Characterisation of Rat Alveolar Cell Line R3/1

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Prohlášení

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány.

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Contents

List of abbreviations

7H6 antigen	tight-junction associated protein antigen
AA	amino acid
ACE	angiotensin converting enzyme
AEC	alveolar epithelial cell
AIC	air-interfaced culture
AJ	adherens junction
ANOVA	analysis of variance
AP	aminopeptidase
APA	aminopeptidase A
APB	aminopeptidase B
APN	aminopeptidase N
APP	aminopeptidase P
APS	ammonium persulfate
ATI	alveolar epithelial type I
ATII	alveolar epithelial type II
ATCC	American Type Culture Collection
BCRP	brest cancer resistance protein
BPA-lectin	*Bauhinia purpurea* lectin
BSA	bovine serum albumin
CALLA	common acute lymphoblastic leukaemia antigen, CD 10
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CPM	carboxypeptidase M
Cx	connexon
DEPC	diethyl pyrocarbonate
DEX	dexamethasone
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
DPP IV	dipeptidylpeptidase IV
DS	desmosome

DTT	dithiothreitol
EC	epithelial cell
EC number	enzyme commission number
EDTA	ethylenediaminetetraacetic acid
ERS	electrical resistance system
EVOM	epithelial volt ohm meter
FBS	foetal bovine serum
GJ	gap junction
GGT	γ-glutamyl transferase
H_2O dd	double distilled water
hAEpC	human alveolar epithelial cells
hTERT	human telomerase reverse transcriptase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPV	human papillomavirus
HRP	horseradish peroxidase
ICAM	inter-cellular cell adhesion molecule
IFM	immunofluorescence microscopy
IL	interleukin
Isc	short-circuit current
JAM	junctional adhesion molecule
K_a	partition coefficient
LCC	liquid-covered culture
MMLV-RT	Moloney murine leukaemia virus reverse transcriptase
mRNA	messenger ribonucleic acid
MRP	multidrug resistance-related protein
MV	microvilli
NEP	neutral endopeptidases, synonym: CD10
OCT	organic cation transporter
P_{app}	apparent permeability coefficient
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	potential difference
PFA	paraformaldehyde
P-gp	P-glycoprotein

PI	propidium iodide
PMA	phorbol myristate acetate
pSVori	plasmid containing SV40 ori region
PVDF	polyvinylidene fluoride
rpm	revolutions per minute
RAGE	receptor of advanced glycation end products
RNA	ribonucleic acid
RNasin	ribonuclease inhibitor
RT	reverse transcription, reverse transcriptase
SBA-lectin	soybean agglutinin (*Glycine max* lectin)
S. D.	standard deviation
SDS	sodium dodecyl sulphate
SP	surfactant protein
SV40	Simian vacuolating virus 40 or Simian virus 40
Taq polymerase	thermostable DNA polymerase originally isolated from bacterium Thermus aquatius,
T1α	plasma membrane protein involved in fluid transport
TBE	buffer containing Tris base, boric acid and EDTA
TEER	transepithelial electrical resistance
TEMED	N,N,N',N'-tetramethylethylenediamine
TJ	tight junction
TOP	thimet oligopeptidase
Tris	tris(hydroxymethyl)aminomethane
TTB	Towbing transfer buffer
UV	ultraviolet
WB	Western blot
ZAK	zonula occludens associated kinase
ZO-1	zonula occludens protein-1
ZO-2	zonula occludens protein-2
ZO-3	zonula occludens protein-3

1. INTRODUCTION

1.1. Lung anatomy

The respiratory system can be anatomically divided into two regions: conducting and respiratory. The conducting airways consist of the air-transmitting passages of the nose, nasopharynx, larynx, trachea, bronchi and bronchioles (Gray 1918, 2000; Itoh, Nishino et al. 2004) the latter three of which form the tracheo-bronchial region of the lung (generations 1-16 in the bifurcating airway model; *Figure 1*). The actual exchange of gases (i.e., fresh oxygen into and carbon dioxide out of the body) occurs across the distal lung respiratory epithelium comprised of the alveolar ducts and saccules (i.e., the alveoli, generations 17-23 in the bifurcating airway model; *Figure 1).*

Figure 1: Structure of the human respiratory tract, adapted from (Weibel 1963)**.**

	generation		diameter (cm)	length (cm)	number	total cross sectional area (cm^2)
conducting zone	trachea	0	1.80	12.0	1	2.54
	bronchi	1	1.22	4.8	2	2.33
		2	0.83	1.9	4	2.13
		3	0.56	0.8	8	2.00
	bronchioles	4	0.45	1.3	16	2.48
		5 ↓	0.35 ↓	1.07 ↓	32 ↓	3.11 ↓
	terminal bronchioles	16	0.06	0.17	$6 \cdot 10^4$	180.0
transitional and respiratory zones	respiratory bronchioles	17	↓	↓	↓	↓
		18				
		19	0.05	0.10	$5 \cdot 10^5$	10^3
	alveolar ducts	20	↓	↓	↓	↓
		21				
		22				
	alveolar sacs	23	0.04	0.05	$8 \cdot 10^6$	10^4

The primary function of the conducting airways is to act as conduit for air movement. Additionally, this region provides filtration, warming and humidification of inhaled air.

Specialised extra-pulmonary organs and tissues of the conducting system also serve other functions. For example, the nose provides a first-pass air filtration system, the pharynx aids in alimentation and the larynx is required for phonation (McDowell, Barrett et al. 1978). The cellular composition of the epithelium varies according to the airway generation (*Table 1*).

Table 1: Cell types of the healthy respiratory epithelium

Lung region	**Bronchi**	**Bronchioles**	**Terminal bronchioles**	**Alveoli**
Type of epithelium	***Pseudostratified columnar***	***Simple columnar***	***Simple cuboidal***	***Simple squamous***
Predominant cell types	***Basal*** **Basal cell** **Kultschitzky cell** ***Superficial*** **Ciliated cell** **Brush cell** **Goblet cell** **Serous cell** **Mucous cell**	**Ciliated cell** **Goblet cell**	**Ciliated cell** **Clara cell**	**Alveolar type I cell** **Alveolar type II cell**

The lining of the tracheo-bronchial airways is composed of several cell types including basal, goblet, ciliated, brush, serous, Clara, and neuroendocrine cells (Jeffery and Reid 1975). A variety of migratory cells such as lymphocytes, leukocytes, and mast cells are also present in the epithelium (Jeffery and Reid 1975). In the terminal bronchioles the epithelium is composed of ciliated cells and Clara cells (Plopper 1996). In the alveolar region epithelial type I and II cells and

alveolar macrophages are present (Plopper 1996). The squamous alveolar type I (ATI) cell covers approximately 96% of the alveolar surface area and has an average cell thickness of 0.26 µm (Stone, Mercer et al. 1992). About 3% of the alveolar surface is covered by the much smaller cuboidal alveolar type II (ATII) cells, which synthesise and secrete surface active materials (Mason and Crystal 1998). The heterogenous composition of the lung epithelium results in a large variation of tight junctional forms with variable tightness (Schneeberger 1980; Godfrey 1997).

The lung and its luminal lining fluid are regularly exposed to a plethora of aerosolised compounds, which include drug molecules, and act as a barrier between air and the body. The lung, therefore, is in an ideal location and is frequently used to biotransform such compounds to reduce their potential toxicity. An important part of this biotransformation feature is the ability to efflux potentially harmful substances or their metabolites out of the body. On the other hand, absorption mechanisms are necessary for catabolic processes, for example, within the airspaces or to re-absorb endogenous compounds from the lining fluid (e.g., surfactant components, serum proteins and neurotransmitters). The respiratory epithelium forms a selective barrier against the entry of compounds into blood *(Figure 2)*.

Figure 2: Possible pathways across the lung epithelial barrier.

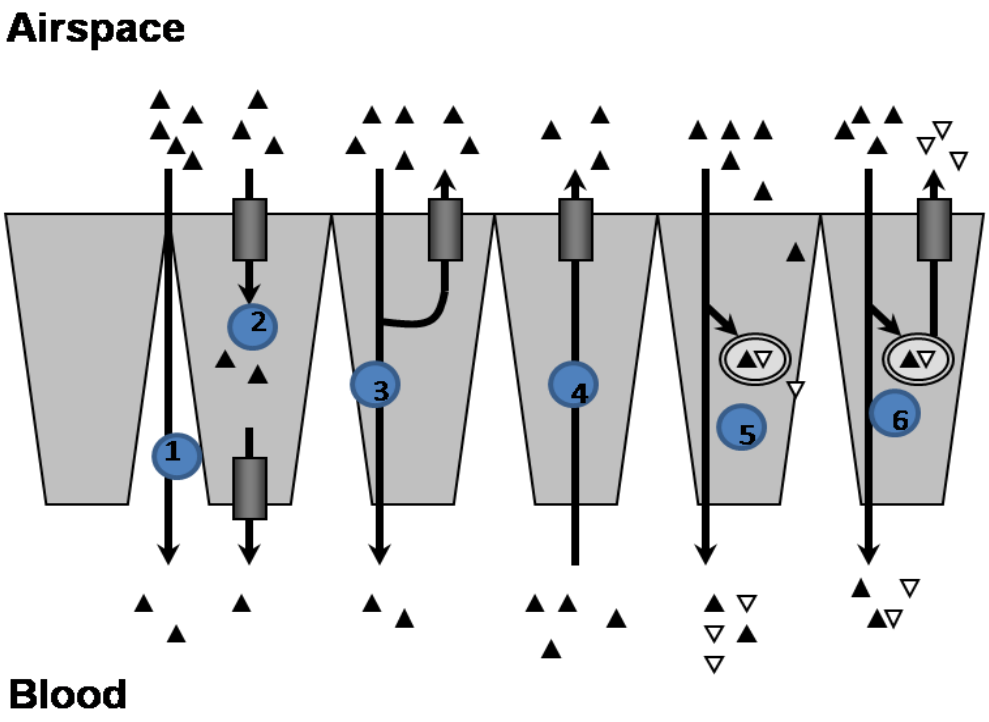


Figure 2 legend: **(1)** Absorption of compounds via the paracellular route is restricted by intercellular tight junctions. **(2)** Carrier-mediated mechanisms at the apical and/or basolateral membranes facilitate the transcellular absorption of certain compounds. **(3)** Efflux transporters at the apical membrane may actively drive compounds back into the lung lumen thus restricting their absorption into blood. **(4)** Apical efflux transporters may also facilitate the pulmonary clearance of compounds that are already present in blood. **(5)** Intracellular metabolising enzymes may modify compounds before they enter the blood. **(6)** Apical efflux transporters and intracellular metabolising enzymes may co-ordinately metabolise and excrete compounds, forming an effective barrier against pulmonary absorption.

In the 1970's and 1980's, Schanker and co-workers conducted a series of studies on the disappearance rate of various compounds from rat lungs after intra-tracheal instillation and aerosol inhalation. Their studies showed that most compounds were absorbed by passive diffusion and that the rate of absorption increased with an increase in lipophilicity for compounds with partition coefficients in chloroform/buffer pH 7.4 ranging from -3 to 2 (Brown and Schanker 1983; Schanker and Hemberger 1983). Recently, systematic generation of *in vitro* and *in vivo* pharmacokinetic data on the absorption of drugs across the rat lung barrier after aerosol delivery has revealed that lipophilicity, molecular polar surface area, and hydrogen bonding potential are the most influential physicochemical properties for pulmonary absorption of structurally diverse, low molecular weight compounds with $\log D_{7.4}$ ranging from –4 to 3 (Tronde 2002). In contrast to the intestinal mucosa and the blood-brain barrier, the pulmonary epithelium was shown to be highly permeable to drugs with a high molecular polar surface area (Tronde 2002).

Absorption of lipophilic compounds is generally considered to occur by membrane diffusion (Effros and Mason 1983) whereas hydrophilic solutes appear to be absorbed by passive diffusion through intercellular junction pores (Crystal 1991). Like hydrophilic molecules, most exogenous macromolecules with a molecular weight less than 40 kDa are also thought to be absorbed from the air space passive diffusion via the tight junctional pathway (Patton 1996; Bur, Huwer et al. 2006). The absorption rate of hydrophilic compounds is inversely related to their molecular weight (range 60-75 kDa) (Schanker and Burton 1976). However, for compounds

weighing less than 1 kDa, the effect of molecular weight on the absorption rate appears to be negligible (Niven 1992). Drug transporters such as peptide transporters (Groneberg, Eynott et al. 2002), P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), multidrug resistance-related proteins (MRPs) (van der Deen, de Vries et al. 2005; Endter, Becker et al. 2007), and organic cation transporters (OCTs) (Ehrhardt, Kneuer et al. 2005; Horvath, Schmid et al. 2007) have been identified in the lung although a greater understanding of transporter impact on drug absorption from the lung is required.

Clearly, whole animal studies cannot be used as a screening tool in the early stages of drug development. Therefore, cell culture models have been employed for permeability screening of new drug libraries that have been generated through combinatorial chemistry and high-throughput pharmacological screening (Artursson and Borchardt 1997). The development of screening models for the intestinal barrier started in the late 1980s (Hidalgo, Raub et al. 1989) and, a couple of years later, the first in vitro models became available for studies of pulmonary drug disposition (Mathias, Yamashita et al. 1996).

1.2.Cell culture models for pulmonary drug disposition *in vitro* studies

1.2.1. In vitro models of the tracheo-bronchial airspace

1.2.1.1.Primary cell cultures

Protocols for the isolation and culture of primary tracheo-bronchial epithelial cells obtained from the lungs of many species have been developed over the last 30 years. These include primary cultures of airway epithelial cells of the mouse (Oreffo, Morgan et al. 1990), hamster (Kaufman 1976), guinea pig (Robison, Dorio et al. 1993), rat (Suda, Sato et al. 1995), ferret (Chung, Kercsmar et al. 1991), rabbit (Mathias, Kim et al. 2004), dog (Welsh 1985), pig (Black, Ghatei et al. 1989), cow (Sisson, Tuma et al. 1991), horse (Sime, McKellar et al. 1997) and human (de Jong, van Sterkenburg et al. 1993). Most of the protocols result in well-differentiated epithelial cells with mixed phenotypes and these primary cell cultures are generally good platforms for drug absorption studies for a couple of subcultures after which

they lose their ability to form tight junctions and their ability to generate high transepithelial electrical resistance (TEER) is lost. The degradation of these cultures is characterised by a linear drop in short-circuit current (I_{sc}) which has been observed over successive passages in human tracheal epithelial cells in primary culture (Galietta, Lantero et al. 1998). These subcultures exhibited minimal rates of active Na^+ and Cl^- transport by passage 3 and beyond passage 4 the cells failed to generate any active ion transport when studied using Ussing chamber techniques. These data suggest that it is important to develop airway epithelial cell lines that retain the ability to differentiate, form tight junctions, and maintain ion channel/pump physiology when grown *in vitro* (Galietta, Lantero et al. 1998).

Ready-to-use culture systems of human tracheo-bronchial cell layers exhibiting well-differentiated ciliated and goblet cell phenotypes are commercially available (Epiairway™ system, Mattek Corporation, Ashland, MA, USA) (Chemuturi, Hayden et al. 2005). Although Epiairway™ is marketed for use in drug delivery studies, little data has been reported to date supporting this use perhaps reflecting low usage rate by pharmaceutical companies due to cost or efficacy, or lack of publication of Epiairway™ use due to confidentiality constraints of the pharmaceutical industry. In general, primary culture is less convenient and economical than the use of cell lines. Hence, primary cultured tracheo-bronchial cell layers have not been widely utilised for biopharmaceutical purposes (Forbes and Ehrhardt 2005).

1.2.1.2.Tracheo-bronchial epithelial cell lines

In addition to significantly reduced costs, a major advantage of using immortalised airway epithelial cell models over primary lung airway epithelial cells is the ability to reduce the variability in cultures that arise from donor to donor differences. In contrast to gastrointestinal *in vitro* testing where Caco-2 cells have emerged as the gold standard, there is no such consensus to date on the preferred cell line(s) for modelling the bronchial epithelium *in vitro*. Several detailed protocols for culture, maintenance, growth and permeability assessment of tracheo-bronchial epithelial cell lines have been published in recent years (Forbes and Ehrhardt 2005; Sakagami 2006), with the most commonly used systems being the Calu-3 and 16HBE14o- cell

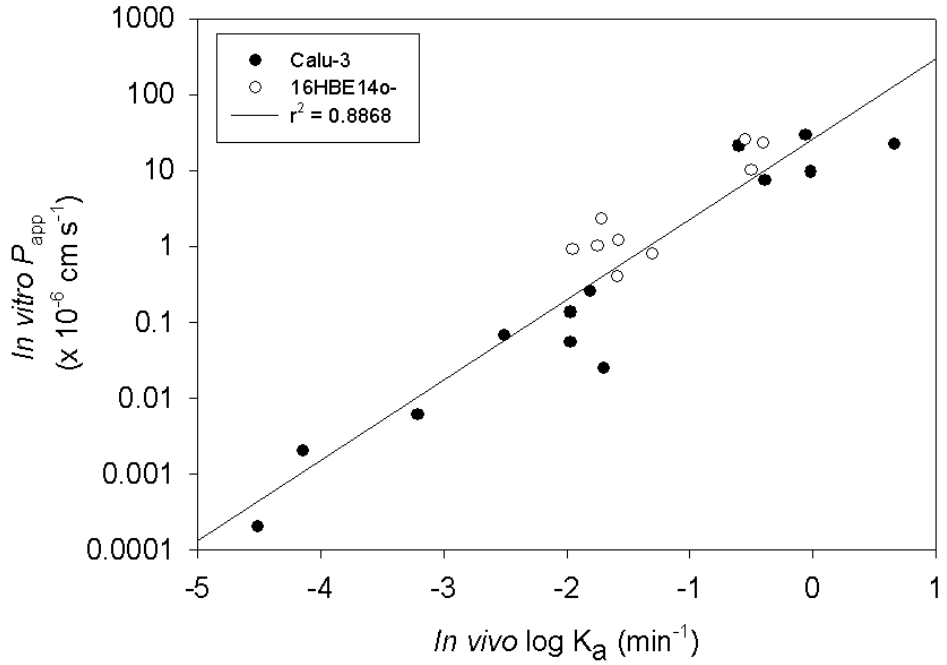
lines. Additionally, the BEAS-2B cell line is frequently used for studies of metabolism and the interaction of cells with xenobiotics. A newly developed cell line, NuLi-1, has also recently been explored for similar purposes (Zabner, Karp et al. 2003).

