Development of suitable *in vitro* models of the bronchial epithelium for inhalation toxicology

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Referent:	Prof. Dr. Thomas Scheper
	Institut für Technische Chemie
	Gottfried Wilhelm Leibniz Universität Hannover
Korreferentin:	Prof. Dr. Cornelia Kasper
	Department für Biotechnologie
	Universität für Bodenkultur (BOKU) Wien, Österreich
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Ich versichere, dass ich diese Dissertation selbstständig verfasst habe und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden. Diese Arbeit wurde nicht als Diplomarbeit oder ähnliche Prüfungsarbeit verwendet.

Hannover, im April 2013

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Abstract

The respiratory tract forms the boundary between the external environment and the body and consequently its lining epithelium is permanently exposed to various pathogens and particles. Major consequences are acute and chronic effects, ranging from simple reversible irritations to persistent inflammation and remodeling of the airway epithelium, which often result in chronic diseases like chronic obstructive pulmonary diseases (COPD), asthma or pulmonary fibrosis. In recent years, the incidence rate of respiratory disorders has steadily increased and they now rank second (after cardiovascular diseases) with regard to morbidity and mortality worldwide.

Numerous cellular and molecular factors are involved in the repair and regeneration of the airway epithelium after damage. Improving our understanding of epithelial function in normal and pathologic conditions may help to develop novel and efficient therapeutics and treatments.

For this purpose, *in vitro* models of the airway epithelium are valuable tools. Unlike animal models, cell and tissue cultures can, in general, be used for a variety of toxicological studies without legislative restrictions and ethical concerns. Moreover*, in vitro* models allow the simultaneous and economic analysis of several cell types specifically under various conditions with numerous analytical methods.

This work deals with the development of *in vitro* tissue equivalents of the bronchial epithelium which exhibit natural differentiation and structural patterns. Critical factors are the use of suitable cell lines and the establishment of appropriate culture conditions. To simulate *in vivo*-like conditions, co-culture system are generated. Here, epithelial cells and fibroblasts are co-cultivated in order to allow their interactions to be comparable to the *Epithelial-Mesenchymal Trophic Unit*. A computer-controlled cultivation system is tested that was specifically designed to enable the maintenance of stable conditions and thus to enhance the reproducibility of well-differentiated 3D models.

To study aspects of acute inflammation and chronic airway diseases, damage of the airway epithelium is tried to be simulated by inducing structural alterations in the developed *in vitro* models using special cultivation conditions.

The developed 3D cell model can be employed for a variety of toxicological studies and may deliver important insight into cellular mechanisms of inflammation, repair and remodeling of the airway epithelium.

Keywords: *In vitro* models, airway diseases, co-culture cell systems, inhalation toxicology

Kurzfassung

Der Atemtrakt bildet die Grenze zwischen der Außenwelt und dem Körper und folglich ist sein auskleidendes Epithel zahlreichen Pathogenen und Partikeln permanent ausgesetzt. Häufige Folgen sind akute und chronische Schäden, die von einer einfachen und reversiblen Irritation bis hin zu chronischen Erkrankungen, wie chronisch obstruktive Lungenerkrankungen (COPD), Asthma oder Lungenfibrose reichen. In den letzten Jahren ist die Häufigkeit von Lungenerkrankungen kontinuierlich gestiegen und sie sind heute die zweithäufigste Todesursache (nach Herz-Kreislauf-Erkrankungen) weltweit.

Zahlreiche zelluläre und molekulare Faktoren sind an der Wiederherstellung und Remodellierung des Atemwegepithels nach Schädigung beteiligt. Ein verbessertes Verständnis der Funktionen des Epithels unter normalen und pathologischen Bedingungen kann dazu beitragen, neue, effiziente Behandlungsmethoden zu entwickeln.

Zu diesem Zweck sind *in vitro* Modelle nützliche Werkzeuge, da sie für zahlreiche toxikologische Untersuchungen eingesetzt werden können, ohne dass gesetzliche Einschränkungen und ethische Bedenken berücksichtigt werden müssen, wie es bei Tierversuchen der Fall ist. Darüber hinaus bieten *In vitro* Modelle die Möglichkeit, verschiedene Zelltypen unter variierenden Bedingungen mit einer Vielzahl von analytischen Methoden gleichzeitig und kostengünstig untersuchen zu können.

Die vorliegende Arbeit befasst sich mit der Entwicklung von *in vitro* Gewebenachbildungen des Bronchialepithels, welche eine natürliche Differenzierung und Struktur aufzeigen. Entscheidende Faktoren hierbei sind zum einen die Verwendung von tauglichen Zelllinien und zum anderen die Erzeugung von geeigneten Kulturbedingungen. Damit *in vivo*- nahe Bedingungen simuliert werden können, werden Co-Kultursysteme erzeugt, bei denen Epithelzellen mit Fibroblasten gemeinsam kultiviert werden. Auf diese Weise sollen Interaktionen zwischen den beiden Zelltypen wie in der *Epithelial-Mesenchymal Trophic Unit* ermöglicht werden. Ein computergesteuertes Kultivierungssystem wird getestet, welches speziell entwickelt wurde, um stabile Kulturbedingungen zu erzeugen und so die Reproduzierbarkeit der ausdifferenzierten 3D-Modelle zu verbessern.

Um Aspekte einer akuten Entzündung und chronischer Atemwegerkrankungen studieren zu können, wird versucht, eine Schädigung des Atemwegepithels zu simulieren, indem

Strukturveränderungen in den entwickelten *in vitro* Modellen gezielt durch spezielle Kulturbedingungen induziert werden.

Die entwickelten 3D Zellmodelle können für eine Vielzahl von toxikologischen Untersuchungen eingesetzt werden und so wichtige Einblicke in zelluläre Mechanismen während Entzündungs- und Reparaturprozessen und daraus resultierenden Strukturveränderungen liefern.

Schlagworte: *In vitro*-Modelle, Co-Kultur Zellsysteme, Inhalationstoxikologie, Atemwegerkrankungen

1 General Introduction

Legislative restrictions on the use of animals for toxicological studies in the European Union require the development of alternative methods for the evaluation of chemicals and complex atmospheres for human health risk assessment.

Despite the construction of technical devices for such *in vitro* studies, the development of tissue and cell models resembling parts of human organs remains a key factor.

The airways are the boundary between the environment and the body and consequently the airway epithelium plays an important role as a protective physical and functional barrier for inhaled pathogens and toxicants. Impairment of the barrier function, e.g. caused by persistent inflammation, may lead to chronic airway diseases, like COPD. The incidence of such diseases is currently increasing and they are already the leading cause of death in many countries [(1)].

The development of realistic *in vitro* models of the human epithelium is of great value for the understanding of barrier properties and physiological processes during inflammation, repair and remodeling of this tissue. The cell models can serve as a complement to or, to a certain degree, as a replacement for traditional animal models in various toxicological studies. Unlike animal models, the effects of different agents on single cell types can be comprehensively analyzed by means of molecular biological or biochemical methods.

Today, cell cultures composed of readily differentiated epithelial cells isolated from different parts of the respiratory tract (upper and lower airways) are commercially available from different suppliers (e.g. Epithelix Sàrl, MatTek Corp.). However, these models generally only consist of epithelial cells, while *in vivo* the airway epithelium comprises different components: The *Epithelial-Mesenchymal Trophic Unit* (EMTU) is an anatomical and functional unit that includes (a) the epithelial layer (b) the basal lamina to which the epithelial cells are attached and (c) the connective tissue (*lamina propria*) with its fibroblasts embedded in an extracellular matrix [(2)]. The *in vitro* simulation of this complex structure is still a major challenge.

The extracellular matrix (ECM) of the connective tissue in the airways is mainly made up of collagen fibers. Fibroblasts, the main cell type of the connective tissue, are scattered in this matrix. These mesenchymal cells have been shown to play a crucial role in epithelial growth,

differentiation and wound repair by secreting cytokines and chemokines in response to various stimuli [2-4]. Thus, to meet *in vivo* conditions as close as possible, *in vitro* models of human airway epithelium optimally include both, epithelial cells and fibroblasts.

The most critical factors for the implementation of 3D tissue equivalents that resemble the airway epithelium, with regard to cell type composition and structure, are the use of appropriate cell lines and the establishment of optimal culture conditions, including nutrition supply and the surrounding atmosphere. Primary epithelial cells isolated from human tissue explants are able to retain their natural characteristics and to form a structure similar to normal epithelia *in vitro* when cultivated under appropriate conditions. However, these cells have a limited lifespan and can only be subcultivated for a few passages until they become senescent. Thus, the reproducibility of established 3D tissue equivalents with these cells might be limited and the use of primary cells obtained from different donors probably leads to heterogeneous results [5-7].

Permanent cell lines are either isolated from carcinomas or they are generated by immortalization of normal cells, e.g. through viral transformation. These cell lines can be subcultivated for a long time and thus establish continuity in the system to be studied. There are several permanent epithelial cell lines of the respiratory tract which are commercially available. However, only a few of them are able to form functional tight junctions and to express characteristics similar to differentiated cells *in vivo* [8].

For the differentiation and realistic structuring of lung epithelial cells into basal, mucus secreting and ciliated cells *in vitro,* the use of certain growth factors, vitamins and hormones and their concentrations are decisive factors [9]. In addition, the type of cultivation plays a key role in the mucociliary differentiation. The air-liquid interface (ALI) has been proven to be essential for airway epithelial cells for mucus secretion and cilia formation (mucociliary differentiation) while the proliferation is enhanced in submerged cultures [5].

In summary, the realization of *in vitro* models simulating the structural and functional characteristics of the airway epithelium is dependent on the choice of appropriate cell lines and a balanced cultivation protocol. Moreover, to study the effects of test substances on the airway epithelium, the interactions between mesenchymal and epithelial cells should be taken into account and thus co-culture systems are indispensable for toxicological studies in order to provide near-realistic conditions.

2 Scope and objectives of the thesis

This work deals with the establishment of realistic and reproducible *in vitro* models of the bronchial epithelium for toxicological studies. The achievement of such cell models showing natural differentiation patterns is strongly dependent on the source of epithelial cells. There are some permanent bronchial epithelial cell lines, either immortalized or tumor-derived, which are described to have retained several important characteristics of their normal (non-cancerous) *in vivo*- counterparts when cultivated under appropriate conditions. Some of the most commonly used lung epithelial cell lines, the 16HBE14o- and Calu-3, and a rather unknown cell line, ChaGo-K1, are tested in this thesis for their suitability to serve as a *in vitro* model of the human bronchial epithelium.

In addition, newly developed immortalized cell lines that have not been characterized before, are comprehensively tested for their (1) general cell viability, (2) life span and (3) ability to develop natural differentiation patterns.

Since primary (non-transformed) cells isolated from the target tissue are the best source for cell culture models with regard to the reflection of realistic *in vivo* conditions, an easy and efficient protocol for the isolation of normal human bronchial epithelial (NHBE) cells from lung tissue is developed. Primary cells are known to show donor-specific variations and thus, all isolated NHBE cells are characterized for their cell viability and differentiation capacity. In order to guarantee the availability of these cells, the suitability of cryopreservation of NHBE cells and bronchial tissue samples is evaluated.

Subsequently, the most suitable epithelial cell lines are used for the generation of 3-dimensional tissue equivalents of the bronchial epithelium. These cell models are composed of bronchial epithelial cells and fibroblasts and are intended to simulate natural interactions of the EMTU. Two different approaches are tested for the implementation of such 3D co-culture models: The Sandwich Cell Model (SCM) reproduces the structure of the bronchial epithelium and provides a direct contact between the two cell types. In the Compartment Cell Model (CCM), on the other hand, only an indirect contact between the lung epithelial cells and fibroblasts is enabled but here, both cell types can be analyzed separately.

The commercial IMR90 fetal human lung fibroblast strain is primarily used as mesenchymal component in the bronchial epithelial tissue equivalents. In addition, a protocol for the isolation of normal human lung fibroblasts (NHLF) from bronchial tissue samples is developed, in order to test whether the co-cultivation with adult fibroblasts shows distinct effects on epithelial cells.

The automated Long Term Cultivation module (CULTEX® LTC) developed by Cultex Laboratories GmbH was specifically designed for the cultivation of cell cultures on insert membranes that are cultivated at the air-liquid-interface. The system enables a computer-controlled medium supply and is intended to increase the stability and reproducibility of the culture conditions. The practicability and suitability of this cultivation method for different cell lines is tested for the first time in this thesis.

The ability to simulate natural pathologic features of airway inflammation after stimulation with certain substances is another requirement for suitable *in vitro* models for toxicological studies. Therefore, a further aim of this thesis is the development of protocols for the induction of inflammation states in bronchial epithelial cells *in vitro.* For this purpose, different signaling molecules (cytokines) or growth factors that are known to be related to inflammation or irritation of the airway epithelium are added to the culture medium during the differentiation phase of the cell cultures. The cellular response to these additives is analyzed by means of several analytical methods.

3 Theoretical Framework

3.1 Toxicological Testing

Toxicology is "the study of the adverse effects of chemical, physical or biological agents on living organisms and the ecosystem, including the prevention and amelioration of such adverse effects" [(10)]. Most developed countries today have enacted laws and regulations for the marketing of drugs, food additives and other substances of toxicological concern. To evaluate the risk to human health or the environment, guidelines for toxicity testing are prescribed to enable public authorities to calculate the risks of particular substances [(11)]. For example, REACH is the European Community Regulation on chemicals and their safe use which came into force on 1 June 2007 ([EC] No 1907/2006). It deals with the **R**egistration, **E**valuation, **A**uthorisation and Restriction of **Ch**emical substances. This regulation places greater responsibility on industry to manage the risks from chemicals and to provide safety information on the substances.

Chemicals can have many types of harmful effects: some cause allergic reactions, others irritate the eyes or skin. More severely, chemicals might also be genotoxic, carcinogenic or reproductively toxic. The latter effects are mostly not immediately detectable but require long-term studies. The most critical factor for the risk assessment of substances is the selection of appropriate toxicity test systems. The use of living animals for the evaluation of adverse effects of chemicals began in the 1920s when the LD_{50} test was introduced by the British pharmacologist J.W. Trevan [(12)]. This test aims to classify of the acute toxicity of substances by determining its single dose that would kill half of the animals exposed to it. Later, animal testing gained more and more attention in the 1940s with the development of the standardized test for eye and skin irritation on albino rabbits, the "Draize test" [(13)]. A few years later, the US National Cancer Institute established the first standardized test for the identification of carcinogenicity of chemicals through the daily exposure of rats and mice to the test substances for up to two years [(11)].

Due to the increasing importance of chemicals and pharmaceuticals during the 80s and consequently the augmentation of animal testing, the Organisation for Economic Co-operation and Development (OECD) devised harmonized test guidelines that should regulate

the use of animals for toxicological assessment internationally. At the same time, the call for alternatives to animal testing for toxicological investigations increased steadily for several reasons. Firstly, the general public and scientific community have raised concerns about the suffering of experimental animals since some of the conventional test methods included highly painful procedures and sometimes consumed large numbers of animals for testing a single substance. The latter aspect has secondly led to a debate about the enormous costs and the low throughput of animal experiments. Another point that has raised concerns is the reliability and relevance of animal-based toxicity tests. Extrapolating the results of toxicity studies performed on animals to the human health risk is a critical point [14-15]. It is impossible to exactly know whether a test on rodents or dogs will provide a reliable prediction of toxic effects in humans [16]. Moreover, the relevance of the tested doses of the substances is questionable with regard to realistic human exposure levels [17].

Alternative toxicological test methods are those that enable the reduction, refinement or replacement of animal experiments [18]. The “Refinement Alternatives” include methods that eliminate or minimize pain and distress, for example through enhancing the experimental design and handling of the animals [19]. “Reduction Alternatives” are strategies that will result in lower number of animals that are necessary to obtain sufficient data [18]. This might be achieved by using novel exposure technologies and by implementing statistical methods to select sample sizes [19-20]. Moreover, duplication of experiments should be avoided by providing public access to the information of animal studies [21]. Acceptable “Replacement Alternatives” are non-animal approaches that allow the classification and labeling of substances in the same way as current animal experiments allow [22]. Here, the most promising test systems are cell and tissue culture models that enable the rebuilding of organs *in vitro*. Advances in cell culture techniques and tissue engineering gave rise to emerging high-throughput technologies, like automated cultivation systems and robotics, providing an cost- and time- efficient methodology for toxicity testing.

The present work focuses on the development of *in vitro* cell culture models of the airway epithelium that can be used for inhalation toxicological studies as an alternative method to current animal models. Inhalation of airborne substances is one of the major contributors to the human health problems. Humans are permanently exposed to particulate, vapor and gaseous contaminants [23]. Due to the fact that the incidence of lung diseases is increasing

[24-25] and is the major cause of lethality in many countries (see section 3.4.3), the studies in the field of inhalation toxicology today mainly concentrate on the understanding of mechanisms of lung injury and repair after exposure to toxicants [26]. Studying the adverse effects of inhaled substances is still a major challenge with regard to the design of exposure devices allowing the generation of controlled atmospheres, as well as the development of appropriate test systems for providing relevant data [27-28].

3.2 Legislative Regulations for Animal Experiments

In 1986, the OECD agreed to promote the refinement and reduction of animal testing and the development of *in vitro* techniques as alternatives for toxicological assessments. *Directive 86/609/EEC* and in particular its revised version *2010/63/EU* [29] regulates the use of laboratory animals for scientific purposes in the EU in concordance with the "Three Rs" principle postulated by Russell and Burch, the ***R**efinement, **R**eduction and **R**eplacement* of animal testing whenever possible [18]. Article 7.2, for example, declares "*an experiment shall not be performed if another scientifically satisfactory method of obtaining the result sought*, *not entailing the use of an animal*, *is reasonably and practicably available".* Moreover, article 47 states that the Commission and the member countries should encourage the development and validation of alternatives to animal testing.

In 1991, the European Centre for the Validation of Alternative Methods (ECVAM) was established to promote the development and to coordinate the validation of such alternative methods in the Member States [30]. In 1997, the USA followed this example and established the Interagency Coordinating Center for the Validation of Alternative Methods (ICCVAM).

In Europe, the Member States also have national organizations that encourage and support the development and implementation of alternative test methods, like the Zentralstelle zur Erfassung und Bewertung von Ersatz- und Ergänzungsmethoden zum Tierversuch (ZEBET) in Germany. Promising methods are submitted as a proposal to the ECVAM which decides if it is suitable to enter first the small-scale prevalidation and, if successful, afterwards the validation phase. This official acceptance requires the existence of an agreed protocol for the method. The validation process includes different stages and for each stage a set of criteria

has to be fullfilled [31]. The starting point is the test definition which includes the description of the scientific purpose of the test and the determination of appropriate endpoints and protocols. The next few stages should show the reliability of the method by evaluating (1) the within-laboratory variability, (2) the transferability and finally (3) the inter-laboratory variability. Subsequently the relevance is tested by applying the test method to reference standards (e.g. results of previous *in vivo* studies) and by determination of the applicability domain. As soon as all stages have been passed successfully, a final independent peer review process is entered [31]. Once an alternative method has been validated, it has to be accepted by ECVAM for regulatory purposes.

International acceptance can be achieved by presenting the ECVAM-validated test methods to the OECD. Here, the method is evaluated according to the OECD validation principles and acceptance criteria to ensure that the new test will allow chemicals to be classified and labeled in the same way as current animal tests [22, 32]. Internationally accepted alternative methods are recorded in OECD guidelines and will be implemented by regulatory agencies in all OECD Member States. The adoption of an alternative method in OECD guidelines is the last stage in the regulatory acceptance process and it is a significant step towards the replacement of animals for this particular purpose.

The development of alternative methods for toxicology has gained more attention since the adoption of the Seventh Amendment to the EU Cosmetic Directive 2003/15/EC which prescribed a phase-out of animals for testing finished cosmetics products from 2004 and for cosmetic ingredients latest from March 2009. A total ban of animal experiments for cosmetic evaluation, including for repeated-dose toxicity, reproductive toxicity and toxicokinetics, has only recently came into force on 11 March 2013. The time estimation of the phase-out was based on the presence of accepted alternative *in vitro* methods for cosmetic-related toxicity testing (e.g. skin sensitization/ corrosion, skin absorption/ penetration, genotoxicity or mutagenicity) or the progress in their development [33].

Several animal rights organizations call for the expansion of the ban of animal testing to other industries, like tobacco products. It is conceivable that the enormous progress being made in the development of alternative methods might lead to the implementation of an animal phase-out also for other industry branches in the near future.

3.3 Alternative *Methods*

Recent advances in tissue and cell culture methods, molecular biology and computational techniques have led to a variety of alternative toxicological test methods that are under validation or have already been accepted by ECVAM or ICCVAM or even internationally by the OECD according to the respective validation criteria. These alternative tests include, among others, *in vivo* methods that require a reduced number of animals compared to previous tests for a particular purpose, for example due to the support of *in vitro* data. Furthermore, there are currently a number of accepted protocols for *in vitro* tests available which make use of reconstituted tissue (i.e. skin models), human or other mammalian cell lines or microorganisms, depending on the respective purpose (e.g. microorganisms are used for testing the genotoxic potential of chemicals).

The use of cell and tissue cultures for studying cellular aspects outside of the animal body started in the early 20th century. Today, cell culture has become one of the major tools used in many different fields of the life sciences. The recent changes in the legislative regulations for toxicological testing have led to an increased usage of cultured cells or reconstituted tissue for a variety of tests even outside the regulatory framework.

The overall aim of toxicology today is to maximize the information of the toxic potential of chemicals while reducing financial costs and testing time as well as minimizing the use of laboratory animals. This can be achieved by so-called *Integrated Testing Strategies* (ITS) which combine a variety of existing data from *in vitro* tests and computational toxicology [(34)].

In the following sections, some of the most important non-animal-based approaches for toxicological assessment are described with special focus on cell culture methods.

3.3.1 Integrated Testing Strategies (ITS)

ITS are combinations of test batteries which include relevant mechanistic steps and are organized in a logical, hypothesis-driven decision scheme [(35)].

The first step of each ITS is to assess all prior data that are related to the chemical to be tested. These data might already lead to the decision whether a chemical can be rejected as likely being too hazardous or not [(34)]. If not, in the next step, the bioavailability of the chemical and the resultant target organs are determined by relevant algorithms or tests on

available co-culture cell systems. This information is then used to define the type of hazard more specifically. This is mostly first performed by computational methods, followed by *in vitro* tests [34]. Only in the ultimate step, if necessary, will animals be employed for a final decision on the toxic potential of the tested substance.

The need for more research on ITS and the usefulness of this approach has recently been discussed [35]. However, the practicability and suitability of ITS, of course, is strongly dependent on the availability of existing data and *in vitro* test systems for the respective chemical or target organ.

3.3.2 Tissue Engineering

Tissue engineering was developed to create biological tissue substitutes that can be used for repair and replacement of damaged tissue and organs [36].

The principle of tissue engineering is to isolate cells and to expand them in culture on an appropriate scaffold. The differentiated tissue constructs are afterwards reimplanted into the body [37].

The scaffold materials have to fulfill different requirements depending on the tissue that is intended to be engineered. In general, they have to be biocompatible and should support the attachment, growth and differentiation of the respective cell type [38]. Different scaffold materials show varying levels of mechanical stability and porosity and are selected according to the requirements of the target organ. Ideally, the materials are biodegradable, which means that they will degrade as cells deposit their extracellular matrix molecules [36].

Engineered skin, cartilage and bone tissue are amongst the most advanced areas of tissue engineering and some products are currently in clinical use [Apligraf® (skin), Dermagraft® (skin), Orcel® (skin)', CARTICEL® (cartilage), Hyalograft® C (bone)]. Several other tissue-engineered products are in clinical trials [36].

However, besides their use for clinical purposes, engineered tissues can also be employed for *in vitro* testing, e.g in toxicology and cell biology. At this point, the fields of tissue engineering and recent cell culture techniques, like 3D cell cultures, overlap.

3.3.3 Cell Culture

Most plant and animal cells can be maintained, expanded and even be induced to express differentiation properties in a culture dish, given appropriate conditions [39].This was first shown in 1885, when Roux achieved the maintenance of embryonic chicken cells in a saline solution outside the animal body [40].

At the beginning of the 20th century, researchers started to use small tissue explants to study cellular behaviour and functions under normal and stress conditions outside the animals. In the following years, the use of disaggregated explanted cells obtained by outgrowth from these isolated tissue cultures became more prominent for these experiments [41].

The first continuous cell line was introduced in 1943 by Earle and colleagues. This cell line was established by isolating single cells from the mouse L cell line and cloning them in culture. The cell line, known as L929 fibroblastic cell line, is today still widely used for standard testing, such as biomaterial testing (see section 3.6.2) [42]. The first human continuous cell line was established from a human cervical carcinoma in 1952 by Gey and colleagues and later became the well-known HeLa cell line [39].

In 1952 Dulbecco described a procedure for the generation of replicate subcultures by using the enzyme trypsin which cleaves the proteins bonding the cultured cells to the dish, so that the cells can be suspended and transferred to fresh dishes. This method for subcultivation of cells and the development of chemically defined media promoted the use of cell cultures routinely for a diversity of testing until today [41] .

Besides clonal cell lines of primary cells, a variety of continuous cell lines, either cancer-derived or immortalized, of different cell types from nearly all organs are commercially available today.

Primary cells are those that have been directly isolated from tissue of an organism without further passaging *in vitro*. Subcultivation of these cells results in so-called secondary cultures which can be, depending on the cell type, repeatedly subcultivated for weeks or months [39]. Primary cells are able to successfully imitate the properties of their *in vivo* –counterparts when appropriate surroundings are established [7]. However, in contrast to cell lines, the number of population doublings is highly restricted in primary cells before they reach replicative senescence [43]. Thus, their availability is considerably limited and, when using

cells from different tissue samples, donor-specific variations are likely to occur [44]. In general, the reproducibility of the experiments performed with primary cells is limited and thus, the use of primary cells for routine testing, like screening assays, is not favorable [7].

Replicative cell senescence is caused by the structure of the telomeres in many human somatic cells. Telomere shortening occurs in cells that are deficient for the enzyme called telomerase and leads to cell cycle arrest. Most cancer cells have regained the ability to produce telomerase and therefore have an almost unlimited proliferation ability [39]. For that reason, isolated cancer cells are often used for *in vitro* studies, especially in standard testing and screening assays. These cells bring continuity into the test system and thus provide reproducible data. However cancer-derived cells often show characteristics distinct from normal cells [45]. For example, they can often proliferate in a higher density in culture dishes and are not dependent on ECM (extracellular matrix) proteins for adherence. In addition, they mostly lack many important differentiation properties [8, 39].

Besides cancer cells, permanent (or continuous) cell lines can also be established from primary cells by genetic transformation of viral oncogenes, like the Simian Virus 40 (SV40) large T antigen, or by introducing the catalytic subunit of human telomerase (hTERT) into the cell [46-48]. These immortalized cell lines have a prolonged life span and are therefore especially suitable for routine tests. However, they might have lost some important characteristics of primary cells and often show a decreased sensitivity to certain agents or conditions. But, given appropriate culture conditions, some of these cell lines show a similar phenotype to primary cells and have been proven to be suitable for particular studies [5].

3.3.3.1 Three-dimensional cell culture

In the conventional 2D cell cultures, the cells are grown on flat surfaces in monolayers. This type of cultivation is easy and convenient and was shown to be a valuable tool for the fast identification of toxic compounds, as in the biomaterial testing (see section 3.6.2). Moreover, for the maintenance of cells in culture, they are also grown under 2D conditions.

However, numerous studies revealed that cells under 2D conditions have some limitations with regard to their physiological relevance [49-50]. *In vivo*, cells grow in a three-dimensional manner and they interact with other cell types or their surrounding environment, like the

extracellular matrix (ECM) [51-52]. 2D cell culture conditions affect the natural phenotype of cells and accordingly also their response to certain stimuli [49].

As a consequence of the increasing recognition of the limitations of 2D cell cultures, a variety of 3D cell cultures has recently been developed. These advanced cell culture systems have been proven to mimic *in vivo* conditions more closely and to considerably enhance toxicological and drug absorption studies [49, 51, 53]. There are different methods for the cultivation of cells in a 3-dimensional manner. The most commonly used systems are multicellular spheroids and hydrogels. Spheroids are spherical clusters of cells that are formed under distinct conditions, for example when the cells are cultivated in spinner flasks, on agar plates or in hanging drop [54-57]. Under these conditions, the cells can survive for several weeks and they develop many properties of normal tissue *in vivo,* amongst others, the formation of a natural ECM [49, 56]. Multicellular spheroids are often employed for cancer research, since they are able to develop distinct regions under certain conditions that are also found in tumors, with a proliferating outer circle, a quiescent intermediate region and a necrotic inner core [49, 55].

As in tissue engineering, 3D cell cultures can also be established by using 3D scaffolds into which the cells can invade. Hydrogels were proven to be highly suitable for the 3D cultivation of many cell types since these crosslinked networks share many properties of the ECM *in vivo* [58]. Hydrogels can be composed of natural components, such as ECM proteins, or prepared synthetically. Synthetic hydrogels, however, lack important biological characteristics and cells grown in these matrices fail to develop certain surface receptors [58].

The use of hydrogels prepared from natural ECM proteins is therefore advantageous in order to promote the natural cell behaviour. Collagens are the major extracellular components of the connective tissue and collagen type I is the most abundant ECM protein in mammals [39]. Matrices prepared from collagen type I are commonly used for the cultivation of various cells in a 3-dimensional manner (see 3.3.3.1.1).

In a more sophisticated approach of 3D cell cultures, different cell types are combined in co-culture systems in order to additionally consider their interactions. For example, to study the function of epithelia under certain conditions, the inclusion of fibroblasts in the *in vitro* models is advantageous, since they play a critical role for the growth, differentiation and regeneration of epithelial cells *in vivo* [3-4]. For that purpose, collagen matrices with

embedded fibroblasts are often applied since this structure resembles the connective tissue which underlies the epithelial layer *in vivo*. This co-culture model was successfully used for reconstruction of the skin and cornea [59-61].

Special co-culture models with cell types of the respiratory system are further detailed in section 3.5.3.

3.3.3.1.1 Collagen matrices for 3D cell culture

Matrices prepared from collagen type I are often used for 3D cell cultures in order to mimic the ECM of the connective tissue where collagen I is a predominant molecule [62].

In vivo, the cells are embedded into the ECM which does not only provide mechanical support but also plays a central role in their proliferation, differentiation and morphology and influences the gene expression [58, 63]. These interactions are mediated through signaling via specific surface receptors, primarily integrin receptors, of the collagen fibrils [64].

There are many published protocols for the isolation and preparation of collagen I from various organisms and tissues. Moreover, a variety of collagen type I products are commercially available. For these reasons, collagen type I provides a good source as a natural scaffold and has been widely used for the establishment of 3D cultures. For example, several studies have shown the suitability of collagen-based hydrogels for the cultivation of fibroblasts. Here, reciprocal interactions between the matrix and the fibroblasts were observed and it was shown that this cell culture system provides a good model for studying matrix remodeling, an event which is important for the maintenance of tissue homeostasis [65-66].

Collagen matrices form a meshwork of fibrils which polymerizes from collagen monomers. The polymerization occurs when the collagen solution is brought to physiological conditions, with a neutral pH and a temperature of 20-37 °C. The preparation and storage of collagen solutions, on the other hand, requires an acidic pH (around 2) and the cooling to 2-8 °C [62].
There are numerous factors that determine the biochemical and physical characteristics of the collagen matrix and consequently influence the cell-ECM interactions. These factors include (a) the collagen source (tissue and species of origin, pepsin or acid extraction) [67], (b) the collagen concentration and (c) the polymerization protocol (pH and temperature variations) [68]. For example, the presence or absence of telopeptides in the collagen

molecules highly influences the fibril growth and assembly. The pepsin extraction of collagen type I cleaves off the telopeptides from the molecules whereas they are preserved in the acid extraction method [62, 64]. Another factor that has to be taken into account is the lot-to-lot and product-to-product variation, due to a lack of standardized requirements for the characterization of collagen products.

In summary, the preparation of suitable collagen matrices for 3D cell cultures is a challenging task and mostly requires a time-intensive optimization of the protocol. However, the use of collagen enables the simulation of a natural environment for cells and is thus valuable for the construction of realistic 3D tissue equivalents *in vitro*.

3.4 The Respiratory System

The respiratory system is mainly designed to deliver inspired air to the parts where gas exchange takes place and to transport gaseous waste, like carbon dioxide out of the body. At the same time, the inspired air is humidified, warmed and filtered [69]. The major parts of the respiratory system through which air passes from proximal to distal are the nasal cavity, the pharynx, the larynx, the trachea, the bronchi, the bronchioles, the terminal bronchioles, the respiratory bronchioles, the alveolar ducts, the alveolar sacs, and finally the alveoli [70].

The lung, with special focus on its conducting portion, the bronchi and proximal bronchioles, will further be described in the following sections.

3.4.1 The Lung

The lung is the boundary between an organism and its surrounding environment. It comprises the bronchi, bronchioles and the alveoli. The bronchi and proximal bronchioles are conducting airways, which means, that their major task is the conduction of air to and from the respiratory portion of the lung. The respiratory airways include the remaining parts of the lung where the gas exchange takes place - the respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli [69]. Here, the inspired oxygen is transferred to the blood circulation.

In humans, the trachea divides into the two main bronchi that enter the roots of the lungs. The bronchi continue to divide like branches of a reversed tree within the lung [71-72]. The

trachea and bronchial tubes are surrounded by cartilage and smooth muscle. The amount of cartilage progressively decreases with continuing branching of the bronchi and is absent in the bronchioles. On the other hand, smooth muscle increases with decreasing cartilage, enabling the constriction of the bronchioles and thus controlling the airflow [73-75].

The next generation of branches following the main bronchi are the lobar bronchi. These bronchi each enter one of the five lung lobes, three into the right and two into the left lung (Figure 3-1). The lobar bronchi divide into the segmental bronchi which supply the lung segments, ten in the right and eight in the left lung. With each branching, the diameter of the bronchi decreases. After further multiple divisions, the bronchi give rise to the bronchioles which have a diameter of 1 mm or less and which in turn divide into bronchioles with a diameter of 0.5 mm or less [71-72].

