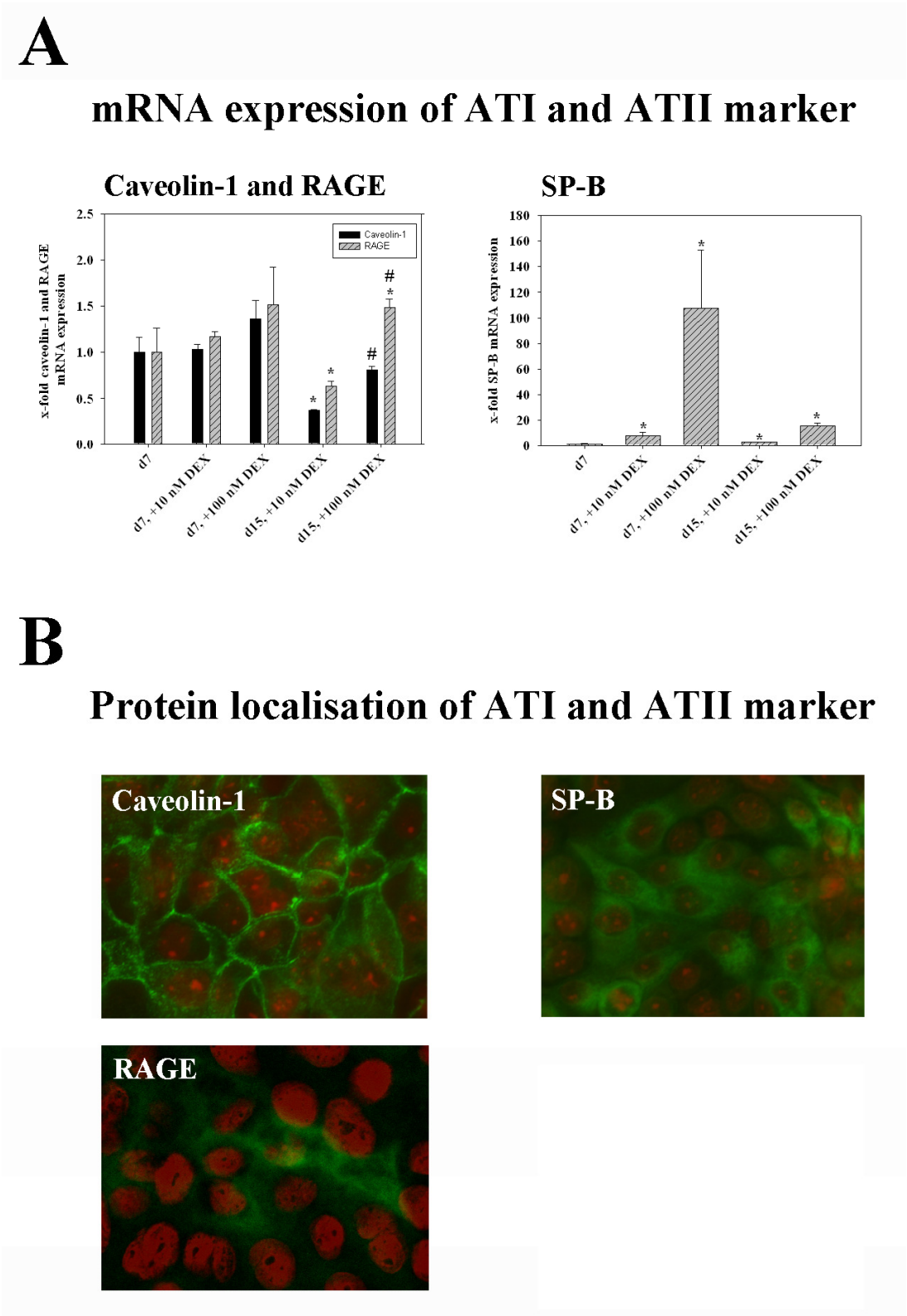
http://dx.doi.org/10.1016/j.diff.2012.08.006

**Figure 2**



Original publication in: Neuhaus et al., Differentiation 84 (2012) 294-304.;
http://dx.doi.org/10.1016/j.diff.2012.08.006