Calu-3 (American Type Culture Collection; ATCC HTB-55) is a human bronchial epithelial cell line derived from an adenocarcinoma of the lung (Fogh and Trempe 1975). This cell line has been shown to exhibit serous cell properties and form confluent monolayers of mixed cell phenotypes, including ciliated and secretory (Shen, Finkbeiner et al. 1994), but the cilia of these cells are highly irregularly and seem to disappear with increasing passage number (unpublished observations CE & BF). Calu-3 cells have shown utility as a model to examine transport (Foster, Oster et al. 1998; Mathia(s), Timoszyk et al. 2002; Grainger, Greenwell et al. 2006) and metabolism in human bronchial epithelial cells for many therapeutic compounds (Florea, Cassara et al. 2003). Furthermore, they have been used in a number of particle-cell interaction studies (Fiegel, Ehrhardt et al. 2003; Cooney, Kazantseva et al. 2004; Amidi, Romeijn et al. 2006).

Another continuous bronchial epithelial cell line, 16HBE14o-, was generated by transformation of normal bronchial epithelial cells obtained from a one-year-old male heart-lung transplant patient. Transformation was accomplished with SV40 large T antigen using the replication defective pSVori- plasmid (Cozens, Yezzi et al. 1994). 16HBE14o- cells can be obtained from Dieter C. Gruenert, Ph.D., at the California Pacific Medical Center (San Francisco, CA). Although this cell lines is a good lung epithelial model, it has been less widely used than Calu-3 most likely because it is not commercial available. 16HBE14o- cells have a non-serous, non-ciliated phenotype and are generally rounder in shape and smaller in size than Calu-3. When grown under liquid-covered culture (LCC) conditions, 16HBE14o- form confluent, polarised cell layers with functional tight junctions and several drug transport proteins are expressed (Ehrhardt, Kneuer et al. 2002; Ehrhardt, Kneuer et al. 2003). In contrast, air-interfaced culture (AIC) conditions lead to cell layers of less desirable phenotypic and morphological traits. Since most epithelial cells that are normally located at an air interface *in vivo* function optimally under AIC conditions for cultivation, the superior performance of the 16HBE14o- cell line

under LCC conditions is an exception for which the exact reasons or mechanism are currently unknown. Like Calu-3, the 16HBE14o- cell line has been utilised for drug absorption and particle-cell interaction studies (Forbes, Lim et al. 2002; Manford, Tronde et al. 2005). *Figure 3* compares absorption rate constants across Calu-3 and 16HBE14o- cell layers with *in vivo* rate constants determined for absorption from the rat lung after intra-tracheal delivery of various molecules (K_a).

Figure 3: Apparent permeability coefficients (Papp) observed in vitro across Calu-3 (●) (Mathia(s), Timoszyk et al. 2002) and 16HBE14o- (○) (Manford, Tronde et al. 2005) cell layers vs. in vivo rate constants determined for absorption from the rat lung after intra-tracheal delivery of various molecules (Ka) (Ehrhardt and Kim 2008).



The BEAS-2B cell line was derived from normal human epithelial cells that were immortalised using an adenovirus 12 and simian virus 40 hybrid (Reddel, Ke et al. 1988). BEAS-2B is available from the ATCC (CRL-9609) and has been popular for use in studies of airway epithelial cell structure and function including phenotyping and mechanistic investigation of cytokine regulation (Atsuta, Sterbinsky et al. 1997). BEAS-2B cells have also been used to evaluate responses to challenges such as tobacco smoke (Sun, Wu et al. 1995), environmental particles (Veranth,

Kaser et al. 2007), and hyperoxia (Odoms, Shanley et al. 2004). Because it is challenging to generate TEER values higher than 100 ohm·cm^2 using BEAS-2B cells (Noah, Yankaskas et al. 1995), this cell line is not frequently in drug permeability studies, but is more commonly used to investigate the expression and activity of drug metabolising enzymes (Proud, Subauste et al. 1994; Eaton, Walle et al. 1996).

A relatively new cell line, NuLi-1, developed from primary airway epithelial cells infected with retroviruses expressing hTERT and HPV-16 E6/E7, has not to date been characterised for its use in biopharmaceutics (Zabner, Karp et al. 2003). When grown on collagen-coated, semi-permeable membranes (Millicell-PCF), NuLi-1 TEER decreased over 30 passages from 685±31 to 389±21 ohm·cm^2. The TEER of NuLi-1 over 30 passages is within reasonable range of that observed in the primary bronchial cultures (532±147 ohm·cm^2). Thus, NuLi-1 cells can form an electrically tight airway epithelial barrier that mimics the active and passive ion transport properties of primary human bronchial epithelial cells (Zabner, Karp et al. 2003).

1.2.1.3. In vitro models of cystic fibrosis airway epithelium

Cystic fibrosis (CF) is an autosomal recessive disease and is the most common lethal genetic disease in the world (O'Dea and Harrison 2002). There are approximately 30,000 patients in the USA and Europe (Boucher 2004; Laube 2005). CF is caused by mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene located at chromosome 7 and is most commonly associated with defective chloride transport in airway epithelial cells. Lung pathology in CF includes abnormally low, and in extreme cases nil, chloride transport, increased mucus viscosity, reduced mucociliary clearance rates, recurrent infections, chronic inflammations and airway damage (Boucher 2004). Because of the significance of the disease, the most visible gene therapy schemes in development are inhalable regimens containing DNA complimentary to CFTR to treat CF by restoration of CFTR function in the airways of the lung.

In vitro models based on CF airway epithelium have been used widely in order to better understand CF pathophysiology, study alterations in airway permeability, and to assess the efficiency of gene vectors. Utilising approaches similar to those for cultivation of cells from healthy tissues, several protocols have been developed to culture airway epithelial cells harvested from CF patients (Gruenert, Basbaum et al. 1990). The development of immortalised cell lines with a CF phenotype has been significantly beneficial for investigators in the CF field by alleviating the major limitation inherent in primary cell culture models: the very limited availability of suitable CF tissues (Gruenert, Finkbeiner et al. 1995; Gruenert, Willems et al. 2004). A number of these CF cell lines have been reported exhibit polarisation of cell layers with the necessary bioelectrical tightness to make them potentially useful for drug permeability studies. Examples of human immortalised CF airway epithelial cell lines are NCF3 (Scholte, Kansen et al. 1989), CFT1 (Olsen, Johnson et al. 1992), CFBE41o- (Ehrhardt, Collnot et al. 2006) and CuFi-3 and -4 (Zabner, Karp et al. 2003). It should be noted that only the CFBE41o- and CuFi cell lines have been characterised and validated regarding their long-term stability as reliable *in vitro* models.

1.2.2. In vitro models of the alveolar airspace

1.2.2.1.Primary alveolar epithelial cell culture models

Due to the paucity of appropriate alveolar epithelial cell (AEC) lines that form functional tight junctions, primary cultures of AEC are used for most *in vitro* studies of alveolar epithelial function (e.g., solute transport and metabolism). Primary mammalian AEC techniques involve isolation, purification, and culture of ATII cells from tissues obtained after lung resections or from isolated perfused lungs. These ATII cells, when plated on permeable supports or plastics under appropriate culture conditions, acquire type I cell-like phenotypes and morphologies (Danto, Shannon et al. 1995; Fuchs, Hollins et al. 2003; Demling, Ehrhardt et al. 2006). Mocular markers and lectins present in AT I and AT II epithelial cells are listed in *Table 2:* Molecular markers and lectins used for differentiation analysis between alveolar type I and type II epithelial cells

Table 2: Molecular markers and lectins used for differentiation analysis between alveolar type I and type II epithelial cells

Alveolar type I cell	Alveolar type II cell
T1α*	Surfactant protein (SP) -A, -B, -C and -D
Aquaporin-4 (AQP-4)	Aquaporin-3
Aquaporin-5 (AQP-5)	γ-aminobutyric acid receptor π-subunit (GABRP)*
Receptor for advanced glycation end products (RAGE)	
Caveolin-1	*Maclura pomifera* agglutinin
Fibroblast growth factor receptor-activating protein-1 (FGFR AP-1)*	*Helix pomatia* lectin
P2 purinergic receptor 7 (P2X7)*	*Sambucus nigra* agglutinin
Interferon-induced protein*	
Bcl2-associated protein*	
Lycopersicon esculentum lectin	
Ricinus communis agglutinin	
Erythrina cristagalli lectin	
Bauhinia purpurea agglutinin	
Soybean lectin	

* not yet confirmed in human pneumocytes

Although isolation of ATI pneumocytes from rat lungs has recently been reported with some success (Borok, Liebler et al. 2002; Johnson, Widdicombe et al. 2002; Chen, Chen et al. 2004), development of a confluent ATI cell monolayer with electrically tight characteristics has not been reported yet. It should be noted that unlike many other cells in primary culture, AEC generally exhibit a very limited proliferation profile and are therefore not suitable for passaging. Thus, a new preparation of cells has to be used for each data set which is tremendously costly. Furthermore, a necessary, reliable normalisation scheme for data observed from each set of cell preparations has not been developed.

Due to the lack of availability of human lung tissues and ethical issues pertaining to use of human tissues, most lung permeability studies have been based on isolation and culture of cells from the lungs of animals including mouse (Corti, Brody et al. 1996), rat (Goodman and Crandall 1982), rabbit (Shen, Elbert et al. 1999) and pig (Steimer, Franke et al. 2007). Since evidence for species differences between human and rodents might be more significant than once assumed (King and Agre 2001), confirmation using human pneumocyte cultures has been performed

for various aspects (Bingle, Bull et al. 1990; Ehrhardt, Kim et al. 2005; Wang, Edeen et al. 2007).

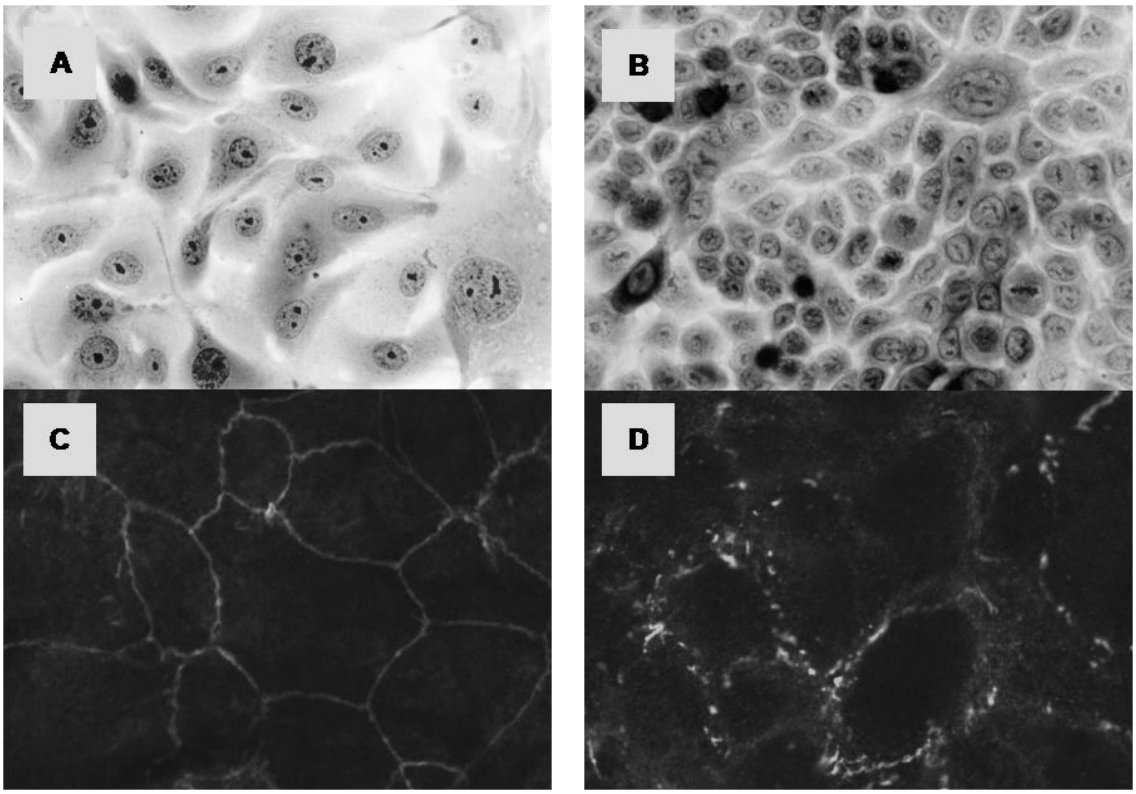
1.2.2.2. Alveolar epithelial cell lines

While a number of immortalised cell lines emanating from different cell types of the airway (i.e., tracheo-bronchial) epithelium of lungs from various mammalian species are available (see above), reliable and continuously growing cell lines that possess alveolar epithelial cell morphology and phenotype have not been reported to date. Most studies have relied on the use of cell lines of alveolar epithelial origin for drug absorption studies with observations that are hard or meaningless to extend to humans.

The most frequently used alveolar epithelial model is the A549 cell line (American Type Culture Collection, ATCC CL-185); a continuously growing cell line derived from a human pulmonary adenocarcinoma that has some morphologic and biochemical features of the human pulmonary alveolar type II cell *in situ* (Lieber, Todaro et al. 1976). A549 cells contain multilamellar cytoplasmic inclusion bodies, like those typically found in human lung ATII cells, although these hallmarks disappear as culture time increases. At early and late passage levels, the cells synthesise lecithin with a high percentage of disaturated fatty acids utilising the cytidine diphosphocholine pathway (Lieber, Todaro et al. 1976). The cell line has been utilised for many biopharmaceutical studies despite the fact that A549 cells lack the ability to form tight monolayers of polarised cells due to their inability to form functional tight junctions (Foster, Oster et al. 1998; Elbert, Schäfer et al. 1999; Kim, Borok et al. 2001; Forbes and Ehrhardt 2005).

Figure 4 highlights the differences in morphology between primary human alveolar epithelial cells (hAEpC) that started as ATII cells and differentiated into ATI-like morphology at 8 days in primary culture (Panel A) and A549 cells on day 5 in culture (Panel B) by light microscopy. In addition, Figures 4C and 4D show results of immunofluorescence microscopy using an antibody against occludin (a tight junctional protein) for the hAEpC monolayer and A549 cells, respectively.

Figure 4: Morphological diferences between human alveolar epithelial cells in primary culture at day 8 (A and C) **and the A549 cell line at 5 days** (B and D). Cells are visualised by light microscopy (A and B) and immunofluorescence microscopy (C and D), (Ehrhardt and Kim 2008).



Despite their obvious unsuitability due to a lack of good transepithelial electrical resistance, some investigators still employed A549 cell layers in drug absorption studies (Kobayashi, Kondo et al. 1995). The absorption data reported by these groups were not able to clearly show differences in transport rates between proteins and peptides of various sizes where the published permeability values were 2-4 orders of magnitude higher than those obtained in tight monolayer systems (e.g., hAEpC monolayers) using identical compounds (Forbes, Lim et al. 2002). Moreover, the reported A549 TEER of ~600 ohm·cm^2 by one group (Kobayashi, Kondo et al. 1995) may be in gross error. Their data indicate that large hydrophilic molecules are translocated across A549 cell layers at rates approaching near-free diffusion limits. In other words, such large fluxes probably took place via large gaps between A549 cells, contradictory to the very large TEER they reported. It should be noted that

the TEER of A549 cells is usually in the range of 20 - 60 ohm·cm^2. These lower, measured TEER values are consistent with the knowledge that no functional tight junctions are present in A549 cell layers (Foster, Oster et al. 1998; Elbert, Schäfer et al. 1999). Notwithstanding, A549 cells might still be a useful model in other areas of biopharmaceutical research, including metabolism or cytotoxicity studies for various substances (Foster, Oster et al. 1998; Forbes, Wilson et al. 1999; Anabousi, Bakowsky et al. 2006).

Other cell lines of an alveolar epithelial origin used in published studies include H441 (human), MLE-12 and 15 (mouse), and L-2 and R3/1 (rat). Of these, the NCI-H441 cell line (ATCC HTB-174), originating from a human lung adenocarcinoma, has been described as having characteristics of both ATII (Duncan, Whitsett et al. 1997; Rehan, Torday et al. 2002) and bronchiolar (i.e., Clara) epithelial (Zhang, Whitsett et al. 1997; Newton, Rao et al. 2006). Studies can thus be designed to characterise metabolism and transport properties of these two particular cell types in a single culture. There is emerging evidence that H441 cells are capable of forming polarised monolayers that exhibit significant transepithelial electrical resistance (Shlyonsky, Goolaerts et al. 2005; Woollhead and Baines 2006), but the cell line has not been used for drug absorption experiments to date.

Wikenheiser and co-workers generated a series of continuous alveolar epithelial cell lines [MLE-7, -12, and -15] from transgenic mice harbouring the SV40 large T antigen under the control of the human SP-C promoter region. These MLE cell lines maintained morphological and functional characteristics of distal respiratory epithelial cells normally lost after isolation and primary culture (Wikenheiser, Vorbroker et al. 1993), which are consistent with those seen in non-ciliated bronchiolar and ATII epithelial cells. However, morphological and functional characteristics associated with an individual cell type do not appear to always co-exist in a clonal cell line.

The L-2 cell line (ATCC HTB-149) is derived from cells isolated from the adult rat lung using clonal culture techniques. These cells appear to retain differentiated functions that are present in ATII cells of intact rat lungs. L-2 cells are diploid,

epithelial cells. They contain osmiophilic lamellar bodies in their cytoplasm and synthesise lecithin by the same *de novo* pathways as in a whole lung (Douglas and Kaighn 1974). It is not known if L-2 cells are capable of forming confluent and electrically tight monolayers. L2 cells have not been systematically investigated for suitability as a model for absorption studies.