Terminal bronchioles branch into respiratory bronchioles which merge into alveolar ducts. Terminal bronchioles mark the end of the conducting airways while the respiratory bronchioles are the beginning of the respiratory division. The alveolar ducts are formed by a series of alveoli clusters, the alveolar sacs. The alveoli are ordered like individual grapes within a bunch. The individual alveoli are tightly wrapped in blood vessels which enable the gas exchange between the lungs and the blood circulation (Figure 3-1) [70]. The alveoli contain neither cartilage nor smooth muscle [69].

The luminal surface of the airways is lined by the airway epithelium. Each region of the respiratory system is characterized by a unique composition of epithelial cell types. The epithelium of the lung, comprising the bronchi, bronchioles and alveoli, is detailed in the following section.

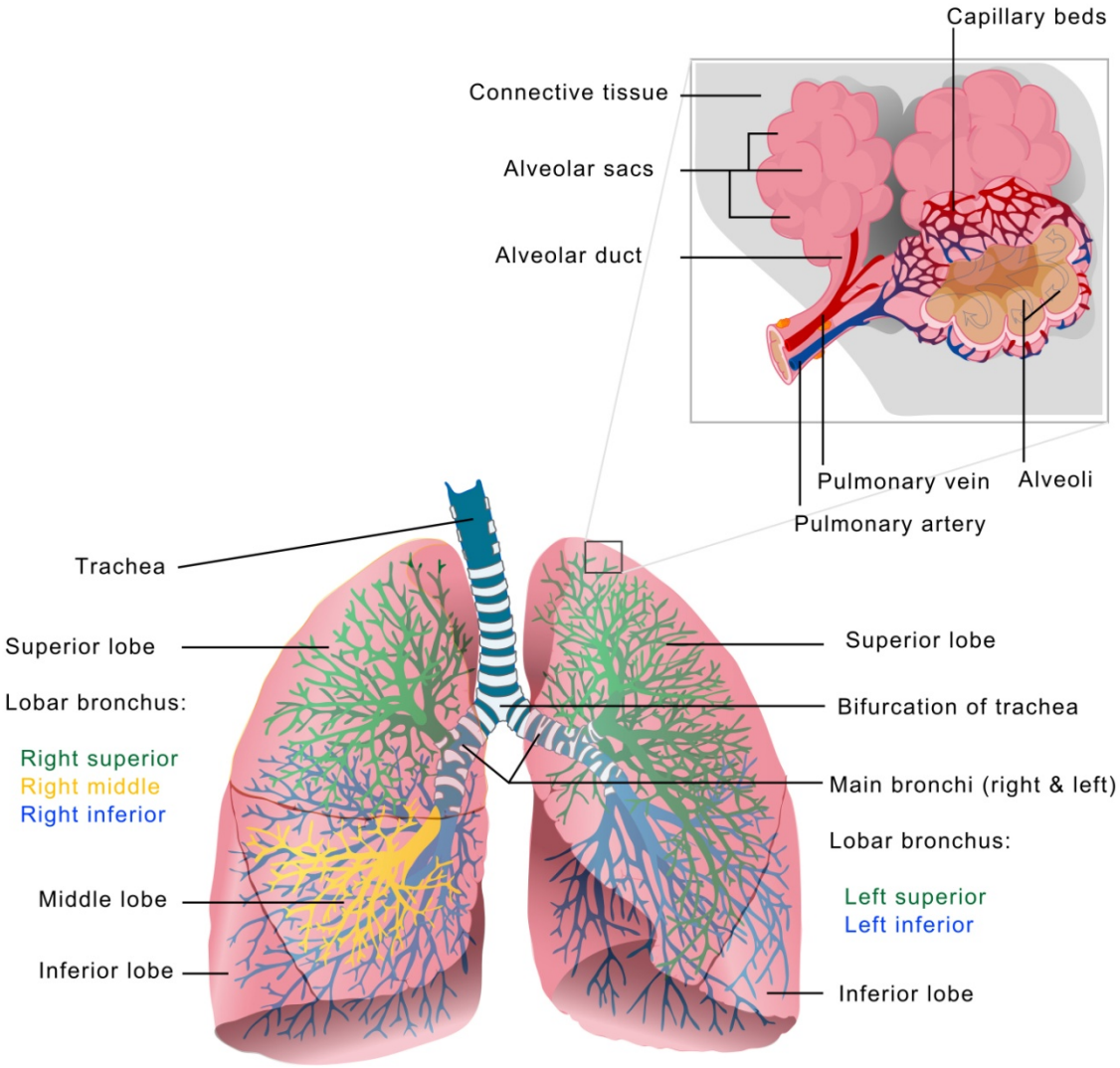

Figure 3-1: Structure of the bronchial tree and the individual alveolar sacs (adapted from Wikipedia).

3.4.2 The lung epithelium

The larger airways, e.g. the trachea and bronchi, are lined by a pseudostratified, columnar epithelium mainly consisting of ciliated cells interspersed with mucus-producing goblet cells and the multipotent basal cells [(76)]. The base of each cell reaches the basement membrane, and therefore it is a simple, not stratified epithelium (Figure 3-2). In healthy humans, the ciliated cells are the predominant cell type in the bronchi followed by the mucus-producing goblet cells [(77)]. During inflammation, this ratio is often found to shift. Here, goblet cells or dedifferentiated squamous cells are mostly more abundant than ciliated cells (goblet cell hyperplasia or squamous metaplasia, see section 3.4.2.1).

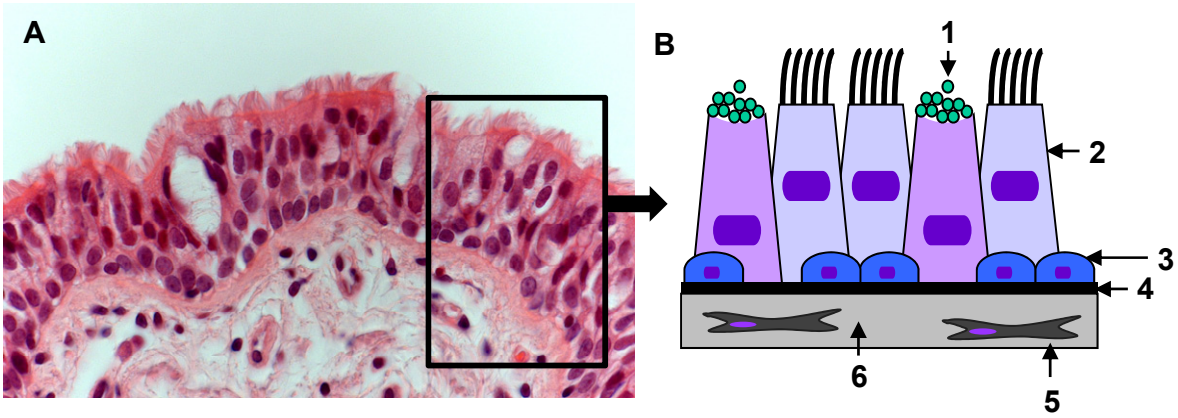

Figure 3-2: Structure of the bronchial epithelium. A: Histological section of the human lung showing the bronchial epithelium [hemalaum-eosin (HE) stained]; B: Schematic drawing of the bronchial epithelium;1: Mucus-secreting goblet cell; 2: Ciliated cell; 3: Basal cell; 4: Basement membrane (collagen type IV); 5: Fibroblasts; 6: *Lamina propria* mainly composed of ECM proteins.

In the small bronchioles (≤ 1mm), the ciliated epithelium is columnar to cuboidal-shaped and shows no pseudostratification [8]. Here, basal cells are infrequent and the number of goblet cells decreases in correlation with the diameter of the bronchiole and are lacking in the terminal bronchioles [69]. By contrast, in these small airways, the epithelium exhibits another secretory cell type, the Clara cells, which are most numerous in the terminal bronchioles [78]. Clara cells produce the surfactant apoproteins A and B and presumably contribute to the host defense and airway regeneration [79].

The bronchial and bronchiolar epithelial cells are anchored to the basement membrane (basal lamina) by adhesion molecules. A major component of the basement membrane is collagen type IV [80]. Besides the anchorage of epithelial cells, the basement membrane has a variety of other functions and plays a central role in the development of the phenotype of the epithelium [76]. The *lamina propria*, a thin layer of loose connective tissue, lies beneath the basement membrane. It is composed of ECM proteins, mainly collagen type I fibers and a cellular component, the fibroblasts, which are embedded in the ECM. The epithelial cells and the *lamina propria*, including the ECM and fibroblasts, form the EMTU which is characterized by complex reciprocal interactions between the three components [2, 81-82].

Beneath the *lamina propria* lies the submucosa, a thick layer of connective tissue which includes the serous und mucous glands. Submucosal glands are complex structures comprising multiple tubules that feed into a large primary collecting duct (Figure 3-3) [83]. The tubules are lined with mucous cells and serous cells. The primary duct passes from the

surface epithelium into the submucosa and collects the secretion products of the mucous and serous cells [84]. Mucous cells of the submucosal glands primarily secrete the mucin 5B while the watery secretory product of the serous cells is rich in antimicrobial agents and antioxidants [83-84]. Submucosal glands contribute greatly to the mucociliary clearance of the airway epithelium (see section 3.4.2.1). Their number progressively decreases from the proximal to distal airways [75].

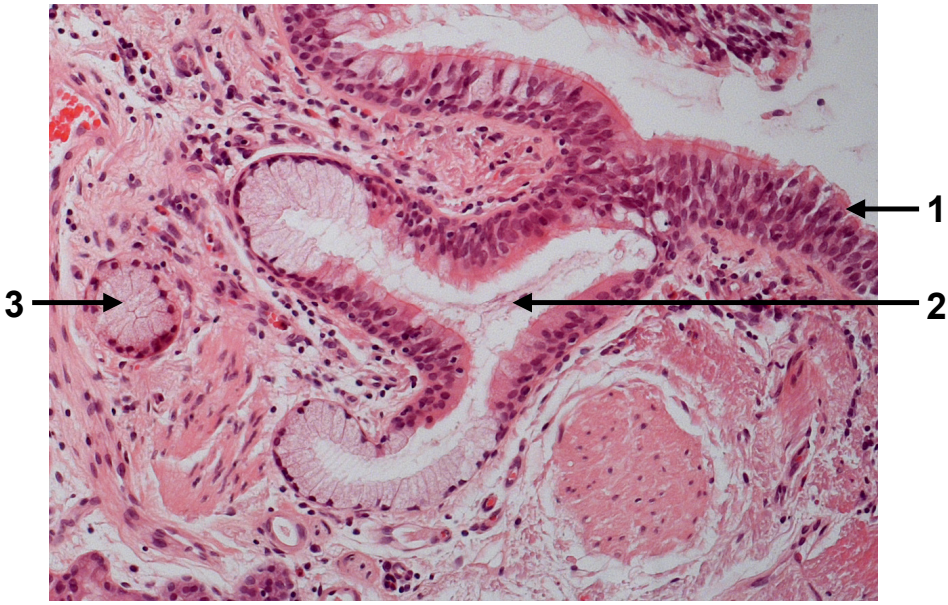

Figure 3-3: Histological section of the human lung showing the bronchial epithelium and a submucosal gland, including a primary collecting duct and secondary tubule (HE staining, Magnification: 200x);1: Bronchial epithelium; 2: Primary collecting duct of submucosal gland; 3: Tubule of submucosal gland

The human alveolar epithelium with its large surface area of approximately 102 m^2 mainly consists of two cell types, type I and type II pneumocytes (alveolar epithelial cells, AEC). Although the AEC type II are more numerous, the AEC type I constitute >93% of the alveolar surface due to their large size and flat morphology and accordingly they form the structure of the epithelium [85]. The AEC type II secrete the pulmonary surfactant that decreases the surface tension of the alveoli and thus prevents them from collapsing and furthermore contains antioxidants and molecules for the defense against microorganisms. The AEC type II also serve as a progenitor for both AEC type I and II [86]. Another cell type found in the alveolar epithelium is the alveolar macrophages that play an essential role in host defense and tissue remodeling [87].

In the alveoli, the basal lamina fuses with that of the capillaries. This ultrathin barrier enables the gas exchange (< 0.5 µm) and is potentially the main portal for pharmaceuticals into the circulatory system.

3.4.2.1 Function and role of the lung epithelium

As a boundary between the outer environment and the respiratory portion of the lung, the epithelium lining the conducting airways forms a barrier for pathogens and particles [88-89].

This barrier function is driven by two major mechanisms, (1) the mucociliary clearance and (2) the formation of a physical barrier by adherence molecules.

Nevertheless, the permanent exposure to airborne substances and pathogens makes the airway epithelium vulnerable to inflammation. Mostly, inflammation processes and remodeling of the epithelium are reversible states. However, a permanent burden may lead to the development of chronic airway diseases, such as Chronic Obstructive Pulmonary Diseases (COPD) and asthma [90].

The following paragraphs give a brief overview of the barrier function mechanisms and important processes during inflammation, repair and remodeling of the airway epithelium.

Mucociliary clearance

The apical surface of the epithelium in the bronchi and proximal bronchioles is lined by a liquid which is composed of two distinct layers, the mucus layer and the periciliary layer [91]. The mucus is a viscoelastic gel which is mainly composed of water (~95%), salts and carbohydrate-rich glycoproteins (mucins) [92]. The main airway mucins are the MUC5AC and MUC5B [93]. MUC5AC is predominantly secreted by the goblet cells in the proximal airways, whereas the secretory cells of the submucosal glands mainly produce MUC5B [94]. The thickness of the mucus layer decreases in correlation with the airway diameter and is thickest on top of the pseudostratified epithelium of large airways (up to 50 µm). The mucus layer functions as a trap for inhaled particles. The periciliary layer, on the other hand, has a low viscosity and thus enables the cilia movement [91]. Ciliated cells account for more than 50% of all epithelial cells in the airways. They each have up to 300 cilia at their apical surface, moving in a coordinated manner from the distal airways to the pharynx, to transport mucus-trapped particulate matter out of the lungs [76]. The effective mucociliary clearance is

essential not only to keep the airways clean but also to maintain the normal mucus balance. Consequently, failure of the mucociliary clearance can cause various airway diseases [91].

Physicochemical barrier

As a complement to the mucociliary clearance, the epithelial cells form a physical barrier for inhaled particles through various cell adhesion molecules which form the cell-cell junctions (intercellular bridges), including tight junctions (TJ), adherence junctions, gap junctions and desmosomes [95-96]. Among them, TJs are most important for maintaining the epithelial integrity [96]. These structures are located at the apicolateral borders of adjacent epithelial cells. Besides providing a physical barrier, TJs are also responsible for the selective regulation of the passage of ions and other molecules through the paracellular space and for the maintenance of an ionic gradient in order to allow the directional secretion of many substances [96-97]. The loss of the epithelial integrity through the disruption of TJs during inflammation may play a critical role in the development of chronic lung diseases [97].

Inflammatory response, repair and remodeling

There are a variety of factors that can cause injury to the lung epithelium, such as pathogen infection, inflammation, allergic reactions, physical trauma or the exposure to xenobiotics, like cigarette smoke or diesel exhaust [98]. Epithelial repair is rapidly induced after injury and the initial response can be highly diverse, depending on the type of injury [98-99]. This response may include the release of cytokines and recruitment of inflammatory cells. A common event that is described to occur early after epithelial injury is the de-differentiation and migration of epithelial cells in order to close the wound. Proliferation is induced afterwards to replace the lost epithelial cells [81, 88, 98-100].

Remodeling is a normal response to injury and inflammation in the airway epithelium [90, 101]. However, repeated events of injury can lead to persistent alterations, which are often precursors for chronic airway diseases [102]. The different diseases are each characterized by specific features, including unique remodeling patterns and cytokine and chemokine secretion profiles [103].

Common transitory structural alterations during repair and regeneration of the injured epithelium are the goblet- or basal cell hyperplasia and squamous metaplasia [104-105]. In the airways of COPD patients, these alterations are persistent [100, 102]

Basal cell hyperplasia is characterized by an extensive increase of basal cells up to a multitude of layers. The superficial layer generally consists of normal differentiated, columnar cells [106-107]. Basal cell hyperplasia may be developed as a response to the exposure to a number of toxins and is very common in the airways of smokers [106-107]. It is thought to be reversible but might also be an early stage of squamous metaplasia [106, 108].

Goblet cell hyperplasia is characterized by an increased number of goblet cells. Together with the enlargement of mucous glands, which is another common feature in COPD, goblet cell hyperplasia leads to a hypersecretion of mucus and consequently to the failure of the mucociliary clearance [90, 109-110]. Mucus hypersecretion is a major cause of airway obstruction in COPD patients [111-112]. The goblet cell hyperplasia accompanied by the enlargement of submucosal glands is also a pathologic feature in mild, moderate and severe asthma and a major cause of airway obstruction in this disease [90, 113-116]. Moreover, the loss of epithelial integrity and shedding of the epithelium are common characteristics in asthma [81, 98, 109].

In airways that exhibit squamous metaplasia, the normal differentiated columnar epithelium is completely replaced by a full thickness squamous epithelium [107, 117]. Although squamous metaplasia is a common response to inflammation and infection, it is also correlated with the severity of pulmonary obstruction in COPD patients and it is believed to be a prestage for the development of carcinoma [102].

Besides alterations of the epithelial layer, remodeling after injury of the airways also extends to other components of the mucosa [90, 98, 109, 118]. The interactions between epithelial cells, fibroblasts and the ECM play a central role in the regulation of inflammation and repair of damaged tissue [3-4, 81, 89]. Fibroblasts, which are in a quiescent state under normal conditions, start to proliferate as a response to injury of the epithelium [90]. This cell type is the primary source of several ECM proteins and consequently the higher number of fibroblasts leads to an increased deposition of ECM proteins [81, 119]. In asthma and COPD, the increased deposition of ECM proteins, like collagen or proteoglycan, is a common pathologic feature, leading to a narrowing of the affected airways [90, 120-121].

Fibroblasts express a variety of proteins of the matrix metalloproteinase (MMP) family. MMPs have a regulatory function in matrix remodeling and degradation [82, 118]. Epithelial cells were also shown to produce some of these proteases, like MMP-9 and MMP-2. MMP-9

is elevated in the airways of COPD and asthma patients, suggesting a contributory role in inflammation processes [122]. Although MMPs are primarily matrix-degrading proteases, recent studies also indicated that they affect various processes during inflammation by influencing several cytokines [123].

Depending on the type of injury or disease, there are numerous signaling molecules, including growth factors and pro-inflammatory cytokines, that regulate the immune response, repair and remodeling in the airway epithelium. Some of the most important and best studied signaling proteins are described in the following paragraphs. The induction or effects of some of these proteins in cultured bronchial epithelial cells were also investigated in this work (see 4.3).

The transforming growth factor-β1 (TGF-β1) is ubiquitously expressed by several cell types but in a latent form [117, 124]. For active signaling, TGF-β1 needs to be activated by dissociation from the latency associated peptide (LAP) to which it is covalently linked in its latent form. This process is highly controlled by different mechanisms. Activated TGF-β1 is critically involved in numerous cellular processes, including differentiation, apoptosis and proliferation. Moreover, it plays a central role in tissue homeostasis, ECM deposition and in the development of several pathogenic conditions [117, 125-127]. For example, TGF-β1 was found to be increased in the lungs of asthma and COPD patients. It is moreover described to be a major factor in driving squamous metaplasia of airway epithelial cells *in vitro* [124-125, 128-131].

Interleukin-13 (IL-13) is a potent mediator for mucus secretion in the airways. This was shown *in vivo* using a mouse model *and in vitro* on cultured human airway epithelial cells [111, 132-135]. The enhanced mucus secretion is associated with the increase of goblet cells while ciliated cells are reduced [132]. IL-13 production is elevated in asthma and is likely to mediate the mucus hypersecretion in this disease [111].

Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are prototypical proinflammatory cytokines that act on respiratory epithelial cells [136]. Secreted IL-1β and TNF-α are central to the events of inflammation and can initiate transcription of chemokine genes via the transcription factor NF-κB (Nuclear Factor κB) activation [137]. A central role of IL-1β and TNF-α in the induction of squamous metaplasia is suggested [102].

The cytokine interleukin-8 (IL-8), which is secreted by a variety of cell types, is primarily a chemoattractant for neutrophils and accordingly it is involved in the immune response [138]. In airway epithelial cells, the IL-8 expression is elevated after the exposure to a variety of environmental factors, including cigarette smoke, allergens and air pollutants [139]. *In vitro,* IL-8 is released as a response to the stimulation of human airway epithelial cells with inflammatory mediators, like TNF- α [140].

Interleukin–6 (IL-6) is a pleiotropic cytokine that is involved in the adaptive immune response by contributing to the determination of the type and intensity of the response [141]. Results from animal studies concerning the role of IL-6 in inflammation processes are contradictory but it was shown that its expression is elevated in patients with severe COPD and in asthmatics [141]. Moreover, the IL-6 secretion was increased in cultured human bronchial epithelial cells after stimulation with TGF-β1 [142]. Together with IL-1β, TNF-α and IL-8, IL-6 is widely used as a biomarker for inflammation.

The interplay of several proteins in complex signaling pathways is critical for tissue homeostasis and for the response to inflammation. Accordingly, studying the effects of the induction of key molecules can deliver important information on the development of acute and chronic lung diseases.

3.4.3 Prevalence and incidence of airway diseases

During breathing, the airways are constantly exposed to external insults, like pathogens or particles, and consequently the respiratory system, or more specifically its lining epithelium, is extraordinarily vulnerable to diseases [53].

In recent years, respiratory disorders, e.g. chronic obstructive pulmonary disease (COPD), asthma, tuberculosis and lung cancer were the leading causes of death worldwide. A further increase in mortality is expected in the future. In Europe, respiratory diseases rank second in terms of mortality (after cardiovascular diseases), incidence, prevalence and cost. In some countries (e.g. the UK), they are already the leading killer [143].

Asthma and COPD are two of the most common chronic diseases affecting millions of people worldwide [144]. COPD, for example, was the fifth leading cause of death in high-income countries and the sixth leading cause of death in low- and middle-income countries in 2001,

as reported by the World Health Organization. Risk factors for both COPD and asthma can be categorized as host and environmental factors. Environmental factors that serve as risk factors for COPD include inhalation exposure to cigarette smoke, occupational dusts and chemicals as well as indoor and outdoor pollution [145]. Cigarette smoke is potentially the major cause for COPD (more than 90% of COPD patients are smokers) but only 15 to 20% of smokers develop this disease [118, 146].

COPD and asthma are complex and multifactorial diseases with many disease phenotypes existing individually or simultaneously [90, 144, 147]. Moreover, there are several overlapping phenotypes of asthma and COPD which makes their discrimination often barely possible [90, 144].

The symptoms of asthma are often treatable with anti-inflammatory medications, like inhalable corticosteroids, which can also lead to a permanent improvement in the airway inflammation. However, asthma is underdiagnosed and undertreated and consequently, the number of people developing severe asthma is still increasing [144, 148]. In contrast, COPD patients generally do not respond to anti-inflammatory medications and permanent damage of the airways is evident in this disease [148]. Moreover, COPD is a progressive disease and continued smoking worsens the symptoms and accelerates the progress of airway destruction [149].

Improving the understanding of the diverse phenotypes of COPD and asthma and identifying early disease markers might help to decrease the number of severe cases in the future. *In vitro* models of the airway epithelium can help to unravel cellular aspects and thus contribute to the prevention and treatment of chronic airway diseases.

3.5 In vitro Models of the Airway Epithelium

Although the number of *in vitro* studies on inhalation toxicology is continuously increasing, there are still no standardized *in vitro* models of the airway epithelium. Consequently, many researchers are currently engaged in the establishment of such models.

Many criteria have to be considered when searching for a suitable cell model. Ideally, the cells should be derived from the species of interest, in order to preclude species-specific characteristics.

Depending on the question to be answered, special additional requirements have to be fulfillled. For example, to study drug absorption or particle deposition, the cell model should show an intact barrier function comparable to the airway epithelium *in vivo,* through the formation of functional tight junctions. Moreover, the development of cilia and the secretion of airway mucins are desirable to simulate the mucociliary clearance which plays a critical role in the protection of the epithelium against toxicants and pathogens and which also represents an additional barrier for drug absorption.

For studies on the repair and regeneration capacity of the airway epithelium, the presence of basal cells and/or Clara cells in the respective *in vitro* model might be required. These cell types are believed to be the progenitor cells for all other cell types in the bronchi or bronchioles, respectively [(150-151)]. Therefore, they potentially play a key role in the regeneration of the lung epithelium after inflammation or mechanical damage.

For high throughput screening (HTS), e.g. for large-scale toxicity testing, a cell line that is characterized by a high doubling rate and robustness is required [(152)]. Moreover, it should have a long life span and its permanent availability has to be guaranteed.

Ready-to-use cell culture systems composed of primary epithelial cells from different portions of the airway epithelium (nasal cavity, trachea or bronchus) are commercially available (EpiAirway™, MatTek Corporation; MucilAir™, Epithelix) [(153-154)]. These models are fully differentiated and exhibit all important features of airway epithelial cells *in vivo*. Moreover, they are made up either of cells from healthy persons, smokers or non-smokers, or from COPD or asthma patients and thus may cover a variety of aspects of interest. A long shelf-life of these cultures is also predicted. However, due to their commercial nature, these models are less economical and sample size is severely limited [(78, 155)]. A lot-to-lot variation may be assumed due to donor-specific variations and consequently the reproducibility of data is not predictable. In addition, the shipping procedure might have adverse effects on the cultures and may lead to false results.

Therefore, the generation of in-house cell cultures either from normal airway epithelial cells or permanent cell lines is advantageous.

3.5.1 Normal human bronchial epithelial cells

Primary cells that have been isolated from normal, non-cancerous tissue provide the most realistic *in vitro* models of the respective organ since, when cultivated under appropriate conditions, they are able to retain most characteristics of their *in vivo* counterparts [156]. However, these differentiation properties get lost during subcultivation within a short time period and the cell viability decreases [69, 157-158]. Airway epithelial cells of the first three passages have been shown to be best suited as *in vitro* models [69, 158]. To overcome limitations in the availability of primary cells in low passages, a comprehensive pool of cryopreserved cells or lung tissue is required. However, donor-specific variations might have an effect on the reproducibility of data and thus, for repeating experiments or routine testing, the use of cell lines is more favorable [45, 69].

3.5.2 Human continuous airway epithelial cell lines

As described in section 3.3.3, permanent (immortal) cell lines, either cancer or immortalized cells, have an extended life span and can thus be subcultivated over many passages. There are several commercial lung epithelial cell lines of the respiratory tract which are available at public cell collections, like ATCC (American Type Culture Collection) or DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen). However, only a few of them have been proven to provide suitable *in vitro* models of the respective part of the airway epithelium in previous studies. For others, data are unavailable or they have been found to be of no use for inhalation or drug absorption studies due to the lack of functional tight junctions or the loss of other important differentiation properties [5, 8]. The most commonly used bronchial epithelial cell lines are the carcinoma-derived Calu-3 and the two virus-transformed cell lines 16HBE14o- and BEAS-2B [69]. The adenocarcinoma-derived A549 is a well-established cell line for studies on AEC type II cells.

The following paragraphs give brief descriptions of the cell lines that were used in this study.

3.5.2.1 16HBE14o-

The 16HBE14o- is an immortalized cell line derived from the 2nd generation bronchus of a one-year old male. Immortalization has been achieved by transformation with the large T antigen of the SV40 virus [46]. Although licensed and not commercially available, these cells have widely been used and are well-established. They show many characteristics of bronchial epithelial cells *in vivo* when cultivated under appropriate conditions [5]. For example, the 16HBE14o- cells are described to form functional tight junctions and to exhibit an active ion transport. Moreover, they show a high viability over many passages. However, their ability to form cilia and to secrete mucus is controversial [46, 69, 157, 159].

3.5.2.2 Calu-3

Calu-3 is an adenocarcinoma-derived cell line with properties of human submucosal gland epithelia cells. The cells are commercially available from ATCC (product no. HTB-55) [160]. The Calu-3 cells form polarized monolayers and exhibit functional tight junctions and an active ion transport comparable to the bronchial epithelium *in vivo* when cultivated under appropriate culture conditions. These characteristics make them valuable, for example, for drug absorption studies [8, 161-162]. Moreover, these cells have been reported to produce mucus and to form apical cilia, although irregularly [69, 157].

3.5.2.3 ChaGo-K1

ChaGo-K-1 is a hyperdiploid human cell line isolated from a bronchogenic carcinoma of a 45-year-old male [163]. The adherent cells have an epithelial morphology and the main characteristic is the production of high levels of MUC1 mucin [164]. The cell line is available at ATCC (product no HTB-168™).

These cells were shown to respond to the exposure to titanium oxide nanoparticles with an increased mucin secretion, the induction of ROS (reactive oxygen species) production and finally with cell death by the apoptotic pathway. Similar reactions occur in normal human airway epithelial cells *in vivo* [165-166].

However, there are no data available on the differentiation properties of this cell line.

3.5.2.4 A549

There are currently no well-established cell lines for *in vitro* models of the alveolar epithelium. The most prominent cell line is A549, an adenocarcinoma-derived cell line isolated from a 58-year old patient in 1972, sharing metabolic and transport characteristics of *in vivo* AEC type II cells [167-168]. However, A549 show barrier properties distinct from *in vivo* AEC type II and they do not undergo transition into AEC type I-like cells [169]. The suitability of A549 as an alveolar absorption model is also questionable due to the fact that more than 90% of the alveolar surface is covered by AEC type I.

Their robustness and high proliferation rate, nevertheless, recommend the A549 cell line for high-throughput screening assays and studies on acute toxicity [27, 170-172].

3.5.3 Three-dimensional cultures and co-culture models of the airways

The "three-dimensional (3D) cell culture" is inconsistently described in the literature. In the case of cultured airway epithelial cells, the term is sometimes used to describe the fully differentiated and heterogeneous population of epithelial cells cultivated at the air-liquid interface and showing a three-dimensional (polarized), pseudostratified and columnar structure, like the ready-to-use cultures MucilAir™ or EpiAirway™ (see section 3.5). However, in other studies, the "3D cell culture" specifies the co-culturing of different cell types which enhances the physiological relevance of *in vitro* tissue models with regard to the complex interactions between the cell types. The latter definition is adopted in this work.

In recent years, various co-culture models simulating different parts of the respiratory tract have been developed. For example, *in vitro* models of the bronchial epithelium have been realized by co-culturing human epithelial cells (cell lines or primary cells) with human fibroblasts. Here different methods have been used. In the simplest model, bronchial epithelial cells are supplied with fibroblast-conditioned medium or vice versa [4, 173]. In a more sophisticated approach, the two cell types are co-cultured, either in direct or indirect contact. An indirect contact is enabled by seeding the fibroblasts on the opposite side of a microporous insert membrane or in wells of an insert receiver plate, while the epithelial cells are grown on the apical side of insert membranes [3, 174-175]. A direct contact is provided, by seeding epithelial cells onto fibroblasts, that were either grown as monolayers or embedded

into a collagen matrix [176-178]. Thus, the natural structure of the airway epithelium is simulated.

There are also co-culture models that mimic the alveolar epithelium, for example by combining A549 cells with cells of the immune system, like macrophages alone or combined with dendritic cells in a triple co-culture system [179-181]. Another approach utilizes endothelial cells for co-cultures with lung epithelial cells, mostly A549, to simulate the air-blood barrier of the alveoli [182-184].

All of the above mentioned studies consistently demonstrated that different results were obtained between co-cultures and their respective monocultures due to natural intercellular interactions. These 3D lung tissue models were shown to be promising tools for providing more realistic conditions in inhalation, toxicological and drug absorption studies *in vitro*.

3.5.4 Cultivation of airway epithelial cell cultures

In vivo, there are complex interactions of numerous physiological parameters, which are responsible for the structure and functions of the respective tissue. To obtain nearly realistic tissue models *in vitro* with well-differentiated and long-living cells, an optimal balanced culture environment, approximating the *in vivo* conditions, is essential.

The air-liquid interface (ALI) was previously shown to be indispensable for the development of an *in vivo*-like phenotype in cultured human airway epithelial cells [44, 158, 185-187]. Here the cell cultures are supplied with nutrients only from the basal side while their apical surface is exposed to air, comparable to the conditions in the body. For that purpose, cell culture inserts are used which have a microporous membrane, mostly made of polyethylene terephthalate (PET) or polycarbonate, on which the cells are grown. The inserts are placed into wells of receiver plates which are filled with cell culture medium. The membrane pores enable the diffusion of the nutrients from the basal side to the cells (Figure 3-4). The ALI promotes the mucociliary differentiation of airway epithelium *in vitro* and is essential for the formation of TJs [44].

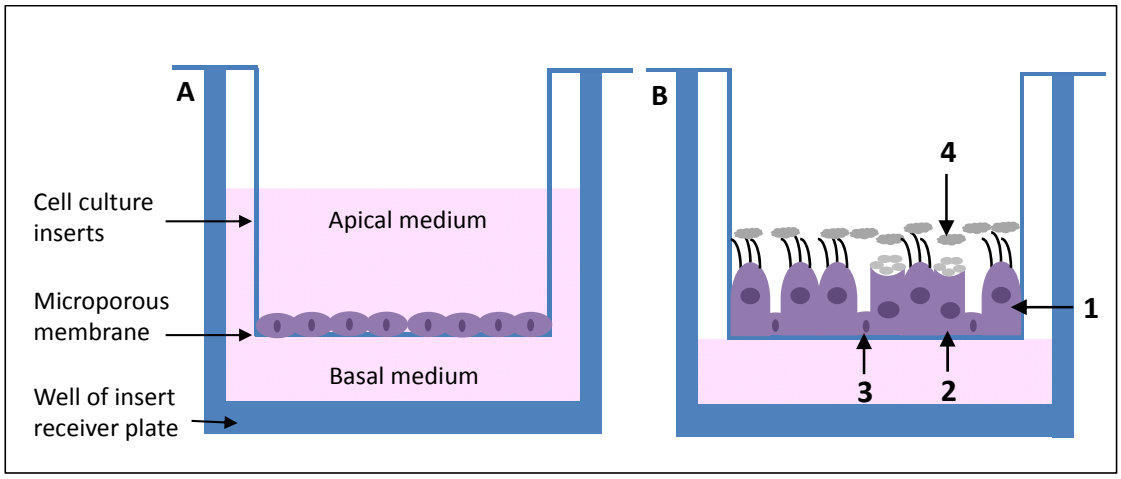

Figure 3-4: Cultivation of airway epithelial cells on microporous membranes of commercial cell culture inserts. A: The cells are cultivated submerged in cell culture medium to enhance the proliferation; B: The air-liquid interface cultivation method is indispensible for the differentiation into ciliated and mucous cells (1= Ciliated cell; 2= Goblet cell; 3= Basal cell; 4= Mucus).