**Figure 3**

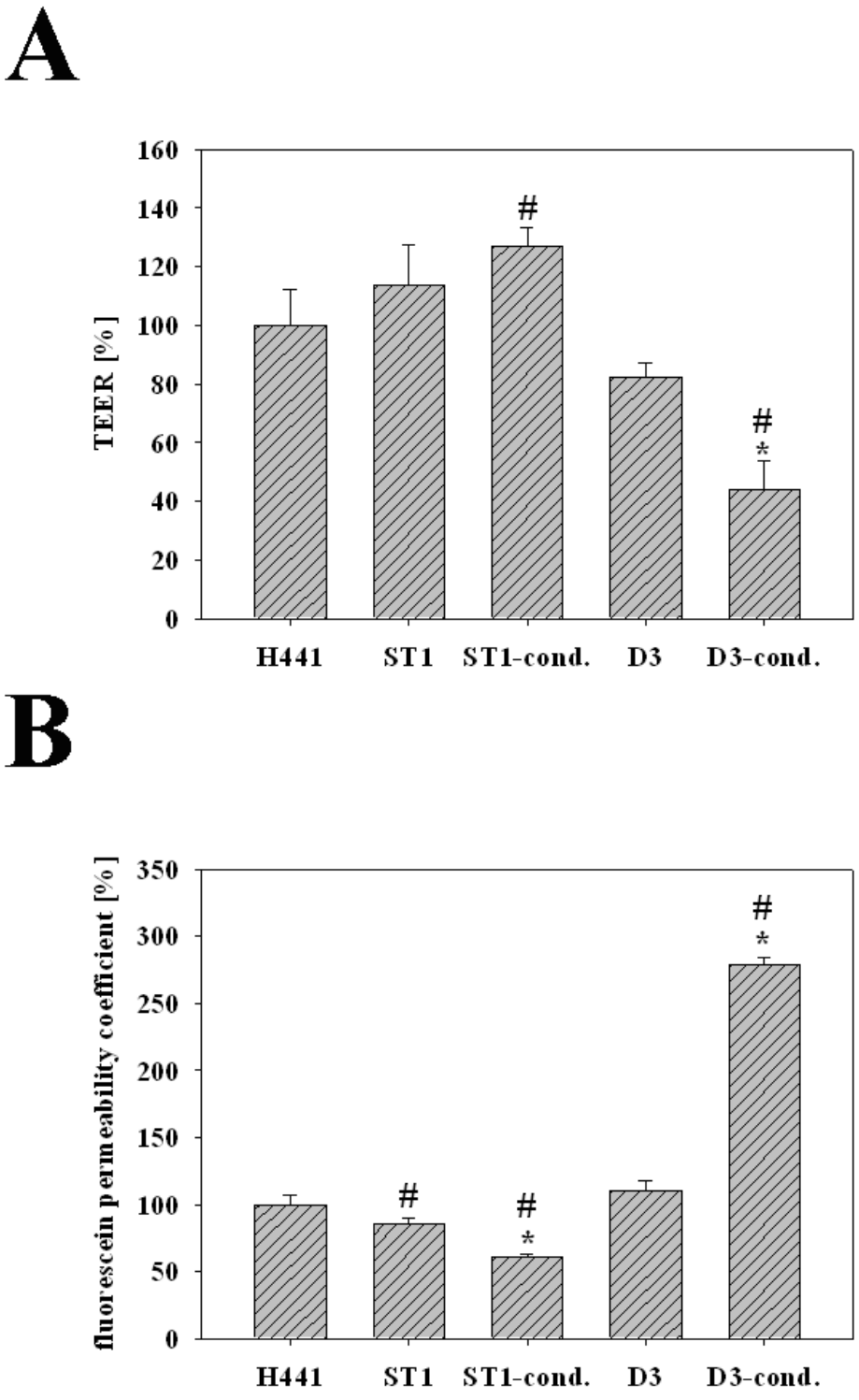


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**Figure 4**

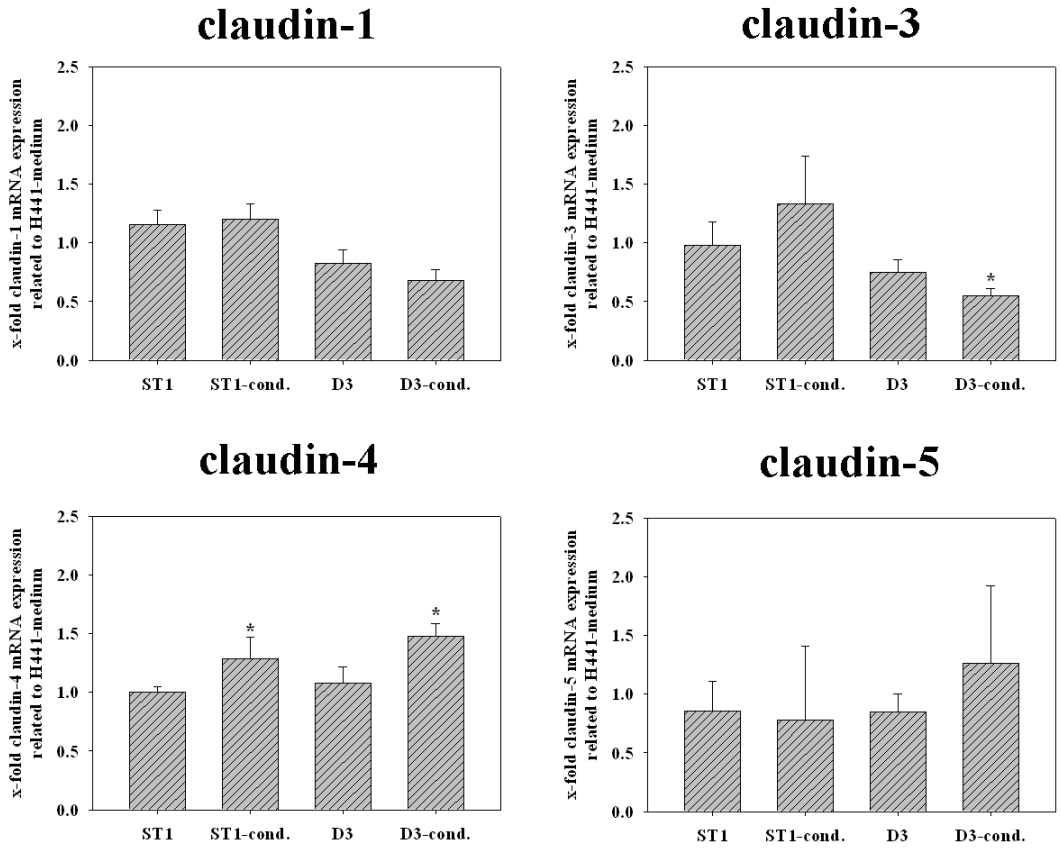