In 2004, the rat cell line R3/1 was established from cells obtained from broncho-alveolar tissues of foetal Han-Wistar rats at 20 days of gestation. This cell line displays a phenotype with several characteristic features of ATI cells. R3/1 cells were analysed to express mRNA and protein markers related to the ATI cell type (i.e., T1α, ICAM-1, connexin-43, caveolins-1 and -2 and receptor for advanced glycation end products (RAGE)). Moreover, lectins *Bauhinia purpure* agglutinin (BPA) and Soybean lectin (SBA) which are characteristic for ATI in normal rat lung were bound to R3/1 cells (Koslowski, Barth et al. 2004; Reynolds, Kasteler et al. 2008). To assess potential ATII cell properties, presence of surfactant proteins SP-A, SP-B, SP-C and SP-D was investigated and compared with normal rat lung tissue. AT II cells in lung tissue showed strong expression of all SPs; R3/1 cells displayed moderate staining for SP-A and SP-B, while SP-C and SP-D were not found. R3/1 cell line was also tested for expression of mesenchymal marker proteins (Barth, Gentsh et al. 2005). R3/1 cells exhibited a mixed phenotype of epithelial (aquporin-5 and cytokeratin-8) and myofibroblast-like (vimentin, α-smooth muscle actin and caveolin-3) features suggesting that this cell line was established from cells at certain stage during epithelial-mesenchymal transition. The AT I-like cell line R3/1 seemed to be a promising *in vitro* model for the study of lung biology.

2. OBJECTIVES OF THE STUDY

The aim of the study was to characterise the rat alveolar R3/1 cell line regarding its property to form tight cell monolayers as necessary for *in vitro* models used for drug absorption studies. Additionally, the presence of peptides degrading enzymes was to be analysed in R3/1 cells to describe the cell line as a possible model for protein stability studies.

In order to characterise the cell line as *in vitro* model for drug disposition studies, R3/1 cells were cultured on filter inserts under various conditions. The transpithelial electrical resistance was measured as a parameter for integrity of cell layers. Molecular biological techniques (RT-PCR, Western blot, immunofluorescence microscopy) were employed to investigate expression of tight junction proteins (occludin, E-cadherin and ZO-1 and ZO-2 proteins) and proteolytic enzymes on mRNA and protein levels.

2.1.Theoretical background

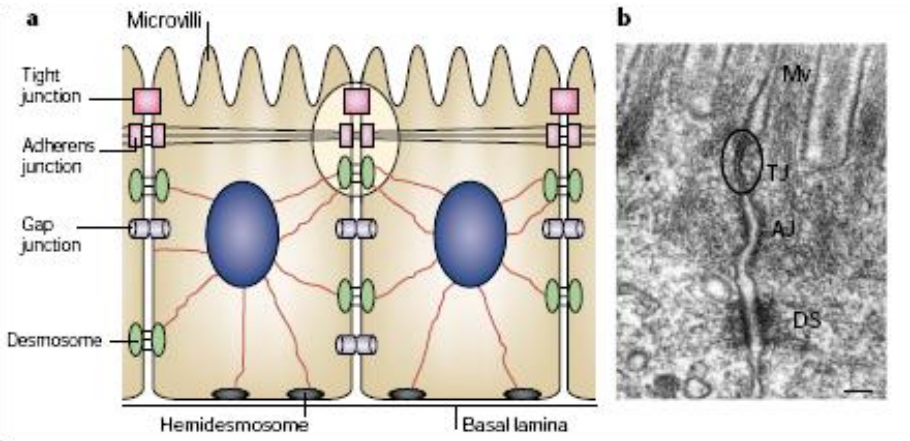
2.1.1. Tight junctions

Two adjacent epithelial cells are tightly connected together to seal paracellular pathway and separate apical and basolateral part of cells in order to allow active transport across cell layers. These cell connections can be divided into four subcategories of junctions *(Figure 5)* tight junctions (TJs), adherens junctions (AJs), gap junctions (GJs) and desmosomes. TJ, AJ and GJ which are located at the most appical region of cells creates the junctional complex (Tsukita, Furuse et al. 2001; Balkovetz 2006).

Figure 5: Junctional complex and tight junction. (Tsukita, Furuse et al. 2001):

a schematic drawing of epithelial cells (intestine)

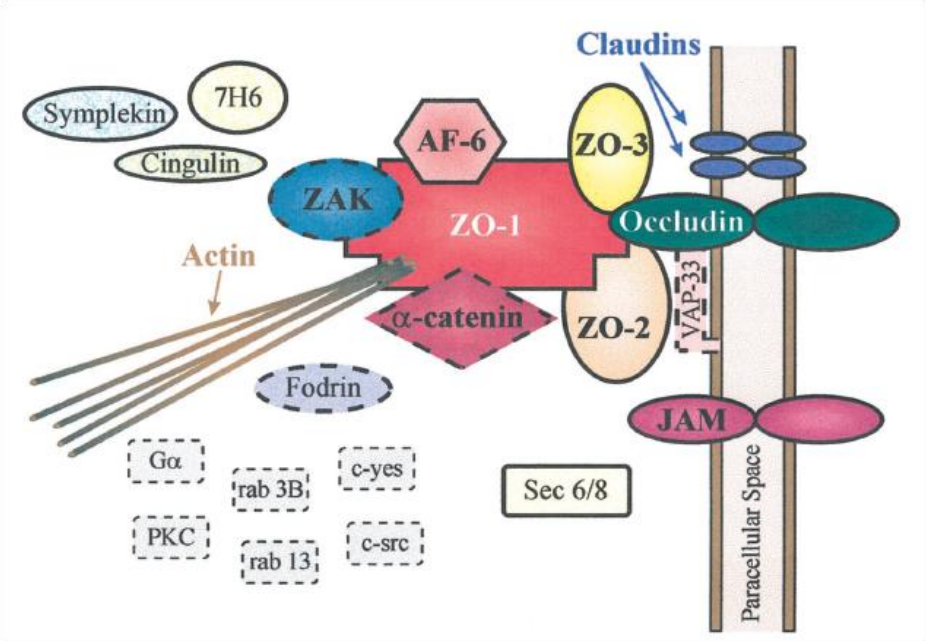
b electron micrograph of the junctional complex.



The junctional complexes consist of number of transmembrane and cytosolic proteins; function of many of them is not completely investigated up to date. Architecture of proteins involved in TJ is shown on *Figure 6.* Some proteins are specific to one type of junction only, others can be found in several types. In TJs, contact between adjacent cells is provided with transmembrane domains of occludin and of claudin family. Number of cytosolic proteins (zonulae occludens proteins 1-3 (ZO-1, -2 and -3, etc), fodrin, etc.) is associated with occludin and claudins (Mitic and Anderson 1998; Fanning, Mitic et al. 1999) and thus a linkage of TJ proteins with cytoskeleton is provided. Junctional adhesion molecules (JAMs; JAM 1-3) are a family of immunoglobuline superfamily proteins colocalized with occludin and claudins at TJ level of epithelial and endothelial cells. JAMs have been shown to bind to TJ-associated cytoplasmic proteins although their role in TJs has still not been identified, JAM protein complex regulates paracellulr permeability (Mandell and Parkos 2005; Vandenbroucke, Mehta et al. 2008).

Adherens junction (AJ) is the most frequent junction in the endothelial barrier (TJs are the most common in the epithelium). The transmembrane part of AJs consists mainly of E-cadherin and nectin transmembrane proteins connected to actin via cytosolic α-catenin and afadin (Niessen and Gottardi 2008).

Figure 6: Organisation of integral membrane proteins in TJs (Fanning, Mitic et al. 1999)



GJs interconnect the cytoplasm of adjacent cells through structures called connexons. Each connexon (Cx) comprises 6 polypeptide subunits (connexins) arranged radially to form a pore (Koval 2002; Yeager and Harris 2007; Alldredge 2008). These pores enable direct diffusion of small molecules and ions from one cell to the neighbour one (Koval 2002).

Cell-cell junctions desmosomes maintain the structural integrity of tissues. Desmosomes connect two adjacent cells in lateral edges. The transmembrane part of desmosome molecular complex is formed by desmogleins and desmocollins; proteins of cadherin family (Stokes 2007; Garrod and Chidgey 2008).

2.1.2. Proteolytic enzymes

The availability of peptides is limited by activity of proteolytic enzymes (Kobayashi, Kondo et al. 1996; Yamamoto, Fujita et al. 1996; Forbes, Wilson et al. 1999). Peptidases are enzymes responsible for cleavage and thus possible inactivation of small protein molecules. They are present on the surface of tissues in the whole body and take part in various processes. Peptidases are classified according to the site of their effect in their substrates, endopeptidases cleave

molecule of the substrate in the middle of the peptide, exopeptidases at the contrary romove aminoacids either from one of the ends of peptide chains. According to the site of action, exopeptidases are divided into group of aminopeptidases cleaving aminoacids (AAs) from N-terminus and carboxypeptidases removing AAs from C-terminus of peptide (Van Der Velden and Hulsmann 1999). From a wide range of peptidases, the interest of this study *(Table 3)* focused mainly on peptidases whose presence in the lung has been shown already although usually without information specifying localization within the lung regions.

Table 3: Peptidases in the lung

Enzyme	Abbrev., EC number Full name	Synonyms	Substrate(s)	Lung region	Literature
NEP	3.4.24.11 neutral endopeptidase	neprilysin enkephalinase CALLA, CD 10	bradykinin angiotensins enkephalins	bronchial EC alveolar EC (AEC)	ABCF
EP	3.4.24.15 endopeptidase 24.15	thimet oligopeptidase	enkephalins	lung	L
APA	3.4.11.7 aminopeptidase A	glutamyl aminopeptidase	angiotensins	lung	AM
APB	3.4.11.6 aminopeptidase B	arginine aminopeptidase	enkephalins leukotrien A	lung	I
APN	3.4.11.2 aminopeptidase N	CD 13	enkephalins IL-8 opioid peptides	bronchi AEC ATI lung EC	AEFKO
APP	3.4.11.9 aminopeptidase P	prolyl aminopeptidase	bradykinin	lung	N
ACE	3.4.15.1 angiotensin-I convering enzyme	kininase II	bradykinin angiotensins enkephalins substance P	lung AEC	ACFO
DPP IV	3.4.14.5 dipeptidyl peptidase IV	CD 26	bradykinin substance P	bronchi	AEF
GGT	2.3.3.2 γ-glutamyl transferase		glutathione	AEC AT II	GH
CPM	3.4.17.12 carboxypeptidase M	kininase I	bradykinin	AEC, ATI	CDF

Summary of the literal sources of Table 3:

Literature	
A	Van Der Velden and Hulsmann 1999
B	Baraniuk, Ohkubo et al. 1995
C	Dragovic, Igic et al. 1993
D	Nagae, Abe et al. 1993
E	Van Der Velden, Wierenga-Wolf et al. 1998
F	Forbes, Wilson et al. 1999
G	Rahman, Bel et al. 1998
H	Joyce-Brady, Takahashi et al. 1994
I	Foulon, Cadel et al. 1999
J	Cadel, Gouzy-Darmon et al. 2004
K	Albiston, Ye et al. 2004
L	Chu and Orlowski 1985
M	Lojda and Gossrau 1980
N	Prechel, Orawski et al. 1995
O	Wang, Toledo-Velasquez et al. 1993

2.2. Principles of methods

2.2.2. *TEER*

The transepithelial electrical resistance (TEER) is determined by formation of tight junctions. According to Ohm´s law, TEE = (*PD*/*Isc*) x *A*; where *PD* is potential difference, *Isc* short-circuit current and *A* is permeation area. Epithelial cells have asymmetric distribution of ion channels on the apical and basolateral membranes, *PD* is equal to the difference between the voltage of the apical and basolateral membranes. *Isc* is defined as the charge flow per time when the cell monolayer is short-circuited, i.e. *PD* is maintained zero value. *A* corresponds with the surface area of filter. Epithelial Volt Ohm Meter (EVOM) *(Figure 7)* measures resistance across the membrane using a pair of chopstick electrodes placed in contact with

media on apical as well as basolateral side of membrane. The advantage of this method is that the checking of membranes is non-destructive.

Figure 7: Example of epithelial voltohmmeter
(http://www.pharmaceutical-int.com/images/companies/1529/wpi17.jpg)

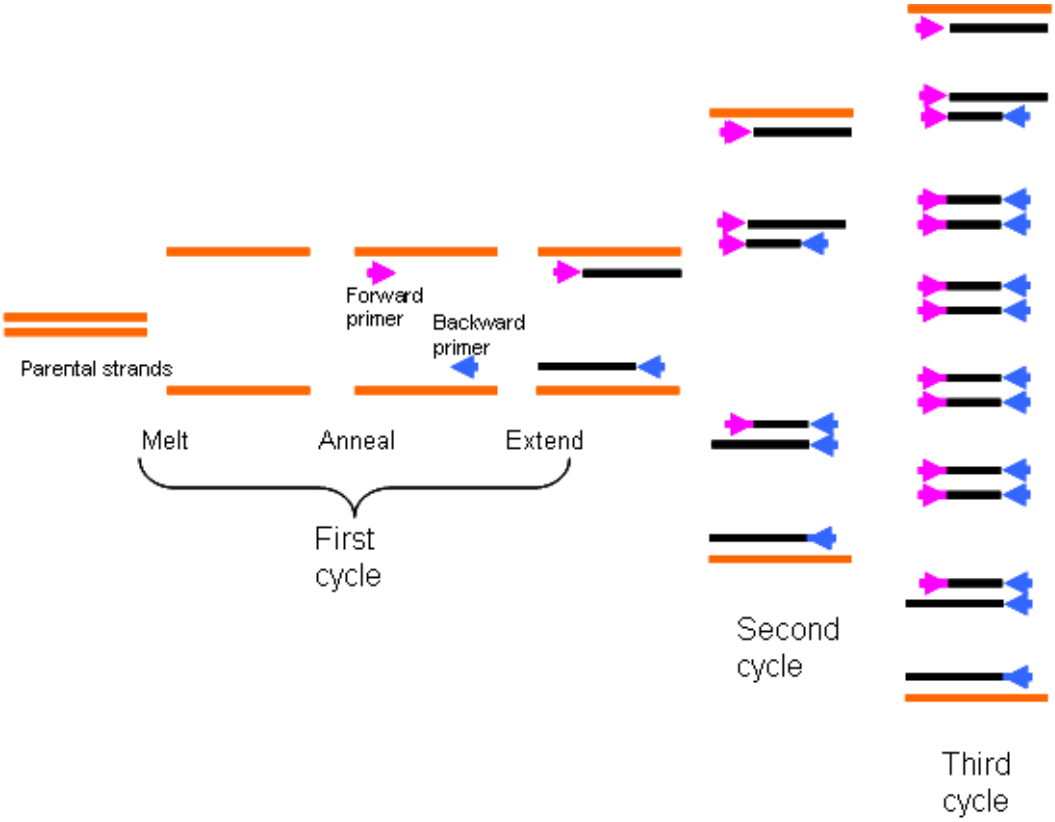
2.2.2. RT-PCR

Polymerase chain reaction (PCR) allows synthesise copies of sequence of particular DNA. A typical cycle of PCR involves 3 steps: denaturation, annealing and elongation step. After initialisation of PCR, i.e. heating reaction mixture for couple of minutes at temperature close to 95°C; denaturation step takes place. During denaturation, strands of DNA and primers are melted by disruption of hydrogen bonds in their structures. After that, primers can align to DNA strand (for this, optimal conditions like temperature or concentration of Mg^{2+} ions for each primer pairs are required). For the last step, primers elongation, optimal temperature is dependent on polymerase enzyme used. Polymerase synthesises new DNA strand

from dNTPs. When the replication of the segment between the two primers (one cycle) is complete, the two new duplexes are heated and denaturated to single strand templates. With increasing number of repetition of cycles of synthesis and denaturation, usually 25-40 times, sequences of DNA of defined length are exponentially amplified and the product of PCR is run on gel electrophoresis to visualise product and its size (*Figure 8).*

Figure 8: Schematic drawing of PCR method

(http://www.obgynacademy.com/basicsciences/fetology/genetics/images/pcr.png)



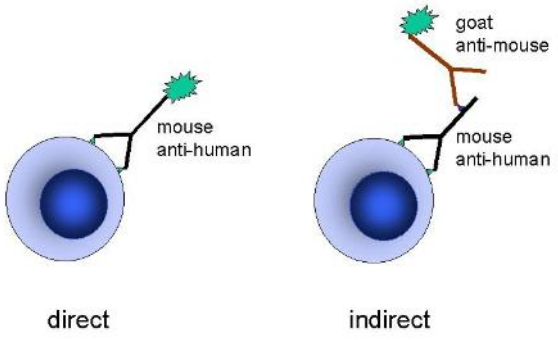
2.2.3. IFM

Cells to be examined with immunofluorescent microscopy (IFM) are grown on solid supports, fixed and permeabilised to allow antibodies to access

to intracellularly located antigen. Fixation can be done either with organic solvents (acetone, methanol) with the mechanism of removing lipids and dehydratation of cells or with cross linking agent (paraformaldehyde) creating intermolecular bridges usually through free amino-acids groups. Fixed and permeabilised specimens are then incubated with antibody. Unbound antibody is washed out and the bound antibody is either detected directly (if the primary antibody is labelled) or indirectly (using a fluorochrome-labelled secondary antibody) (*Figure 9*). Stained preparations are evaluated using a fluorescent or confocal laser scanning microscope.