Other critical parameters for the cultivation of epithelial cells are the (1) environmental conditions (temperature, humidity), (2) nutrition composition and supply (3) and substrate (culture surface).

Environmental conditions simulating the physiological conditions of the body with regard to temperature and humidity can today easily be established in the laboratory by means of incubators that are available from several companies. These incubators additionally allow the adjustment of a physiologicalyl relevant CO_2 level which is also important for maintaining a constant pH in the cell culture medium. The pH is a critical factor and therefore most cell culture media contain buffers which are dependent on certain CO_2 levels [188].

The airway epithelium is characterized by a diverse phenotype with several specialized cell types and functions (see section 3.4.2). Thus, the recreation of this complexity *in vitro* is challenging and requires a balanced interplay of various factors and an optimal medium composition.

Retinoic acid (RA) is an oxidized derivative and active form of vitamin A. RA is known to influence numerous processes in the cell and therefore plays an important role in cell growth and differentiation both *in vivo* and *in vitro* [158, 189-190]. Especially in the airway epithelium, retinoic acid is crucial for mucociliary differentiation [158, 190-191]. Vitamin A deficiency, on the other hand, leads to the development of squamous metaplasia and keratinization [190, 192-195]. However, retinoic acid is also known to reduce or inhibit

proliferation in some cells, including human bronchial epithelial cells in concentrations higher than 1 µM [196-197]. Accordingly, the use of low concentrations of RA during the growth phase is advantageous while higher concentrations are required for the induction of the mucociliary differentiation in the later differentiation phase. Mostly, a concentration of 50-100 nM is used for the induction of differentiation in airway epithelial cells [9].

Calcium is known to affect cell signaling, differentiation and proliferation of certain cell types [198]. Low concentrations of $CaCl_2$ were found to enhance cell proliferation but to suppress the differentiation of airway epithelial cells *in vitro*. Therefore, medium with a low $CaCl_2$ concentration is used during the growth phase while the concentration is later increased to promote the differentiation.

Epidermal growth factor (EGF) promotes the growth of epithelial cells but has an inhibitory effect on the normal differentiation of airway epithelial cells. High EGF concentrations were furthermore shown to induce basal or goblet cell hyperplasia in cultured airway epithelial cells [199-200]. Consequently, low concentrations are required to enhance the development of cilia and the mucus secretion [9, 158, 198, 201].

There are several other factors that promote the growth and differentiation of cultured airway epithelial cells, including insulin, transferrin and hydrocortisone. Optimized cell culture media that were specifically designed for the growth of airway epithelial cells are commercially available from different suppliers. Ideally, the supplements are provided in individual tubes, like the BEGM™ Bulletkit™ provided by Lonza. In this case, the concentrations of critical factors for the cell differentiation can be added in user-defined concentrations. Optimal concentrations of these supplements resulting in well-differentiated cultures of airway epithelial cells can be found in the literature [9].

Animal serum is commonly used as a supplement to the cell culture medium since it contains numerous hormones, growth and attachment factors that are essential for the cultivation of many cell types [202]. The most widely used sera are derived from fetal bovine calves [156, 203]. There are some reasons that promote the use of serum-free cell culture media. Besides the ethical concerns, serum is a potential source for microbiological contaminations, like bovine viruses or mycoplasma. Moreover, the composition of serum is highly variable and consequently differs between lots [156]. Several serum substitutes are available from different suppliers, like Ultroser® G (Pall Corp.) that was also used in this work. Although these serum

substitutes are also delivered as an undefined mixture, their composition is highly consistent between lots.

Epithelial cells are adherent growing cells. Their adherence to the cell culture vessel depends on the nature of the surfaces. *In vivo*, the attachment of the cells to their substrate or to each other is enabled through the ECM which is composed of a variety of proteins secreted by cells organized into a structural network to which the cell surface integrin receptors bind [58]. The ECM also promotes the growth and differentiation of epithelial cells *in vivo*. The extracellular matrix is mainly composed of different types of collagens. Further important components are attachment molecules, like fibronectin or laminin [188].

In cell cultures, normal attachment, growth, and differentiation of many cell types also depend on attachment factors and other ECM components [188, 204]. While some cells are able to synthesize these components, others require an exogenous source, particularly when grown in serum-free cultures. Thus, many cell cultures are seeded on surfaces that are coated with ECM components [45, 188, 198]. Collagen type I is predominantly used for this purpose because it is the most common protein in the human body and it can easily be isolated, for example from rat tails or bovine skin, and is thus economically available. For the cultivation of primary airway epithelial cells, collagen type IV is also frequently used as main component of the basal membrane to which the epithelial cells are attached *in vivo* [201]. Many protocols recommend a mixture of collagen and fibronectin and/or laminin for surface coating.

The optimal conditions for the adherence, growth and differentiation were established individually for each cell line and cell type used in this work in a stepwise process.

3.6 Automated cultivation systems

The maintenance of cell cultures is laborious work made up of many repetitive steps that are necessary to guarantee the provision of viable cells. This routine work is vulnerable to person-specific variations which might be disadvantageous for the reproducibility of cell culture studies. Moreover, the static medium supply leads to a nutrition gradient over the cultivation time and shifts in the pH of the medium. To increase the consistency of cell

culture processes and to establish standardized conditions, automated cell culture systems are increasingly employed, especially in pharmaceutical companies [205].

There are different fields of application for automated cell culture systems, each demanding the automation of particular working processes [206]. For example, for high-throughput screening assays, the large-scale expansion and subcultivation of cell lines as well as the preparation of high numbers of cell cultures in multiwell plates are required. Here, fully-automated cultivation systems with integrated pipetting robots are advantageous, like the CompacT SelecT provided by TAP Biosystems [205, 207].

For the maintenance and expansion of cells or tissue-engineered cultures under stable conditions, bioreactors are often used. These systems allow a dynamic medium supply and enable the control of pH, oxygen and transport of metabolic waste [38, 208]. Moreover, the stirring or agitation of the cell culture medium not only offers an optimal nutrition and oxygen supply but also induces a mechanical stimulation and thus provides more realistic environmental conditions which enhance the growth and differentiation of the cells [209]. Many different types of bioreactors are available which are either suitable for suspension cultures (e.g. spinner flasks) or, in the case of anchorage dependant cells, for cell cultures on special matrices (e.g. the rotation bed bioreactor) [38, 209].

There are two automated cultivation systems that were specifically designed for the small scale cultivation of cell cultures grown on 3D scaffolds [210-211]. These systems are based on a modular concept, whereby the cell cultures are cultivated in individual chambers that can be connected to each other [211]. A continuous medium supply through each chamber is provided leading to a constant provision of nutrients and, at the same time, to a mechanical stimulation of the cells. Thus, a more realistic culture environment is established [210-211].

Despite the great advances made in the field of automated cell cultivation, there are currently no systems that enable the controlled cultivation of cells on cell culture inserts and, moreover, that are suitable for the air-liquid interface cultivation which is indispensable for the differentiation of airway epithelial cells.

3.6.1 The CULTEX® Long-Term Cultivation (LTC) System

The manual cultivation of cell cultures grown on insert membranes is a time-consuming task and, for the successful and reproducible establishment of fully differentiated cell layers, a multitude of factors has to be considered.

For example, the handling of a large number of cell culture inserts requires the frequent opening of the incubator which causes repeated fluctuations in temperature, humidity and CO_2 content. Moreover, the manual renewal of the cell culture medium may lead to slight deviations in the adjusted medium level which could have severe effects on the integrity of cell layers in the case of air-liquid interface cultures. In addition, a static medium supply at defined time-points leads to variations in the nutrition supply and pH throughout the cultivation period, which further depends on the metabolic rate of the respective cell type.

To overcome the numerous difficulties of the complex manual cultivation of insert cell cultures at the air-liquid interface, Cultex Laboratories GmbH developed and constructed a system that was specifically designed for the computer-controlled long-term cultivation of such cultures, the CULTEX® LTC.

In order to establish dynamic and stable cultivation conditions, the CULTEX® LTC module incorporates a computer-controlled medium supply and mixing device. The system provides a controllable intermittent or continuous individual medium supply for three insert cell cultures. The medium circulation through one insert holder is driven by two peristaltic pumps. The medium enters each vessel via the medium inlet and is sucked out through the medium outlet. At the base of the vessels, the medium passes through a perforated metal plate which enables the mixing of the medium and is involved in providing a mechanical stimulus induced by a teflon membrane beneath the metal plate. The teflon membrane can be moved up and down by a lifter that is driven by an electromagnet (Figure 3-5). The medium can thus be stirred thoroughly and, at the same time, a mechanical stimulation is provided. The medium level is controlled by an ultrasonic sensor that is able to detect level deviations of ≥0.1mm (Figure 3-5). The adjustment of the medium levels, the medium renewal rate and the mixing frequency are computer-controlled by special software.

The cell culture medium and medium pumps are located outside the incubator and are connected to the CULTEX® LTC inside the incubator via silicone tubing and cables. This setup

allows the control of the cultivation system without interfering with the adjusted conditions of the incubator.

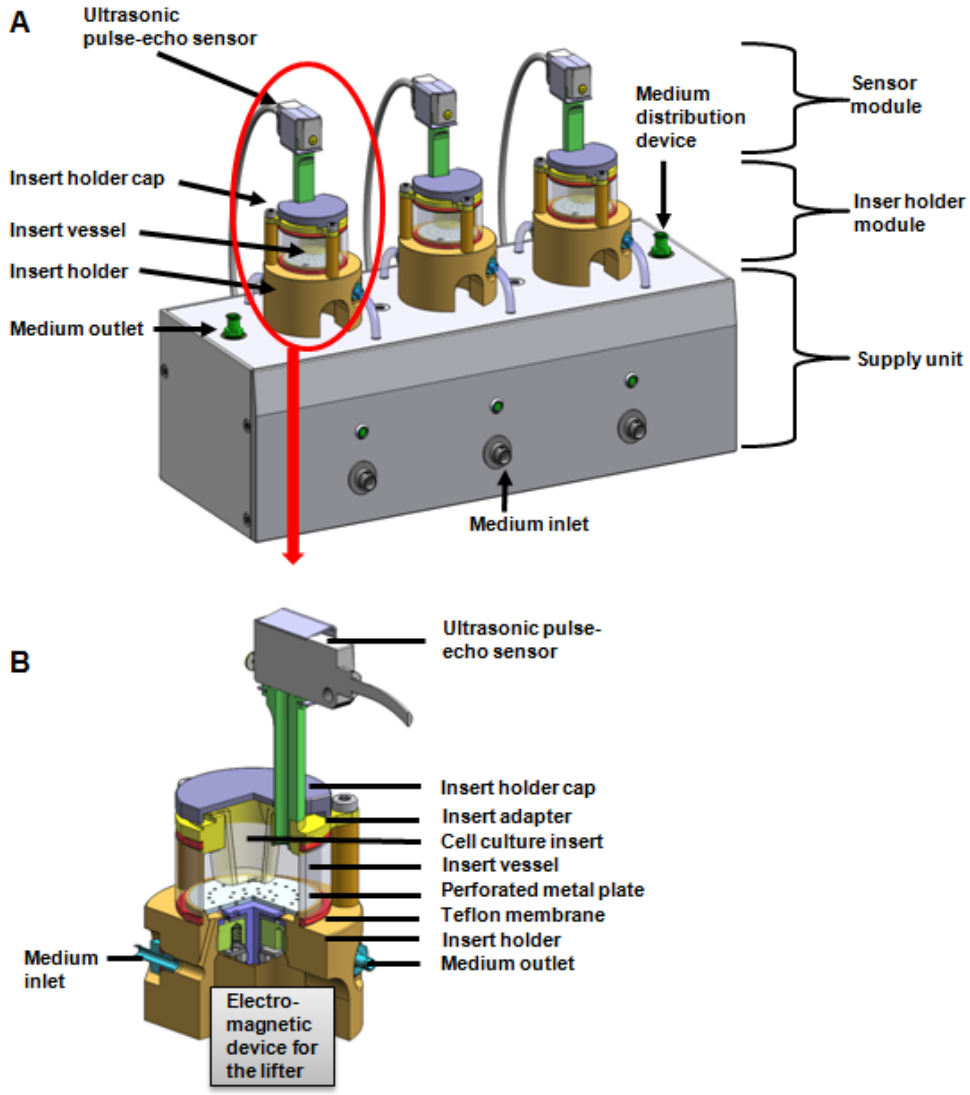

Figure 3-5: Overview of the CULTEX® LTC (Long-Term Cultivation) module (A) and a detailed description of the individual automated vessels for the cell culture inserts (B). (Illustration provided by Halter Engineering GmbH)

The integration of this technical device should optimize several processes of the cultivation of cell cultures on insert membranes at the air-liquid interface. The intermittent or continuous medium renewal and the mixing of the medium lead to a stable nutrition supply and pH. At the same time, a mechanical stimulation is provided which reflects the *in vivo* situation and may thus have a positive effect on the growth and differentiation of the cell cultures. The computer-controlled cultivation increases the maintenance and reproducibility of stable conditions by allowing the exact adjustment of the medium level and by eliminating

the necessity to open the incubator. Last but not least, such a system is a time-saving alternative to the manual handling of cell cultures cultivated on insert membranes.

3.6.2 Testing of materials for *in vitro* devices

There are many factors that have to be considered when constructing devices for *in vitro* techniques, especially when utilizing living cell or tissue cultures. For example, exposure devices should optimally be designed with special regard to providing stable and reproducible conditions and physiologically relevant exposure times and concentrations depending on the respective test agent [212-213]. Moreover, especially for long-term exposure experiments, sterility and physiological conditions (temperature, pH) have to be guaranteed. The latter parameters are also most relevant for automated cultivation systems.

Another critical point is the use of appropriate materials. The necessity to test the materials used for the construction of *in vitro* devices to exclude any influence on the cell cultures has already been declared in the late 1950s by Giardinello and colleagues [214].

The International Standard (ISO) 10993-5:2009(E) describes three test methods to assess the biocompatibility of materials *in vitro* using cell cultures. This standard was developed to meet the requirements of testing materials for medical devices which is regulated in the European Directive 93/42/EEC and its amendments [215-216].

The “direct contact method “provides the most realistic and efficient test procedure. Here, the material sample is placed in the culture vessel and incubated with the cell culture during the entire cultivation time under standard conditions. Potential toxic components may thus be released into the culture medium and can be detected through alterations in the cell cultures. This method generally enables the qualitative (cell morphology) and quantitative (cell viability) analysis of cytotoxicity. However, movement of the specimens might cause a physical trauma to the cells and thus hinder the quantitative interpretation [217].

The “indirect contact method” typically utilizes an agar layer to separate the specimen from the cells (agar diffusion test), thus preventing a physical impact. Here, the selection of a suitable agar is crucial. A low melting temperature is required to avoid a thermal shock to the cells and the molecular weight must be appropriate to allow the diffusion of all extractable chemicals. The agar layer should be constant since the diffusion distance might

affect the concentration of toxicants in the cells [217]. This test method allows qualitative analysis but not quantitative assessment of cytotoxicity.

In the “elution test”, extracts of the test materials are prepared. For that purpose the samples are incubated in cell culture medium for a defined time [218]. The medium should then contain all leachables from the test material and can be used for the cultivation of the cell cultures. This method also allows the qualitative and quantitative analysis of cytotoxicity. However it is more time-intensive than the direct contact test since the extract has to be prepared prior to testing.

In this thesis, an improved variant, which combines the advantages of all three standardized test methods, is established. This procedure can be used for the rapid evaluation of biocompatibility of materials that are intended to be used for the construction of newly developed *in vitro* devices.

4 Experimental Work

The experimental work of this thesis started with the characterization and evaluation of different available human airway epithelial cell lines and commercial primary cells of the respiratory tract. Later, a protocol for the isolation of bronchial epithelial cells from human lung tissue was developed and these cells were then also included in the analyses. The aim was to find an appropriate cell model of the airway epithelium that (1) reflects important *in vivo* characteristics and (2) is suitable for routine toxicological testing.

In the next step, selected appropriate airway epithelial cell models were combined with lung fibroblasts- which mostly were embedded in a collagen matrix- in order to reflect the reciprocal interactions of the EMTU. Here different protocols for the implementation of such 3D co-culture systems were developed. The mesenchymal component was mostly represented by a common human fetal lung fibroblast strain, the IMR90, which is commercially available. However, to also include adult fibroblasts, a protocol for the isolation of fibroblasts from human lung tissue was developed.

For the analyses of the 3-dimensional growth structure and the presence of certain differentiation characteristics, several analytical methods, like histology, immunohistochemistry and immunofluorescence were applied. Since these techniques were originally developed for tissue cultures (histology and immunohistochemistry) or cells grown in a 2-dimensional manner on glass or plastic (immunofluorescence), the protocols had to be adapted specifically for cell cultures grown on microporous membranes of cell culture inserts.

The selected airway epithelial cell models were afterwards further used to study the effects of important signaling molecules that are associated with inflammation processes. The aim of these experiments was to investigate whether the *in vitro* models are capable of developing natural inflammation and remodeling features which is an important requirement for suitable cell models for toxicological studies.

In the last step, the CULTEX® LTC was used for the cultivation of the developed *in vitro* models, to evaluate the effects of such an automated system for cell growth and differentiation as well as for its suitability to deliver highly reproducible cell cultures.

4.1 Evaluation of cell lines and primary cells of the bronchial epithelium

Different permanent lung epithelial cell lines and primary cells were characterized for their ability to develop important differentiation patterns. The most important functional characteristics of the bronchial epithelium are the mucus secretion, cilia beating, barrier property (tight junctions) and self-renewal capacity (presence of basal cells).

All cultures were prepared on cell culture inserts in 12-well-format. They were cultivated under submerged conditions in their cell-specific growth medium within the first days of cultivation until the cells formed a confluent layer. Then the apical medium was removed and the cells were cultivated at the air-liquid interface with the respective differentiation medium. Most cultures were supplied with the basic differentiation medium with the following composition:

BEBM (Lonza)/ DMEM (incl. 1g/l glucose, HEPES), at a ratio of 1:1

+ BEBM *Single Quots*® (without retinoic acid)

- Hydrocortisone
- Insulin
- Transferrin
- Epinephrin
- Triiodothyronine
- Human epidermal growth factor (hEGF)
- Bovine pituitary extract (BPE)
- Gentamicin/amphotericin B (GA)

+ 1mM $CaCl_2$

+ 2% Ultroser®

+ 100 nM retinoic acid

The exact concentrations of the additives provided as SingleQuots® by Lonza are unknown, but they are described to be specifically adapted to the requirements of bronchial epithelial cells.

In order to analyze the cell cultures for the presence of the desired differentiation characteristics, several analytical methods were applied frequently. The growth status and the sterility of the cultures were checked by *in situ* microscopy (inverted microscope), several times a week. To learn more about the differentiation progression of the respective

cell line over the cultivation time, the different inserts representing one cell culture were taken for histology at different time points. For that purpose, the cells were fixed with 10 % formaldehyde and, after dehydration, embedded in Paraplast®. Histological sections (5 – 7 µm) were routinely stained with hematoxylin-eosin (HE), a standard staining for the visualization of different cell components. For the detection of certain differentiation markers, immunohistochemical staining was applied. To get an overview of the distribution of special differentiation characteristics, e.g. cilia, throughout the entire culture, immunofluorescence analyses were performed sporadically on normal, non-embedded cell cultures after a short fixation with 4% formaldehyde. Table 4-1 gives an overview of the markers (antibodies) that were routinely analyzed for the detection of the most important differentiation features or pathological alterations of bronchial epithelial cells by immunohistochemical analyses or occasionally by immunofluorescence. For immunohistochemistry, the DAB (3,3'-Diaminobenzidine) chromogen was used for the detection of antibody binding and thus, positive staining signals were indicated by a brown colour (see 6.1.5.2).

Table 4-1: List of routinely used markers (antibodies) for the detection of important differentiation characteristics of bronchial epithelial cells by immunohistochemistry or immunofluorescence

Marker (antibody)	**Detection of**
MUC5AC	Mucin 5AC
MUC5B	Mucin 5B
Tumor protein p63	Basal cells
Acetylated alpha-tubulin	Cilia
Ki-67	Proliferating active cells
Cytokeratin 6 (CK6)	Basal cells and squamous cells
Cytokeratin 13 (CK13)	Squamous cells

The formation of functional tight junctions was determined by measuring the TEER [transepithelial electrical resistance (Ω/ cm^2)] of the cultures. The higher the TEER values the tighter were the intercellular connections through the development of tight junctions.

Protocols and further information on the analytical methods as well as the cultivation procedures for each cell line are given in chapter 6 (Appendix).

The results of the analyses were used for evaluating the suitability of the respective cell line to serve as a *in vitro* model of the bronchial epithelium.

4.1.1 Continuous epithelial lung cell lines

4.1.1.1 ChaGo-K1

The ChaGo-K1 is a cancer-derived cell line which is not well characterized. Since no data on the differentiation properties of these cells are available in the literature, the ChaGo-K1 was included for evaluation in this thesis.

The major benefits that recommend this cell line are its permanent availability (deposited in a public cell collection) and the extraordinarily high growth rate and cell viability over many passages (at least up to 20 passages after arrival from ATCC). Moreover, ChaGo-K1 cells grow on uncoated surfaces and do not demand any special medium additives. These characteristics are highly advantageous for routine and high-throughput testing in toxicology.

The cells were cultivated in cell culture flasks with a growth area of 75 cm^2 and with RPMI 1640 supplemented with 10 % FBS. They showed a high metabolic activity which was indicated by a rapid acidification of the culture medium visible through the color change of the phenol red indicator to yellow.

For further characterization, the ChaGo-K1 were seeded onto microporous insert membranes with 0.4 or 1.0 µm pores that were either uncoated or coated with collagen type I or IV (see 6.1.1.4). The varying coatings or pore sizes of the membrane had no obvious effects on the growth and differentiation of the cells. The ChaGo-K1 were seeded at a density of 2 × 10^5 cells/ cm^2 and reached confluency after two days under submerged conditions. The cultures were then cultivated at the air-liquid interface and supplied with the basic differentiation medium.

It soon became obvious that the ChaGo-K1 cell line does not provide a suitable *in vitro* model of bronchial epithelial cells. *In situ* microscopy initially suggested that the roundish ChaGo-K1 cells formed a dense monolayer and a remarkable amount of a mucus-like substance was detectable on top of the cultures already after eight days which increased in correlation with the culture age. However, the TEER values were around zero (after subtraction of the blank value), indicating a lack of functional tight junctions. This assumption was further verified by histology. The histological sections revealed that the cells formed a multilayered network of individual cells without intercellular connections. The basal layer was tightly adhered to the membrane, whereas the upper layers became detached during handling. This growth pattern was observed in all ChaGo-K1 cell cultures (Figure 4-1).

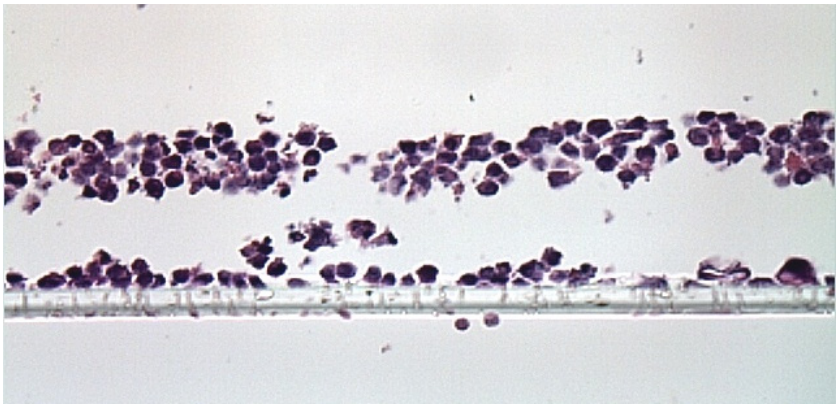

Figure 4-1: Growth pattern of ChaGo-K1 cells cultivated on collagen IV coated microporous membranes (0.4 µm pores) after a total cultivation time of 14 days at the air-liquid interface with basic differentiation medium (HE stained histological section; magnification: 200x).

The cells were small in size and had a high nuclear to cytoplasmic ratio (N/C >1; Figure 4-2). These characteristics are mostly found in cells of small cell carcinoma [(219)].

Immunohistochemical analyses with antibodies against the major airway mucins, MUC5AC and MUC5B, showed no positive signals and cilia-like structures were also not detected in ChaGo-K1 cultures.

Conclusions

In summary, the ChaGo-K1 cells were lacking most of the important properties of normal bronchial epithelial cells and thus do not provide a realistic *in vitro* model of the airway

epithelium. No further investigations on the metabolism of these cells were performed during this thesis. Due to the fact that they failed to form tight junctions, it is not likely that the cultures might reflect properties of a functional epithelial barrier.

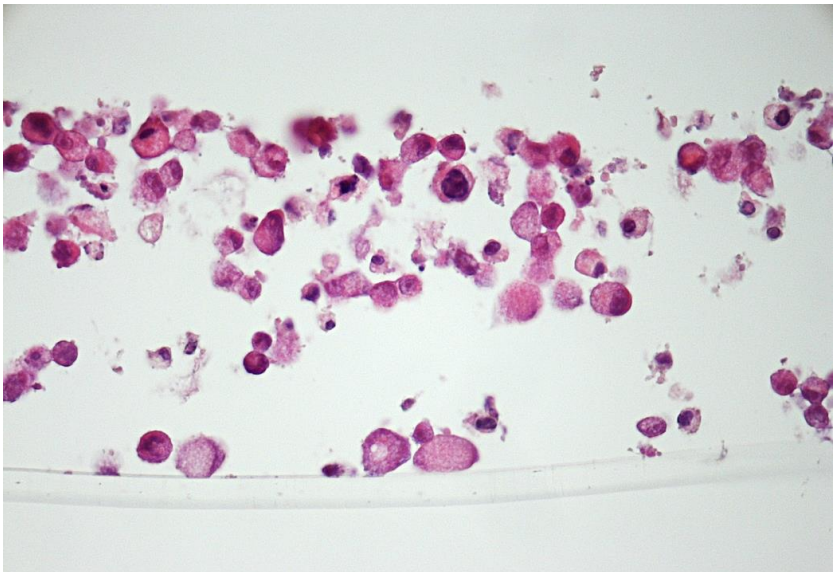

Figure 4-2: Morphology of ChaGo-K1 cells cultivated on collagen IV coated microporous membranes (0.4 µm pores) at the air-liquid interface with basic differentiation medium after a cultivation time of 20 days (HE stained histological section; magnification: 630x).

4.1.1.2 Calu-3

A second cancer cell line was subsequently tested - the Calu-3. In contrast to the ChaGo-K1, Calu-3 cells are well-established and characterized. Previous studies have proven their suitability to study aspects of ion transport, drug transport and metabolism and the functions of tight junctions in the airway epithelium [220-222]. Unlike many other cancer-derived cell lines, Calu-3 is described to be heterogeneous and therefore might represent different cell types of the airway epithelium when cultivated under appropriate conditions [223]. Their permanent availability and high cell viability over many passages recommend the Calu-3 cells for routine toxicological testing.

The cells were cultivated in Eagle´s Minimum Essential Medium (EMEM), supplemented with 10 % FBS and in uncoated culture flasks with a growth area of 75 cm^2. Their growth pattern reflects the cancerous origin of the cells. In contrast to primary cells (see 4.1.2.3), the Calu-3

show a patchy growth pattern. Single cell clusters fuse during the exponential growth phase and finally form a dense cell layer (Figure 4-3).

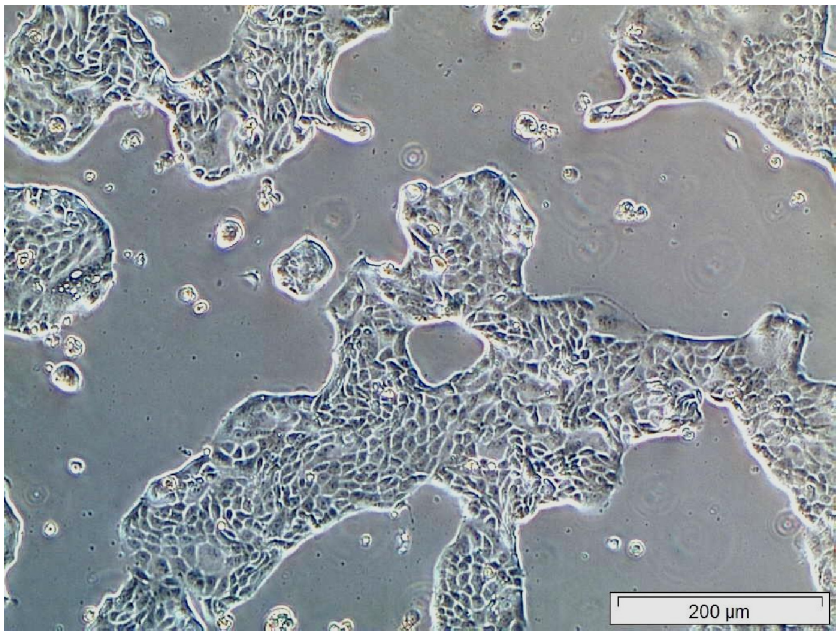

Figure 4-3: Growth pattern of Calu-3 cells cultivated in cell culture flasks (75 cm^2) with EMEM including 10 % FBS

Interestingly, although derived from tumors, these cells have a relatively low growth rate, especially when compared to the ChaGo-K1. Therefore, the subcultivation of the Calu-3 cells was only performed once a week. However, due to their dense growth, the cell yield obtained by passaging the cells of one culture flask (75 cm^2) with a confluency of approximately 80% was extraordinarily high (up to 1.5×10^7 cells/ 75 cm^2).

When the cells were seeded onto collagen type I coated microporous insert membranes (with 0.4 µm pores) at a density of 2.5×10^5 cells/ cm^2, a confluent cell layer was detectable after five to seven days under submerged conditions. After establishing ALI conditions and feeding the cells with basic differentiation medium, they soon formed monolayers with columnar-shaped cells and exhibiting high TEER values between 1330 and 3300 Ω/cm^2, depending on the culture age and amount of mucus secretion. These high TEER values clearly indicate the presence of functional tight junctions. The morphology of Calu-3 cells under these conditions was highly similar to normal bronchial epithelial cells but cilia were lacking (Figure 4-4).

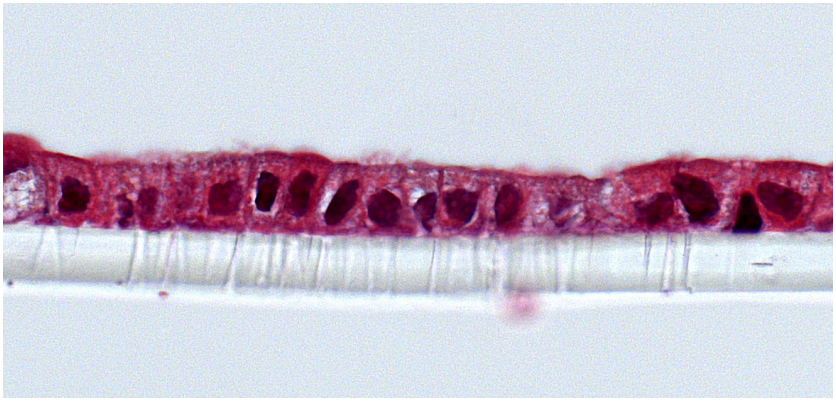

Figure 4-4: Morphology and growth pattern of Calu-3 cells cultivated on collagen I coated microporous insert membranes (0.4 µm) at the air-liquid interface with basic differentiation medium after a cultivation time of 31 days (25 days under ALI conditions; HE stained histological section; magnification: 400x).

Secretion of a mucus-like substance was detectable after three to four days under ALI conditions which increased with culture age. The secretion activity was higher when the Calu-3 were cultivated on membranes with 1.0 µm pores but the life span of these cultures was reduced compared to those that were cultivated on membranes with 0.4 µm pores. Accordingly, the inserts with 0.4 µm pores were constantly used for the cultivation of Calu-3 cells. Under these conditions, the cultures had a life span of about 28 - 35 days and, in a few cases, of up to 50 days before drastic changes in their cell morphology (e.g. large vacuoles) were detectable by *in situ* microscopy and histology (Figure 4-5).

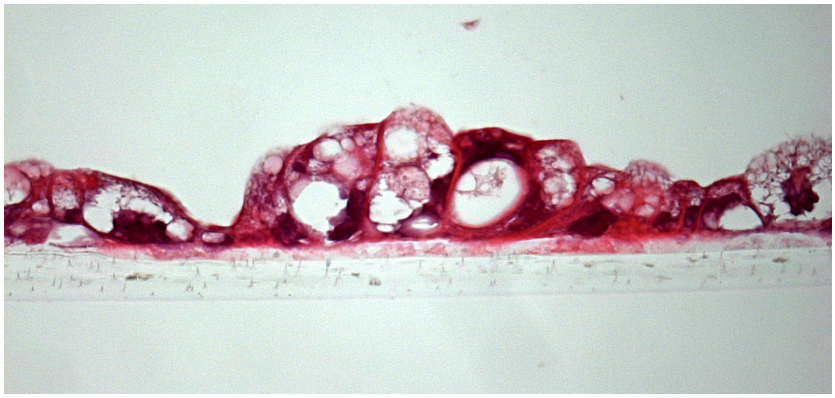

Figure 4-5: Morphological changes of Calu-3 cells grown on microporous membranes (collagen I coated; 0.4 µm pores) after a total cultivation time of 63 days (56 days under ALI conditions) with basic differentiation medium (HE stained histological section; magnification: 630x).