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**Figure 5**

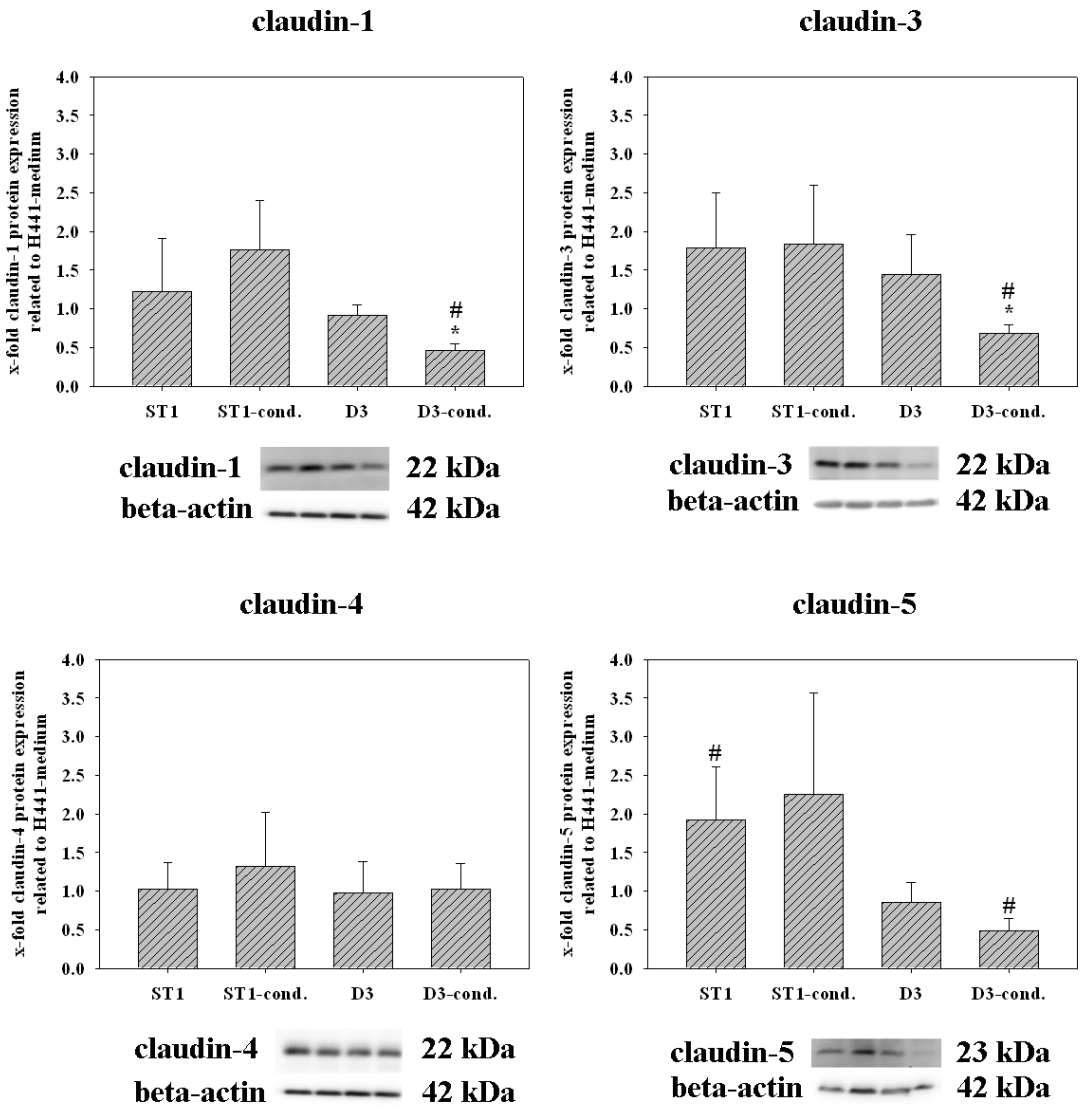

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**Figure 6**