Figure 9: Direct and indirect immunofluorescence

(http://uk.geocities.com/mgormerod@btinternet.com/Figure4-1.jpg**)**



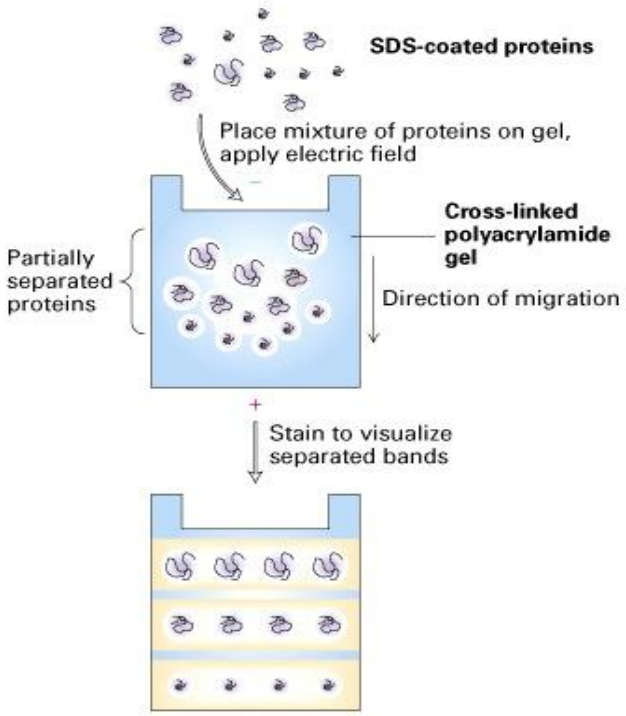
2.2.4. Immunblotting

Western blot (WB) is an immunoblot technique by which proteins can be detected in tissue or other samples. At the first stage, samples precede gel electrophoresis, in most cases SDS-polyacrylamide gel electrophoresis (SDS-PAGE); the mobility of proteins through gel matrix is dependent on their size and concentration of acrylamide. Before electrophoresis itself, samples are treated with solution containing β-mercaptoethanol and SDS (sodium dodecyl suplhate). β-mercaptoethanol cleaves disulfide bonds while SDS (anionic detergent) denaturates proteins and gives them negative charges. This negative charge dominates over intrinsic charge of protein, so during electrophoresis migration of proteins depends only on their sizes (*Figure 10*). Proteins resolved by electrophoresis are transferred

onto cellulose or polyvinylidene fluoride (PVDF) membranes. Proteins are negatively charged (SDS coating effect) and move from negative side (gel) towards positive electrode side, represented by membrane. Protein molecules are identified by probing the blot with antibody to specific protein (*Figure 11a*).

Figure 10: Gel electrophoresis

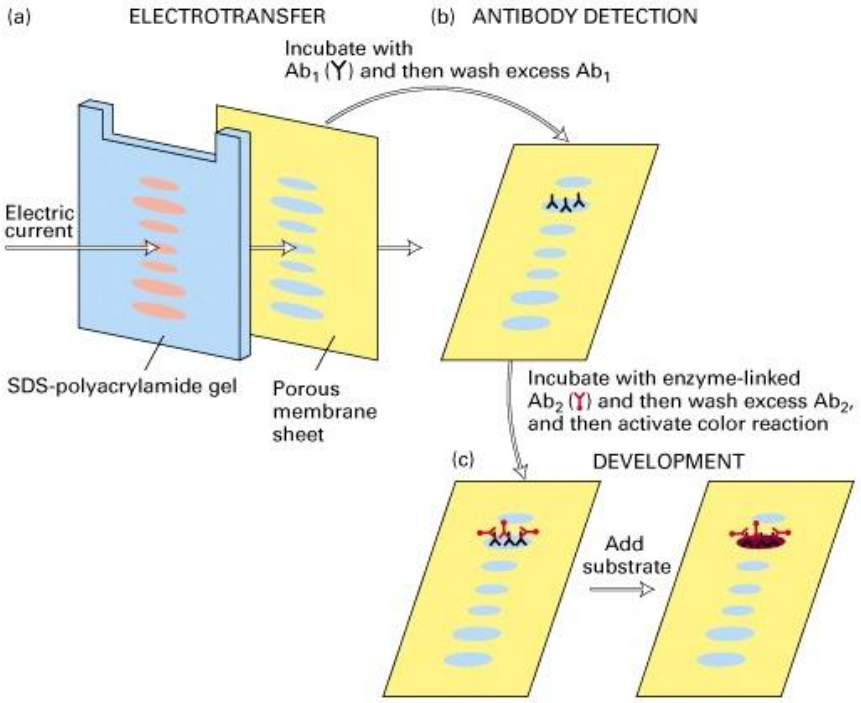
(http://www.life.umd.edu/classroom/bsci423/song/F03-41.jpg)



Membranes are first incubated with blocking agent that blocks non-specific protein binding sites and then blotted with primary antibody specific for the protein of interest. Unbound primary antibody is washed away and membranes are incubated with secondary antibody which is specific for primary antibody and conjugated with horseradish peroxidase (HRP) *(***Chyba! Chybný odkaz na záložku.***b).* HRP substrate is added after washing and HRP catalyses reaction in which colourless substrate is transformed into coloured product which visualise the protein band (*Figure 11c*).

Figure 11: Protein transfer, detection and development

(http://www.life.umd.edu/classroom/bsci423/song/Lab12.html)



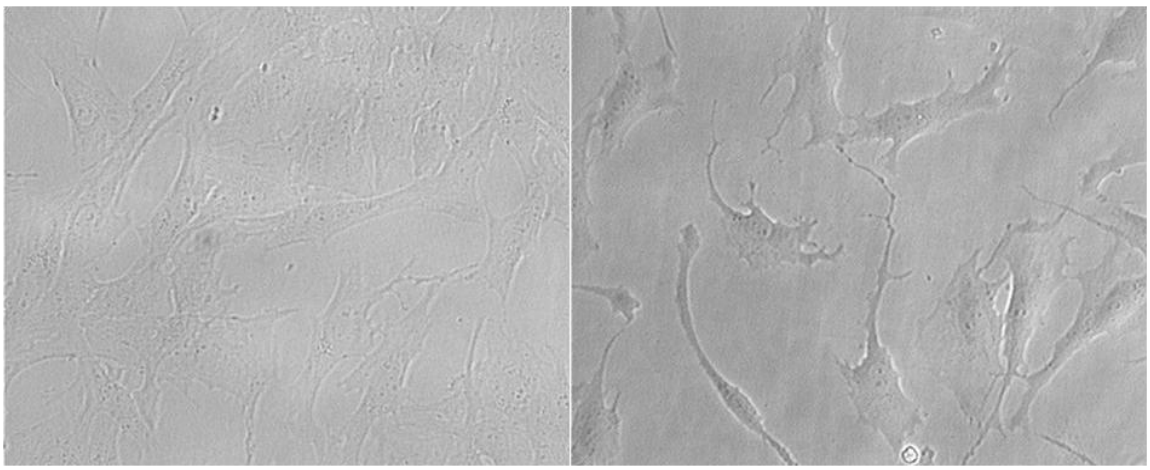
3. MATERIALS AND METHODS

3.1. Cell culture

3.1.1. R3/1 cell line

R3/1 cells of passage numbers 40-59 were grown in 75 ml flasks in an 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham´s F-12 medium (Sigma, Dublin, Ireland) at 37°C in 5% CO_2 atmosphere *(Figure 12).*

Figure 12: Culture of R3/1 cells



The DMEM/Ham's F-12 was supplemented with 10% (v/v) foetal bovine serum (FBS), 1% (v/v) non-essential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 mM HEPES. Moreover, RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin was used as an alternative medium.Cells were fed with 14 ml of fresh media every 2 days. Cells were split when confluent by trypsin EDTA solution, before trypsination cells in flask were washed twice with 10 ml of PBS. Washed cell layers were topped with 3 ml of trypsin/EDTA solution and the flask was placed into incubator (37°C, 5% CO_2) for 3 minutes to allow cell detachment. After that, trypsin/EDTA solution was inactivated with 7 ml of media and cell suspension was homogenised by pipetting several times up and down. Cells were counted manually using a Neubauer-type hematotocytometer, a calibrated counting chamber with identically duplicate counting areas. A cover glass placed over the chamber provides a depth of 0.1 mm.

Each chamber area is divided into squares of equal sizes, 50 μm on each side. 50 μl of homogenised cell suspension was pipetted into sterile Eppedorf tube and mixed with the same volume of tryptan blue solution (Sigma). Tryptan blue staining method is based on the principle that polar dye can hardly cross viable cell membranes but damaged or leaky membranes allow to the dye to intracellular proteins. Under the detection under a light microscope, tryptan blue gives blue colour to the dead cells while living cells stay transparent. Non-stained cells were counted in both parts of chamber and if this numbers did not differ more than 10%, average number from these two countings was used for further calculation. According to parameters of hematocytometer, cells concentration per 1 ml was obtained when multiplying the number of cells counted in chamber by 20.000.

To study the influence of media composition on R3/1 cell monolayer integrity, cells were seeded on Transwell Clear polycarbonate permeable filter inserts (Fisher Scientific, Dublin, Ireland) at densities of 0.5 - 8×10^5 cells/cm^2 and cultured under liquid-covered culture (LCC) or air interfaced culture (AIC) conditions. The effect of dexamethasone (DEX, 0.1 μM) and FBS (0-20%) in the culture medium on the cell function was assessed.

3.1.2. Primary alveolar epithelial cells

Specific pathogen-free adult Sprague-Dawley male rats weighing 120-150 g were euthanized with sodium pentobarbital (2.5 mg/kg, i.p.). Rat lungs were perfused *in situ* with 0.9% NaCl solution and alveolar type II (AT II) cells were isolated from the lungs following elastase digestion (Kim, Cheek et al. 1991). The crude cell mixture was filtered sequentially through 100, 40, and 10 μm meshes, followed by plating onto IgG-coated bacteriological plates. After 1 h incubation, type II cells were collected and centrifuged at 150 *g* for 10 min for further enrichment. Purified rat type II pneumocytes were resuspended and either used directly or seeded onto Transwell® filter inserts at 1.2×10^6 cells/cm^2. Culture medium consisted of DMEM/Ham's F-12 1:1 supplemented with 10% (v/v) newborn bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were fed on day 3 and every other day thereafter. These monolayers transdifferentiated into cells bearing type I

cell-like morphology and phenotype (Borok, Hami et al. 1995). Total RNA was isolated from freshly isolated ATII cells or on day 8 of culture utilising type I cell-like monolayers.

3.2. TEER

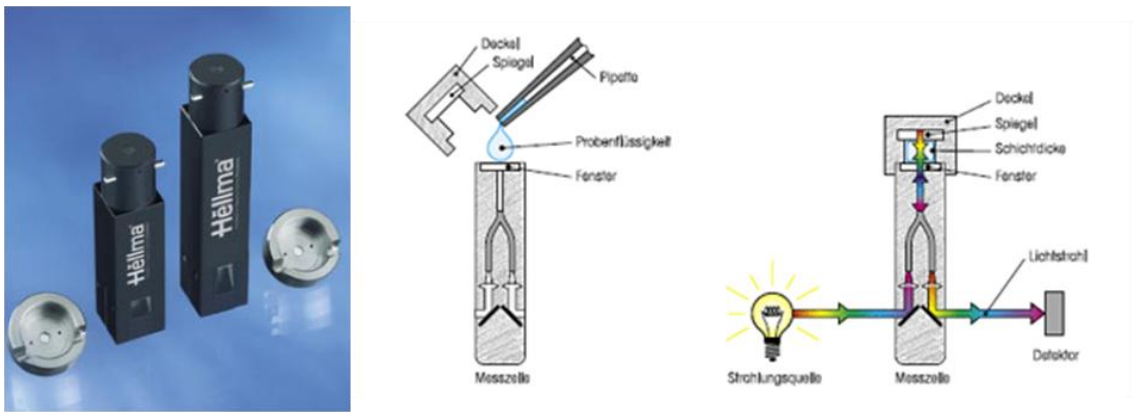
TEER was measured as a parameter of integrity cell monolayer. Cells were seeded on Transwell® Clear permeable filter inserts at densities $2 – 8 \times 10^5$ cells/ cm^2 and cultured under LCC or AIC conditions. Cells were fed every second day with fresh media as follows: LCC grown cells were fed with 1,5 ml of media into basolateral part of filter and 0.5 ml of media into the apical compartment. Media for AIC cells were added into basolateral compartment only, in this case 650 µl was used for each filter. Cells were grown in three different culture media (RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin; DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml and streptomycin; and DMEM/F-12 Ham 1:1, complemented as above. Furthermore, 0.1 µM dexamethasone was added to the media. TEER was measured daily using a Millicell-ERS epithelial voltohmmetre (Millipore, Carrigtwohill, Ireland) fitted with chopstick electrodes. Obtained values were corrected for background resistance contributed by filter and medium. TEER of Transwell filters with LCC culture was measured directly, cells cultured on filters under AIC had to be filled with media up to the volume of LCC conditions, i.e. up to 0.5 ml in apical and 1.5 ml in basolateral compartmen, and the TEER measurement itself was carried out at least 15 minutes after volumes were adjusted.

3.3. RT-PCR

To assess the transcription of tight junction proteins as well as catabolic enzymes in R3/1 cells, the total mRNA was isolated using an RNeasy Mini kit (Quiagen, Crawley, UK). R3/1 cells were plated in 6-well cell culture plates at density 20.000 cells/cm^2, fed every second day with 3 ml of fresh media and harvested at day 6 after seeding. Cells were lysed directly in 6-well plates with 350 µl of RLT buffer (supplemented with 10 µl of β-mercaptoethanol per 1 ml of RLT buffer), detached

with cell scraper and the lysate was homogenized by passing 5 times through needle fitted to a RNase free needle. The homogenized lysate was mixed with the same volume of 70 % ethanol and transferred on RNeasy spin column placed in 2 ml microcentrifuge tube and centrifuged for 15 s at 10.000 rpm. The flow-through was discarded and centrifugation was repeated with RW1 buffer followed by two centrifugations with RPE buffer. As a last step, 30 µl of RNase free water was added to the columns to elute mRNA. Messenger RNA was quantified by UV absorption at 260 nm using a spectrophotometer NanoDrop ND 1000, (NanoDrop Technologies, Wilmington, DE). 1 µl of each sample was pipetted onto the TrayCell – Fibre-Optical Ultra-Micro Measuring Cell (Hellma, Essex, UK), cap was fitted on the cell prior the measurement *(Figure 13).* The cap of the cell creates defined optical light path and due to integrated beam deflection and the use of fibre-optic cables, it was possible to measure the sample directly on the surface of the optical window. The measurement of mRNA concentration of particular sample was repeated 3 times with the same 'drop' of sample, the cap prevented drying out of the samples or sample enrichment due to evaporation of the solvent.

Figure 13: Hellma TrayCell (www.traycell.com/en/img/traycell_1_2.png)



The purity of RNA was estimated by comparison absorption at 260 nm and 280 nm (the A260/A280 ratio), moreover quality of all mRNA samples were checked on 1 % agarose gel electrophoresis containing ethidium bromide. Samples were diluted with RNase free water to equal concentration (100 or 200 µg/ml) and potential

contaminating DNA was removed with Turbo DNA-free™ (Ambion, Austin, TX). For 50 µl of reaction, 5 µl of TURBO DNase Buffer and 1 µl of TURBO DNase (2U/µl) were added to the RNA sample and this mixture was incubated for 30 min at 37°C using a Primus 96 Advanced Gradient termocycler (Peqlab, Erlangen, Germany). 5 µl of DNase Inactivation Reagent was put into the same tube and mixed occasionally at the room temperature. Tubes were then spun down and supernatant transferred into new tube and reverse transcription (RT) step followed immediately.

20 µl of RT mix contained 6.5 µl of water, 4 µl of 5x first strand buffer supplemented with DTT (0.1M), 2 µl of dNTP Mix (10 mM each), 0.25 µl of random hexamers (50ng/ml), 5 µl of RNA, 0.5 µl of RNasin (40U/ µl) and 1 µl of MMLV-RT (200U/µl) (Bioscript™, Bioline, London, UK). Negative controls (i.e. MMLV-RT was replaced with water) were run to eliminate samples with DNA contamination. RT-PCR amplification was performed using oligo primers (Eurofins MWG Operon, Ebersberg, Germany) as listed in *Table 4.*

Table 4: Primer sequences

Primer	Sequence		Product size	Optimal temperature
β-actin	forward	GTCGTACCACTGGCATTGTG	181	59°C
	reverse	CTCTCAGCTGTGGTGGTGAA		
E-cadherin	forward	CCTAGCTGGAATCCTGTCCA	164	62°C
	reverse	CACCAACACACCCAGCATAG		
Occludin	forward	TCTCAGCCGGCATACTCTTT	162	62°C
	reverse	ATAGGCTCTGTCCCAAGCAA		
ZO-1	forward	CACCACAGACATCCAACCAG	230	59°C
	reverse	CACCAACCACTCTCCCTTGT		
ZO-2	forward	GGCCTGGACCATGAAGACTA	232	59°C
	reverse	GTTCATAGCGGGTCTCTGGA		
NEP 24.11	forward	CATTGCCGCAAGAACTCATA	171	59°C
	reverse	TGTGAATTTCCCCCAAGAAG		
EP 24.15	forward	GGTCCTGCACACACAGACAG	198	59°C
	reverse	TTGAAGCGTGTGTGGAACAT		
DPP IV	forward	GAGGCAGCTTGGAACATAGC	225	59°C
	reverse	TGCTAAATGACCAGGCAACA		

CPM	forward	GATTCGAAGCCGTCAAGAAG	185	58.4°C
	reverse	ATGGAGATTCGCAGAGAGGA		
APM	forward	TTGTCAGACTGCCAGACACC	199	59°C
	reverse	TGTGCCCTGTTGATTCTTTG		
ACE	forward	AGTGGGTGCTGCTCTTCCTA	188	59°C
	reverse	ATGGGACACTCCTCTGTTGG		
GGT	forward	AAGACTCGGCACCACCATAC	179	59.8°C
	reverse	GTCCCACTCTCGTCTCTTGG		
APP	forward	TCCTCTCCCCAACTGTGAAC	242	56.2°C
	reverse	TCAGAGTCTGCCCACACAAG		
APA	forward	AAACCAGGATCACCAAGCTG	156	62°C
	reverse	TGGTCAGCCGATAGACACTG		
APB	forward	CTTCGAGATCCTGCACCTG	247	56.2°C
	reverse	GAAGGGCTGTGTGTGGAAAG		

Primer pairs were designed and picked from Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and aligned in BLAST database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) to confirm their unique presence within target species. Each PCR reaction contained 40 ng of cDNA, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 1.25 µl Taq buffer, 0.1 µl Biotaq^{TM} DNA polymerase (Bioline) (5U/ µl), 10pmol of each forward and reverse primer and DEPC treated water up to of final volume 12.5 µl. To facilitate strand separation in cases PCR amplification did not produce acceptable results, dimethyl sulfoxide (DMSO) in final concentration 1-5% was added to the reaction mixture. PCR program started with 5 min at 95°C, followed by 35 amplification cycles (denaturation at 94°C for 30 s, annealing at optimal temperature for concrete primer pair for 45 s and extension at 72°C for 45 s). After the last cycle finished, the reaction was terminated with final extension step for 10 min. at 72°C. At the beginning, each of designed primer pairs was optimized over the temperature gradient (54 - 62°C) using either rat genomic DNA or rat cDNA obtained from transcription of mRNA isolated from rat lung or duodenal tissue. Negative and positive controls were run in the same procedure; HyperLadder IV (Bioline) was used as a DNA size marker. The PCR products were mixed with 3 µl of 5x loading buffer (Bioline) and separated by 2% agarose gel electrophoresis containing ethidium bromide (0.2 µg/ml gel) in 0.5 x TBE buffer.