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The secretion of the main airway mucins MUC5AC and MUC5B was verified in Calu-3 cultures by immunohistochemical analyses, although positive signals were sparse (Figure 4-6). However, the secreted products are normally distributed on top of the cell layer

and are mostly washed away during the fixation and dehydration of the cultures prior to the paraffin embedding. Therefore, it is likely that the secretion of MUC5AC and MUC5B was much higher than the immunohistochemical staining suggested.

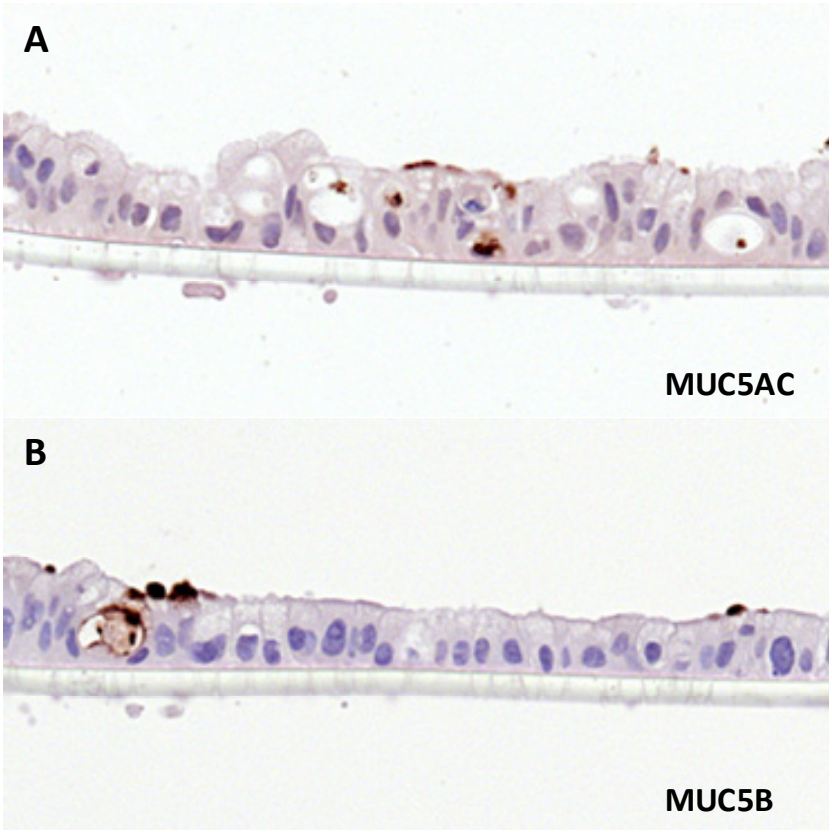

Figure 4-6: Immunohistochemical staining of the mucins 5AC (A) and 5B (B) in a Calu-3 culture cultivated on collagen I coated microporous insert membranes (0.4 µm) at the air-liquid interface with basic differentiation medium after a cultivation time of 27 days (21 days under ALI conditions). The cell nuclei were counterstained with hematoxylin (Magnification: 400x).

Basal cells were lacking in all cultures, which was verified by the negative immunohistochemical staining with the antibody against the tumor protein p63 which is a marker for basal cells in pseudostratified epithelia, like the bronchial epithelium (Figure 4-7) [224].

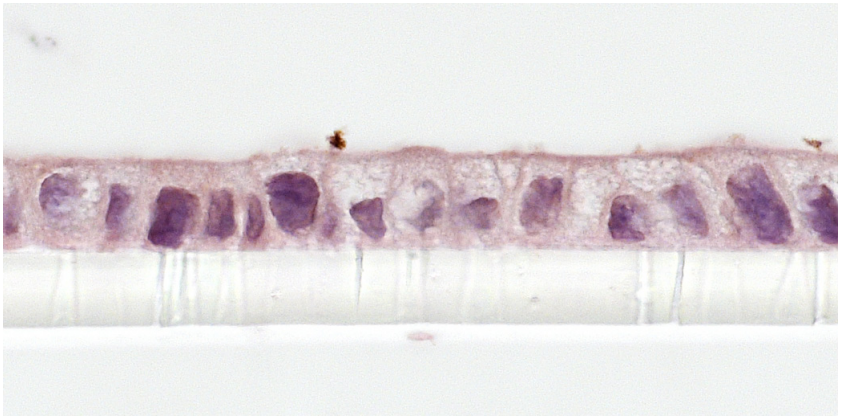

Figure 4-7: Immunohistochemical staining of the basal cell marker p63 in a Calu-3 culture cultivated on collagen I coated microporous insert membranes (0.4 µm) at the air-liquid interface with basic differentiation medium after a cultivation time of 31 days (25 days under ALI conditions). The cell nuclei were counterstained with hematoxylin (Magnification: 1000x).

Cilia-like structures were detected neither *by in situ* microscopy nor in the histological sections. The acetylated α-tubulin which was used as cilia marker in this thesis was strongly expressed throughout the entire cells but not specifically at their apical side, which further indicates a lack of cilia (Figure 4-8). The acetylated α-tubulin is not exclusively expressed in cilia but it is also a component of the microtubules in mammalian cells. In Calu-3 cultures, the expression of this cytoskeletal protein was found to be considerable high.

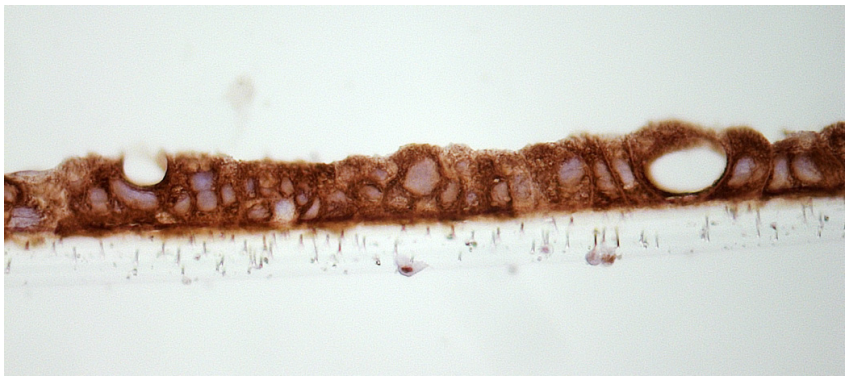

Figure 4-8: Immunohistochemical staining of the cilia marker acetylated α-tubulin in a Calu-3 culture cultivated on collagen I coated microporous insert membranes (0.4 µm) at the air-liquid interface with basic differentiation medium after a cultivation time of 50 days (43 days under ALI conditions). The cell nuclei were counterstained with hematoxylin (Magnification: 630x).

Most remarkably, the variations between different inserts belonging to one cell culture (cultivated under identical conditions), were very low in the case of Calu-3 cells. The growth

pattern, cell morphology, life span, amount of mucus secretion and even the TEER values were highly similar within Calu-3 cultures.

Conclusions

The preliminary characterization of the Calu-3 cell line revealed that these cells might provide a good *in vitro* model of the bronchial epithelium for toxicological studies. Besides their similar morphology, they also showed many properties of differentiated bronchial epithelial cells, like the secretion of the two predominant airway mucins, MUC5AC and MUC5B. Moreover, the Calu-3 cultures were characterized by high TEER values which indicated the formation of functional tight junctions. Their high viability over many passages and the superior cell yield as well as the consistency between the different inserts of one Calu-3 culture and thus, the high reproducibility, recommend these cells for routine testing of substances or high-throughput screening.

However, on average, the TEER values of the Calu-3 cultures exceeded those observed in NHBE cultures (see 4.1.2.4). Therefore, it is likely that Calu-3 cultures might be less sensitive to certain agents and show barrier properties distinct from the airway epithelium *in vivo*. The lack of cilia is another factor that has to be considered when extrapolating the *in vitro* results obtained from toxicological studies with Calu-3 cultures to the *in vivo* situation. Moreover, no basal cells are present in Calu-3 cultures. The implications of the lack of basal cells on the regeneration capacity of the cultures after injury were not analyzed during this thesis.

Nevertheless, due to the numerous beneficial characteristics, the Calu-3 cells were further used in this thesis for the development of 3D co-culture models.

4.1.1.3 16HBE14o-

The 16HBE14o- is a well-established permanent cell line. In contrast to the ChaGo-K1 and Calu-3, the 16HBE14o- is not derived from a tumor but was established from normal human bronchial epithelial cells by immortalization through transformation of the large T antigen of the SV40 virus [(46)].

There is much information available on the optimal cultivation and the differentiation ability of these cells [(225)]. However, the development of certain differentiation characteristics, like

cilia or the mucus secretion, are controversially described in the literature [46, 69, 157, 174]. It is also not clear under which conditions the 16HBE14o- are able to form functional tight junctions [226].

The cells are not publicly available and their use is licensed. For this thesis, the 16HBE14o- cell line was obtained from Professor Gruenert (Cardiovascular Research Institute, University of California, San Francisco) via a Material Transfer Agreement (MTA) for a half-year test period.

The 16HBE14o- cells are grown on surfaces that are coated with a mixture of collagen and fibronectin (see section 6.1.1.4). They were supplied with Minimum Essential Medium (MEM) supplemented with 10 % FBS and cultivated in 75 cm^2 culture flasks. The 16HBE14o- showed a patchy growth similar to Calu-3 cells but with lower density (Figure 4-9). The growth rate was relatively high compared to the Calu-3 cells and therefore the subcultivation could be performed twice a week, resulting in a high cell yield. The cell viability was constant over many passages (at least up to 30 passages after arrival from the University of California).

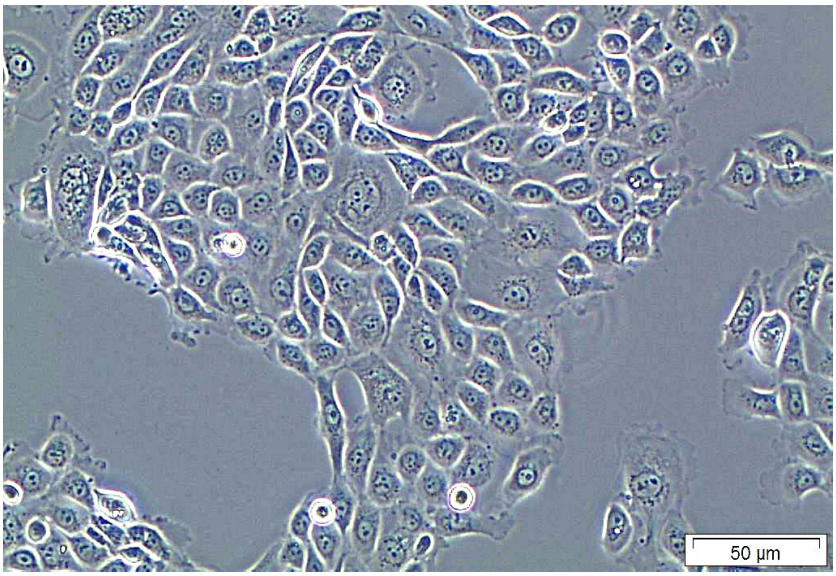

Figure 4-9: Growth pattern of 16HBE14o- cells cultivated in cell culture flasks (75 cm^2) with MEM including 10% FBS

For further characterization, the cells were seeded on collagen/fibronectin coated insert membranes (0.4 µm pores) at a density of 2.5 × 10^5 cells/ cm^2. A confluent cell layer was

observed after approximately three to four days under submerged conditions. The cultures were then cultivated at the air-liquid interface and supplied with basic differentiation medium. Histological sections revealed that, under these conditions, the 16HBE14o- did not form polarized monolayers but grew in a multilayered manner. The cell morphology was distinct from the columnar-shape of normal human bronchial epithelial cells (Figure 4-10).

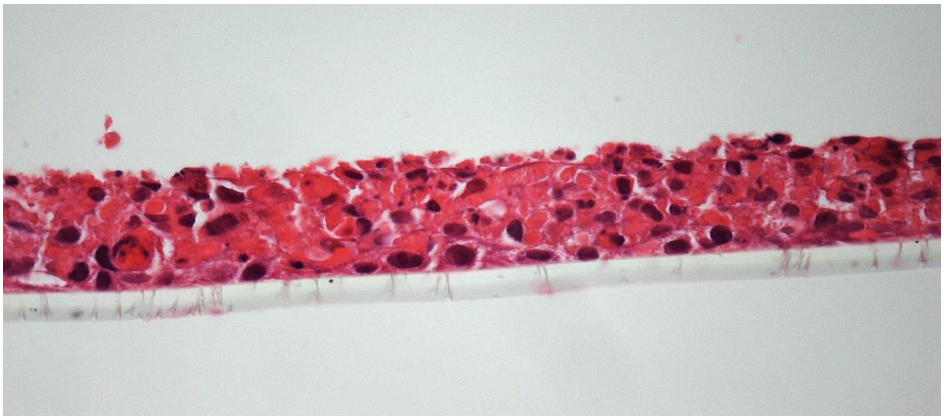

Figure 4-10: Morphology of 16HBE14o- cells cultivated on collagen/fibronectin coated microporous insert membranes (0.4 µm) at the air-liquid interface with basic differentiation medium after a cultivation time of 21 days (14 days under ALI conditions; HE-stained histological section; magnification: 400x).

The 16HBE14o- cultures were further characterized by an extensive secretion of a highly viscous substance whose amount increased with culture age and which often led to a reduced stability of the cultures. The secreted material accumulated between the cells and thus the intercellular contacts were obviously disrupted. In many cases, the upper cell layers became detached from the basal layer while the latter remained attached to the membrane (Figure 4-11).

When the apical surface of the cultures was rinsed with PBS once or twice a week, the secretory material was reduced and the culture stability and life span could thus be increased (Figure 4-12).

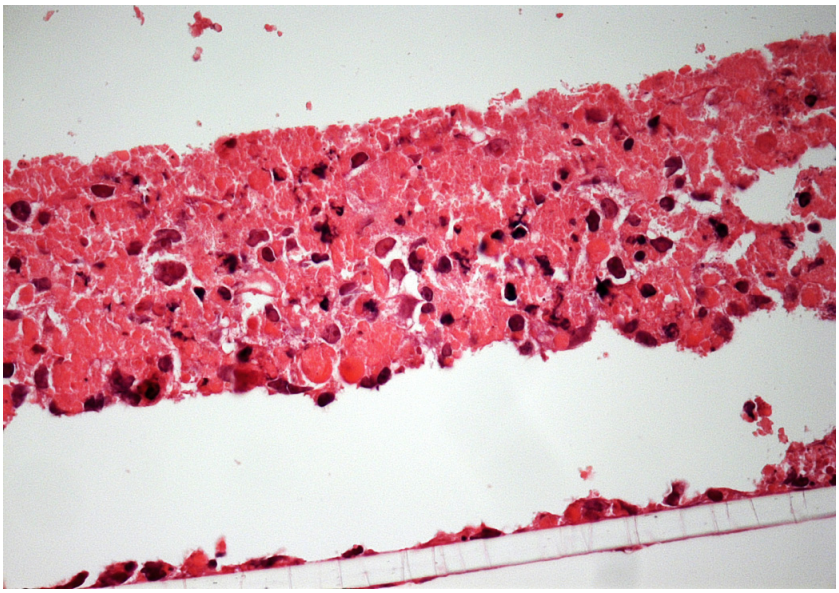

Figure 4-11: Increase of secretory material of 16HBE14o- cells cultivated on collagen/fibronectin coated microporous insert membranes (0.4 µm) at the air-liquid interface with basic differentiation medium after a cultivation time of 31 days (24 days under ALI conditions; HE-stained histological section; magnification: 400x).

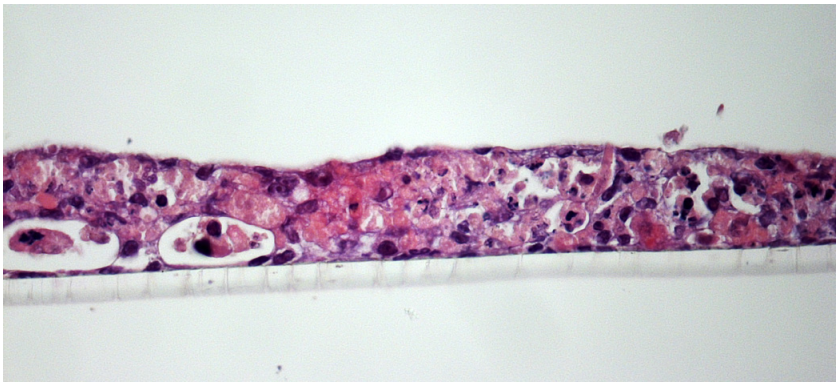

Figure 4-12: Increased stability and life span of 16HBE14o- cultures after rinsing with PBS once or twice a week. The cultures were cultivated on collagen/fibronectin coated microporous insert membranes (0.4 µm) at the air-liquid interface with basic differentiation medium after a cultivation time of 56 days (50 days under ALI conditions; HE stained histological section; magnification: 400x).

Interestingly, although a large amount of secreted substances was detected in all 16HBE14o- cultures, immunohistochemical analyses revealed a sparse staining for MUC5AC and a lack of MUC5B, indicating that the main component of the secretory product of 16HBE14o- cells is not represented by these two prominent airway mucins (Figure 4-13).

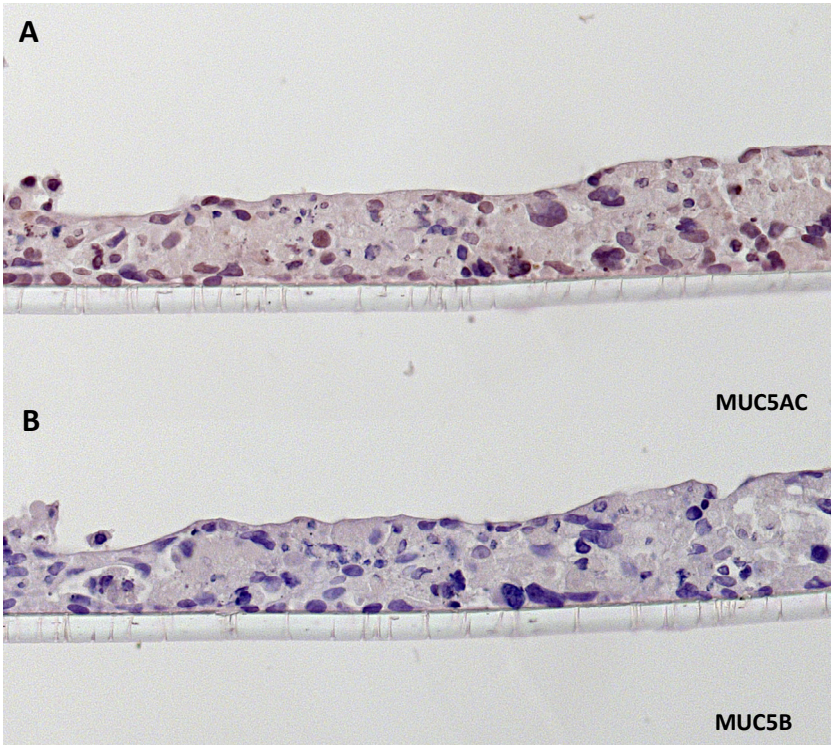

Figure 4-13: Immunohistochemical staining of the major secreted airway mucins MUC5AC (A) and MUC5B (B) in a 16HBE14o- culture cultivated on collagen/fibronectin coated microporous insert membranes (0.4 µm) at the air-liquid interface with basic differentiation medium after a cultivation time of 56 days (51 days under ALI conditions). The cell nuclei were counterstained with hematoxylin (Magnification: 400x).

The secreted material was also found to be barely soluble in PBS or water, which is not typical for secreted mucins of the airway mucus. *In vivo*, the mucus layer of the airways is viscoelastic and has a water content of about 95% in which the other components are dissolved (see section 3.4.2.1). The presence of large amounts of highly viscous and water-insoluble substances, like mucin 2 (MUC2), would greatly impair the mucociliary clearance [(227)]. No further characterization of the secreted products of 16HBE14o- cells was performed in this work. However, it is likely that this secreted material severely limits the suitability of this cell line for toxicological studies since it might act as a protection shield and thus presumably reduces the sensitivity of the cells.

Although the 16HBE14o- cells were initially described to be able to develop cilia [(46)], a recent study refuted this assumption [(174)]. During this thesis, cilia were detected neither by *in situ* microscopy nor by immunohistochemical analyses of the acetylated α-tubulin marker (Figure 4-14).

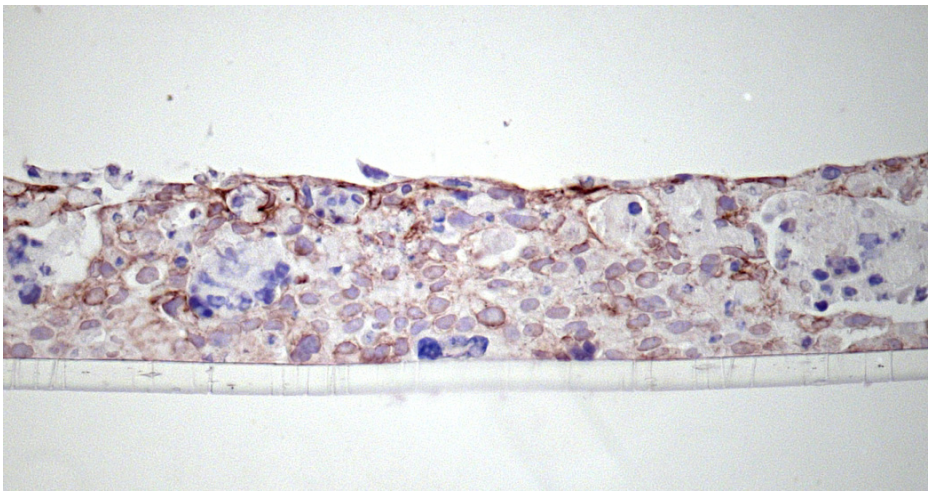

Figure 4-14: Immunohistochemical staining of the cilia marker acetylated α-tubulin in a 16HBE14O- culture cultivated on collagen/fibronectin coated microporous insert membranes (0.4 µm) at the air-liquid interface with basic differentiation medium after a cultivation time of 56 days (51 days under ALI conditions). The cell nuclei were counterstained with hematoxylin (Magnification: 400x).

In the differentiated bronchial epithelium, the proliferative activity is restricted to the basal cells whereas the ciliated and secretory cells rest in a quiescent state. Immunofluorescence staining of the proliferation marker Ki-67 on histological sections of a 16HBE14o- culture after a cultivation time of 37 days revealed that proliferating cells are spread over all layers of the multilayered culture, which further reflects the unordered growth and a lack of functional structuring within the cultures.

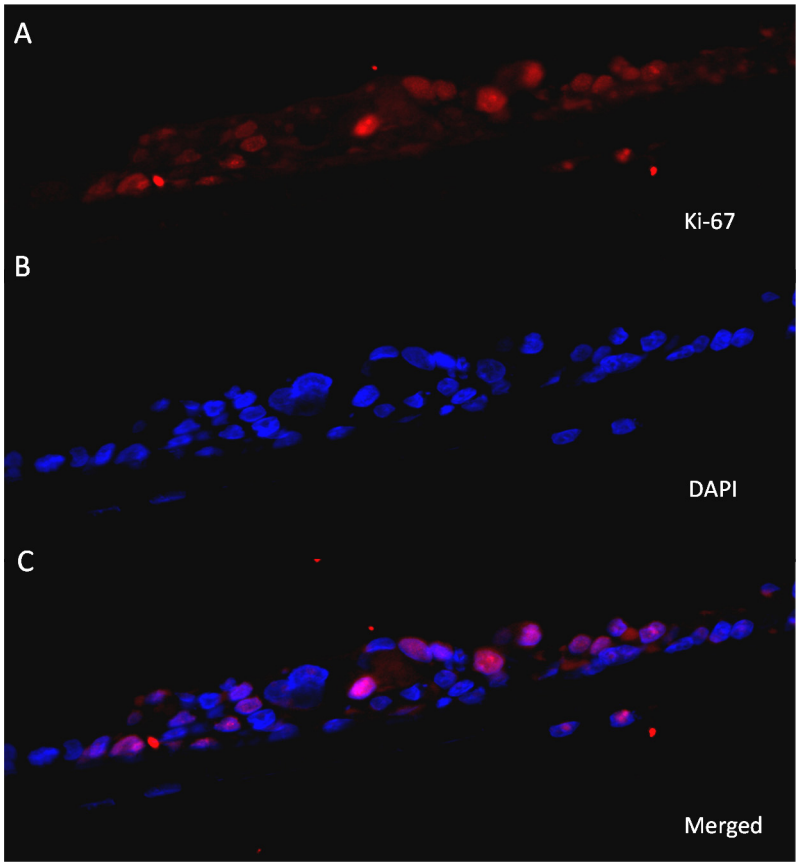

Figure 4-15: Immunofluorescence analyses of the proliferation marker Ki-67 on a histological section of a 16HBE14o- culture cultivated on collagen/fibronectin coated microporous insert membranes (0.4 µm) at the air-liquid interface with basic differentiation medium after a cultivation time of 37 days (32 days under ALI conditions). The Ki-67 staining was visualized through a TRITC-labelled secondary antibody (red; A). The cell nuclei were stained with DAPI (blue; B). Signals of both stainings were merged to obtain an overview of the ratio of proliferating, Ki-67 positive cells (purple; C) to the total number of cells.

The ability of the 16HBE14o- cells to form functional tight junctions is inconsistently described in the literature. Erhardt et al. (2002) suggested that the development of TJs in these cells might depend on the cultivation conditions. According to this study, 16HBE14o- cells only develop TJs under submerged conditions, but not when cultivated at the air-liquid interface [(226)]. To verify this assumption in this thesis, the TEER value of six 16HBE14o- insert cultures were frequently monitored over a time period of 18 days. This was enabled by the use of the Millicell ERS-2 Volt-Ohm-meter (Millipore) which provides a noninvasive technique for the measurement of TEER values (see section 6.1.5.4). For the cultivation of the test cultures, the regular cultivation cycle was applied, meaning that the cells were grown under submerged conditions with their cell-specific growth medium (MEM incl. 10 % FBS) until they formed a confluent layer, which was reached after eight days. Starting from

day nine, the cultures were cultivated at the air-liquid interface. To further study the influence of the medium composition on the formation of TJs, the growth medium was not replaced by the basic differentiation medium until the cells were cultivated for three days under ALI conditions. Accordingly, the cell cultures were supplied with the growth medium during the first three days under ALI conditions. Three of the six insert cultures were not adapted to the differentiation medium afterwards, but were treated with 200 µl of a 0.5 % Triton X-100 solution for 10 min at room temperature. Triton X-100 is known to rapidly induce apoptosis. The TEER values of these cultures were determined 2 h after the addition of Triton X-100 and they were used as blank values.

The results revealed that the TEER values in all six insert cultures were highest between days seven and eight. At this time point, the cells nearly reached 100 % confluency and were still cultivated under submerged conditions. The highest TEER values were between 580 and 800 Ω/ cm^2 which is similar to those observed in NHBE cells (see 4.1.2.4). However, as soon as the cultures were adapted to the ALI conditions, the TEER values dropped immediately to low values around 100 Ω/ cm^2 which can be regarded as no transepithelial electrical resistance. The adaptation to the differentiation medium at day eleven had no effect on the TEER values (Figure 4-16).

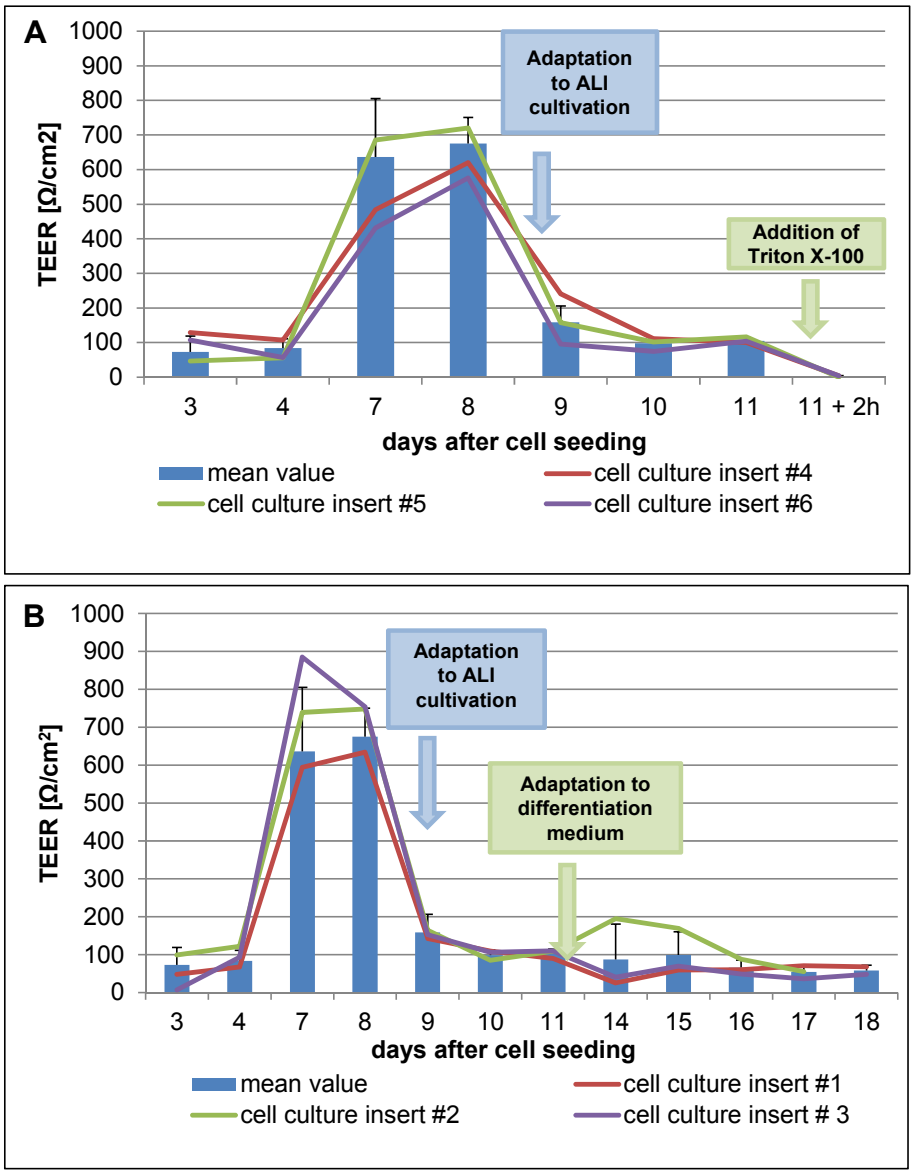

Figure 4-16: Analysis of tight junction formation in six 16HBE14o- insert cultures by monitoring the TEER values over a culture period of 11 (A) and 18 days (B). The cells were cultivated under submerged conditions with cell-specific growth medium (MEM + 10 % FBS) until they formed a confluent layer after 8 days. Starting from day nine, the cultures were cultivated at the air-liquid interface but still supplied with the growth medium until day 11; A: At day 11, three 16HBE14o- insert cultures were treated with Triton X-100 to induce apoptosis. The TEER values were determined 2 h after the addition of Triton X-100 and these values were considered as blank values. B: The other three insert cultures were adapted to the basic differentiation medium (BEBM/ DMEM (1:1) including SingleQuots® (w/o retinoic acid), 1 mM $CaCl_2$, 2 % Ultroser® G and 100 nM retinoic acid) at day 11 and monitoring of the TEER was continued until day 18.

This experiment clearly demonstrated that the 16HBE14o- cells are able to form functional tight junctions but only when cultivated under submerged conditions. This type of cultivation, however, prevents the cell differentiation of airway epithelial cells.

Conclusions

Although the 16HBE14o- cell line has been widely used for studying aspects of drug absorption, ion transport and toxicological effects of substances in airway epithelial cells, the data presented does not support its suitability to serve as a realistic *in vitro* model of the airway epithelium. The stratified growth pattern as well as the lack of TJs under ALI conditions does not reflect the integrity of the airway epithelial barrier *in vivo*. Moreover, the highly viscous secreted material with its large amount of water-insoluble components does not resemble the properties of the airway mucus and might provide a protection shield for the 16HBE14o- which possibly reduces the sensitivity of these cells to certain agents and stimuli. Moreover, the restricted use and high licensing costs do not recommend this cell line as a universal *in vitro* model for toxicology.

4.1.1.4 Uncharacterized immortalized clonal cell lines

In the next step, a set of nine immortalized bronchial epithelial cell lines was analyzed with special regard to the cell viability over many passages and the ability to develop natural differentiation characteristics. These clonal cell lines have been immortalized by the German biotechnology company Sirion Biotech GmbH in Munich (Germany) in order to test the efficiency of the respective technique for the generation of permanent cell lines.

The company specializes in the genetic modification of mammalian cells, including the establishment of immortalized cell lines. Sirion Biotech recently transfected NHBE cells with a lentiviral vector expressing the Human papillomavirus oncogene E6/E7 (HPV E6/E7). Transformation with this oncogene was often successfully applied to immortalize human keratinocytes [(228)].