5x TBE buffer (stock solution)

Tris Base	54.0 g
Boric acid	27.5 g
$Na_2EDTA.2H_2O$	3.7 g
H_2O dd	Up to 1000 g

Bands were visualised under UV light using a ChemiDoc^{TM} Bio-Rad system.

3.4. Immunofluorescence Microscopy

The mouse monoclonal anti-occludin antibody (Zymed, San Francisco, CA) was diluted 1:100 in PBS containing 1% (w/v) bovine serum albumin (BSA, Sigma-Aldrich, Dublin). Mouse IgG1 (clone MOPC-21, Sigma) was used as an isotypic control. Transwell-grown R3/1 cells were stained on day 6 after cell plating. Filters were washed twice with PBS (apical and basolateral side). Cells were fixed for 10 min with 2% (w/v) paraformaldehyde (PFA). To prepare PFA solution, 1 g of PFA was added into 50 ml of PBS. Solution was warmed up in water bath with temperature not exceeding 70 °C in a fume hood. Few drops of 1M NaOH solution were added to facilitate the solubilisation, solution was cooled down in room temperature and pH was adjusted to 7.4. PFA is then blocked for 10 min in 50 mM NH_4Cl, followed by permeabilisation for 8 min with 0.1% (w/v) Triton X-100. After a 60 min incubation with 100 µl of the diluted primary antibody, the cell layers were washed three times with PBS before incubation with 100 µl of a 1:100 dilution of an Alexa Fluor 488-labelled goat anti-mouse $F(ab')_2$ fragment (BioSciences, Dun Laoghaire, Ireland) in PBS containing 1% (w/v) BSA. Propidium iodide (PI; 1 µg/ml) was then added to counterstain cell nuclei. After 30 min incubation, the specimens were washed three times with PBS. Filters were cut out and embedded in FluorSave anti-fade medium (Calbiochem, San Diego, CA). Specimens were stored in dark place for at least one hour prior microscopy analysis. Images were obtained on a fluorescence microscope (Zeiss Axiovert 200, Gőttingen, Germany).

3.5. Western blotting

R3/1 cells were treated with FBS free medium for 24 h. Medium was collected and used for analysis of soluble enzymes. For analysis of membrane bound enzymes, cells were washed twice with cold PBS and lysed with Cell Extraction Buffer (BioSciences) supplemented with proteases inhibitors leupeptin (10 μg/10ml of buffer) and aprotinin (60 μg/10 ml of buffer) (Sigma). Cells were detached with cell scraper; detachment was monitored by light microscopy. Cells were transferred into Eppendorf tube and membranes were disrupted with ultrasound needle (Microson ultrasonic cell XL-2000, Misonix Incorporated, Farmingdale, NY) – 2 bursts of 10 s each separated by 30 s cooling on ice. Samples from rat kidney and lung tissues passed through similar procedure with 4 bursts with ultrasound needle in total. After centrifugation (20 min at 10.000 rpm, 4°C), supernatant was collected into new Eppendorf tube and kept on ice. Concentration of proteins in all samples was carried out in a protein assay (Bradford 1976) assay using BSA as a standard (Bio-Rad Protein Assay Kit, Bio-Rad, Hercules, CA). Samples were diluted to the same concentration, western blot loading buffer (containing SDS and β-mercaptoethanol) was added (18 μl of sample + 6 μl of loading buffer), and samples were boiled for 3 min and spin down. In meantime, gels containing polyacrylamide and methylenbisacrylamide (separating/resolving gels) were prepared. Concentration of polyacrylamide in resolving gel differed from 5 to 15 % according to the size of target protein.

Separating gel (8% polyacrylamide):

30% acrylamide/0.8% bisacrylamide solution	4000 μl
4x Tris.Cl/SDS; pH 8.8	3750 μl
H_2O dd	7250 μl
10% ammonium persulfate (APS)(= 0.1 g APS + 960 μl H_2Odd)	50 μl
N,N,N',N'-tetramethylethylenediamine (TEMED)	10 μl

This amount was enough for 4 gels of mini blot size (8.6 x 6.8 cm). Mixture was poured between two glass crystals and topped with water to prevent oxidation and allow polymerisation to start. After polymerisation, sharp border between gel and water occurred and water from the top of polymerised gel was removed. Stacking gel was poured instead of water, comb creating pockets was inserted and gel was left to polymerise.

Stacking gel (for 4 gels):

30% acrylamide/0.8% bisacrylamide solution	1300 µl
4x Tris.Cl/SDS, pH 6.8	2500 µl
H_2O dd	6000 µl
10% APS	50 µl
TEMED	10 µl

When the gel became solid, comb was carefully removed, two gels were assembled together and gap between them was filled with 1x tank buffer.

10x Tank buffer:

Tris base	30.2 g
Glycine	144 g
SDS	10 g
H_2O dd	700 ml
Adjust to pH 8.3	
H_2O dd	up to 1000 ml

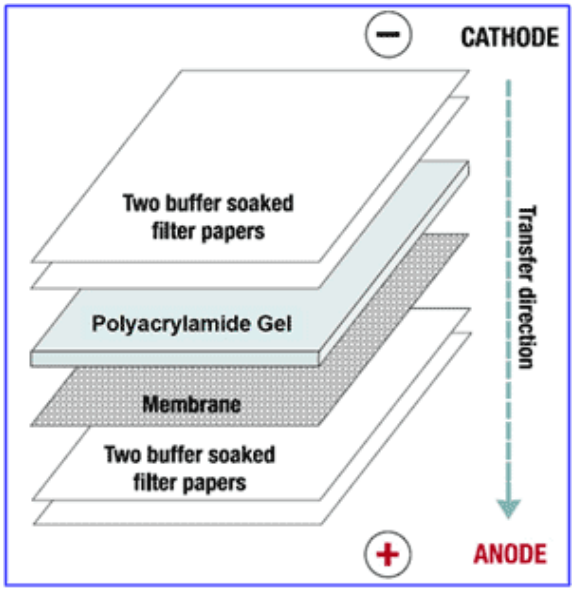
20 µl of the boiled sample mixture (containing WB loading buffer) was loaded into pocket, 10 µl of Precision plus Protein standard (Bio-Rad) was used as a ladder. Gel electrophoresis was run in 1x Tank buffer (150 V, time of electrophoresis run was dependent on percentage of acrylamide in gels and size of target protein). When electrophoresis finished, stacking part of the gel was cut off and the gel was washed while rocking for half an hour in Towbing transfer buffer (TTB).

TTB:

Tris base	12.12 g
Glycine	57.6 g
SDS	2 g
Methanol	800 ml
H_2O dd	Up to 4000 ml

PVDF membranes (Immun-Blot PVDF Membrane, Bio-Rad) were activated by submersion for 30 s in methanol, activated membranes as well as Blot papers (Extra thick blot paper Mini blot size, Bio-Rad) were left in TTB for at least 5' prior transfer. Each WB sandwich consisted of blot paper, PVDF membrane, polyacrylamide gel and another blot paper *(Figure 14).*

Figure 14: Semi-dry WB transfer



Proteins were transferred from gels onto membrane using TRANS-BLOT® Semi-Dry Electrophoretic Transfer Cell (Bio-Rad), at 25 V for 30 min. When transfer

finished, membranes were rocked overnight at 4°C in blocking buffer (5% of dry fat free milk in washing buffer).

Washing buffer:

20 % TWEEN 20	5 ml
2M Tris-HCl	5 ml
NaCl	5.844 g
H_2O dd	Up to 1000 ml

Next day, membranes were warmed up to room temperature and washed for 2 h in primary antibodies solution in blocking buffer. For detailed dilution of primary antibodies see *Table 5*.

Table 5: Primary antibodies working concentrations

Primary Antibody	**Working dilution**
Goat polyclonal anti-aminopeptidase A	1:100 – 1:200
Mouse monoclonal anti-CD10	1:100
Goat polyclonal anti-TOP	1:200
Mouse monoclonal anti-β-actin	1:1000

Antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA (anti APA, anti-CD 10 and anti-TOP), anti-β-actin antibody (clone AC-15) which was employed as loading control was purchased from Sigma Aldrich, Dublin, Ireland. After 2 h incubation, membranes were washed 4 times 5 min in washing buffer, incubated in secondary antibodies solutions: Anti-goat IgG peroxidise antibody for APA and TOP and Anti-mouse IgGs peroxidase antibody for CD10 and β-actin detection. Secondary antibodies solutions were prepared in dilution 1:4000 in blocking buffer). Membranes were washed again 4 times 5 min. Visualisation was done by chemiluminescence; equal parts of two solution of Millipore Immobilon Western Chemiluminescent HRP substrate kit were mixed together, and

each membrane was covered with 1 ml of the solution for 5 min. Excess of liquid was drought out, membranes were placed into transparent plastic foil, the luminescent signal was detected on photographic film and photographs were developed. Presence of protein was quantified using a ChemiDoc^{TM} Bio-Rad system. β-actin signal was detected on the same membranes; after developing picture, wet membranes were immediately washed in washing buffer 2 times 10 min and blocked overnight at 4°C in blocking buffer while rocking. Incubations with primary and secondary antibodies specific for β-actin were repeated.

3.6. Statistical methods

Significance ($p < 0.05$) of differences in the group mean values for TEER determined by one way analysis of variances (ANOVA), followed by Student-Newman-Keuls post-hoc tests. Each data point in figures 8-11 represents means ± S.D. ($n \geq 3$) from 3 different passages.

4. RESULTS

4.1.Monolayer integrity

TEER results are expressed as a mean ± S.D.

When cultured on Transwell Clear filters, R3/1 cells at no time yielded monolayers with TEER values comparable to those of primary cultures of rat pneumocytes typically in the region of > 1500 $\Omega \cdot cm^2$ (Cheek, Kim et al. 1989). The peak TEER value was recorded to be 99±17 $\Omega \cdot cm^2$ after 5 days in culture. Variation of seeding density between 50,000 and 200,000/cm^2 as well as growth under AIC conditions showed no significant changes of TEER of monolayers (*Figure 15* and *Figure 16).*

Figure 15: TEER of R3/1 cells under different seeding densities: Variation of seeding density in interval of 50,000 to 200,000 cells/cm^2 had no impact on TEER values of R3/1 cell monolayers. Cells were grown in RPMI 1640 medium under LCC conditions.

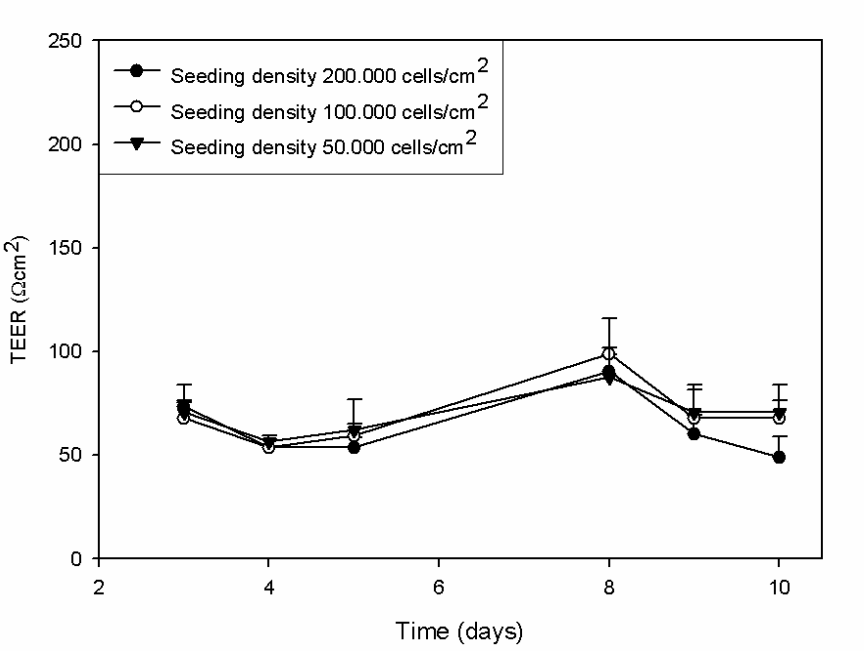
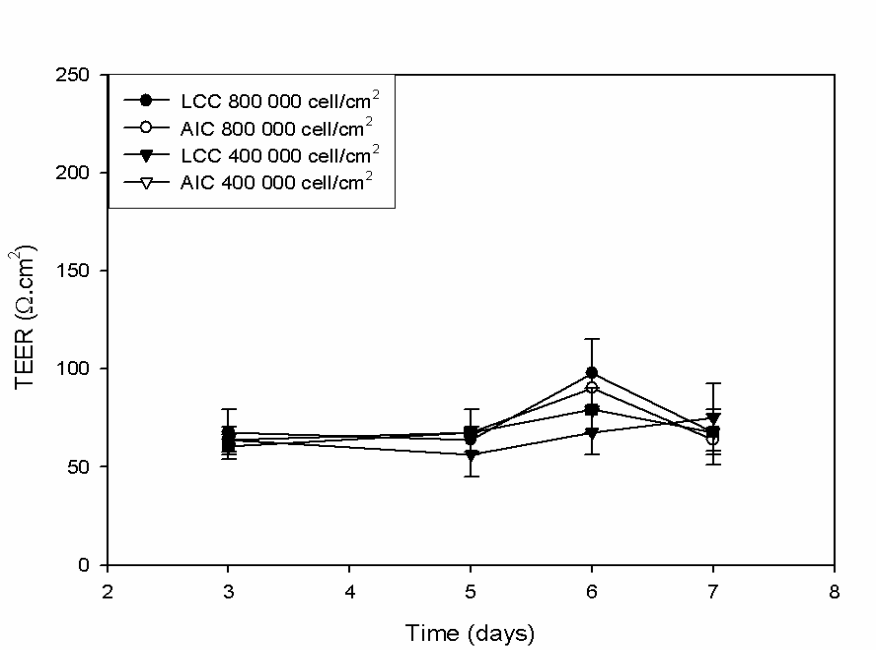
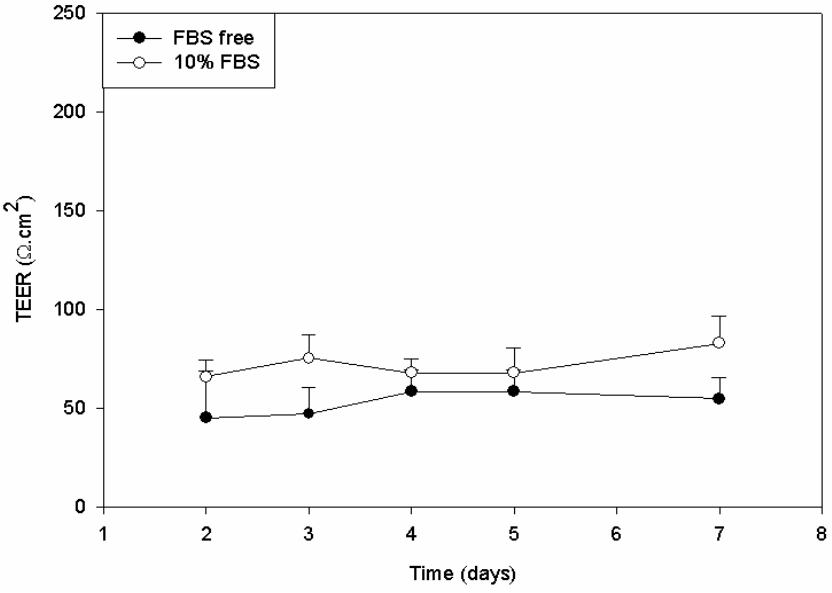


Figure 16: TEER under LCC and AIC condition: Shifting from LCC to AIC conditions (in RPMI 1640 culture medium, seeding densities 400,000 and 800,000 cells/cm^2) did also not cause significant differences in TEER values.