The immortalized cell lines derived from NHBE cells were established by Sirion as follows:

The original NHBE cells were obtained from Lonza as frozen stocks in passage 2. Two different lots were used for immortalization. After thawing, the cells underwent two additional subculture cycles before the transfection was performed. After the transfection, the lentivirus-transduced cells with effective integration of the transgene were selected for stable gene expression by antibiotic selection using G418 (Geniticin). This selection is possible through the presence of the geneticin resistance gene which was included in the

lentiviral vector. The expression of the integrated transgene was further confirmed by qRT-PCR analyses. From this stable cell pool, a single cell cloning was performed by means of the limiting solution method in 96-well plates. The clonality of each cell line was verified using the Clone Select Imager (Genetix). The clonal cell lines were then expanded and characterized for cell viability, morphology and the expression of the Clara cell protein 10 (CC10). The latter analysis was performed due to the fact that CC10 is a specific marker for Clara cells which are the progenitor cells in the small airways and are thus able to differentiate into ciliated as well as secretory cells. Accordingly, the expression of high amounts of CC10 might indicate a Clara cell-specific character of the respective cell line and thus the potential to differentiate into other cell types of the airway epithelium.

As a final step, frozen stocks of each clonal cell line were prepared and stored in liquid nitrogen until use. The ability of these cell lines to develop natural differentiation characteristics, like cilia formation or mucus secretion, was not tested by Sirion. Therefore, during this thesis the cells were characterized with regard to their cell viability and the development of certain differentiation characteristics.

After thawing, the cells were transferred to cell culture flasks with a growth area of 25 cm^2 and cultivated in BEGM (BEBM + SingleQuots®; Lonza) including 40 µg/mL G418). The addition of G418 is essential to specifically expand cells with a stable expression of the transgene. The growth pattern and cell morphology of the immortalized cells was highly similar to normal (non-transformed) bronchial epithelial cells (Figure 4-17). The cells were subcultivated when they reached approximately 70 - 90 % confluency according to the same procedure as the NHBE cells. At least one vial of each cell line was prepared for storage in liquid nitrogen in the next passage after thawing.

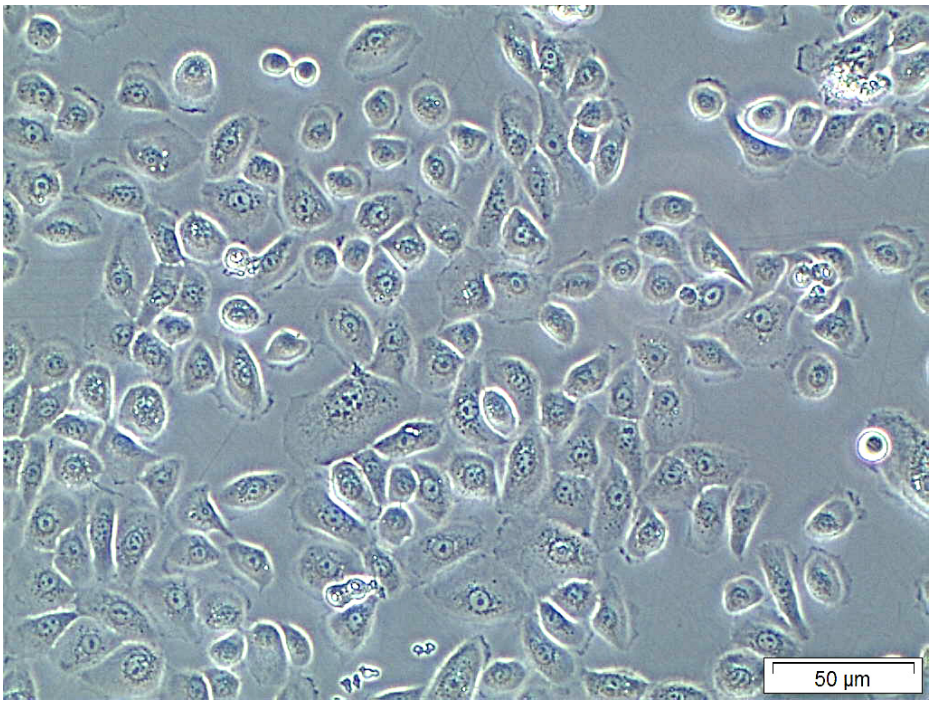

Figure 4-17: Growth pattern of immortalized NHBE cells of clone 1 (7F4167), passage 7, cultivated in cell culture flasks (25 cm^2) with BEGM including G418

The cell clones showed a high variability in their cell viability and proliferation activity. Out of the nine cell lines tested, only five could successfully be cultivated on cell culture inserts. The others did not form a confluent monolayer. Two cell lines (of different donors) showed a high viability over many passages, while the viability of the others greatly decreased with increasing passage number. When grown on cell culture inserts (under identical conditions as NHBE cells, 4.1.2.4), the cells of all five cell clones were lacking many differentiation characteristics of normal bronchial epithelial cells, like the development of cilia or the pseudostratified growth. A similar stratified structure was observed in all cultures but the cell morphology differed between the cell lines (Figure 4-18).

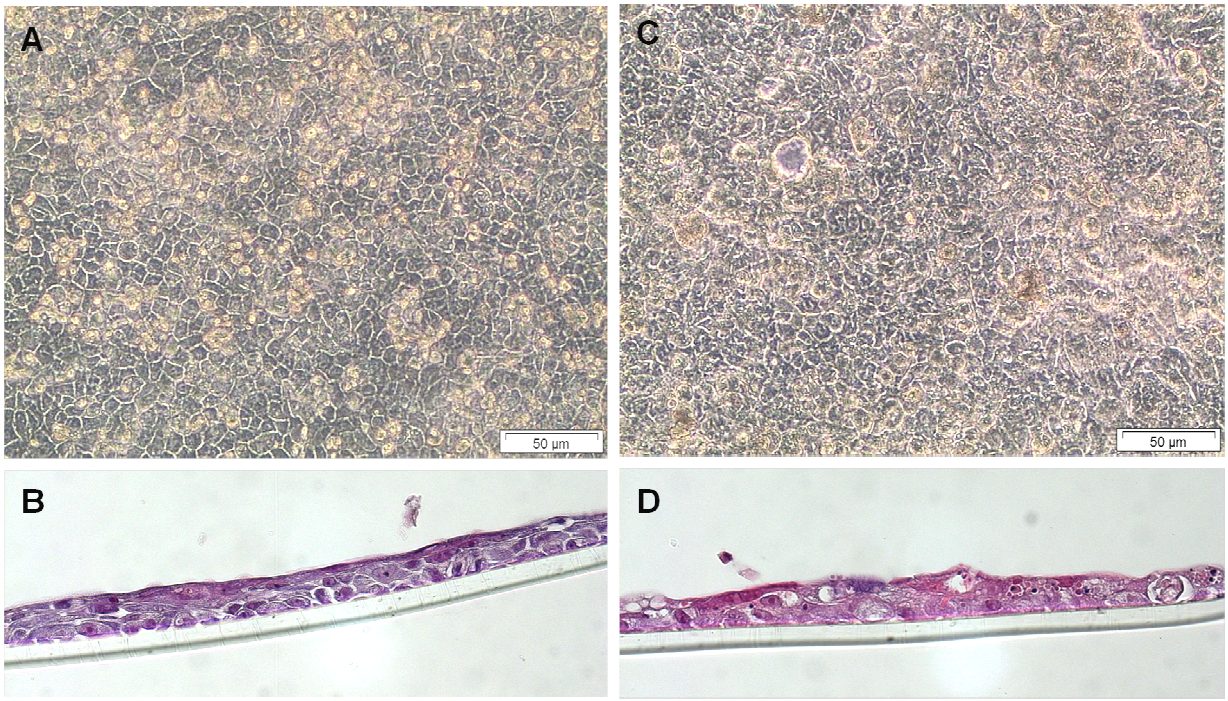

Figure 4-18: Inverse microscopic images (A+C) and HE stained histological sections (B+D) of cultures of two clonal cell lines. The cell morphology of the two clonal cell lines differs while the stratified growth is similar. A+B: Monocultures of clone 1 (P07) after a cultivation time of 11 (A) or 8 days, respectively (B). C+D: Monocultures of clone 13 (P07) after a cultivation time of 12 days (Magnification B+D: 400x)

Secretion was detected in some cultures by inverse microscopy, but no mucin 5AC was verified in immunohistochemical analyses, indicating a lack of goblet cells. All cultures were characterized by a high number of p63-positive cells, but obviously these cells did not function as airway epithelial basal cells since they did not differentiate into ciliated and mucus-producing cells (Figure 4-19).

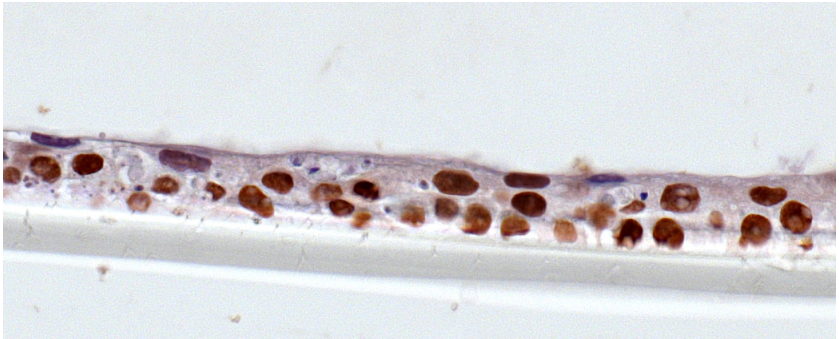

Figure 4-19: Immunohistochemical staining of the basal cell marker p63 in a culture of the clonal cell line 7 (99498) cultivated on collagen IV coated microporous insert membranes (0.4 µm) under ALI conditions with basic differentiation medium after a cultivation time of 29 days (23 days under ALI conditions). The cell nuclei were counterstained with hematoxylin (Magnification: 630x).

The TEER measurement revealed values similar to those observed in NHBE cells, indicating the presence of a barrier function (Table 4-2). However, TEER values of stratified and pseudostratified cultures are hardly comparable.

Table 4-2: TEER values of cultures prepared from different immortalized clonal cell lines dependent on passage number and culture age.

Cell line (donor)	**Passage**	**TEER (Ω/ cm^2)**	**Culture age (days)**
Clone 3 (99498)	7	1020	21
Clone 3 (99498)	9	1209	8
Clone 7 (99498)	7	790	12
Clone 7 (99498)	7	820	12
Clone 23 (7F4167)	7	1010	8
Clone 13 (7F4167)	7	1137	14
Clone 13 (7F4167)	7	1505	19

Conclusions

By testing these immortalized clonal cell lines, the suitability of the immortalization induced by the HPV E6/E7 transduction for human bronchial epithelial cells was evaluated.

The results demonstrated that the technique was successful for immortalizing NHBE cells, since at least two out of nine cell clones showed an increased life span with a high cell viability over at least up to 20 passages. In 2D cultures (cultivated in culture flasks), the cells showed a normal epithelial morphology and a proliferation rate comparable to NHBE cells. The fact that no mucociliary differentiation was achieved under ALI conditions is not necessarily related to the immortalization technique. There are some other possible reasons for the lack of normal differentiation characteristics. For example, as discussed below (4.1.2.4), cryopreservation can potentially cause adverse effects in non-transformed human bronchial epithelial cells. The cells used for the immortalization were obtained as cryopreserved vials from Lonza. Consequently, it is conceivable that these cells had already lost their differentiation ability before immortalization due to the cryopreservation and/or shipping procedure. Moreover, commercial primary cells are generally obtained in passage 2

and after arrival they are further cultivated in cell culture flasks until the next subcultivation. As stated below in section 4.1.2.4, NHBE cells of the first and second passage provide the best source with regard to the differentiation ability.

The data presented here suggest that the immortalization with the HPV E6/E7 oncogene might be a suitable technique for NHBE cells, but the use of freshly isolated NHBE cells of passage 1 is strongly recommended. Moreover, the cells should be tested prior to the immortalization for their ability to develop the most important differentiation characteristics.

4.1.2 Normal human bronchial epithelial cell lines

The characterization of several permanent cell lines, either cancer-derived or immortalized, revealed that they all have distinct advantages and disadvantages. However, all tested cell lines lack certain important characteristics of differentiated normal human bronchial epithelial cells.
Therefore, in the next step, normal (non-transformed) human bronchial epithelial cells were tested for their suitability to serve as appropriate *in vitro* models of the airway epithelium.

4.1.2.1 Commercial normal human bronchial epithelial cells (HBEpC)

The testing of normal human bronchial epithelial cells was initially performed with commercial cells which can be obtained from different providers. Depending on availability, cells from donors of different ages and with different medical backgrounds as well as from smokers or non-smokers can be selected. In this thesis, two different lots of these cells were purchased from Promocell GmbH:

1. Lot 9082701.10, passage 2 (HBEpC908); Donor: Male, 68 years, Caucasian, non-smoker
2. Lot 8121902.18, passage 2 (HBEpC812); Donor: Male, 21 years, Caucasian, smoker

The cells were obtained in proliferating state (not cryopreserved) in culture flasks and the first subcultivation was performed 24 h after arrival. They were then cultivated in cell culture flasks with a growth area of 25 cm^2 and supplied with the recommended serum- and

antibiotic-free culture medium provided by Promocell (Airway Epithelial Cell Growth medium).

In order to guarantee the availability of these cells for future studies, a comprehensive cell pool of both lots of the passages three to five was prepared for storage in liquid nitrogen. Due to the limited number of cells, no experiments were performed with fresh cells before cryopreservation.

For evaluation of the cell viability after cryopreservation, one vial each of frozen cells of passage three was thawed and subcultivated over several passages in 25 cm^2 cell culture flasks until they became senescent. The cell seeding density of each passage was 3.5 × 10^5/ 25 cm^2. The number of viable cells was determined with the CASY Cell Counter and Analyzer System (see 6.1.1.2).

After two subcultivation cycles, it became obvious that the two lots differed in their growth rates when cultivated in cell culture flasks. The cells of the lot 9082701.10 (HBEpC908) showed a higher viability and reached confluency earlier than the HBEpC812. The HBEpC908 showed no decrease in cell viability until passage nine. But in passage ten, the growth rate and cell viability was considerably reduced and morphological alterations were observed (Figure 4-20). The majority of cells showed an increased size and some cells exhibited long extensions indicating the beginning of the senescent state (Figure 4-21).

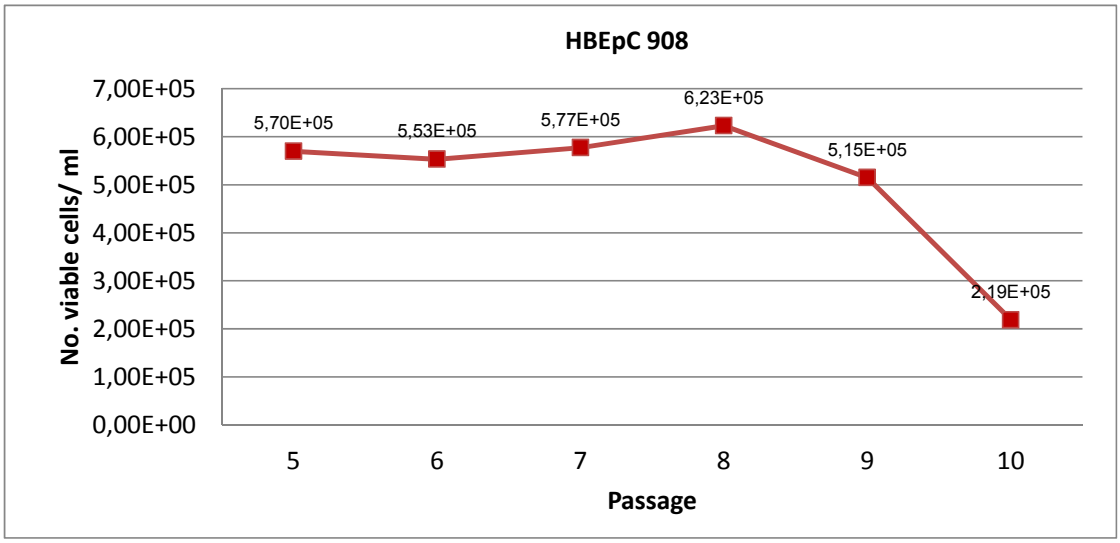

Figure 4-20: Test of cell viability of HBEpC908 in different passages. The cells were cryopreserved in passage 5 and, after thawing, subcultivated until passage 10. The cells were cultivated in 25 cm^2 cell culture flasks at a seeding density of 3.5 × 10^5/ 25 cm^2. Subcultivation was performed every 7 days and the number of viable cells was determined using the CASY Cell Counter + Analyzer System.

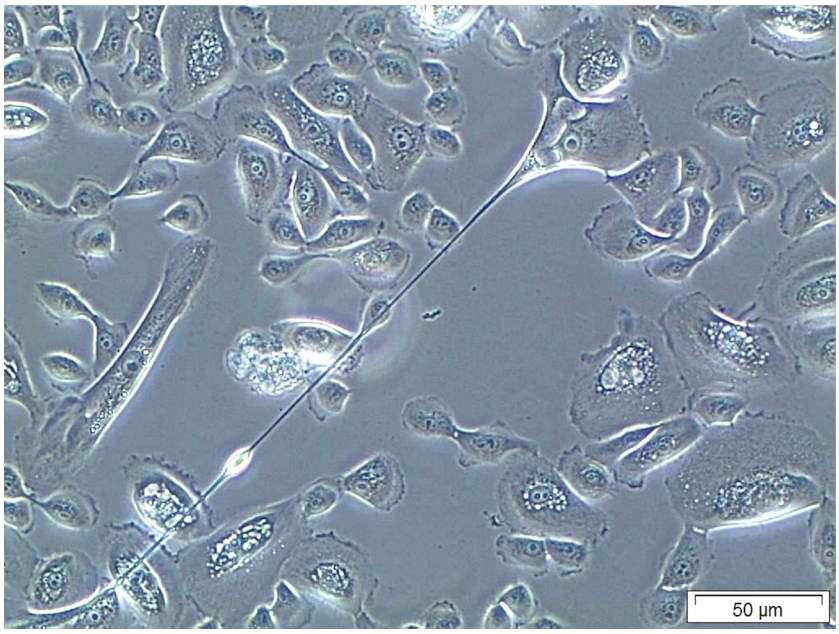

Figure 4-21: Altered morphology of HBEpC908 cells of passage 10, cultivated in a 25cm^2 culture flask and supplied with Airway Epithelial Cell Growth Medium (Promocell). The cells were frozen in liquid nitrogen in passage three, thawed after several weeks and subcultivated for another 7 passages.

For further characterization, the cells of both lots were seeded on collagen type IV- coated insert membranes (0.4 µm pores). For that purpose, cells of the passage three were used. The cell seeding density was found to be a critical factor for HBEp cells in order to form a confluent layer or a natural pseudostratified structure. The optimal seeding density was determined by trial and error and was found to be around 1.0 - 1.5 × 10^5/ cm^2. It was important that the cells reached confluency of nearly 100 % before starting the ALI cultivation, since the life span of the cultures was otherwise dramatically reduced and no cell differentiation was achieved. Similar negative effects were observed when the growth phase under submerged conditions was too long (beyond 100 % confluency). In the latter case, the cells already started to detach and a confluent layer was thus not achieved.

Due to the fact that the recommended growth medium of these cells is serum-free, the omission of the serum substitute Ultroser® G, which was normally added to the basic differentiation medium, was initially tested. Under these conditions, the cells of both lots showed a stratified growth and an undifferentiated squamous morphology (Figure 4-22).

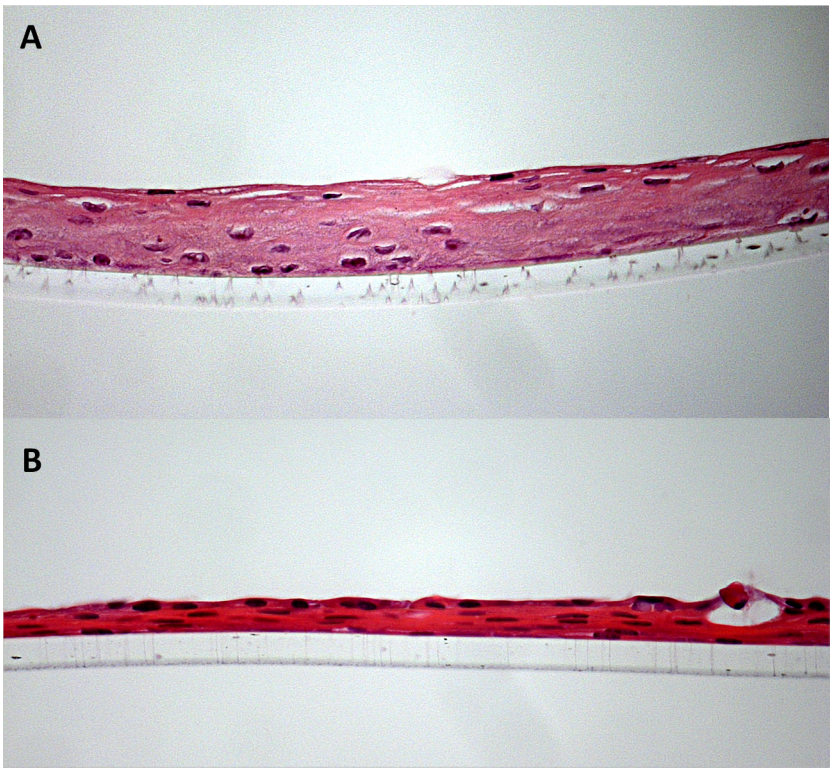

Figure 4-22:Growth pattern and cell morphology of HBEpC812 (A) and HBEpC901 (B) cultures cultivated on collagen IV coated microporous membranes (0.4 µm pores) at the air-liquid interface with basic differentiation medium without Ultroser® G after a cultivation time of 13 (A) or 28 (B) days. (HE stained histological sections; magnification: 400x).

Immunohistochemical analyses with an antibody against cytokeratin 6 (CK 6) revealed an extremely strong signal in every cell throughout the cultures (Figure 4-23). CK6 is a marker for squamous cells and also strongly expressed in basal cells but not expressed in differentiated columnar cells of the pseudostratified airway epithelium [229].

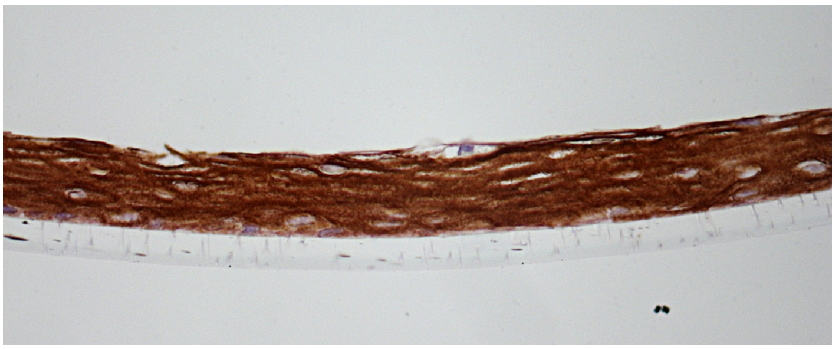

Figure 4-23: Immunohistochemical staining of the squamous cell marker cytokeratin 6 (CK6) in a HBEpC812 culture cultivated on collagen IV coated microporous insert membranes (0.4 µm) at the air-liquid interface with basic differentiation medium without Ultroser® G after a cultivation time of 13 days (9 days under ALI conditions). The cell nuclei were counterstained with hematoxylin (Magnification: 400x).

Since none of the cultures showed natural differentiation characteristics under these initial conditions, in the next step Ultroser® G was added to the differentiation medium with a final concentration of 2 %. The addition of this serum substitution agent was shown to greatly enhance the differentiation capacity of the HBEpC908 cells. Histological sections revealed a pseudostratified structure with columnar-shaped cells in some of the HBEpC908 cultures (Figure 4-24). Moreover, by means of immunohistochemical analyses the presence of basal cells was detected (Figure 4-25).

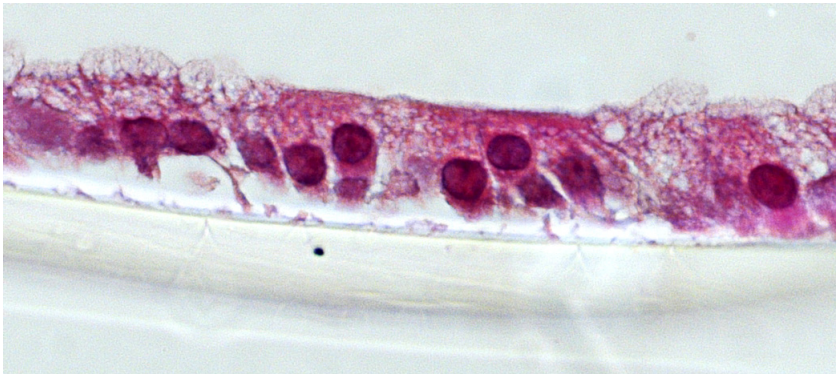

Figure 4-24: Pseudostratified structure of a HBEpC901 culture cultivated on collagen IV coated microporous membranes (0.4 µm pores) at the air-liquid interface with basic differentiation medium including 2 % Ultroser® G after a cultivation time of 54 days. (49 days under ALI conditions; HE stained histological sections; magnification: 1000x).

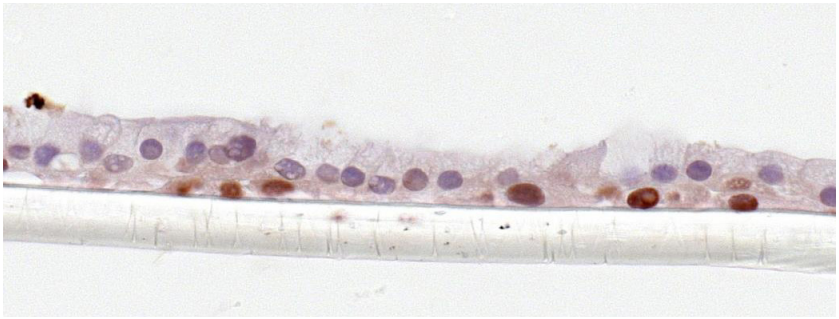

Figure 4-25: Immunohistochemical staining of the basal cell marker p63 in a HBEpC908 culture cultivated on collagen IV coated microporous insert membranes (0.4 µm) at the air-liquid interface with basic differentiation medium including 2 % Ultroser® G after a cultivation time of 54 days (49 days under ALI conditions). The cell nuclei were counterstained with hematoxylin (Magnification: 400x).

By *in situ* microscopy, strong cilia beating and secretion activity were furthermore observed in these HBEpC908 cultures after a cultivation time of approximately 28 days. The presence of cilia was verified with the acetylated α-tubulin marker in immunohistochemical analyses (Figure 4-27). Moreover, the secretion of MUC5AC was also verified and the

immunohistochemical staining of this marker revealed that the MUC5AC positive cells shared the characteristic morphology of the secretory goblet cells of the bronchial epithelium (Figure 4-27).

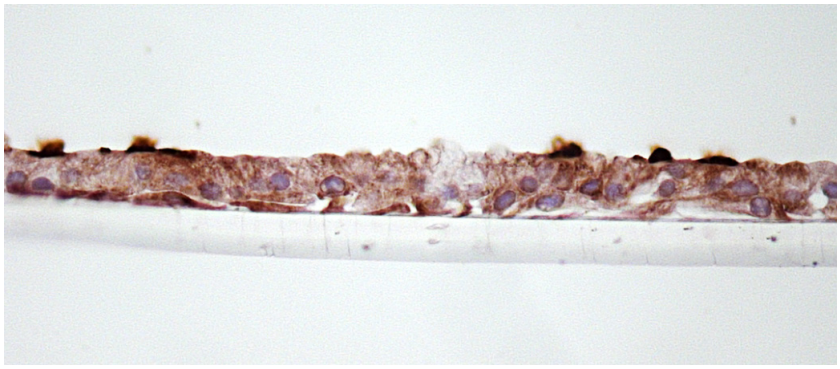

Figure 4-26: Immunohistochemical staining of acetylated α-tubulin in a HBEpC908 culture cultivated on collagen IV coated microporous insert membranes (0.4 µm) at the air-liquid interface with basic differentiation medium including 2 % Ultroser® G after a cultivation time of 54 days (49 days under ALI conditions). The cell nuclei were counterstained with hematoxylin (Magnification: 400x).

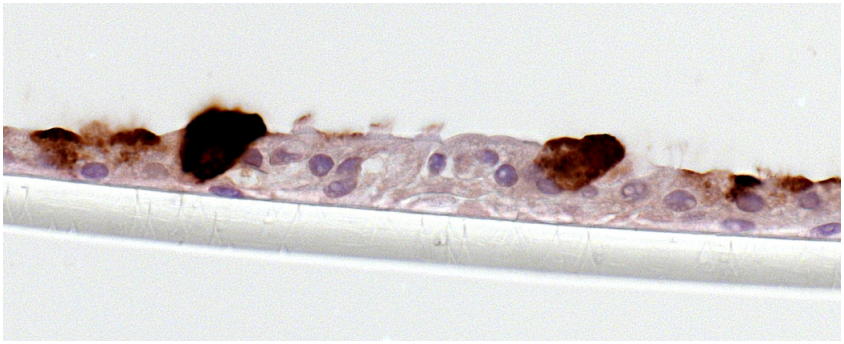

Figure 4-27: Immunohistochemical staining of the mucin 5AC in a HBEpC908 culture cultivated on collagen IV coated microporous insert membranes (0.4 µm) at the air-liquid interface with basic differentiation medium including 2 % Ultroser® G after a cultivation time of 54 days (49 days under ALI conditions). The cell nuclei were counterstained with hematoxylin (Magnification: 630x).

However, above mentioned differentiation properties were only observed in a few inserts of the HBEpC908 cultures while others showed a similar flattened morphology and stratified growth as the cultures that were cultivated without Ultroser® G. A high variability was even observed between different inserts of the same cultures (cultivated under identical conditions in the same receiver plate) ranging from well-differentiated to completely undifferentiated cultures. The differences in the phenotypic characteristics correlated with the TEER values. While the well-differentiated cultures had TEER values between 600 and 1000 Ω/ cm^2, the values determined for the stratified and undifferentiated cultures were

considerably lower (300 – 400 Ω/ cm^2). The TEER values of the differentiated cultures were comparable to those that were found in other studies in NHBE cultures (700 – 1200 Ω/ cm^2) in which functional barrier properties were verified [230-231].

Interestingly, the differentiation was not achieved in any culture when using HBEpC908 beyond the passage four, although no decrease in the cell viability was detectable until passage nine.

The HBEpC812 cells did not show natural differentiation characteristics of bronchial epithelial cells, under none of the tested cultivation conditions.

Conclusions

In general, it was shown that isolated normal human bronchial epithelial cells may retain their ability to develop a phenotype similar to their *in vivo* counterparts and thus provide the most realistic *in vitro* model. The differentiated cell population showed a pseudostratified structure and included ciliated, goblet (secretion of MUC5AC and MUC5B) and basal cells. Moreover, the cell layer formed functional tight junctions with a normal TEER. However, the formation of these properties was strongly depended on appropriate culture conditions and the HBEp cells were shown to be highly sensitive to small deviations from these adjusted conditions.

One out of two tested lots was even shown to be completely unsuited as *in vitro* model due to a lack of the capability to differentiate into a pseudostratified cell layer with mucus producing and ciliated cells, indicating that lot- or donor-specific variations in the differentiation patterns are likely to occur.

In summary, the data suggest that the differentiation of non-transformed bronchial epithelial cells presumably depends on a variety of factors and conditions. These decisive factors might include the handling of the cells after isolation but also the medical and personal background of the respective donor.

Later in this thesis, it is shown that cryopreservation is another critical factor for the differentiation ability of normal human bronchial epithelial cells. Moreover, only cells of the passages one and two were found to be highly suitable for representing realistic *in vitro* models, in general, since in higher passages the ability to develop differentiation characteristics drastically decreased and was greatly varying between different lots.

Considering these crucial factors, the use of commercial normal human airway epithelial cells is problematic in many respects:

1. First, the cells are generally provided in passage two by the supplier. Thus, experiments can only be started at least with cells of passage three. The ability of these commercial cells to develop realistic features after their arrival in the laboratory is therefore questionable and presumably highly lot-specific.
2. Second, commercial cells are mostly shipped as frozen cell stocks. Freezing and thawing of cells generally bears the risk of damage. Moreover, temperature shifts are critical for frozen cells and might lead to a severe decrease in the cell viability after thawing. Therefore the shipping of frozen cells is another risk factor.
3. Third, to ensure the availability of cells of the same lot for later experiments, frozen cell stocks have to be prepared. Here again, only cells of passage three or higher can be used. After thawing, they have to undergo another subcultivation cycle before they can finally be employed for the respective studies. Thus, they will probably not be able to develop realistic differentiation characteristics at this stage.

4.1.2.2 Isolation of bronchial epithelial cells from normal human lung tissue

In order to overcome the limitations of commercial normal bronchial epithelial cells and to ensure their availability in sufficient quantity, a protocol for the isolation of cells from human lung tissue was developed.

The lung tissue was frequently provided by the Thoracic Surgery Department (KRH Klinikum Oststadt) from cancer patients that underwent a lobectomy. The study was notified to the Ethics Commission by the hospital and tissue samples were only taken from patients that have signed an agreement form. Extraction of the bronchus pieces from the surgical tissue was usually performed within the first hour after the surgery. The samples were taken from areas distant from the carcinoma. The age of the donors varied between 50 and 84. Two to five samples were taken from each donor, comprising bronchi of different generations. The bronchi had thus varying diameters between approximately 3 – 11 mm. The length of the isolated pieces was between 5 and 10 mm. The samples were stored in ice cold PBS and placed on ice for transportation.

After arrival at the laboratory, the samples were washed twice with PBS and, in some cases, one of the three pieces was subsequently stored in 4 % formaldehyde for fixation at 4 °C for 12 – 14 days. This reference sample was used for the generation of histological sections while the other pieces were prepared for the immediate cell isolation or for storage in liquid nitrogen for later cell isolation. The isolation procedure was basically adopted from Bals and colleagues with some modifications [232]. First, the bronchi were isolated from the residual surrounding tissue and opened longitudinally by means of a scalpel. The samples that were intended for storage in liquid nitrogen were cut into smaller pieces of about 5 mm x 5 mm. All samples were subsequently placed into plastic tubes each containing 25 - 30 mL of the incubation medium (approximately 3 - 4 pieces per tube). The incubation was performed at 4 °C on a rocking platform for 24 h. The incubation medium contained several antibiotics that are effective against the most common bacteria in the human lung and an antifungal agent. It was additionally supplemented with DNAse I to digest the nucleic acid that leaks from damaged cells and DTT (dithiothreitol) that helps to solubilize proteins. Both agents prevent an increase of viscosity in the medium during the incubation.