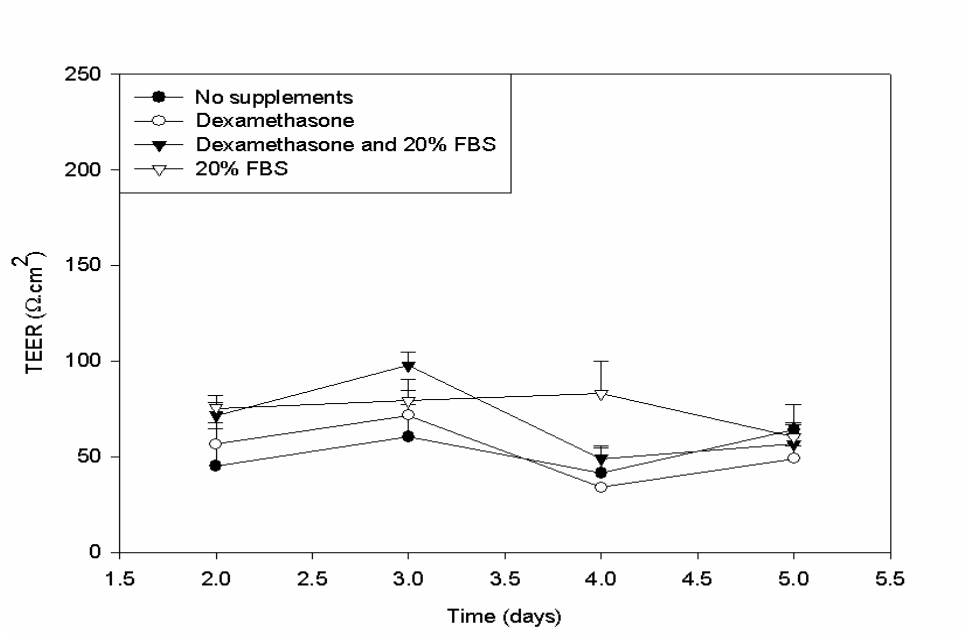
Withdrawal of FBS reduced the TEER to a value of 52±11 Ω·cm^2 *(Figure 17).*

Figure 17: Effect of FBS on TEER: TEER values of R3/1 cells grown under LCC conditions (200,000 cells/cm^2) without FBS were reduced in comparison to cells cultured in the presence of FBS.



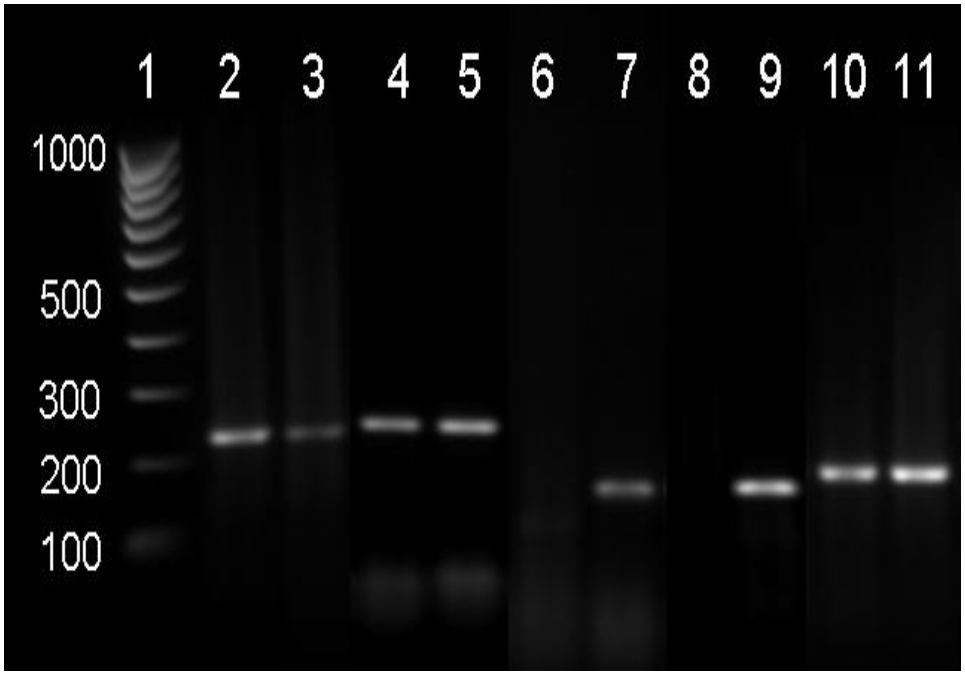
FBS at concentration of 20% in addition of dexamethasone (DEX, 0.1 μM) in the culture medium resulted in an increase of TEER by 65%. However, increased concentration of FBS to 20% only or addition of DEX alone caused no significant change in TEER values (*Figure 18).*

Figure 18: Effect of FBS and dexamethasone on TEER: Doubled concentration of FBS in addition to 0.1 μM dexamethasone increased TEER values of R3/1 seeded in density 200,000 cells/cm^2 under LCC conditions in DMEM - Ham's F12 medium by 65%. When only FBS concentration was increased or only DEX was added, no significant effect on TEER was observed.



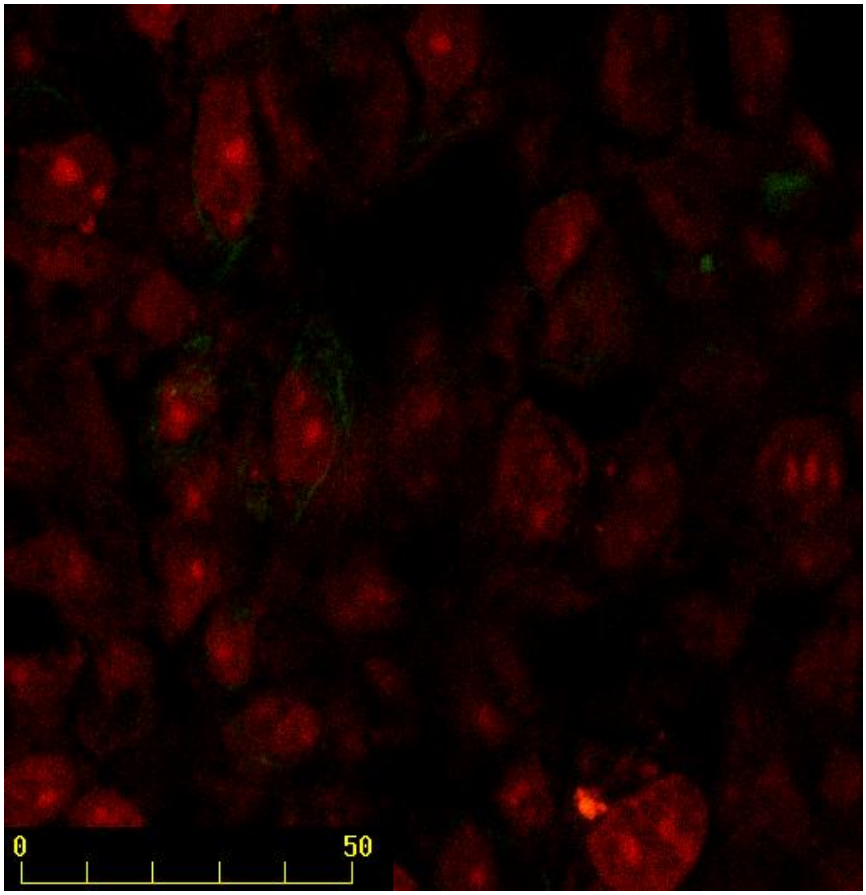
RT-PCR revealed the absence of transcripts encoding E-cadherin and occludin. However, ZO-1 and ZO-2 mRNA transcripts were found *(Figure 19)*.

Figure 19: Representative gel of tight junction proteins in R3/1 cells: Representative agarose gel of the RT-PCR analysis of tight junction proteins in R3/1 cells and rat lung tissue. Lane 2, ZO-1 in R3/1 cell line; Lane 3, ZO-1 in lung; Lane 4, ZO-2 in R3/1 cells; Lane 5, ZO-2 in rat lung; Lane 6, E-cadherin in R3/1 cells; Lane 7, E-cadherin in rat lung, Lane 8, occludin in R3/1 cells; Lane 9, occludin in rat lung. Lanes 10 and 11 show internal standard β–actin (in R3/1 cells and rat lung respectively). Lane 1 contains DNA size marker.



IFM using a monoclonal antibody against occludin revealed the absence of the antigen in R3/1cells *(Figure 20)*. R3/1 cells were processed for immunofluorescence microscopy using mouse monoclonal anti-occludin antibody and Alexa Fluor 488-labelled goat anti-mouse secondary antibody (green). DNA was stained with propidium iodide (red).

Figure 20: Immunofluorescence microscopy image. No signal representing presence of occludin in R3/1 cells was observed.



4.1.**Expression of proteolytic enzymes**

The qualitative results from the RT-PCR analysis of peptidases in R3/1 and primary rat pneumocytes are summarised in *Table 6*, pictures of PCR products separated by gel electrophoresis can be found in Appendix. From the range of investigated proteolytic enzymes, mRNA transcripts encoding aminopeptidase A and aminopeptidase B as well as endopeptidase 24.11 and endopeptidase 24.15 were detected. Transcripts for those peptidases as well as transcripts for aminopeptidase N, carboxypeptidase M and γ-glutamyl transferase were also present in rat alveolar epithelial type I cells in primary culture. In ATII primary cells genes encoding aminopeptidases A, N and P, carboxypeptidase M, γ-glutamyl transferase, and endopeptidases 24.11 and 24.15 were identified by RT-PCR. A weak signal was also obtained for angiotensin converting enzyme. This week expression could be caused by contamination with lung capillary endothelial cells.

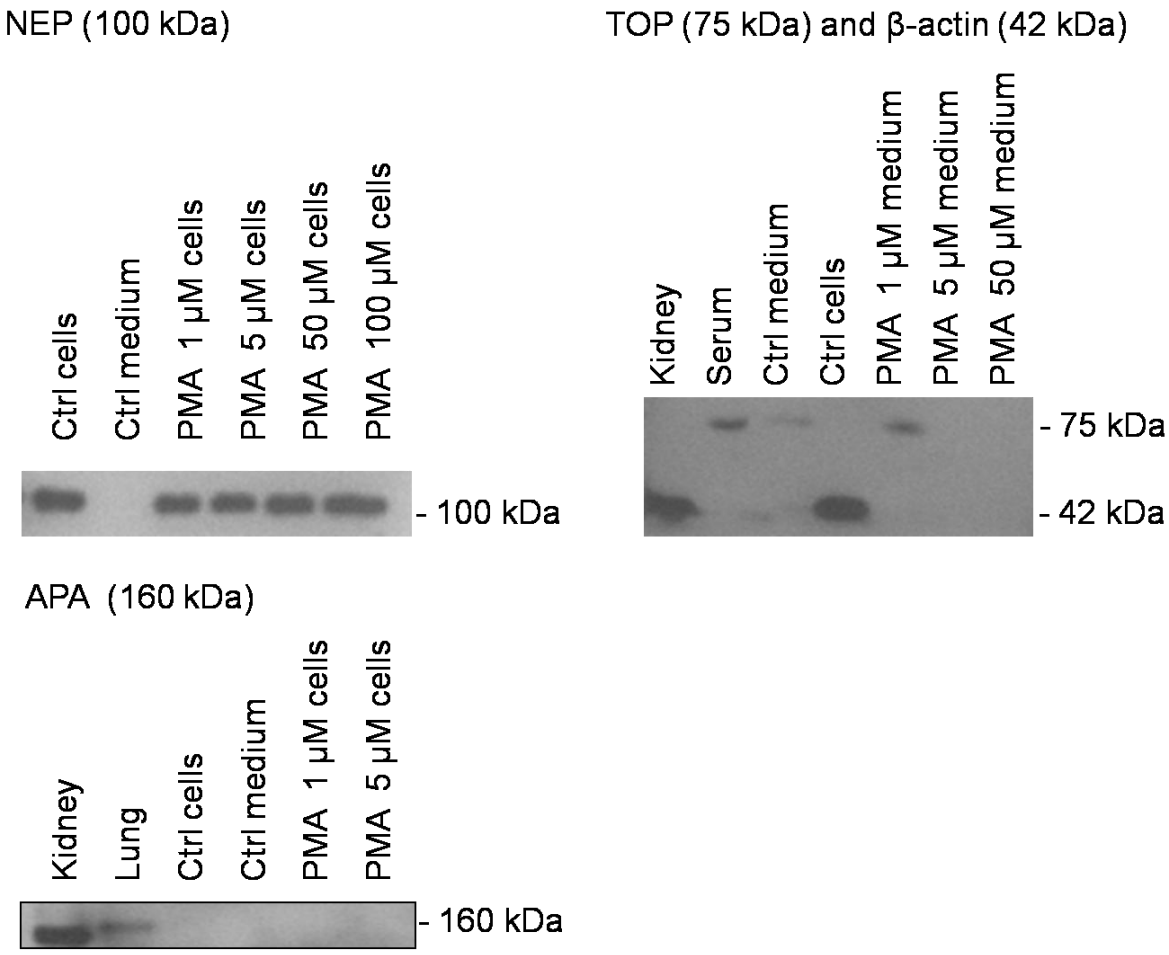
Table 6: Expression patterns of proteolytic enzymes in the R3/1 cell line as well as in AT I and AT II pneumocytes

	R3/1	AT I	AT II
CPM	-	+	+
APA	+	+	+
APB	+	+	-
APN	-	+	+
APP	-	-	+
GGT	-	+	+
ACE	-	-	(+)
DPP IV	-	-	-
EP 24.11	+	+	+
EP 24.15	+	+	+

Western blot confirmed presence of EP 24.11(=NEP; CD10) and EP 24.15 (TOP) in R3/1 cells, APA was not detected *(Figure 21)*. Homogenates of cells or serum free supernatant were investigated. To increase protein expression, cells were stimulated with phorbol myristate acetate (PMA) at concentrations of 1-100 μM. Tissue extracts

from rat kidneys and lungs and serum were used as positive controls. Presence of APB was not investigated; antibodies against rat APB are not commercially available.

Figure 21: Representative Western blots of NEP, EP 24.15 and APA: β-actin was used as loading control. PMA treatment did not influence expression of investigated enzymes.



5. DISCUSSION AND CONCLUSION

Several cell culture models of the tracheo-bronchial epithelium have been characterised and are widely used for drug absorption studies. These include cell lines of human origin, such as Calu-3 and 16HBE14o-. Moreover, continuously growing airway cells with characteristics of cystic fibrosis epithelium are available. Although protocols for the primary culture of human tracheo-bronchial epithelial cells (both healthy and CF phenotypic) into well defined and differentiated confluent cell layers were published over 20 years ago (Gruenert, Finkbeiner et al. 1995), complicated maintenance, low reproducibility of absorption data, and high costs have prevented the application of these *in vitro* models at a larger scale. It should, nevertheless, been noted that none of the cell lines which are currently used as surrogates has been properly investigated with regards to their expression pattern of drug transporter proteins and/or metabolic enzymes. Similar investigations have been conducted in the area of intestinal drug disposition where the gold standard, the Caco-2 cell line, has been extensively and successfully compared to the enterocytes of the small intestine (Hilgendorf, Ahlin et al. 2007). Considering the potentially important role that active transport mechanisms play in the respiratory mucosa, it is quite surprising how little is known about the expression pattern and spatial distribution of transporter proteins in the lung. The extent to which cell lines or primary cultures can model the absorption of actively transported compounds remains to be shown. Existing data in this area is diverse and more systematic studies are underway. The evaluation of cell systems in critical *in vitro-in vivo* and other benchmarking studies will permit the rational selection of tracheo-bronchial or alveolar epithelial cell cultures for preclinical applications. Accordingly, the use of such well characterised epithelial cell systems with validated protocols for studying drug disposition will enhance our understanding of drug transport and metabolism in the lung.

Major hurdles also still remain for the successful development of passageable alveolar type I and type II epithelial cell lines, either as separate or mixed cell types of ATI and ATII coexisting in a single-monolayer setting, that mimic the *in vivo*

setting. Moreover, the co-culture models of alveolar epithelium and capillary endothelium of the mammalian lung are in their infancy at best. With further refinement of such co-culture systems, additional cell types (e.g., macrophages, lymphocytes, dendritic cells, and/or polymorphonuclear cells) can be added to the culture to study various cell biology-related questions in the lung field (including drug metabolism and modulation of properties of lung air-blood barrier).

Although most of the pioneering work in respiratory epithelial biology was based on isolation and culture of cells from the lungs of small laboratory animals including mouse, rat, and rabbit, the requirement for an organ specific cell system that is of human origin is paramount. However, due to the lack of availability of human tissue and ethical issues pertaining to the use of human tissues in certain countries, not much information on species differences in this specific area of cellular research is available yet.

The advantages and limitations of using a simple culture system compared to one that recreates to a greater extent the epithelial structure and function *in vitro* should be considered according to the preclinical application required. This choice is complicated by the lack of comparative data, both between the different cell systems and for *in vitro-in vivo* correlation, upon which to base such decisions. However, no meaningful *in vitro- in vivo* correlation has been established to date for lung alveolar epithelial drug transport/metabolism and data reported for bronchial cell lines and primary models is in all cases based on intra-tracheal instillation into rodents, which is certainly not applicable to the *in vivo* situation in humans. Therefore, these data should be interpreted with extreme caution.

Notwithstanding the difficulties and challenges surrounding various aspects of *in vitro* respiratory epithelial models, mechanistic studies of pulmonary drug delivery using tracheo-bronchial and alveolar mucosae are expected to provide us with a wealth of information in the coming years that will enable us to (1) devise newer and more efficient methods to treat lung-specific diseases using targeting approaches and (2) help improve the bioavailability of those therapeutics that yield very poor absorption via other routes.

We have shown that, R3/1 cells express some markers typical for type I pneumocytes (Koslowski, Barth et al. 2004) including T1α, ICAM-1, connexin-43, caveolins-1 and -2. In contrast to primary rat pneumocytes, these cells do not form electrically tight monolayers. Therefore, R3/1 cells cannot be considered as a reliable *in vitro* model for alveolar absorption studies. However, our data indicate that R3/1 cells may be suitable for stability assays of inhaled proteins (Patton 1996). This study established that, although R3/1 cells express certain markers typical for type I pneumocytes (e.g., T1α, ICAM-1, connexin-43, caveolins-1 and -2 , their inability to form electrically tight monolayers prevents them from being a reliable *in vitro* model for alveolar absorption studies.

Pulmonary delivery of macromolecule aerosols has been considered an attractive non-invasive way to overcome frequent injections. The recent termination of development of inhaled insulin by Nektar, Pfizer, Novo Nordisk and Eli Lilly might be a drawback for research in the area of insulin delivery, but other protein drugs may have a brighter future. However, due to their high molecular mass and large size, proteins may have severe difficulties to cross epithelial barriers. Moreover, they might be degraded by proteases and/or removed by alveolar macrophages, as soon as they reach the alveoli (Yamahara, Lehr et al. 1994; Patton 1996). To address these issues, *in vitro* models of alveolar epithelium can be useful in predicting the fate of peptides and proteins administered to the lung (Yamahara, Lehr et al. 1994; Williams 2003; Bur, Huwer et al. 2006). Hence, it was important and relevant to compare the protease expression pattern of R3/1 cells with primary rat pneumocytes in vitro as this continuous cell line potentially could enable in vitro screening of enzymatic stability of proteins and peptides. From the range of proteolytic enzymes investigated in our study APN, CPM and GGT were differently expressed in R3/1 cells and ATI-like cells in primary culture, whereas CPM, APB, APN, APP, GGT and ACE were differently expressed in R3/1 and ATII cells.

The distribution of proteolytic enzymes in ATI *vs.* ATII cells *in vivo* as well as *in vitro* in different cell types/cell lines of lung epithelial origin has not been extensively investigated. The transdifferentiation from ATII to ATI-like cells *in vitro*

has been reported to go along with increased CPM activity and a decrease of DPP IV expression (Nagae, Abe et al. 1993; Forbes, Wilson et al. 1999; Williams 2003).

In contrast, presence of GGT has been controversial. Some reports describe the enzyme as a typical feature of ATI cells (Ingbar, Hepler et al. 1995) while others suggests GGT to be characteristic for the ATII phenotype (Joyce-Brady, Takahashi et al. 1994). In our study we found CPM and GGT in both ATI-like cells and freshly isolated ATII cells RT-PCR, while DPP IV was not detectable. The activity of ACE was first reported by Forbes and co-workers to be associated with the ATII phenotype and this was confirmed in our study. However, it cannot be completely ruled out this signal might have been generated by contaminating endothelial cells in the ATII population in both studies (Forbes, Wilson et al. 1999). Investigations of catabolic peptidases in the A549 cell line have confirmed the presence of CPM, APN, DPP IV and EP 24.11 (Forbes, Wilson et al. 1999). This expression pattern resembles neither ATI nor ATII cells. In contrast, the pattern of proteolytic enzymes found in the R3/1 cell line showed more similarities to ATI than ATII pneumocytes. Since ATI cells cover the vast majority of the pulmonary epithelial surface, it can be suggested that this cell type might be of most interest when it comes to protein absorption and degradation.

It can be concluded that R3/1 cells are unlikely to be used as an *in vitro* screening model for alveolar absorption. However, the cells are suitable for an *in vitro* screening model for protein and peptide stability studies.

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7. LIST OF PAPERS

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8. SUMMARY

Buněčná řada R3/1 odvozená z krysích embryí, vykazuje několik fenotypových znaků vlastních buňkám alveolárního epitelu I. řádu. Cílem této práce bylo dále popsat vlastnosti R3/1 buněčné linie vzhledem k jejímu případnému využití jako *in vitro* drug disposition model, tj. model popisující veškeré procesy zahrnující absorpci, distribuci, metabolismus a exkreci léčiva do organismu. R3/1 buňky byly kultivovány na Traswell filtrech a transepitheliální elektrický odpor (TEER) byl měřěn jako parametr integrity buněčných vrstev. Přítomnost proteinů důležitých pro vytvoření funkčních buněčných spojení (tight junction, TJ) - E-cadherinu, occludinu a proteinů ZO-1 a ZO-2 byla analyzována na úrovni mRNA pomocí polymerázové řetězové reakce s reverzní transkripcí (RT-PCR) a na úrovni antigenu pomocí imunofluorescenční mikroskopie (IFM). Dále byla sledována přítomnost katabolických peptidáz, konkrétně karboxypeptidázy M, aminopeptidáz A, B, N a P, γ-glutamyltransferázy, dipeptidylpeptidázy IV, angiotensin konvertujícího enzymu a endopeptidáz 24.11 a 24.15. Zároveň s R3/1 buňkami byly tyto peptidázy stanoveny v primárních kulturách alveolárního epitelu potkanů a výsledky byly porovnány.

Hodnoty TEER dosáhly maximální hodnoty ~99±17 Ω•cm^2 pátého dne kultivace, obohacení živného media o dexametazon (v koncentraci 0.1 μM) společně s dvojnásobným množstvím séra v médiu (zvýšení z 10 na 20 %) mělo za výsledek vzestup TEER o 65 %. I přesto se TEER R3/1 buněk nepřiblížil k hodnotám, které vykazovaly primární kultury krysích pneumocytů.

Výsledky RT-PCR odhalily absenci transkriptů kódujících E-cadherin and occludin, oproti tomu byly nalezeny mRNA transkripty pro ZO-1 a -2. IFM používající monoklonální protilátku proti occludinu potvrdila nepřítomnost antigenu v R3/1 buňkách. Ze spektra proteolytických enzymů analyzovaných metodou RT-PCR byla zjištěna přítomnost mRNA transkriptů pro aminopeptidázy A a B a rovněž pro endopeptidázy 24.11 a 24.15. Při porovnání peptidáz přítomných v R3/1 buněčné řadě s peptidázami nalezenými v krysích pneumocytech I. a II. řádu je patrná podobnost R3/1 buněk s epitelem I. řádu.

Ačkoli R3/1 buněčná řada disponuje několika charakteristickými znaky typickými pro alveolární epiteliální buňky I. řádu, jako je T1α, ICAM-1, connexin-43, caveoliny-1 a -2, není schopná vytvářet dostatečně pevná TJ a tudíž nemůže být vhodným *in vitro* modelem pro studování absorpce léčiv. Tato buněčná linie může najít uplatnění jako model pro stabilitní studie inhalovaných léčiv – peptidů.

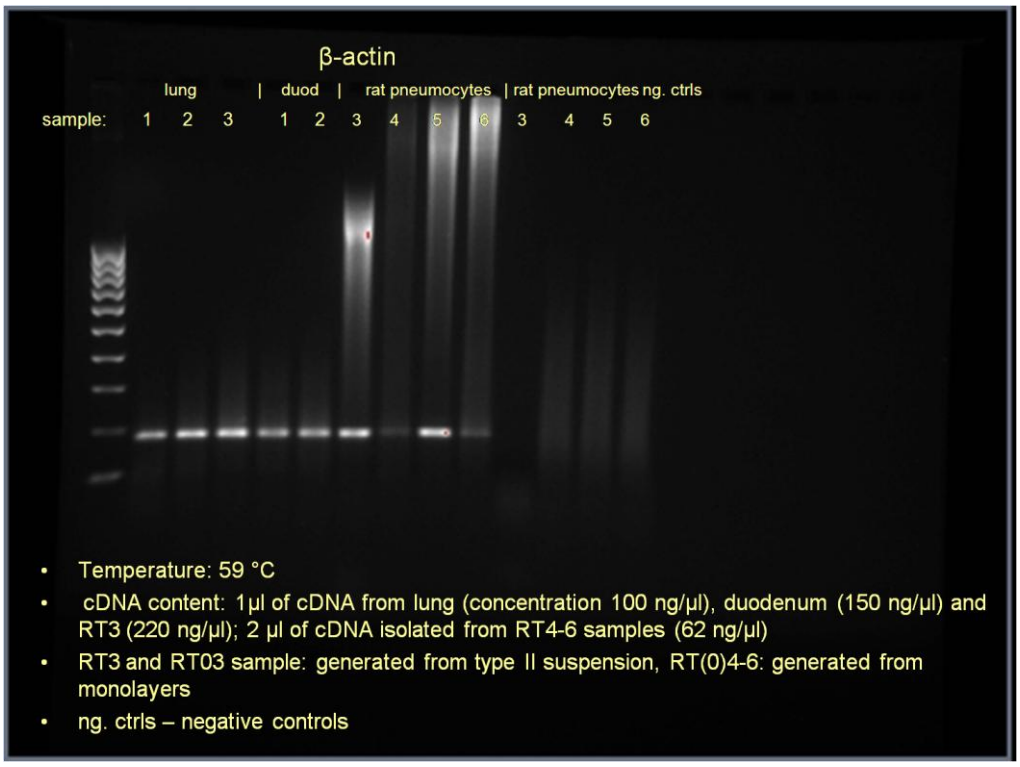
The rat cell line R3/1 displays several phenotypical features of alveolar epithelial type I cells. In order to characterise the cell line as an *in vitro* model for drug disposition studies, R3/1 cells were cultured on Transwell filters and the transepithelial electrical resistance (TEER) was measured as a parameter for the integrity of cell layers. Presence of cell junctional proteins (i.e., E-cadherin, occludin, ZO-1 and ZO-2) was studied on mRNA (by reverse transcriptase-polymerase chain reaction, RT-PCR) and antigen level (by immunofluorescence microscopy, IFM). Moreover, the expression pattern of catabolic peptidases (carboxypeptidase M, aminopeptidases A, B, N and P, γ-glutamyltransferase, dipeptidylpeptidase IV, angiotensin-converting enzyme, and endopeptidases 24.11 and 24.15) was analysed in R3/1 cells and compared to rat alveolar epithelial I-like cells in primary culture.

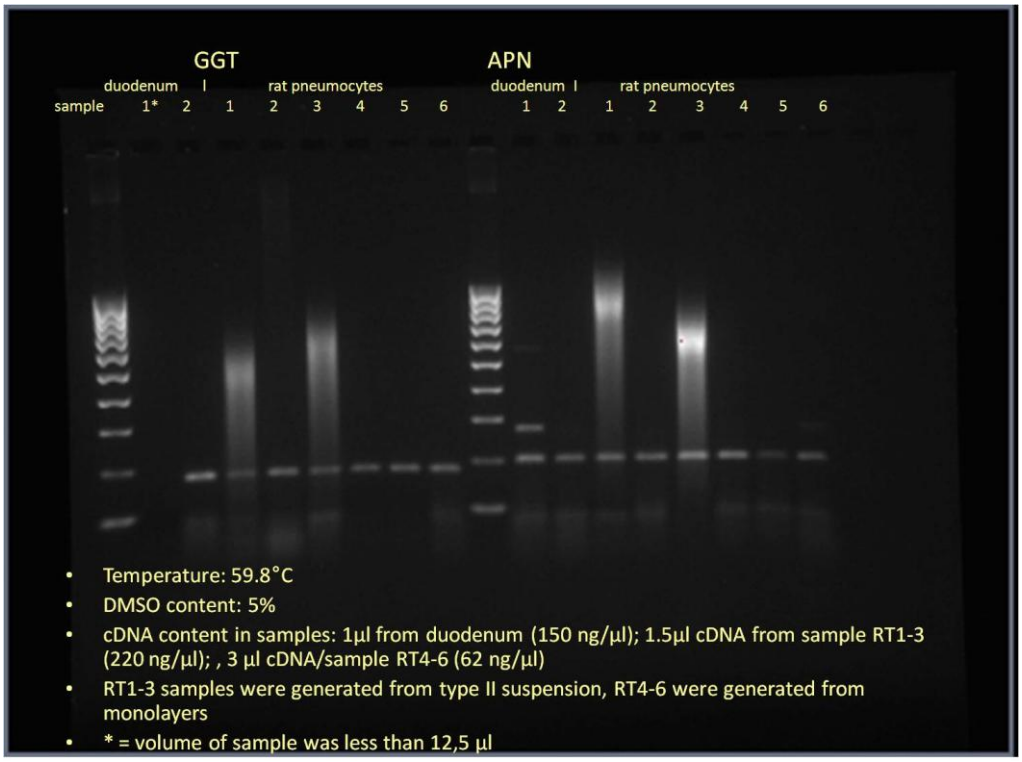
TEER values were peaking at ~99±17 $\Omega \bullet cm^2$ after 5 days in culture. Addition of 0.1 μM dexamethasone together with foetal bovine serum at 20% increased TEER by 65%. However, none of culture conditions used in our study yielded monolayers with TEER values comparable to those of primary cultures of rat pneumocytes. RT-PCR revealed the absence of transcripts encoding for E-cadherin and occludin. However, ZO-1 and -2 mRNA transcripts were found. IFM using a monoclonal antibody against occludin confirmed the absence of the antigen in R3/1 cells. From the range of investigated proteolytic enzymes, mRNA transcripts encoding aminopeptidases A and B as well as endopeptidases 24.11 and 24.15 were detected; a pattern similar to rat alveolar epithelial I-like cells in primary culture.

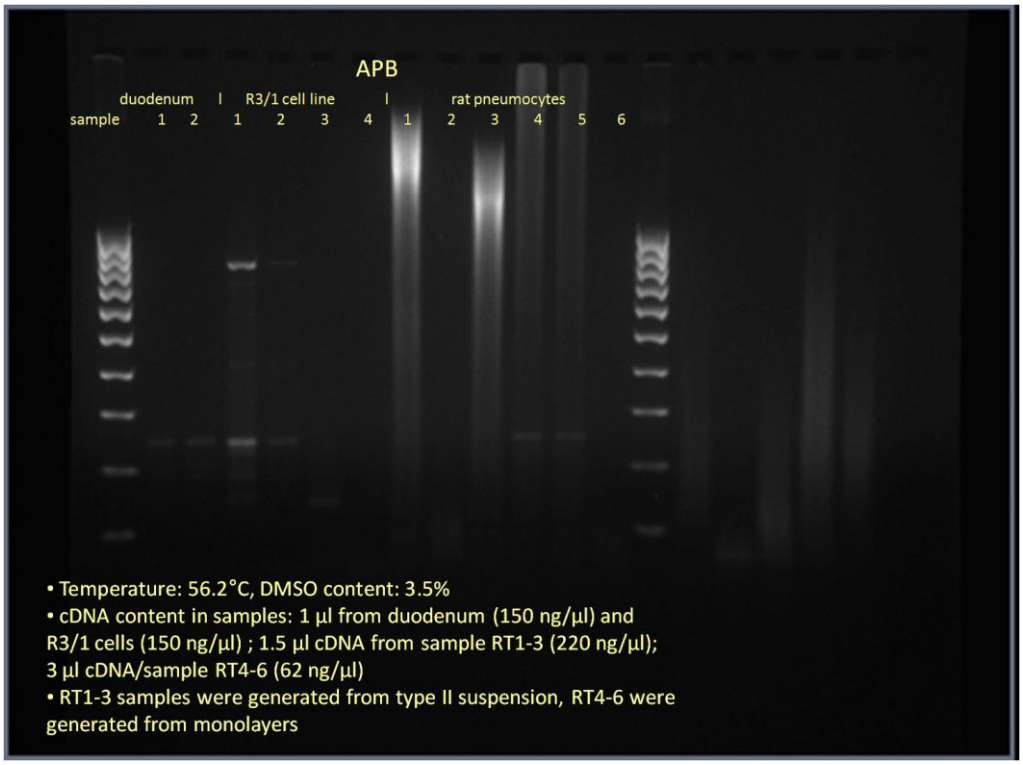
Although R3/1 cells express certain markers typical for type I pneumocytes (e.g., T1α, ICAM-1, connexin-43, caveolins-1 and -2), their inability to form electrically tight monolayers prevents them from being used as an *in vitro* model for alveolar absorption. The R3/1 cell line might be useful for stability assays of inhaled proteins.

9. APPENDIX

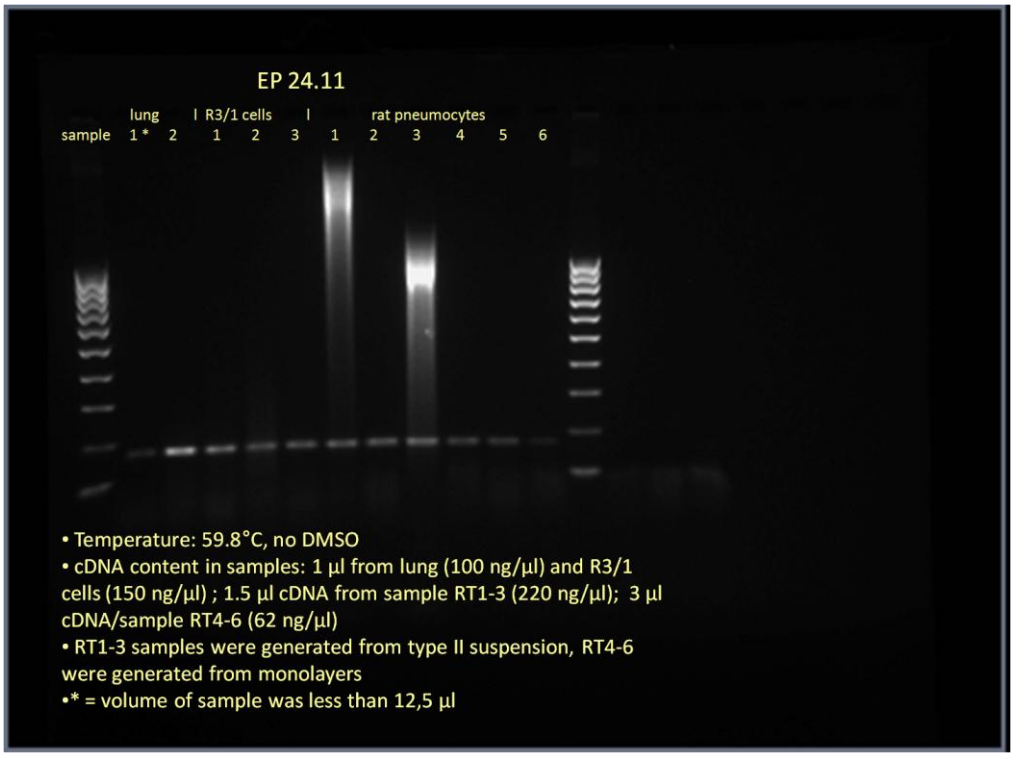
RT-PCR products separated by agarose gel electrophoresis.