After this first incubation step, the sample pieces that were intended for the storage in liquid nitrogen were transferred to cryogenic vials containing 1.8 mL of the cold Recovery™ Cell Culture Freezing Medium (Life Technologies) or homemade freezing medium (see section 6.1.1.2) and incubated in the “Mr. Frosty” Freezing container (Nalgene) at –80 °C for approximately 24 h before they were transferred to the liquid nitrogen tank. The cryogenic vials and the freezing container were pre-cooled at 4 °C for 1 h before use.

For thawing of the tissue, the cryogenic vials were dipped into a 37 °C water bath until the majority of the Freezing Medium was unfrozen. The medium and the frozen tissue piece were transferred to a Petri dish containing PBS (pre-warmed at room temperature) and washed twice with fresh PBS.

The next steps were either performed with fresh tissue or with tissue pieces that were recovered from liquid nitrogen storage. The specimen was incubated in dissociation medium for another 24 h at 4 °C on a rocket platform. The dissociation medium had the same composition as the incubation medium but was further supplemented with protease XIV, an extremely non-specific proteolytic enzyme that can be used for the dissociation of various tissues. The exact compositions of the used media are described in detail in section 6.1.1.1.

Following the incubation steps, the tissue samples and the dissociation medium were transferred to Petri dishes with a diameter of 9 cm. The bronchus pieces were fixed with forceps at one end while their luminal surface was scraped with a scalpel to remove the epithelial cells from the tissue underneath. The scalpel was rinsed afterwards in the dissociation medium to release the cells into the Petri dish. The cell suspension was subsequently centrifuged for 10 min at 170 x g and the supernatant was discarded. The cell pellet was resuspended in 5 – 10 mL BEGM (Lonza), depending on the size of the tissue sample and the expected cell yield.

After the isolation of epithelial cells, the tissue pieces were further used for the isolation of fibroblasts (see 4.1.3.2).

The suspension containing the isolated epithelial cells was centrifuged again for a few seconds at 310 x g (centrifugation stopped when the speed of 310 x g was reached) to separate the cells from larger tissue fibers. The supernatant (a small residual volume was left in the tube) was then transferred to the collagen/fibronectin coated wells of a 6-well plate. The total volume of the cell suspension per well was between 3 and 4 mL (depending on the approximate number of cells). The final yield of NHBE cells was strongly dependent on the viability of the cells in the tissue sample and was highly variable between the different specimens. The amount of cells showing a strong cilia beating was found to be a reliable measure for the cell viability and predictive for the cell yield. In the case of a high viability, numerous ciliated cells, which were detected through their fast movement, were detectable with an inverted microscope directly after the isolation. The cilia beating of isolated cells usually decreased within a few min and the cells lost their columnar shape and became roundish (Figure 4-28). Cells in cell aggregates retained a strong cilia beating and their columnar shape generally longer- for up to 48 h.

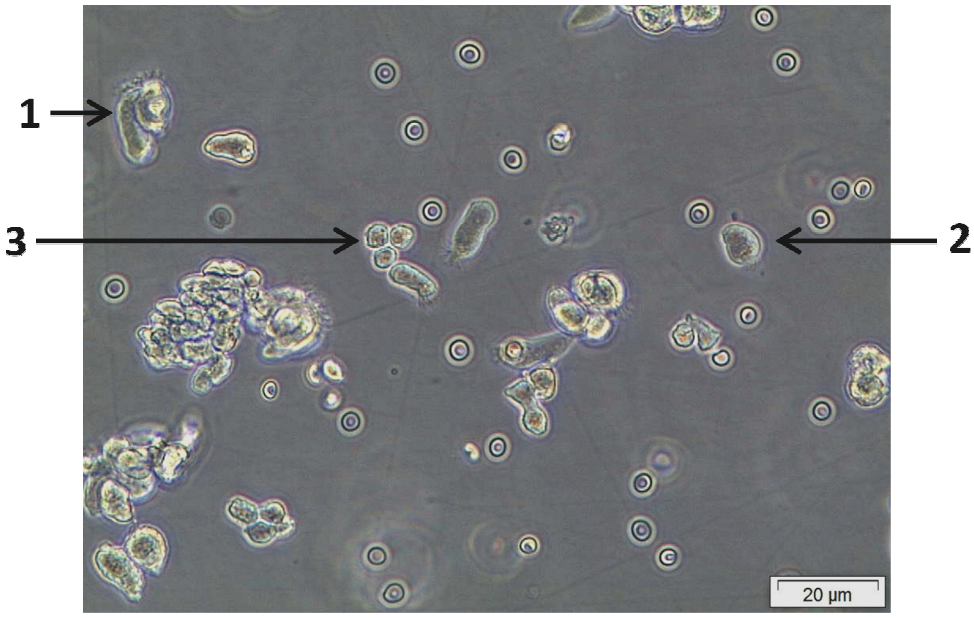

Figure 4-28: Normal human bronchial epithelial cells, resuspended in BEGM, approx. 15 min after isolation from fresh lung tissue. The cells underwent three morphological stages before they adhered to the culture vessel. Cells with strong beating cilia (1) were only detectable within the first 30 min. Afterwards the beating slowed down and the cells became more roundish (2). In the last stage, the cilia were lost and the cells had a round shape (3).

The isolated cells in the wells were supplied with nutrition by partially renewing the medium every other day. Within the first three to four days after isolation, the removed medium was transferred to new collagen/fibronectin-coated wells and all wells were filled up to 3 - 4 mL again with fresh BEGM. With this procedure, the cells were supplied with fresh medium without losing any unattached cells.

Remarkable differences in cell viability, cell yield and time until the cells adhered to the culture vessels were observed between fresh and cryopreserved tissue pieces. When using fresh tissue, the cells usually still formed cell aggregates or were trapped in fibres of the connective tissue after scraping them off the bronchial surface, while only few isolated cells were detectable. The cell viability was mostly very high, meaning that many cells exhibited strong cilia beating. Moreover, the number of epithelial cells was generally also high. However, the most crucial factor for the final yield of NHBE cells was their adherence to the culture vessels. Generally, only isolated cells or cells of small cell aggregates adhered to the culture surface. Larger aggregates, on the other hand, were floating in the medium for many days and did not adhere. The time until the adherence occurred was found to be critical since the early adhering cells usually showed the highest viability and the strongest differentiation ability in later stages while the cells that adhered only after more than eight

to ten days often resulted in NHBE cells with a lower viability or with unusual cell morphology. The adherence of the isolated epithelial cells was further strongly influenced by the level of contamination with erythrocytes (Figure 4-29). The presence of these blood cells generally negatively influenced the attachment of cells due to a lack of space, since the erythrocytes accumulated near the bottom of the wells. The number of isolated erythrocytes highly differed between the tissue samples. However, their number was constantly reduced by transferring parts of the cell suspension from well to well, since these non-nucleated cells are not able to divide.

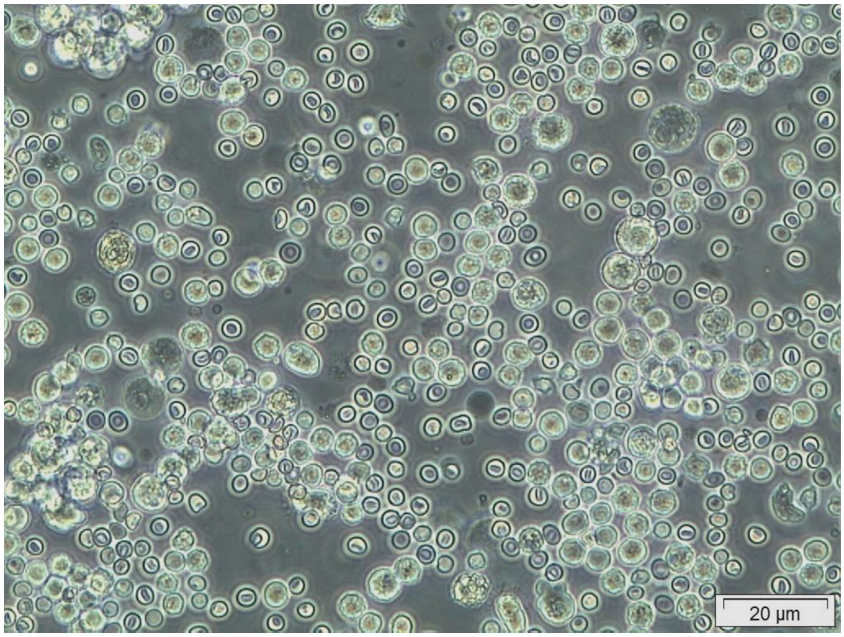

Figure 4-29: Normal human bronchial epithelial cell culture in BEGM, approx. 2 h after isolation from fresh lung tissue. The culture is highly contaminated with erythrocytes (round biconcave disc-shaped cells) accumulating at the bottom of the culture vessel.

When using cryopreserved tissue, the number of bronchial epithelial cells after the isolation procedure was considerably lower, as well as the cell viability, indicated by a highly reduced number of beating cilia. However, in contrast to fresh tissue, the cells were mostly separated from each other and seldomly trapped within tissue fibres or cell aggregates. Moreover, the cell suspension was free from erythrocytes.

An additional dissociation step was mostly performed when fresh tissue samples were used, in order to “rescue” single cells from cell clumps which were floating in the medium. This procedure was adapted from Fulcher *et al.* [201]. For that purpose, within 48 h after the cell

isolation, a large part of the cell culture medium containing most of the cell aggregates was removed from some of the wells and transferred to a centrifugation tube. After centrifugation at 220 x g for 7 min, the BEGM was discarded and the cell pellet was resuspended in 10 mL of the “Rescue Medium”. This medium contained collagenase, a protease that specifically dissociates collagen fibrils. Residual collagen fibrils of the connective tissue and basal lamina might be the cause for the strong cohesions of the isolated cells within the cell clumps. The cell suspension was transferred to a 25 cm^2 cell culture flask and the dissociation was performed at 37 °C and monitored carefully with an inverse micrsocope. The incubation of the cells in the “Rescue Medium” should not exceed 1 h and should be stopped when the dissociation was completed [(201)]. The dissociation was generally stopped after 10 - 20 min by adding 10 % FBS (1 mL). The cell suspension was afterwards centrifuged for 7 min at 220 x g and the cell pellet was resuspended in BEGM. The cells were transferred to an appropriate number of collagen/ fibronectin-coated wells and cultivated as mentioned above. When the cell aggregates were incubated in this special dissociation medium for no longer than 20 min (an incubation of 10 min was mostly sufficient), this procedure was highly efficient to increase the NHBE yield. The cells generally started to adhere shortly after the treatment and within the following 24 h, the majority of cells was attached to the bottom. In contrast, in the wells with “non-rescued” cells, only a minor number of cells adhered and divided. Consequently, the “rescued” cells reached a confluent layer earlier than the “normal” cells and were generally the first to be subcultivated (Figure 4-30). Although dissociation enzymes, such as collagenases, might cause damage to cells, the viability of the NHBE cells which underwent the additional dissociation procedure, (determined by the CASY Cell Analyser System; see 6.1.1.2) was generally comparable to the non-treated cells.

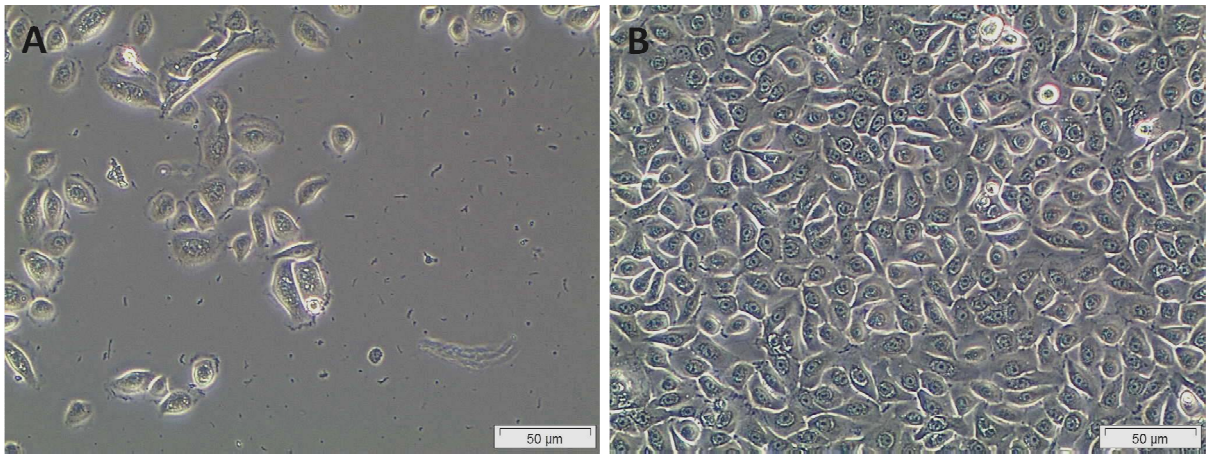

Figure 4-30: Normal human bronchial epithelial cells adhered to collagen/fibronectin coated culture vessels 16 days after isolation. Cells that underwent an additional dissociation step with "rescue medium" (B) formed a confluent layer earlier than the "non-rescued" cells (A) in some cases.

The additional dissociation step was occasionally also performed with cells obtained from frozen tissue, although large cell aggregates were rare. Here it was shown that for some samples, this dissociation step was even essential for obtaining any adhered NHBE cells, since in these few cases, the "normal" procedure did not result in adherent and dividing cells. This observation suggests that the collagenase modifies the cell surface and thus enhances the cell adherence. However, in other cases, the treatment with the rescue medium led to an altered morphology of the cells and a reduced viability.

Besides the "rescue" procedure, a further centrifugation of floating cell aggregates and single cells supported the adherence and increased the cell yield in some samples. Here, the medium of the wells (containing "non-rescued" and non-adhered cells) was centrifuged for 5 min at 500 x g, resuspended in BEGM (the volume was adjusted to the estimated cell density) and transferred to new collagen/fibronectin coated wells. Soon after the centrifugation, single cells started to adhere. After 48 h, even some cell clumps were attached to the bottom and a few cells started to grow out from the aggregates. These outgrowing cells generally differed in their appearance and seemed to be less viable since they carried large vacuoles (Figure 4-31). However, cells with a low viability were mostly lost during the subcultivation procedure and thus did not contribute to the final cell pool. Therefore, the viability of the cells that have undergone the additional centrifugation step was similar to the "normal" (non-centrifuged) cells.

The isolated cells were termed NHBE cells (Normal Human Bronchial Epithelial) and the respective sample number was added to the name of each NHBE lot.

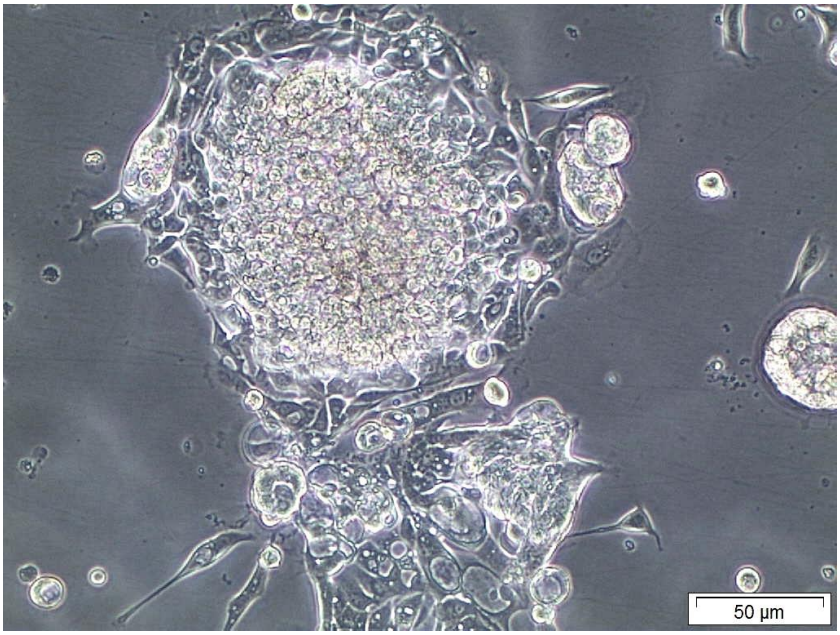

Figure 4-31: An additional centrifugation of the cell suspension of normal human bronchial epithelial cells within approx. 5 days after isolation often resulted in the adherence of large cell aggregates. The cells growing out from these adhered cell aggregates often had an altered morphology

Although the above-mentioned approach was effective to increase the number of adherent cells, the cell yield and viability in general varied strongly between the tissue samples. There are several possible reasons for these differences:

A. Donor-specific variations

   → depending on age, personal and medical background

B. Tissue-specific variations

   → depending on handling and sampling time after surgical removal

C. Medium-specific variations (enzyme/antibiotic activity)

   → depending on date of medium preparation

The latter point could be excluded by only using freshly prepared media, while the other factors could not be influenced and thus, tissue specific differences in the yield and viability of NHBE cells existed.

In summary, the use of cryopreserved tissue generally led to a reduced cell yield compared to fresh tissue. However, it was shown that some fresh samples also resulted in a low cell

yield which was due to tissue specific variations. On the other hand, the NHBE isolation procedure was more straightforward with cryopreserved specimens because interfering erythrocytes and large cell aggregates were lacking. And, the cell yield could be increased by using more than one tissue piece for the cell isolation. Moreover, the cryopreservation of the tissue enabled the delay of the NHBE isolation to a time point when fresh cells were required.

4.1.2.3 Establishment of normal human bronchial epithelial (NHBE) cell lines

The isolated NHBE cells were subcultivated as soon as they formed a dense layer in the wells (Figure 4-32).

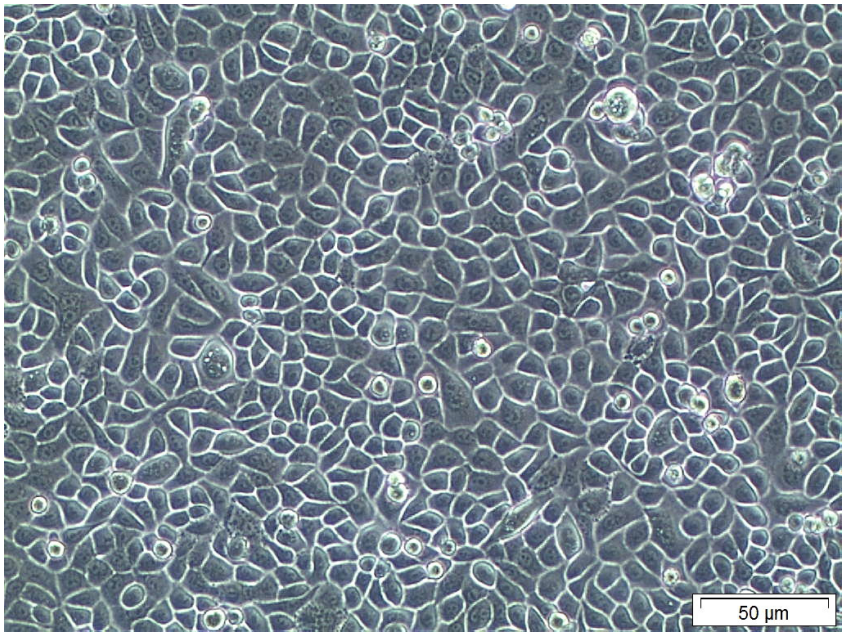

Figure 4-32: NHBE cells forming a confluent cell layer in wells of a 6-well plates approximately 17 days after isolation from tissue.

In some cases, they had already reached approx. 100 % confluency in the middle of the wells, but not at the outer edges. The cells were then subcultivated anyway, to prevent a growth arrest of the confluent cells. The detachment of the cells was achieved with 1 mL trypsin/EDTA (0.05 %/ 0.02 %) per well after rinsing the cells twice with PBS (w/o Mg^{2+} and Ca^{2+}). The trypsinization was performed at room temperature for several min until the major part of the cells became detached. The enzyme activity was stopped with 1 mL trypsin inhibitor from soy bean (1 mg/mL in PBS) mixed with 2 mL BEGM. If a large amount of the cells was still attached to the culture vessel, the trypsinization was repeated. After

detachment, the number of viable cells was determined with the CASY Cell Counter and Analyzer System (see 6.1.1.2). The cell suspension was then centrifuged for 5 min at 220 x g to remove the trypsin. Dependent on the subsequent approach, the cell pellet was either resuspended in BEGM or in freezing medium. The medium volume was adjusted to the desired cell number per milliliter. The cells were used for subcultivation in culture flasks, for the establishment of mono- and co-cultures on microporous insert membranes or for storage in liquid nitrogen. For the preparation of cell stocks for storage in liquid nitrogen, only cells of the first two passages were used. Here, the cells were resuspended in a defined amount of Recovery™ Cell Culture Freezing Medium (Gibco®, Life Technologies Corp.) or homemade freezing medium (see section 6.1.1.2).

For the general cultivation of the NHBE cells, culture flasks with a growth area of 25 cm^2 or occasionally with 75 cm^2 were used (Figure 4-33). In the case of cells of the passage 1, the culture surface was previously coated with collagen/fibronectin to support their adherence and thus to increase the cell yield. The cells were seeded at a density of 3 – 3.5 × 10^5/ 25 cm^2 and fed with BEGM (BEBM including all enclosed SingleQuots™, Lonza). Subcultivation was performed once a week.

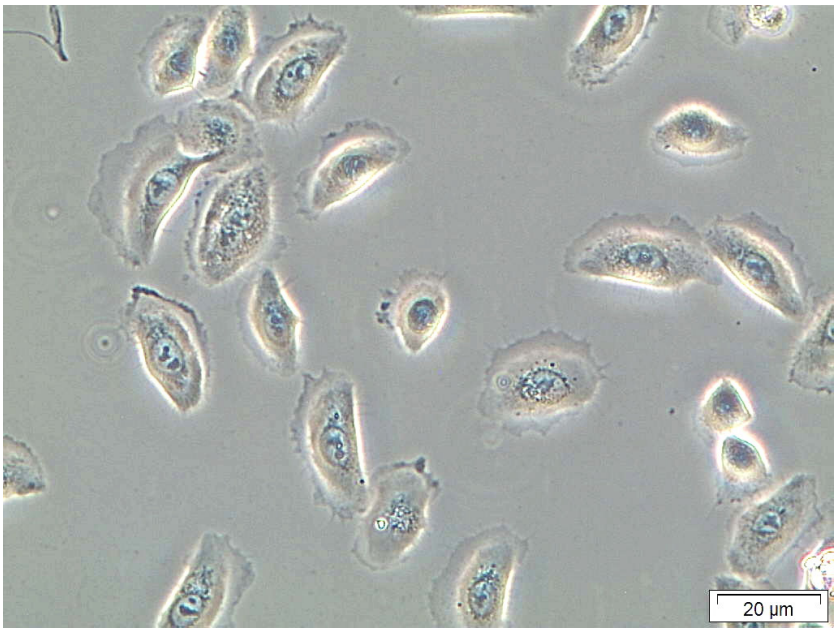

Figure 4-33: Normal human bronchial epithelial cells of passage 1 cultivated in a collagen/fibronectin coated culture flasks (25 cm^2) and supplied with BEGM.

Comparable to the commercial HBEp cells, no remarkable differences in the proliferation rate and cell viability were observed in NHBE cells when cultivated in cell culture flasks over at least eight passages. But, after this time point, the growth rate started to decrease until it came to a complete arrest, approximately in passage 10.

4.1.2.4 Characterization of normal human bronchial epithelial (NHBE) cell lines

For characterization, freshly isolated NHBE cells between the passages 1 and 5 were grown on inserts with collagen type IV or type I coated microporous membranes (0.4 µm pores). No obvious differences between the two coating types were observed. However, the collagen type IV coating was mostly used due to its higher physiological relevance through mimicking the biochemical properties of the basement membrane *in vivo*.

Similar to the HBEpC, the seeding density was found to be a crucial factor also for NHBE cells, in order to obtain a confluent cell layer. The optimal density was between $1 – 1.5 \times 10^5$/ cm^2. The turnover from the submerged cultivation to the air-liquid interface was optimally performed when the cells showed a confluency of 100 % and latest 24 h after reaching this state of complete confluency. Any deviations from this cultivation mode or the appropriate seeding density resulted in a lack of differentiation properties and structural alterations accompanied by a decrease of the culture life span.

The work on commercial human bronchial epithelial cells already revealed that these non-transformed cells are able to form cell populations with ciliated, mucus-producing (MUC5AC and MUC5B) and basal cells and to develop tight junctions with realistic TEER values (600 – 2200 Ω/ cm^2) when cultivated at the air-liquid interface and supplied with the basic differentiation medium, including Ultroser® G. However, these previous studies suggested that the differentiation strongly depends on the passage number and is highly variable between different lots.

In freshly isolated NHBE cells, natural differentiation patterns with goblet, ciliated and basal cells were achieved in nearly all cultures under identical culture conditions when using cells of the passages one and two, independent of donor-specific characteristics (Figure 4-34 - Figure 4-36). Once differentiated, the cultures were also characterized by a long shelf life of at least 12 weeks.

However, the ability to develop *in vivo*-like characteristics decreased dramatically in higher passages. Moreover, the differentiation capacity of "older" cells (beyond passage two) showed high donor-specific variations. Here, some lots retained the differentiation ability up to passage 4, while others showed no natural differentiation characteristics and resulted in cultures with a decreased life span.

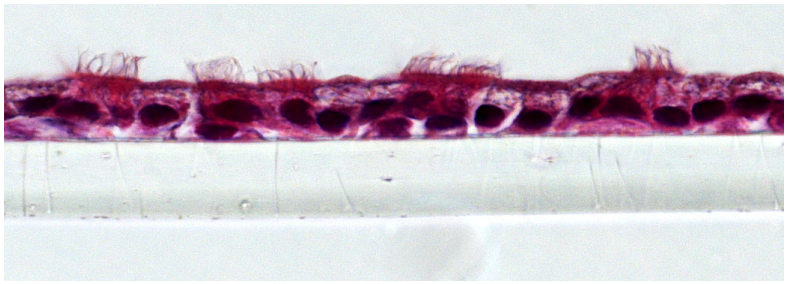

Figure 4-34: Differentiated culture of NHBE cells of passage 2 cultivated on collagen IV coated microporous membranes (0.4 µm pores) at the air-liquid interface with basic differentiation medium after a cultivation time of 42 days (38 days under ALI conditions). The cell population is characterized by a pseudostratified structure with basal cells, ciliated cells and non-ciliated columnar cells (potentially secretory cells; HE stained histological sections; magnification: 1000x).

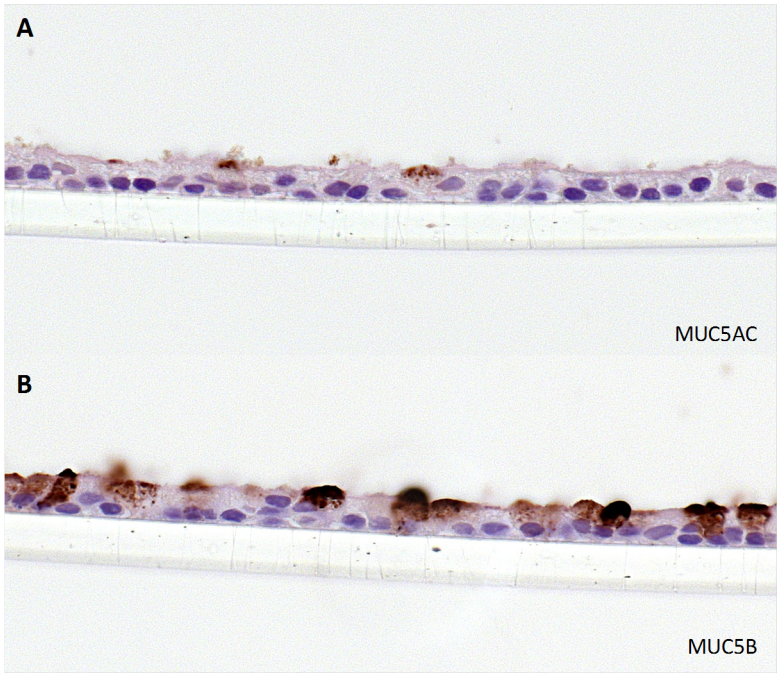

Figure 4-35: Immunohistochemical staining of the mucins 5AC (A) and 5B in a NHBE culture (passage 2) cultivated on collagen IV coated microporous insert membranes (0.4 µm) at the air-liquid interface with basic differentiation medium after a cultivation time of 42 days (38 days under ALI conditions). The cell nuclei were counterstained with hematoxylin (Magnification: 400x).

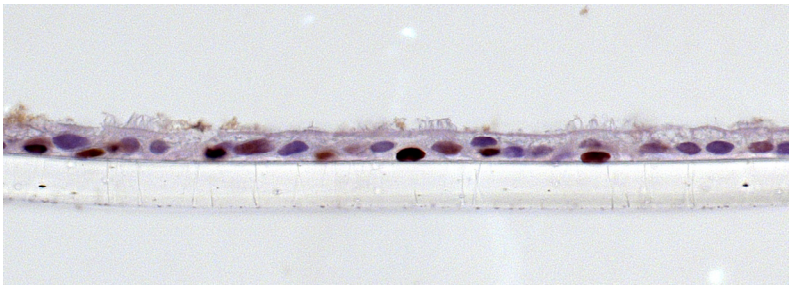

Figure 4-36: Immunohistochemical staining of the basal cell marker p63 in a NHBE culture (passage 2) cultivated on collagen IV coated microporous insert membranes (0.4 µm) at the air-liquid interface with basic differentiation medium after a cultivation time of 42 days (38 days under ALI conditions). The cell nuclei were counterstained with hematoxylin (Magnification: 400x).

The fact that no decrease in the cell viability was observed until passage eight when cultivated in cell culture flasks (see 4.1.2.3) clearly demonstrated that the decrease in the differentiation ability cannot be associated with the general cell viability of NHBE cells.

Although, NHBE cells of the passages one and two were mostly able to develop the desired natural differentiation properties, independent on the original sample, lot-specific morphological variations in the differentiated cell populations were nevertheless abundant. These variations comprised the distribution of the different cell types within the cell layers as well as the shape of the cells and structure of the cultures. In cultures of some NHBE lots, a higher secretion activity was observed, pointing to an increased number of goblet cells, while in other cultures a higher number of ciliated cells were detected. The columnar-shape of the ciliated and goblet cells as well as the pseudostratified structure was also found to be more pronounced in some distinct cultures. Since these variations were reproducibly obtained, the occurrence of donor- or tissue-specific differences in NHBE cells was clearly proven in this thesis.

The availability of NHBE cells of passage one and two was the limiting factor for the performance of experiments with cultures of these cells. The time from the isolation to the final use of NHBE cells was between three and five weeks and the number of cells was highly varying between the samples. Therefore, the suitability of cryopreserved NHBE cells was also tested. For that purpose, different lots of NHBE cells that were frozen in liquid nitrogen in the passages one or two were cultivated under the same conditions as fresh cells that never underwent cryopreservation. In most cases, the cryopreserved cells did not retain the ability to show the natural differentiation characteristics, like the formation of cilia or a pseudostratified structure, as they did before freezing (Figure 4-37). The cultures mostly had

a stratified growth and the cells showed a squamous morphology. Moreover, the shelf life of these cultures was reduced to approximately four weeks until the cells partially started to detach and holes within the cell layer became visible.

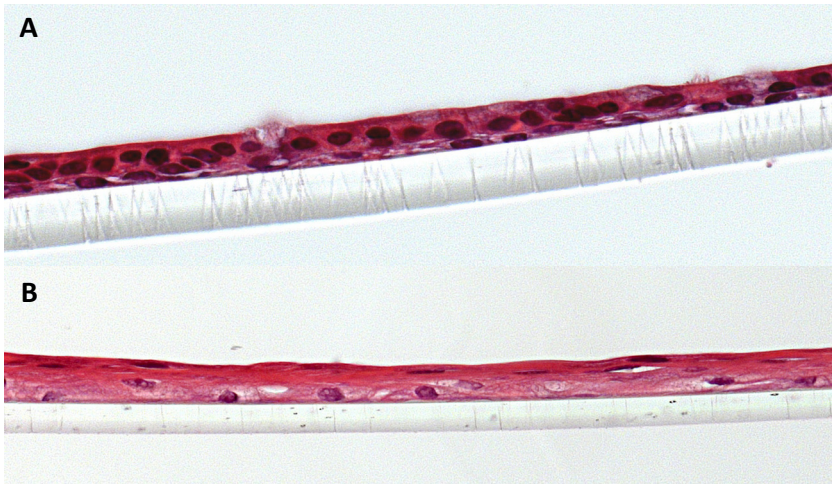

Figure 4-37: Two cultures of the same NHBE lot before (A) and after cryopreservation (B). Both cultures were cultivated under identical conditions (on collagen IV coated insert membranes, at the air-liquid interface and with basic differentiation medium) for 26 days (21 days under ALI conditions; HE stained histological sections; magnification: 400x).

Conclusions

Freshly isolated NHBE cells in passage one and two provide the most reliable and realistic *in vitro* model of the airway epithelium since they are able to differentiate into all common cell types of the bronchial epithelium, including ciliated, goblet and basal cells. In addition, the formation of functional tight junctions in these cultures was verified by TEER values which ranged between 600 and 2000 Ω/ cm^2. Moreover, in contrast to the commercial HBEp cells, the development of the natural differentiation characteristics was highly reproducible in NHBE cultures when using cells of the passages one and two.

However, the restricted availability of cells in passage one and two as well as the loss of the differentiation ability after cryopreservation and the donor-specific variations limit the suitability of NHBE cells as universal *in vitro* model for toxicological studies, especially in the case of large scale assessments. Nevertheless, whenever possible, freshly isolated normal human bronchial epithelial cells should be the first choice for *in vitro* studies on the bronchial epithelium or they should be employed for verification of the results obtained with permanent cell lines.

Cryopreservation of lung tissue was found to be a valuable method to guarantee the availability of NHBE cells at a defined time point.

4.1.3 Human lung fibroblasts

In order to realize 3D co-culture systems mimicking the EMTU of the bronchi, a suitable human lung fibroblast line was additionally required. For that purpose, the commercial fetal fibroblast line IMR90 was initially used. Later adult fibroblasts were also included which were isolated from human bronchial tissue.

4.1.3.1 IMR90- fetal human lung fibroblasts

The IMR90 human fibroblast cell line is derived from a 16-week-old female fetus with a normal karyotype. The IMR90 is a commercial cell line (ATTC CCL-186) and thus permanently available. Moreover, it is described to have a long life span, of up to 50 passages [(233)]. These characteristics recommend the IMR90 as a cell line for the routine work in order to provide reproducible conditions. Accordingly, IMR90 fibroblasts have already been used in various co-culture studies [(178, 234-235)].

The cells were obtained in passage 10 and showed a stable and high viability up to 15 more passages. A pool of frozen cell stocks has been established and the IMR90 cells were used between the passages 13 and 25.

The IMR90 cells were cultivated in 75 cm^2 culture flasks with a seeding density of 2×10^6 cells/ 75 cm^2 and supplied with EMEM (MEM Eagle, Lonza 125F) supplemented with 2mM L-glutamine, 10 % FBS and 1 mM sodium pyruvates. Subcultivation was performed twice a week.

4.1.3.2 Establishment of normal human lung fibroblast cell lines (NHLF)

Due to the fact that the IMR90 fibroblasts are derived from a fetus and might therefore show properties that are distinct from adult cells, the inclusion of adult fibroblasts for verifying the results obtained with IMR90 is advantageous. For that purpose, a protocol for the isolation of fibroblasts from the human bronchial tissue samples was developed.

In the first approaches, only fresh lung tissue was used. Later, the fibroblast isolation from cryopreserved samples was also tested. Here, the same tissue pieces that were previously used for scraping off the epithelial cells were “recycled”. The isolation of normal human lung fibroblasts (NHLF) was performed according to the outgrowth procedure where the tissue was attached to a culture surface and, under appropriate culture conditions, the fibroblasts grew out from the samples. In general, the first fibroblasts were visible around the tissue piece earliest after seven days (Figure 4-38).

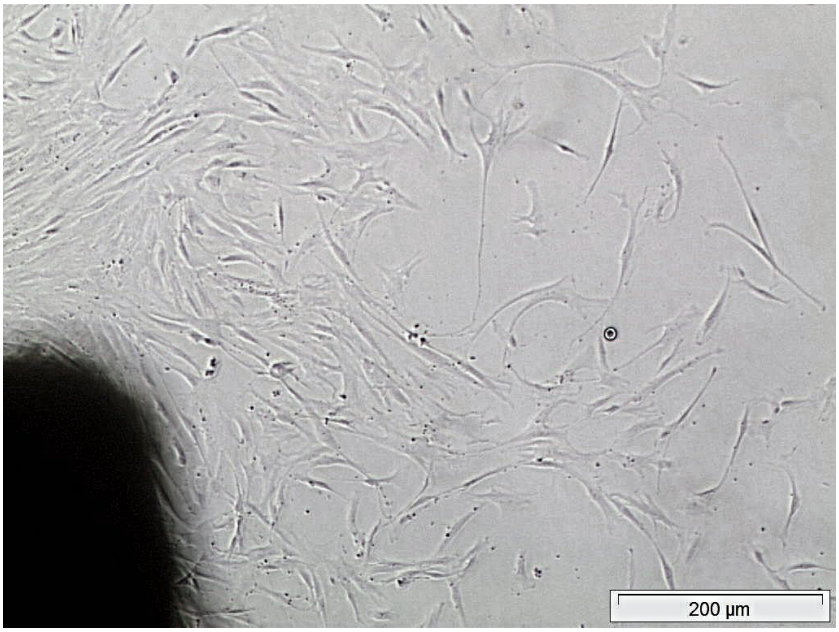

Figure 4-38: Fibroblasts growing out from human bronchial tissue cultivated in Petri dishes (3 cm diameter) for 20 days.

The tissue was then further cultivated for another seven to ten days before it was removed and the cells were cultivated in the absence of the tissue until reaching the desired cell density. During the first attempt of fibroblast isolation, the importance of the following parameters was evaluated:

- Size of tissue samples
- Diameter of Petri dishes
- Medium composition

These investigations revealed that the optimal tissue size for the isolation of fibroblasts was around 3 x 3 mm. Sharp cutting edges were shown to promote the fibroblast outgrowth

which were obtained by using a scalpel. For unknown reasons, the cell yield was significantly higher when using Petri dishes with a diameter of 3 cm compared to 9 cm Petri dishes. The medium level played a crucial role for the adherence of the tissue pieces on the culture surface. Best results were obtained when the medium level covered approximately half of the tissue piece. If the medium volume was too high, the tissue detached from the surface.

In the first approaches with fresh lung tissue samples, the specimens and isolated fibroblasts were supplied with RPMI 1640 supplemented with 10 % FBS, L-glutamine (2 mM), sodium pyruvate (1 mM) and penicillin/streptomycin (10 U/ 10 µg/mL).

In later approaches the isolation of pure fibroblast cultures under above mentioned culture conditions sometimes failed. Here, another cell type was predominantly isolated. Besides a few epithelial cells and fibroblasts, a third cell type was outgrowing from the tissue in high numbers. Most likely, these cells were macrophages, since this cell type is described to be the first to grow out from tissue [(236)]. The cell morphology also supports the presumption of macrophage isolation. The supposed macrophages accumulated directly beneath the tissue pieces and were thus only detectable after removing the samples. In contrast, the fibroblasts migrated to the outer edge of the tissue and were visible alongside the sample (Figure 4-39).

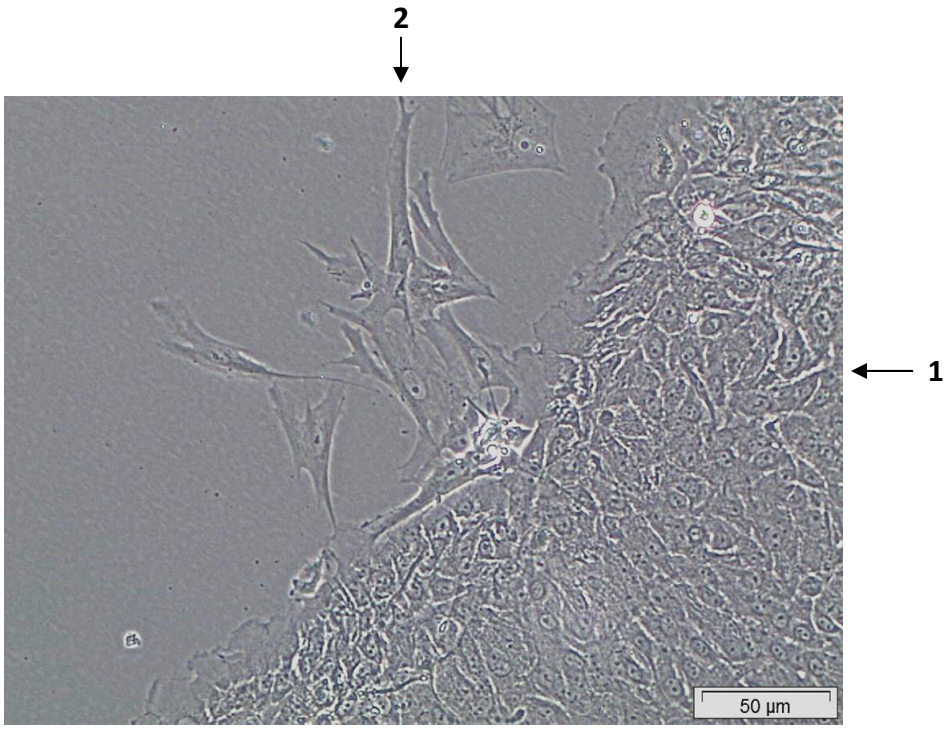

Figure 4-39: Two different cell types growing out from human bronchial tissue cultivated in Petri dishes (3 cm diameter) for 14 days. After removal of the tissue sample, a large number of presumed macrophages (1) were detected, while only a few fibroblasts (2) grew out from the tissue at the outer edges.

Isolated macrophages are described to have a low proliferation rate and to contribute little to the final culture after trypsinization [236]. In fact, the freshly isolated macrophages started to die at the outer edges of the outgrowth population a few days (2 - 3) after the removal of the tissue piece. However, due to their large number, they were still predominant and the few isolated fibroblasts were often suppressed. If a mixed cell population composed of assumed macrophages and fibroblasts was subcultivated and transferred to cell culture flasks, none of the cell types could be expanded.

In later approaches, the utility of cryopreserved tissue samples for the fibroblast isolation was tested. Under the above mentioned culture conditions, the outgrowth of fibroblasts was not achieved. But when using culture medium with a higher concentration of D-glucose [4.5 g/L; DMEM (Biochrom) incl. L-glutamine, sodium pyruvate, supplemented with 10 % FBS and penicillin/streptomycin (10 U/ 10 µg/mL)], the isolation of fibroblast was successful with some of the tested tissue samples. Compared to fresh lung tissue, the time until the first cells were detectable at the edges of the samples took at least 14 days. No macrophage contamination was observed when using cryopreserved tissue.

Interestingly, once the fibroblast outgrowth was achieved, the particular tissue piece yielded a large number of fibroblast over several weeks when transferred to new Petri dishes from time to time. After removal of the tissue, the fibroblasts were cultivated for another five to seven days until subcultivation.

For maintenance and expansion, the cells were cultivated in 75 cm^2 culture flasks with a seeding density of $1 – 2 \times 10^6$ cells/ 75 cm^2 and supplied with RPMI (incl. 10 % FBS, L-glutamine, sodium pyruvates and penicillin/ streptomycin). The subcultivation was performed twice a week and the cells showed a stable viability at least until passage ten. A pool of frozen cell stock was prepared from each lot between the passages two and four.

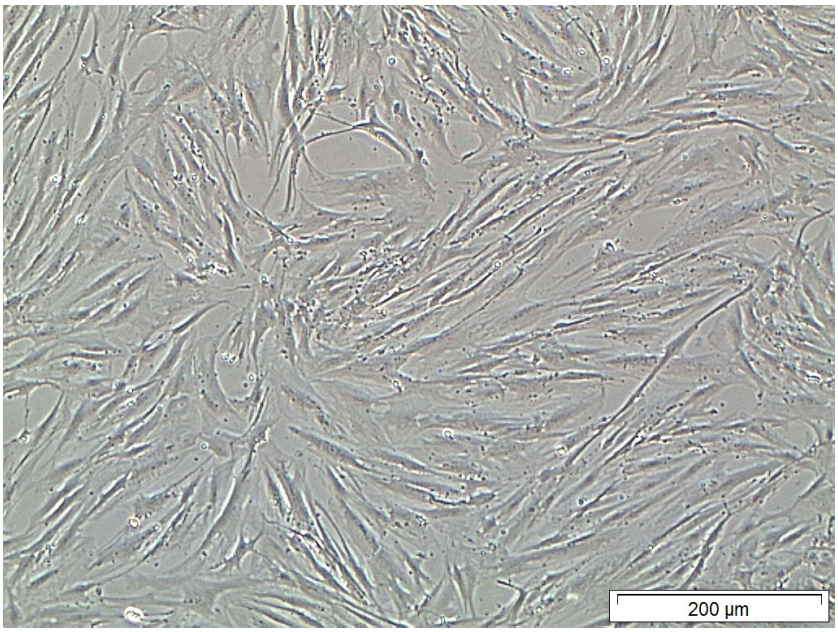

Figure 4-40: Normal human lung fibroblasts of passage 2 after isolation from lung tissue cultivated in 75 cm^2 culture flasks and supplied with RPMI incl. 10 % FBS, L-glutamine, sodium pyruvates and penicillin/streptomycin

Conclusions

The results revealed that the success of isolating fibroblasts from normal human bronchial tissue varied significantly between different samples. When using fresh lung tissue, the NHLF yield was strongly dependent on the extent of macrophage contamination which was found to differ between tissue samples of different donors. On the other hand, the outgrowth of NHLF from cryopreserved tissue was only achieved in some of the tested samples.

However, if the NHLF isolation was successful, the cell yield was generally very high and the cell viability was stable for at least ten passages.

4.2 Development of 3-dimensional in vitro models of the airway epithelium

In order to simulate the natural interactions between the bronchial epithelial layer and the underlying connective tissue, including the ECM and fibroblasts, as they take place *in vivo,* two different 3D co-culture models were developed in this thesis.

4.2.1 Sandwich Cell Model (SCM)

The Sandwich Cell Model (SCM) is based on a protocol that has originally been developed for the construction of 3D skin equivalents [237]. It provides a realistic *in vitro* replication of the bronchial epithelium. Similar to the *in vivo* structure, the SCM is composed of an epithelial cell layer and the underlying *lamina propria* - a layer of loose connective tissue whose main components are fibers of ECM proteins in which cells are embedded. Fibroblasts represent the predominant cell type and collagen type I the most abundant protein in this type of connective tissue. The SCM is accordingly composed of epithelial cells, fibroblast and a collagen type I matrix. The practical implementation of the natural airway epithelial structure was realized by the following basic protocol: Fibroblasts were suspended in a collagen solution which was previously brought to a neutral pH by adding a NaOH-containing buffer solution (see 6.1.2). An appropriate volume of this collagen-fibroblast solution was applied onto microporous membranes of cell culture inserts and incubated at 37 °C for polymerization of the collagen. Afterwards, the epithelial cells were applied onto the hardened collagen matrix and the cultures were cultivated under submerged conditions until the epithelial cells formed a confluent layer. To promote the differentiation of the epithelial cells, the cultures were subsequently supplied with the basic differentiation medium and cultivated at the air-liquid interface (Figure 4-41).

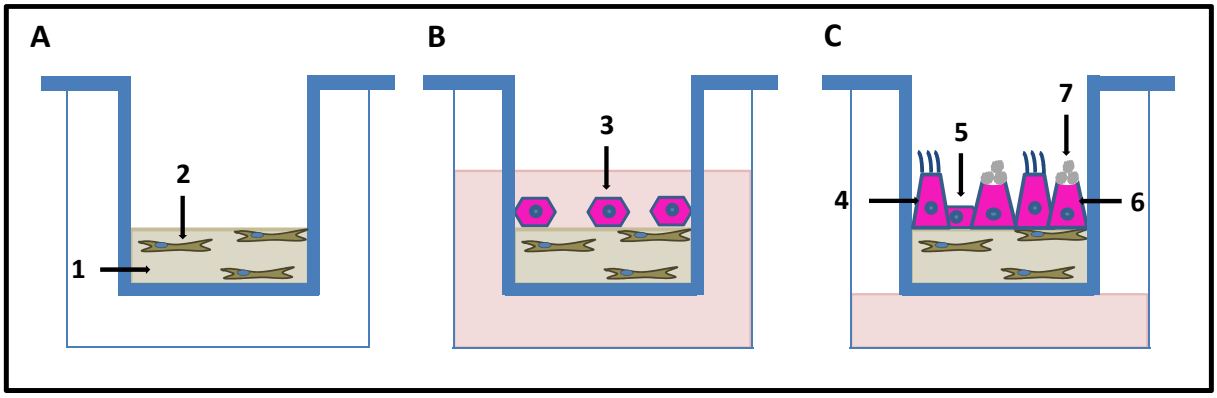

Figure 4-41: Basic protocol for the Sandwich Cell Model (SCM). A: Lung fibroblasts are suspended in a pH neutral collagen solution and applied onto the membrane of a cell culture insert. B: After the complete polymerization of the collagen at 37°C, lung epithelial cells are applied on the collagen matrix and the co-cultures are cultivated under submerged conditions. C: As soon as the epithelial cell layer reached 100% confluency, the cultures are cultivated at the air-liquid interface to promote the cell differentiation (1= Collagen matrix; 2= Fibroblasts; Undifferentiated epithelial cells; 4= Ciliated cell; 5= Basal cell; 6= Goblet cell; 7= Mucus).

As described in section 3.3.3.1.1, there are many commercial collagen type I products with different properties. In the first approaches, acid soluble collagen I isolated from rat tails (Serva Electrophoresis GmbH) was used for the SCM cultures. This type of collagen has also been used for the generation of bronchial epithelial *in vitro* models in other studies [177, 238].

Initial experiments revealed that the most critical factor for the successful construction of SCM cultures is the achievement of a stable collagen matrix. A frequently occurring problem was the contraction of the polymerized collagen matrix a few days after preparation, leading to instability of the cultures.

In vivo, contraction of the connective tissue is an important event during wound healing. Fibroblasts are described to mediate collagen contraction both *in vivo* and *in vitro* [238]. *In vitro* studies furthermore revealed that epithelial cells enhance the fibroblast-mediated collagen contraction [177, 238].

In the SCM cultures, the contraction was either starting shortly after changing the cultivation conditions from submerged to ALI or within the first ten days under ALI conditions, depending on other applied parameters (see below). During contraction, the collagen matrix detached from the housing of the cell culture insert and occasionally also from the membrane. The detachment mostly started at one side of the culture and then expanded stepwise. The extent of contraction was differing between cultures and also between inserts of the same culture (cultivated under identical conditions; Figure 4-42).

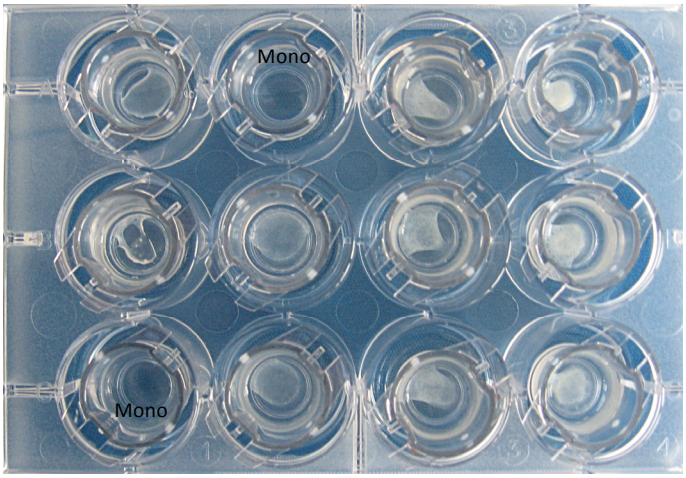

Figure 4-42: 12-well receiver plate with ten cell culture inserts carrying SCM cultures prepared from different epithelial cells (HBEpC, 16HBE14o-) and two HBEpC monocultures (marked). All SCM cultures clearly show contraction of the collagen matrix.

In most cases, the shrinkage highly reduced the surface of the culture and made it impracticable for the generation of intact histological sections or immunofluorescence analyses. Moreover, the development of a natural structuring of the epithelial layer was hindered by the collagen contraction. A wave-like structure with an uneven multi-layered growth was formed as a consequence of the non-uniform contraction and by compression of the epithelial cells due to the sudden lack of space.

Besides the collagen contraction, another crucial point of the SCM cultures was found to be the viability of the fibroblasts which were embedded in the collagen matrix. Observations by *in situ* microscopy and histological sections revealed that the fibroblasts often stopped proliferation or even died. Both, the collagen contraction and the viability of cells embedded in collagen were potentially strongly related to the physical properties of the matrix [(239)]. For example, the pH of the matrix and the thickness and arrangement of collagen fibers seem to influence the cell viability and morphology of fibroblasts. In addition, the fiber characteristics are dependent on the incubation temperature and time [(240-241)].

In order to obtain a stable collagen matrix with viable fibroblasts, different modifications of the original protocol with regard to the pH, incubation temperature and duration as well as the thickness of the collagen matrix were tested. The influence of the pH was tested by adding different amounts of the NaOH buffer solution. This approach also led to variations in the collagen concentration. It was found, that an increase of the pH from originally 7 - 7.5 to approximately 8 - 8.5 by adding a higher volume of NaOH buffer solution improved the stability of the collagen matrix and delayed the contraction. Nevertheless, after 7 - 14 days the collagen matrix started to contract also in these cultures.

The time point when the contraction started, as well as its extent, was depending on the respective epithelial cell line. The SCM cultures prepared with the two permanent cell lines 16HBE14o- and Calu-3 showed a less shrinkage of the collagen matrix and the contraction started later than in cultures with HBEpC.

In further approaches, the effects of the incubation temperature and duration as well as the thickness of the collagen matrix were tested. For that purpose, the collagen solution was applied onto the membranes in different amounts (150 - 300 µl) and incubated either at 4 °C, room temperature or 37 °C for varying time intervals (2 – 5 h or overnight). However, none of the tested variations delivered reproducible SCM cultures without collagen

contraction. But, modifications of the temperature resulted in differences in the cell viability of the fibroblasts. The highest viability of this cell type was observed when the collagen matrix was immediately incubated at 37 °C.

SCM cultures that were stable for up to four weeks (showing no collagen contraction) were only obtained sporadically and in these cases histological sections revealed that either the epithelial cells did not form a confluent layer or the fibroblasts were lost by cell death or both. These observations clearly suggest that both cell types play a pivotal role in the collagen contraction *in vitro*.

In order to investigate the role of the collagen source for producing stable collagen matrices, collagen type I solutions from different suppliers and isolated by different techniques and/or from other animals and organs were tested in the next steps.

Two collagen solutions prepared from bovine skin by acid-based extraction methods (3 mg/mL, BD Biosciences and Sigma-Aldrich) delivered similar results as the previously used rat tail collagen. None of the tested approaches resulted in stable and reproducible SCM cultures. Moreover, contraction of the collagen matrix was even more pronounced than in previous cultures that were prepared with the rat tail collagen solution. Thus, the two bovine skin collagen solutions were also precluded from further approaches.

Next, two collagen solutions isolated from porcine tendons were tested (Cellmatrix I-A and I-P, Nitta Gelatin Inc.). Both products contain 3 mg/mL of collagen type I and have a pH of 3.0. The difference is that the Cellmatrix I-A solution contains acid-soluble collagen while the Cellmatrix I-P consists of pepsine-solublized collagen. As stated by the manufacturer, the two collagen solutions are specially designed to culture cells on top or embedded within the collagen matrix. A comprehensive instruction for the preparation of the collagen matrices was furthermore provided. The basic protocol was as follows: The collagen solutions were each mixed with NaOH neutralizing buffer (see 6.1.2) and 10X MEM medium at a ratio of 8:1:1. When cultivating cells embedded in the polymerized collagen, the manufacturer recommends the preparation of a basal collagen layer as shown in Figure 4-43.

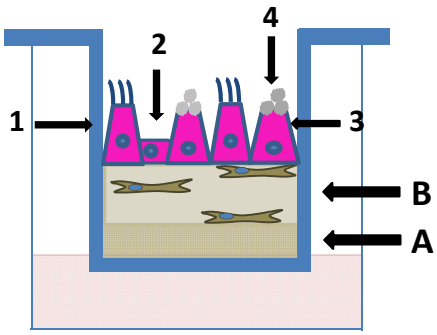

Figure 4-43: Schematic representation of a SCM culture composed of a basal collagen layer (A: 100 µl collagen solution); and a main collagen layer in which the fibroblast are embedded (B: 170 – 200 µl collagen solution; 1= Ciliated cell; 2= Basal cell; 3= Goblet cell; 4= Mucus).

Different combinations of the two collagen solutions for the matrix preparation were tested. For the base layer, the Cellmatrix I-A or I-P were used alternatively. For the main collagen layer (with embedded fibroblasts) a 1:1 mixture of both collagen solutions was additionally tested. Best results with regard to matrix stability and fibroblast viability were obtained when using the combination of Cellmatrix I-A and I-P solutions. The use of Cellmatrix I-A alone resulted in a decreased stability and a contraction of these matrices was observed more often. The Cellmatrix I-P seemed to produce matrices with a higher stiffness. A higher ECM matrix stiffness was suggested to be beneficial for the proliferation and gene regulation of fibroblasts [(242)]. The use of a 1:1 mixture of I-A and I-P, resulted in a collagen/fibroblast layer with a high stability for up to six weeks without a visible contraction, at least when cultivated in the absence of epithelial cells. Moreover, these results were highly reproducible.

The preparation of a base collagen layer between the membrane and the main collagen matrix was found to be not beneficial. On the contrary, these SCM cultures showed a decreased stability and thus this additional layer was omitted in later cultures. The purpose of this layer was to prevent the fibroblasts from attaching to the membrane and growing there. However, since histological section revealed that the fibroblasts were uniformly distributed in the main collagen matrix, the base layer was not required.

For the main collagen matrix, a volume of 200 – 250 µl of the collagen/fibroblast solution was found to be optimal. The number of fibroblasts should rather be low (4 – 5 x 10^5/ culture), since they generally showed a steady proliferation during the cultivation time. Although the fibroblasts were only sporadically detected in the histological

section of SCM cultures (5 – 7 µm thickness), their growth and morphology could be well monitored by *in situ* microscopy.

In order to find optimal conditions for the polymerization of the collagen matrix, different incubation times and temperatures were applied. However, these parameters had no obvious impact on the matrix stiffness and stability but only on the duration of the complete polymerization. The final protocol includes an incubation step at 37 °C for 3 - 5 h before the epithelial cells are applied onto the polymerized collagen matrix (see section 6.1.2).

In initial experiments, Calu-3 cells were used for the generation of SCM cultures with the Cellmatrix collagen solutions since previous approaches with rat tail collagen revealed that these cells induced a less intense contraction of the collagen than the HBEpC. The SCM co-cultures composed of Calu-3 and IMR90 cells and a collagen matrix prepared from Cellmatrix I-A and I-P (1:1) were stable for at least four weeks. No contraction of the collagen matrix was observed during this time but a slight degradation of the collagen starting after approximately 20 days. The Calu-3 cells showed their typical monolayered growth and columnar shape. Moreover, mucus secretion was observed by inverse microscopy and verified by immunohistochemical analyses of MUC5AC in these cultures (Figure 4-44).

The limiting factor of these SCM cultures was the life span of the Calu-3 cells. As described in section 3.5.2.2, the Calu-3 cell line is characterized by an early differentiation of the cells after approximately three days under ALI conditions but also by a limited life span. In SCM cultures, the cells showed an altered morphology after four to five weeks. At the same time, a partial degradation of the collagen was detected. The cultures had a milky to cloudy appearance and histological sections revealed small crumbles within the collagen matrix.

As discussed earlier in this thesis, normal, non-transformed human bronchial epithelial cells (passage 1 and 2) provide the best *in vitro* model of the bronchial epithelium since they retained most of the natural characteristics of their *in vivo* counterparts. Moreover, once differentiated, the cultures were characterized by a long life span of several months. Thus, they are well suited for long term toxicological studies. Co-culture systems composed of NHBE or HBEp cells and fibroblasts embedded in a matrix of ECM proteins provide an even better reflection of the natural structuring and might furthermore enable the simulation of interactions taking place within the EMTU (see section 3.4.2). Accordingly, these models are one step closer to a realistic *in vitro* model.

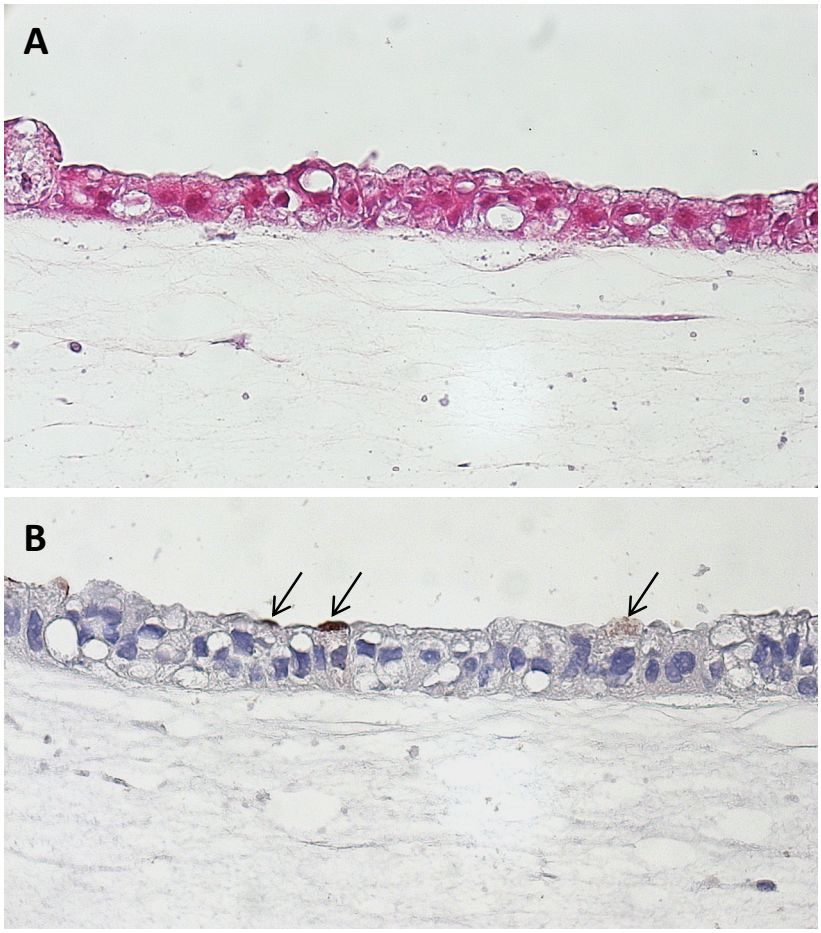

Figure 4-44: SCM cultures composed of Calu-3 and IMR90 cells (embedded in collagen I) after a cultivation time of 26 days (20 days under ALI conditions). The monolayered Calu-3 cells are characterized by a columnar-shape. A: slight degradation of the collagen is indicated by small crumbles within the matrix (HE stained histological sections). B: The presence of mucus producing cells was verified by immunohistochemical analyses of MUC5AC (see arrows; the cell nuclei were counterstained with hematoxylin; magnification: 400x)

But, first SCM cultures of HBEpC prepared with rat tail collagen I revealed a low stability and reproducibility, due to a strong contraction of the collagen matrix. The contraction was more pronounced than in cultures of Calu-3 or 16HBE14o- indicating the presence of factors that promote matrix remodeling. However, since normal human bronchial epithelial cells (HBEpC or NHBE) in general showed extensive passage- and donor-specific variations in their differentiation patterns, their suitability for the generation of stable and reproducible SCM cultures might potentially also differ. Therefore, for further evaluation and optimization of the SCM co-culture system with the Cellmatrix I-A and I-P, NHBE cells from different donors and of different passages were used in addition to the HBEpC.

Although the use of Cellmatrix I-A and I-P (1:1) for the preparation of the collagen matrix highly enhanced the stability of the SCM cultures with NHBE or HBEp cells, a contraction was nevertheless observed in some cultures.

Overall, NHBE cells of passage one and two resulted in the most stable SCM cultures. Here, mostly no contraction of the collagen matrix was observed for at least up to 25 days and the cultures were well reproducible when using cells of the same donor and passage. However, donor-specific variations in the SCM cultures became evident, since a few cultures prepared with NHBE cells of passage one still showed a contraction already after 14 days under ALI conditions. When using NHBE cells in higher passages (non-cryopreserved) for the preparation of SCM cultures, contraction of the collagen was observed more frequently.

Degradation of the collagen was observed in all cultures, starting after 3-4 weeks, and its degree increased with increasing culture time (Figure 4-45).

The differentiation ability of NHBE cells was considerably reduced in SCM cultures. The epithelial cells mostly formed a stratified layer with two distinct regions: One to three layers of cells with a “normal” morphology which stained positive for the basal cell marker p63 and an upper layer composed of cells with a flattened morphology. Cytokeratin 13 (CK13), which is a marker for squamous cells, was only detected sporadically in few cells and to a low amount, suggesting that no squamous metaplasia-like phenotype, as shown in section 4.3, was developed in these cultures (Figure 4-46).

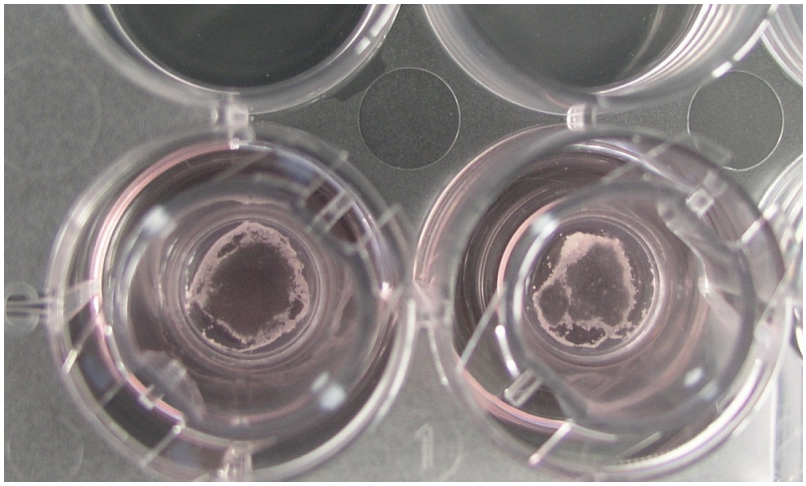

Figure 4-45: SCM cultures prepared with NHBE cells of passage 1 after a cultivation time of 29 days. The cultures did not show a contraction of the collagen matrix but a high degradation or drying out of the collagen.