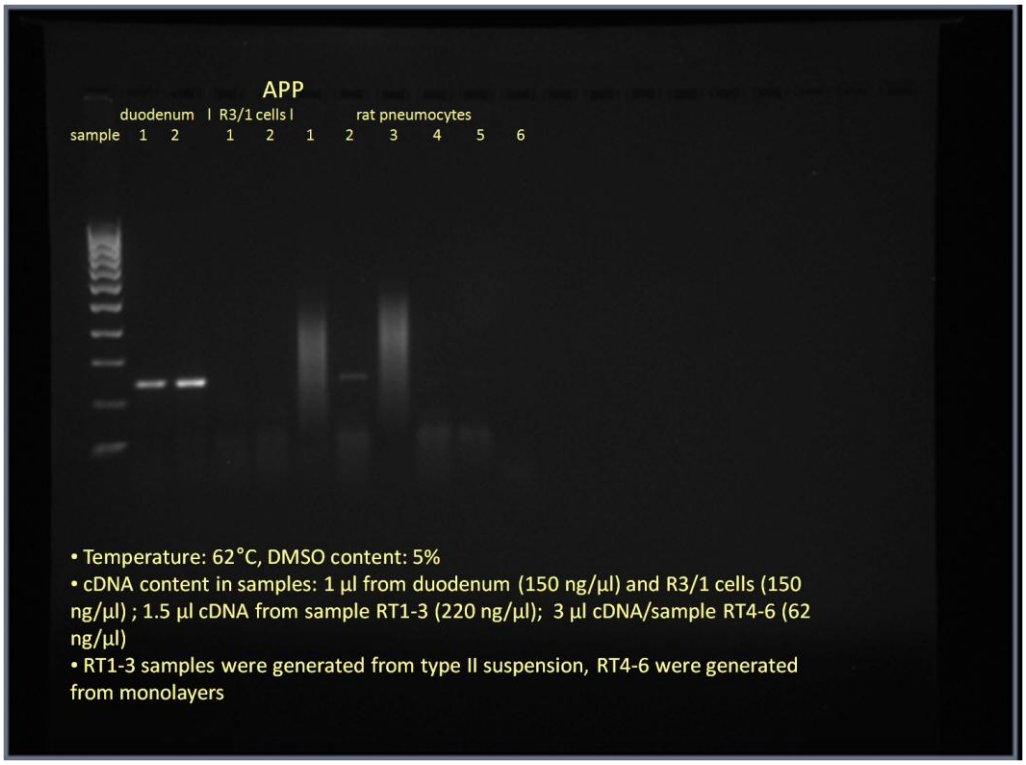




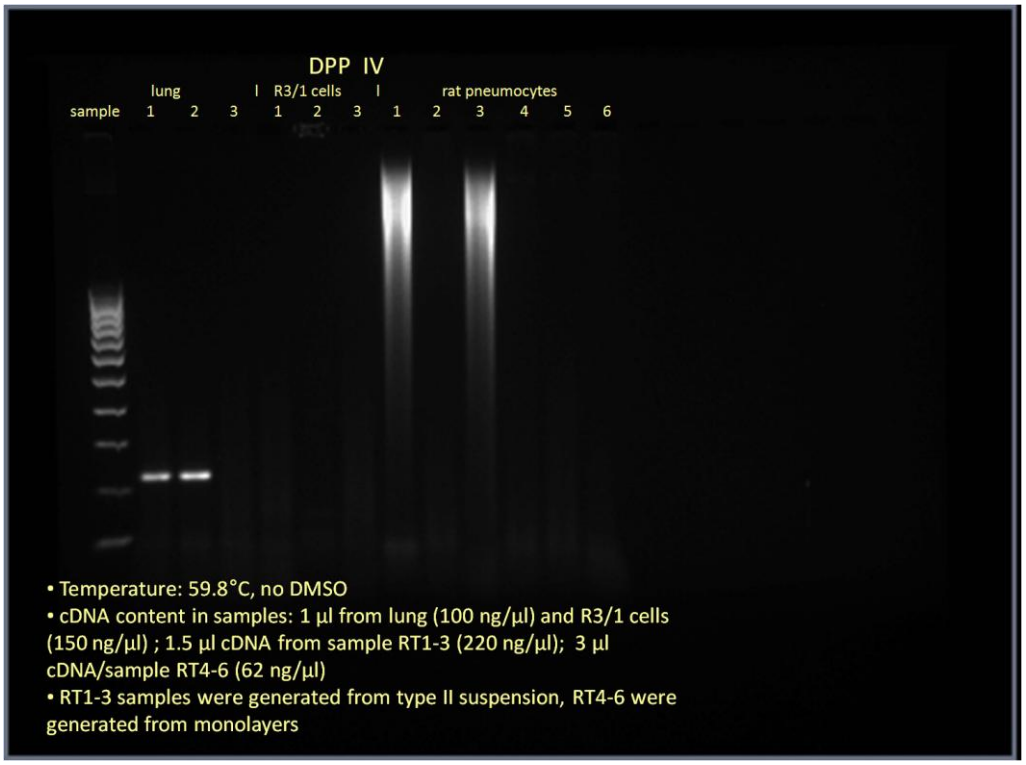
• Temperature: 56.2°C, DMSO content: 3.5%
• cDNA content in samples: 1 µl from duodenum (150 ng/µl) and R3/1 cells (150 ng/µl) ; 1.5 µl cDNA from sample RT1-3 (220 ng/µl); 3 µl cDNA/sample RT4-6 (62 ng/µl)
• RT1-3 samples were generated from type II suspension, RT4-6 were generated from monolayers



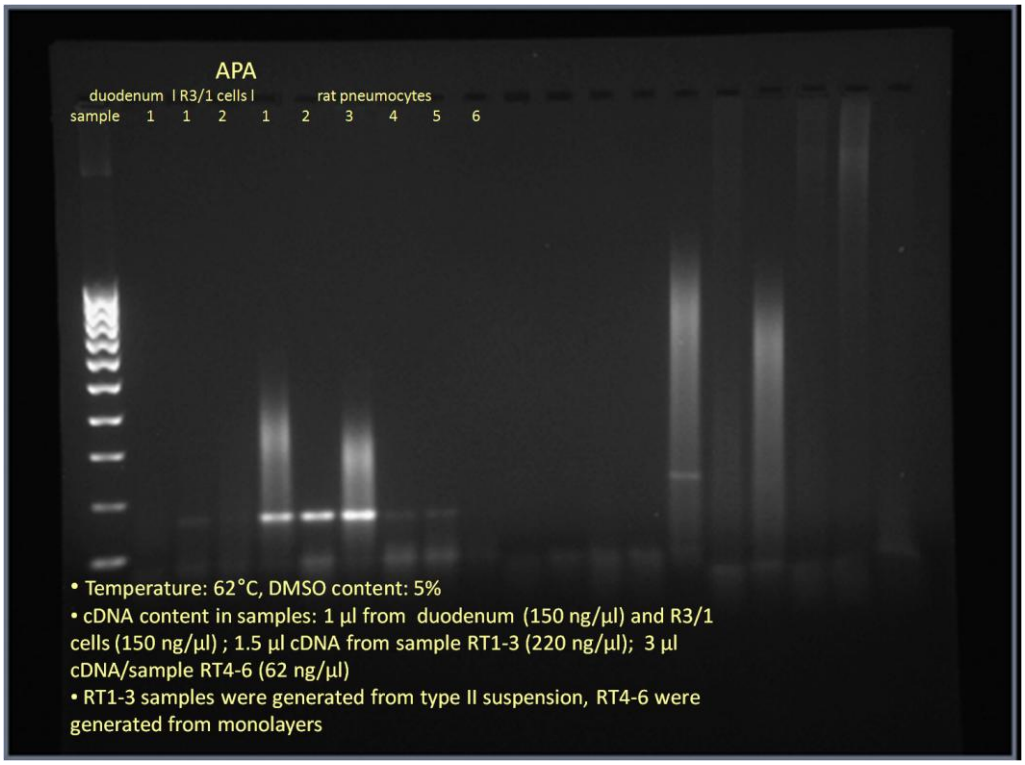
• Temperature: 59.8°C, no DMSO
• cDNA content in samples: 1 µl from lung (100 ng/µl) and R3/1 cells (150 ng/µl) ; 1.5 µl cDNA from sample RT1-3 (220 ng/µl); 3 µl cDNA/sample RT4-6 (62 ng/µl)
• RT1-3 samples were generated from type II suspension, RT4-6 were generated from monolayers
•* = volume of sample was less than 12,5 µl



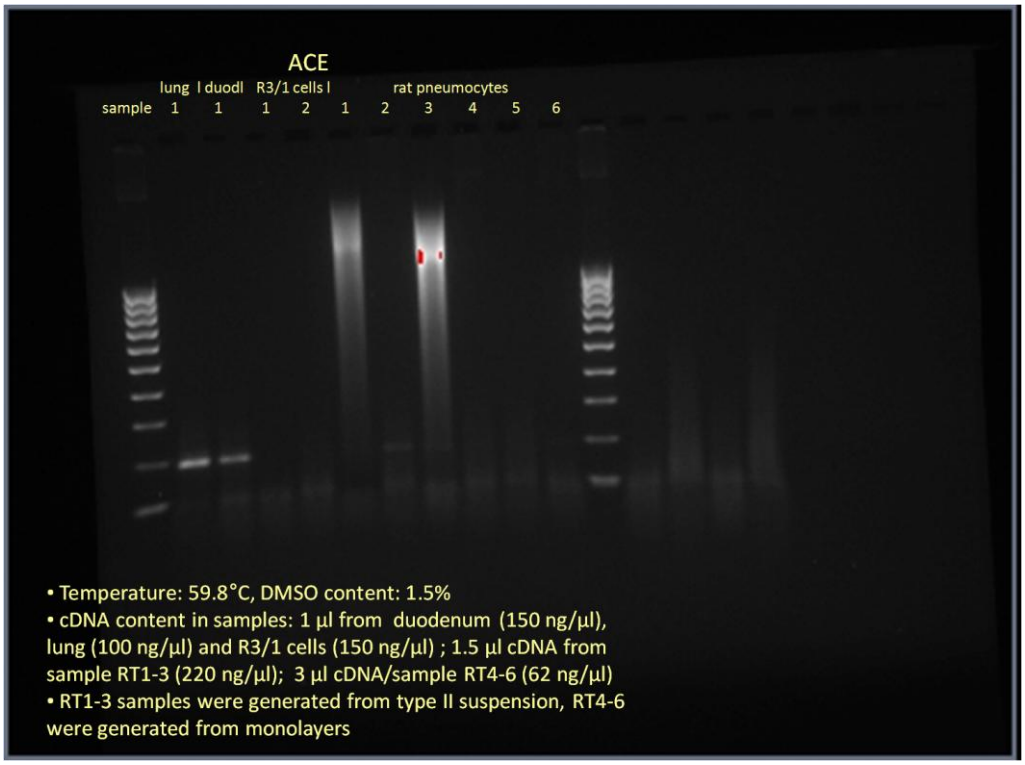
• Temperature: 62°C, DMSO content: 5%
• cDNA content in samples: 1 µl from duodenum (150 ng/µl) and R3/1 cells (150 ng/µl) ; 1.5 µl cDNA from sample RT1-3 (220 ng/µl); 3 µl cDNA/sample RT4-6 (62 ng/µl)
• RT1-3 samples were generated from type II suspension, RT4-6 were generated from monolayers



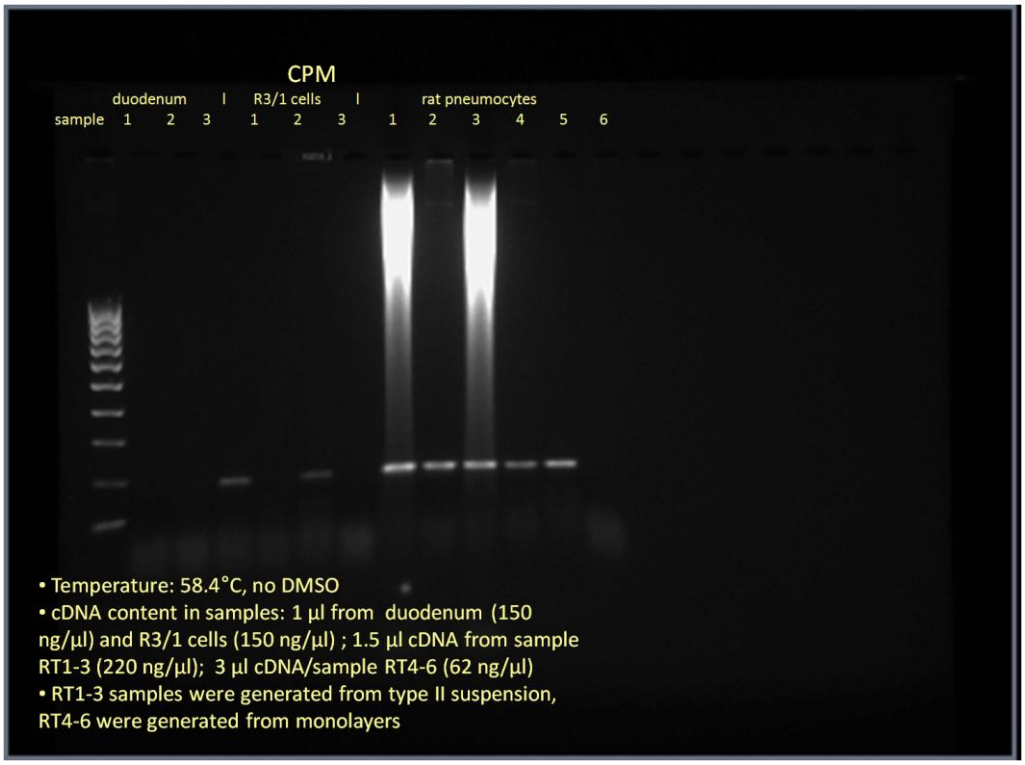
• Temperature: 59.8°C, no DMSO
• cDNA content in samples: 1 µl from lung (100 ng/µl) and R3/1 cells (150 ng/µl) ; 1.5 µl cDNA from sample RT1-3 (220 ng/µl); 3 µl cDNA/sample RT4-6 (62 ng/µl)
• RT1-3 samples were generated from type II suspension, RT4-6 were generated from monolayers

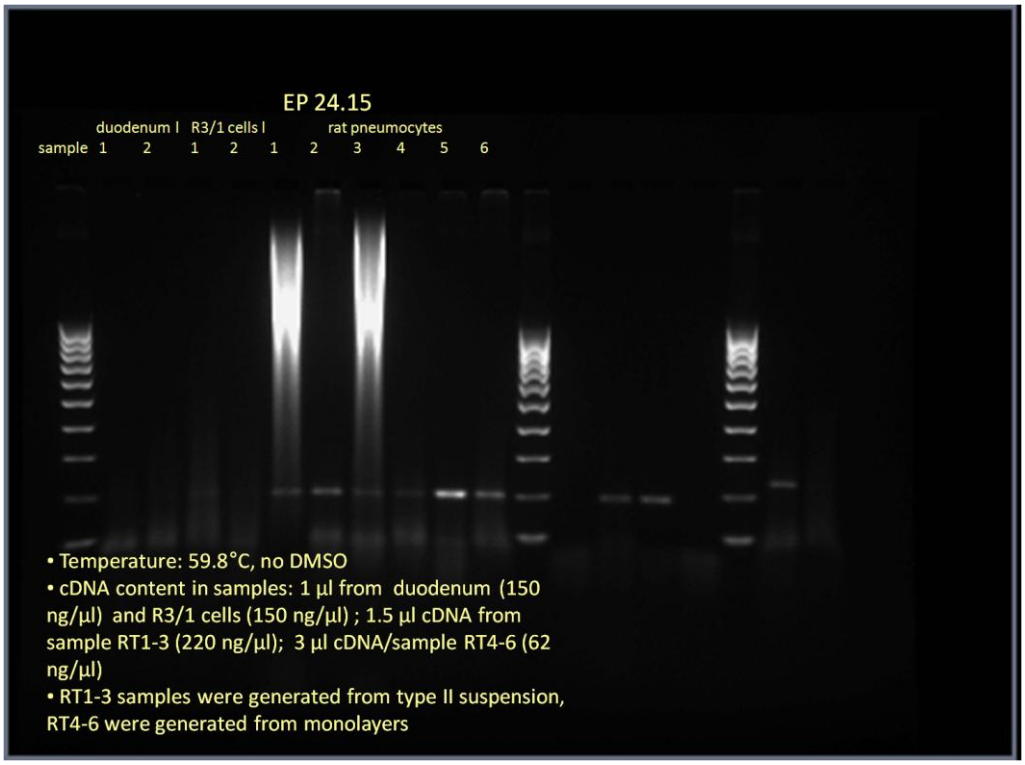


• Temperature: 62°C, DMSO content: 5%
• cDNA content in samples: 1 µl from duodenum (150 ng/µl) and R3/1 cells (150 ng/µl) ; 1.5 µl cDNA from sample RT1-3 (220 ng/µl); 3 µl cDNA/sample RT4-6 (62 ng/µl)
• RT1-3 samples were generated from type II suspension, RT4-6 were generated from monolayers



• Temperature: 59.8°C, DMSO content: 1.5%
• cDNA content in samples: 1 µl from duodenum (150 ng/µl), lung (100 ng/µl) and R3/1 cells (150 ng/µl) ; 1.5 µl cDNA from sample RT1-3 (220 ng/µl); 3 µl cDNA/sample RT4-6 (62 ng/µl)
• RT1-3 samples were generated from type II suspension, RT4-6 were generated from monolayers


CPM
duodenum
R3/1 cells
rat pneumocytes
sample 1 2 3 1 2 3 1 2 3 4 5 6
• Temperature: 58.4°C, no DMSO
• cDNA content in samples: 1 µl from duodenum (150 ng/µl) and R3/1 cells (150 ng/µl) ; 1.5 µl cDNA from sample RT1-3 (220 ng/µl); 3 µl cDNA/sample RT4-6 (62 ng/µl)
• RT1-3 samples were generated from type II suspension, RT4-6 were generated from monolayers


EP 24.15
duodenum
R3/1 cells
rat pneumocytes
sample 1 2 1 2 1 2 3 4 5 6
• Temperature: 59.8°C, no DMSO
• cDNA content in samples: 1 µl from duodenum (150 ng/µl) and R3/1 cells (150 ng/µl) ; 1.5 µl cDNA from sample RT1-3 (220 ng/µl); 3 µl cDNA/sample RT4-6 (62 ng/µl)
• RT1-3 samples were generated from type II suspension, RT4-6 were generated from monolayers