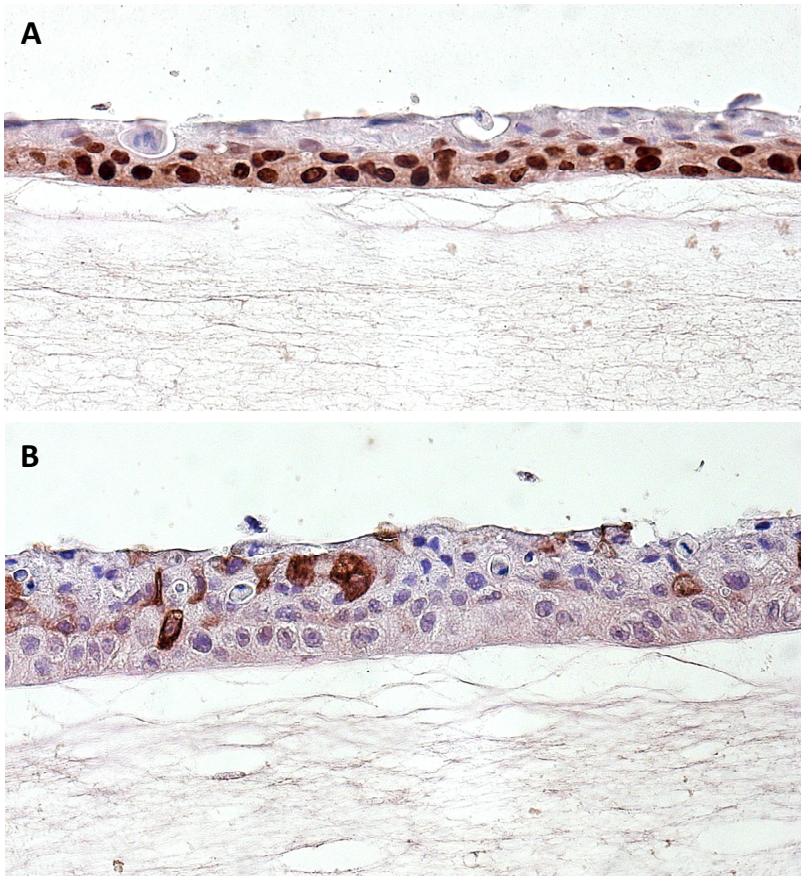

Figure 4-46: Immunohistochemical analysis of the basal cell marker p63 (A) and the squamous cell marker CK13 (B) in SCM cultures prepared from NHBE cells of passage 2 and IMR90 cells embedded in collagen type I, after a cultivation time of 18 days (14 days under ALI conditions). A: One to three layers of basal cells were detected by immunohistochemical analyses of the p63 marker. B: CK13 was detected only sporadically in few cells, suggesting that no squamous metaplasia-like phenotype was developed (the cell nuclei were counterstained with hematoxylin; magnification: 400x)

The contraction of the collagen matrix enhanced the differentiation of the NHBE cells. Although, a multilayered structure was also found in these cultures, the upper layer exhibited cells with a normal columnar shape (Figure 4-47). However, this structure was only found in some areas of the cultures and the reproducibility of these contracted SCM cultures was highly limited.

The use of different fibroblast lines - either NHLF of different donors or IMR90 - had no influence on the matrix remodeling or on the growth and differentiation patterns of the epithelial cells. Moreover, the seeding density of the epithelial cells was also found to neither effect the induction and extent of the collagen contraction nor their differentiation ability (Figure 4-48).

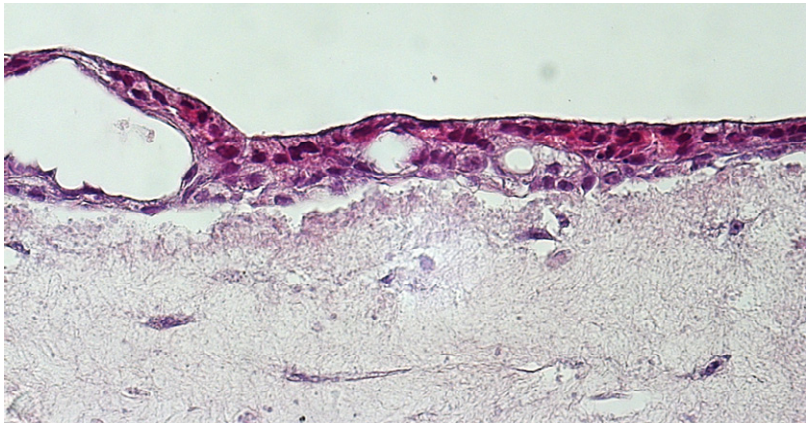

Figure 4-47: SCM culture prepared with NHBE (P03) and IMR90 showing a strong contraction of the collagen matrix after a cultivation time of 18 days (14 days under ALI conditions). The NHBE cells form two layers. The upper layer shows cells with a natural columnar shape. (HE stained histological sections; magnification: 400x)

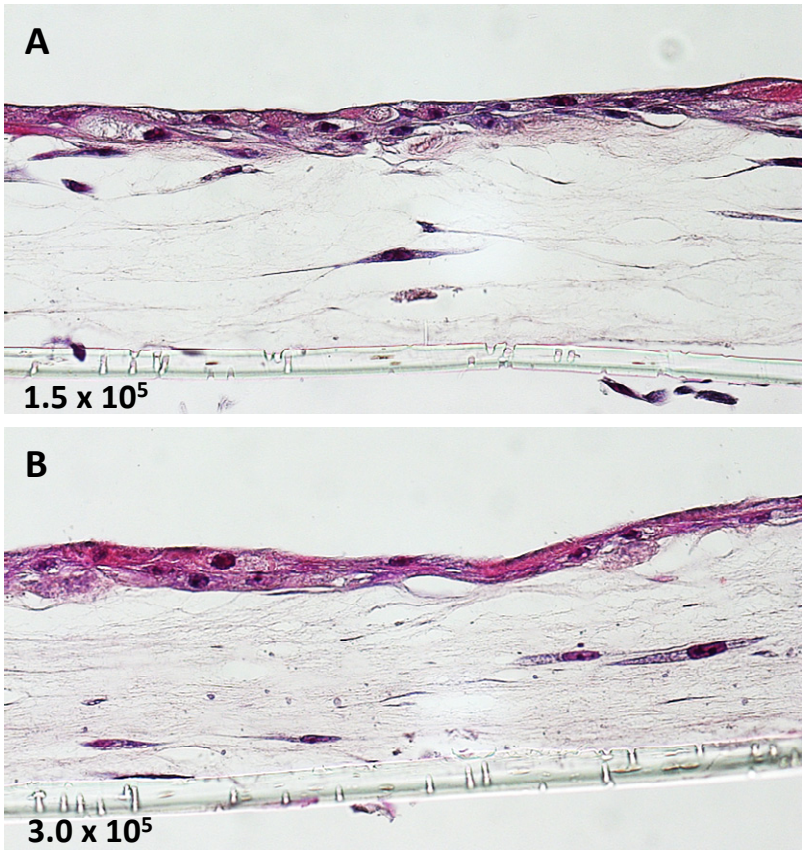

Figure 4-48: SCM cultures prepared with HBEpC (P05) and IMR90 after a cultivation time of 20 days. The HBEpC were applied with different seeding densities, 1.5 x 10^5 (A) and 3.0 x 10^5 (B) which had no obvious effect on the stability of the collagen matrix or on the differentiation pattern. (HE stained histological sections; magnification: 400x).

In order to enhance the differentiation capacity of NHBE cells in SCM cultures, the collagen matrix was complemented with other ECM proteins which are known to promote the differentiation of bronchial epithelial cells *in vivo*.

The ECM proteins laminin, fibronectin and collagen IV were added alternatively to the collagen solution in physiologically relevant concentrations:

Laminin: 4.4 µg/mL (approx. 1 µg/ insert culture)

Fibronectin: 9.2 µg/mL (approx. 2 µg/ insert culture)

Collagen type IV: 27.3 µg/mL (approx. 6 mg/ insert culture)

In the cultures prepared with laminin, a strong contraction of the collagen matrix was observed after 14 days. The generation of histological sections from these cultures was difficult and thus no concluding results about the effect of laminin on the differentiation of NHBE cells in SCM cultures are available.

The addition of fibronectin also reduced the stability of the SCM cultures. While the control cultures (without fibronectin) were stable up to at least 22 days, a contraction of the collagen matrix was detectable after 18 days in cultures with fibronectin. Moreover, no obvious improvement of the cell differentiation was observed when adding fibronectin (Figure 4-49).

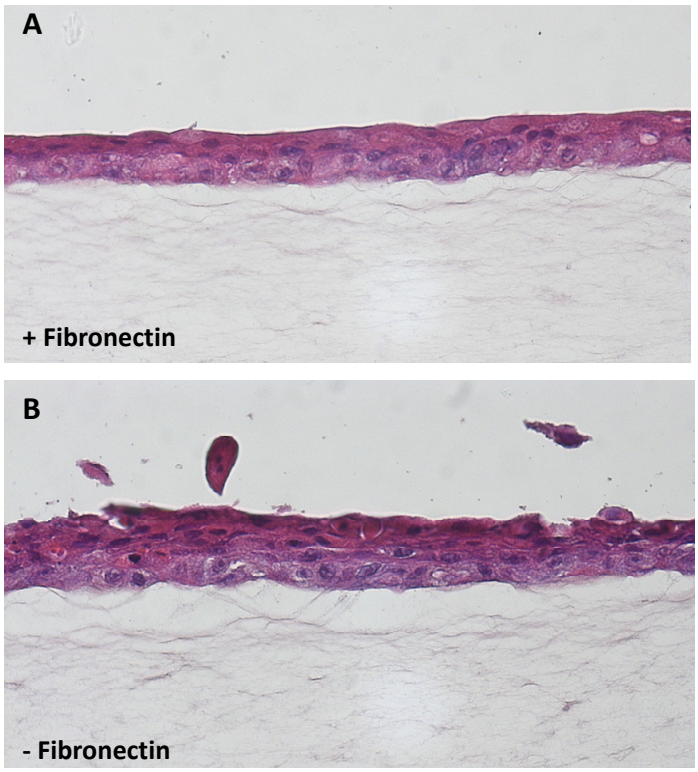

Figure 4-49: SCM cultures prepared with NHBE cells of P02 and IMR90 after a cultivation time of 22 days (18 days under ALI conditions). The collagen matrix was either supplemented with 2 µg (total concentration) fibronectin (A) or not (B). No remarkable differences in the structure and cell morphology were detected between these cultures. (HE stained histological sections; magnification: 400x).

The SCM cultures containing collagen type IV as additional ECM component did not show a contracted collagen matrix for up to four weeks. But here a strong degradation of the collagen was observed starting after approximately 20 - 23 days. The differentiation pattern of the NHBE cells did not show any obvious differences to the control cultures (without collagen IV addition). They again formed two distinct layers composed of a basal layer with "normal cells" covered by a second layer with flattened cells as observed in previous studies. Ciliated cells were not detected in any SCM cultures but mucus secretion (MUC5AC) was verified in the cultures prepared with and without collagen type IV after 14 days (Figure 4-50).

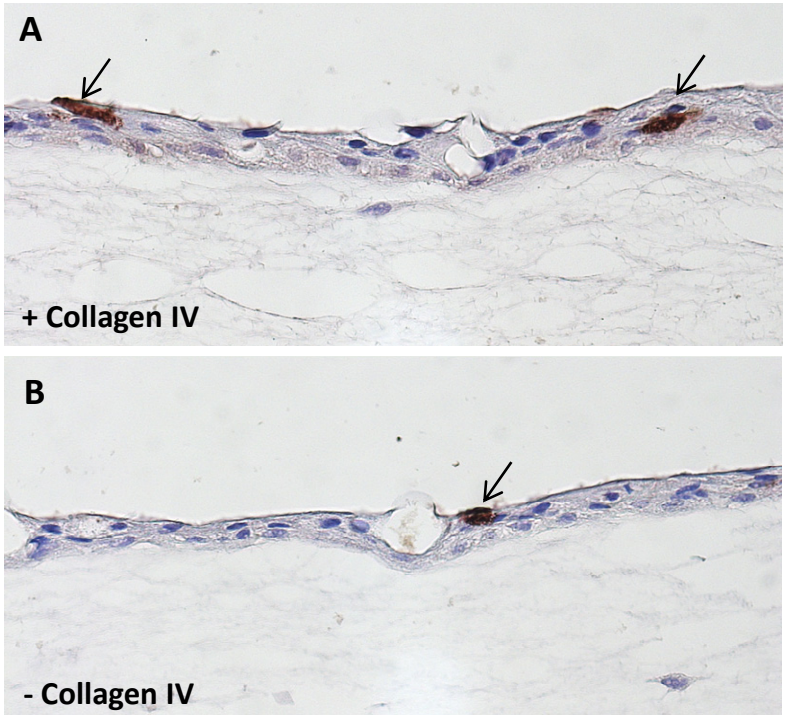

Figure 4-50: Immunohistochemical analysis of MUC5AC in SCM cultures composed of NHBE cells (P01) and IMR90 with (A) and without (B) collagen IV added to the collagen I matrix (after a cultivation time of 22 days; 18 days under ALI conditions). The epithelial cells of both cultures formed two distinct layers with normal shaped basal cells covered by flattened cells. A few MUC5AC producing cells were detected in both cultures (arrows; Magnification: 400x)

In summary, the use of the Cellmatrix I-A and I-P (1:1) collagen solutions enhanced the practicability of the SCM as an *in vitro* model of the bronchial epithelium. These SCM cultures were characterized by a high stability of the collagen matrix and accordingly by an improved reproducibility when prepared with Calu-3 cells as epithelial component. On the other hand, when using NHBE cells, a contraction of the collagen was sometimes still observed. Moreover, degradation of the collagen was found in all SCM cultures, but it was highest in those with NHBE or HBEp cells as epithelial component. No degradation and contraction of the collagen was observed in cultures that were solely composed of fibroblasts embedded in collagen and that were lacking the epithelial layer. Therefore, these matrix remodeling events can clearly be attributed to the bronchial epithelial cells.

As described in section 3.4.2.1, matrix metalloproteinases (MMP) are known to play a central role in matrix remodeling and degradation. While fibroblasts express a variety of proteins of the MMP family only some of them are also produced by epithelial cells, like MMP-9 and MMP-2. MMP-9 is elevated in the airways of COPD and asthma patients [(122)] and since these airway diseases are associated with remodeling of the connective tissue in the

airways, investigations on the role of MMP-9 in collagen contraction and degradation in the SCM cultures were performed by immunohistochemical staining.

MMP-9 expression was verified in both Calu-3 and NHBE cells. Moreover, positive staining signals were also found throughout the collagen matrix. In NHBE cells, the MMP-9 expression was restricted to the basal cell layer. Since the Calu-3 cells were characterized by a monolayered growth without basal cells, the staining pattern of MMP-9 differed in these cultures. However, interestingly the Calu-3 cells showed a clear polarization and MMP-9 expression was here also restricted to the basal side of the cells. (Figure 4-51).

There were no obvious differences in the staining intensities and accordingly the levels of MMP-9 expression seemed to be similar in SCM cultures of NHBE and Calu-3 cells. This suggests that MMP-9 is not responsible for the observed differences in collagen contraction and degradation and it can be concluded that there are other main inducers of the collagen remodeling events in SCM cultures that need further investigation.

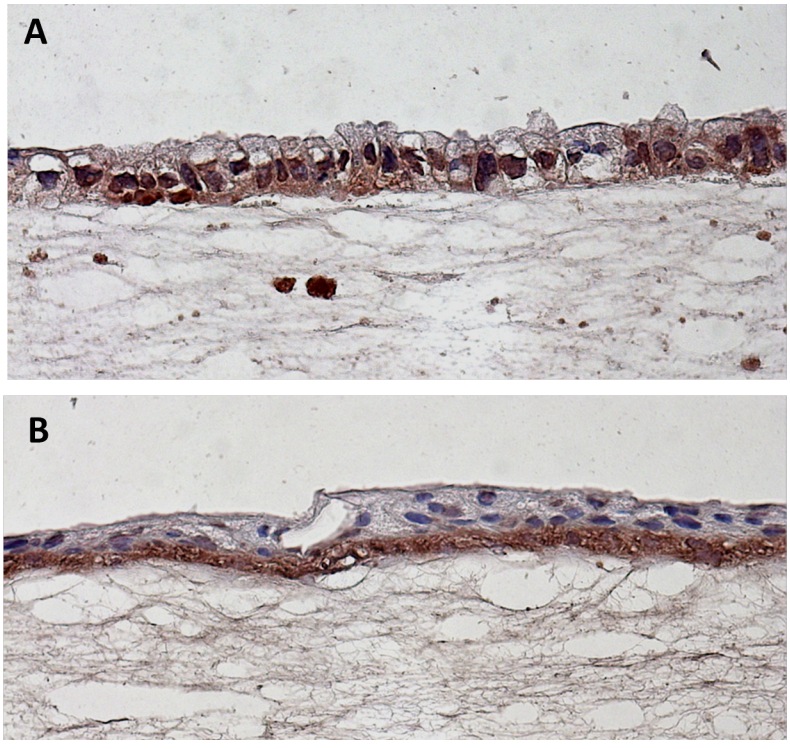

Figure 4-51: Immunohistochemical analyses of MMP-9 expression in SCM cultures prepared with Calu-3 (A) or NHBE cells (B). A: The expression is strongest on the basal side of the polarized Calu-3 cells and throughout the collagen matrix. B: MMP-9 expression is restricted to the basal cell layers and the collagen matrix, while no MMP-9 expression was detected in cells of the upper layer (Maginification: 400x).

Conclusions

Although the Sandwich Cell Model provide a good *in vitro* simulation of the bronchial epithelium regarding the structure and potential interactions between epithelial cells, fibroblasts and the extracellular matrix, its practicability and suitability was shown to be highly limited, due to the following reasons:

1. Contraction is a frequently occurring event when working with collagen matrices [177, 238]. Although matrices prepared from a 1:1 mixture of the Cellmatrix I-A and I-P collagen solutions highly increased the stability of the matrices, a contraction was nevertheless observed in many SCM cultures when using normal (non-transformed) human bronchial epithelial cells. While the differentiation of the epithelial cells was partially enhanced in SCM cultures with contracted matrices, the reproducibility was considerably reduced. Moreover, since the collagen shrinkage greatly reduced the culture surface (up to approximately 60 %), their suitability for exposure experiments is thus limited and the paraffin embedding for histological analyses was complicated. The induction of the collagen contraction was potentially donor- and passage-specific but it was also sometimes varying between inserts of the same culture (prepared from the same cells and cultivated under identical conditions) and therefore not predictable.

2. Degradation of the collagen was another common occurrence in SCM cultures. Histological sections revealed that this degradation was starting at the upper end of the collagen matrix, directly beneath the epithelial cells. Moreover it was not detected in cultures that were only composed of fibroblasts embedded in the collagen matrix. This clearly suggests that degradation is also mainly induced by the epithelial cells. The collagen degradation was another factor leading to a reduced reproducibility and stability of the SCM cultures. Moreover, the life span of the cultures was thus considerably decreased.

3. The differentiation ability of NHBE and HBEp cells was highly diminished in SCM cultures. A realistic structure and morphology comparable to the bronchial epithelium was not achieved under none of the tested culture conditions. Mucus secretion was only detected sporadically in a few cells and cilia were completely lacking in these cultures. Moreover, the cells showed an abnormal stratified growth. Therefore the suitability of these cultures to serve as a reliable *in vitro* model is restricted. Only the Calu-3 cells showed a normal growth

and differentiation in this co-culture model. But, Calu-3 cultures, in general, are not suited for long-term studies.

4. The application of several analytical methods is complicated or not feasible in the SCM co-culture system. For example, the two cell types can barely be monitored separately by *in situ* microscopy. Contraction and degradation additionally hinder the analysis by *in situ* microscopy and complicate the paraffin embedding procedure for histology. The determination of TEER values was found to be impracticable in SCM cultures with the Millicell ERS-2 Volt-Ohm Meter (Millipore). In addition, individual analyses of epithelial cells and fibroblast, like the measurement of their cell viability or gene expression analyses by RT-PCR are not feasible since they cannot be separated from each other. In summary, many commonly analyzed endpoints are not applicable and consequently the use of SCM cultures for toxicological testing is rather impracticable.

4.2.2 Compartment Cell Model (CCM)

In order to overcome the limitations of the SCM, another co-culture system composed of fibroblasts and epithelial cells was developed during this thesis. The principle of this 3D culture method was published by Pohl et al. in 2009. In contrast to the SCM, the two cell types are spatially separated in this co-culture model and can only interact via soluble factors that are released into the cell culture medium [(174)].

In the original protocol according to Pohl and colleagues, the two cell types are cultivated on opposite sides of the microporous membranes of cell culture inserts. This protocol was initially adopted in this thesis. Briefly: The insert was turned by 180 °C and the fibroblast suspension was applied on the basolateral side of the membrane. After an incubation time of approximately 3 hours to allow the fibroblast to adhere to the membrane, the inserts were repositioned in the wells of the receiver plate and the epithelial cell solution was added onto the apical side of the membrane which was previously coated with collagen type I or IV. The cultures were cultivated under submerged conditions until the epithelial cells formed a confluent cell layer. Afterwards, the epithelial cells were cultivated at the air-liquid interface while the fibroblast remained submerged in the cell culture medium (basic differentiation medium; Figure 4-52).

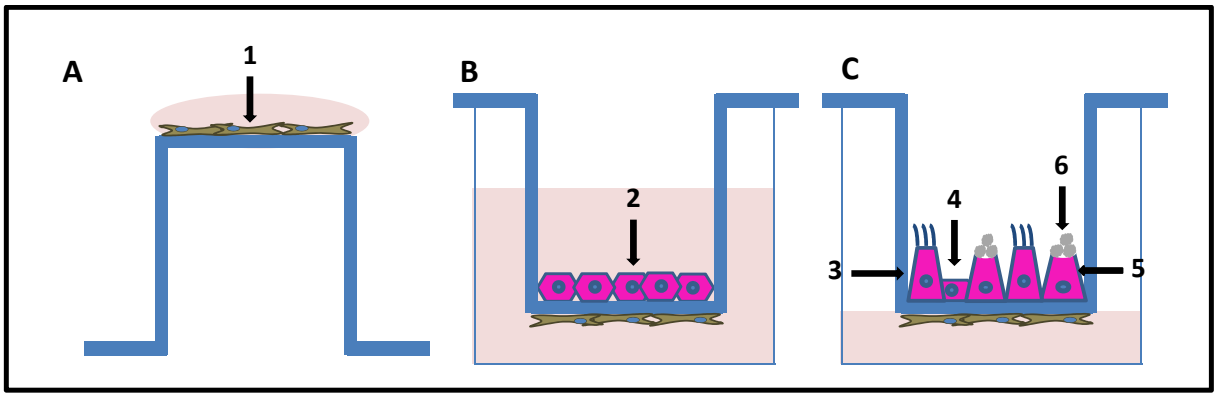

Figure 4-52: Schematic representation of the original protocol of the CCM (according to Pohl *et al.* 2009). **A**: The fibroblasts are seeded on the basolateral side of the microporous membrane of cell culture inserts. **B**: The epithelial cells are applied on the apical side of the membrane and thus, the two cell types are spatially separated but can interact through secreted molecules that are released into the cell culture medium. The cultures are cultivated under submerged conditions until the epithelial cells formed a confluent layer. **C**: The epithelial cells are afterwards cultivated at the air-liquid interface to promote their differentiation while the fibroblasts remain submerged in the culture medium (1= Fibroblasts; 2= Undifferentiated epithelial cells; 3= Ciliated cell; 4= Basal cell; 5= Goblet cell; 6= Mucus).

It became soon evident that this co-culture model is not suitable for long-term cultivation. Histological sections revealed a gradual detachment of the fibroblasts from the membrane in all cultures starting already after a cultivation time of eight to twelve days. In some cases, the detached fibroblasts were removed during the medium renewal, but mostly they adhered to the bottom of the multiwell plate and grew there. Thus, they were still able to interact biochemically via the cell culture medium.

The latter observation gave rise to the CCM *variant I,* a modified protocol of the CCM, which enhanced the co-culture system with regard to an easier preparation and more convenient handling. Here, the IMR90 fibroblasts were seeded on the bottom of 12-well receiver plates at a density of approx. 1 x 10^4 cells/well and supplied with their specific growth medium (EMEM including 10 % FBS). The epithelial cells were applied onto the membranes of cell culture inserts (coated with collagen I or IV) and cultivated under conventional, cell line-specific conditions in a separate receiver plate. After the epithelial cells nearly reached 100 % confluency, the inserts were transferred to the fibroblast-containing wells and the growth medium was replaced by differentiation medium. After another two days, the air-liquid interface was established to promote the differentiation of the epithelial cells (Figure 4-53).

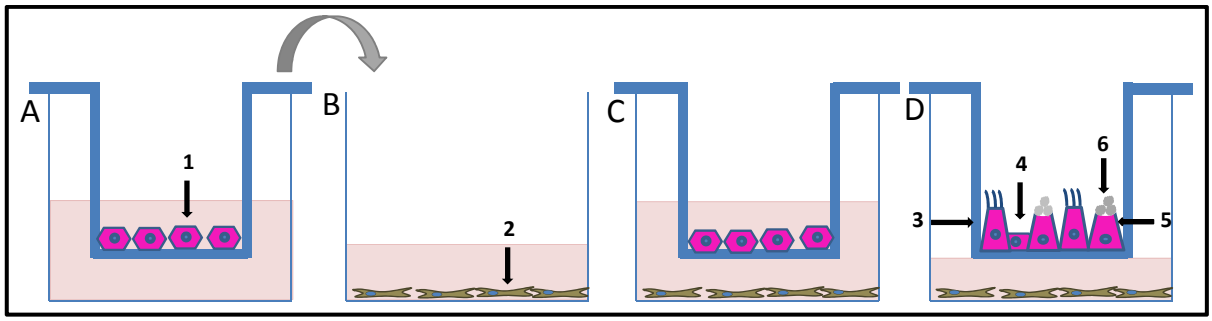

Figure 4-53: Schematic representation of the CCM *variant I*. **A**: The epithelial cells are seeded on microporous membranes of cell culture inserts and cultivated under conventional, cell-specific conditions until reaching nearly 100 % confluency. B: The fibroblasts are seeded on the bottom of insert receiver plates and cultivated separately under cell-specific conditions for several days. C: The inserts with the epithelial cells are placed into the fibroblast-containing receiver plate and the growth medium is replaced by the differentiation medium, but the cultures are still cultivated under submerged conditions for another 2 days. **D**: The epithelial cells are afterwards cultivated at the air-liquid interface to promote their differentiation while the fibroblasts remain submerged in the culture medium (1= Undifferentiated epithelial cells; 2= Fibroblasts; 3= Ciliated cell; 4= Basal cell; 5= Goblet cell; 6= Mucus).

For comparison, monocultures of both cell types were cultivated under identical conditions. The protocol was tested with NHBE cells and IMR90 fibroblasts or the Calu-3 cell line with IMR90.

Histological sections of NHBE cells cultivated in CCM *variant I* cultures revealed that this co-culture system promoted their differentiation. Ciliogenesis was observed earlier and was more pronounced in co-cultures than in the corresponding monocultures of the NHBE cells (Figure 4-54).

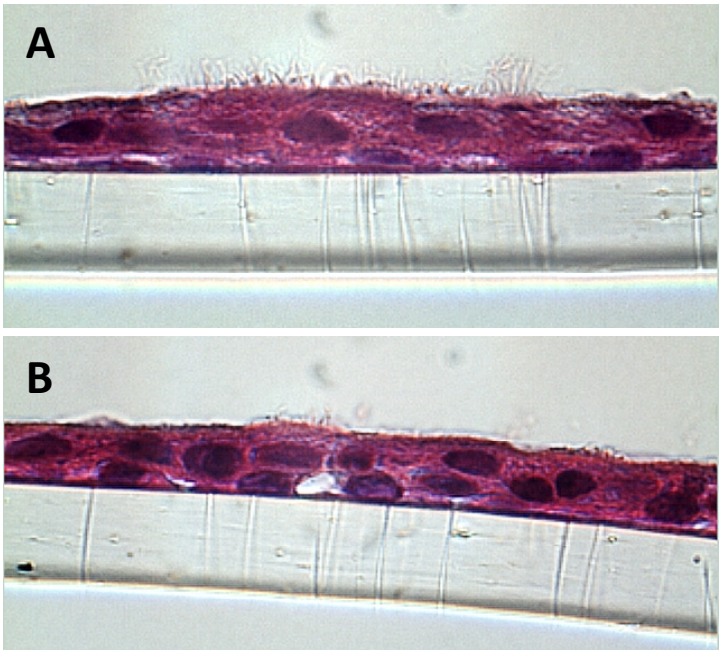

Figure 4-54: Differentiated NHBE cells that were either cultivated in CCM *variant I* co-cultures with IMR90 fibroblasts (A) or as monoculture (B) for 24 days (19 days under ALI conditions). The ciliogenesis was enhanced in co-cultures (HE stained histological sections; magnification: 1000x).

Moreover, the TEER values were considerable higher when the NHBE were co-cultivated with fibroblasts in CCM *variant I* co-cultures, indicating the presence of an enhanced barrier function in these cultures (Table 4-3). The inserts were placed in new receiver plates for TEER measurement, to exclude influences of the fibroblasts on the TEER values.

Table 4-3: Comparison of TEER values of each three inserts of NHBE cells either cultivated in CCM *variant I* co-cultures with IMR90 fibroblasts or as monoculture after a cultivation time of 19 days.

	NHBE in co-culture with IMR90	NHBE monoculture	Culture age (days)
TEER value [Ω/cm2]	>900	<600	19

In Calu-3 cells no considerable differences in cell morphology or their differentiation ability were observed between CCM *variant I* co-cultures and monocultures. However, when regarding the TEER values, it was shown that the development of tight junction was here also enhanced in the co-cultures.

Table 4-4: Comparison of TEER values of Calu-3 cells either cultivated in CCM *variant I* co-cultures with IMR90 fibroblasts or as monoculture after a cultivation time of 15 and 29 days. One insert each was analyzed.

	Calu-3 in co-culture with IMR90	Calu-3 monoculture	Culture age (days)
TEER value [Ω/cm^2]	1733	763	15
	3047	2192	29

Overall, interactions between fibroblasts and bronchial epithelial cells were clearly observed in the CCM *variant I.* The fibroblasts seemed to promote and enhance the differentiation of the epithelial cells in both cancer-derived and normal cells. However, a highly critical point for this co-culture system was the frequent replacement of the fibroblast before they reached 100 % confluency. Confluent fibroblasts express genes that are involved in cellular senescence, like the transcription factor p16 [(243)]. Under these conditions, the secretion products of the fibroblasts might be altered and consequently their effect on the epithelial

cells possibly differs. Indeed, alterations in cell morphology or structure were observed in NHBE cells in CCM *variant I* cultures when the fibroblast formed a confluent cell layer. To overcome this difficulty, the fibroblasts were frequently replaced by a new receiver plate with sub-confluent fibroblasts before complete confluency was reached. The inserts with the epithelial cells were transferred to this new plate one to three days after seeding the fibroblasts.

However, this procedure hindered the continuous monitoring and analysis of the fibroblasts over the complete cultivation time. It is conceivable that the interactions between fibroblasts and epithelial cells are reciprocal and might therefore also have an effect on the fibroblasts. Moreover, a frequent replacement of the “old” with “new”, unaffected fibroblasts might disturb the ongoing process of interactions between the two cell types.

These considerations, led to the development of a second modified protocol of the CCM, the CCM *variant II.*

Here, the fibroblasts were embedded in a collagen matrix, similar to the SCM, allowing a three-dimensional growth of the cells. The collagen matrix was prepared according to the same protocol as for SCM cultures (with Cellmatrix I-A and I-P). The collagen/fibroblast solution was poured into the wells of the receiver plate and incubated for approx. 3 h at 37 °C until the polymerization of the collagen was completed. Afterwards these fibroblast cultures were cultivated with the specific growth medium (EMEM (IMR90) or RPMI (NHLF) incl. 10 % FBS) until starting the co-cultivation. The cell culture inserts were placed into the receiver plate when the epithelial cells nearly formed a confluent cell layer and the co-cultures were supplied with differentiation medium under submerged conditions for one day. The inserts were then raised to the air-liquid interface (Figure 4-55).

The studies on the CCM *variant II* cultures were accompanied by monocultures of the respective epithelial cell line and of collagen-embedded fibroblasts, as a reference.

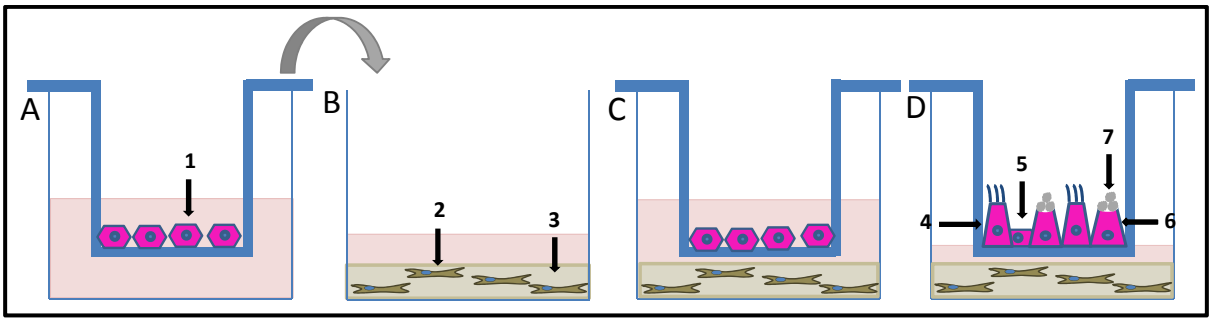

Figure 4-55: Schematic representation of the CCM *variant II*. A: The epithelial cells are seeded on microporous membranes of cell culture inserts and cultivated under conventional, cell-specific conditions until reaching nearly 100 % confluency. B: The fibroblasts are solved in a pH neutral collagen solution and applied on the bottom of insert receiver plates. After polymerization of the collagen (37 °C, approx. 3 h), they are supplied with their cell-specific growth medium and cultivated separately for two to seven days. C: The inserts with the epithelial cells are placed into the fibroblast-containing receiver plate and the growth medium is replaced by the differentiation medium, but the cultures are still cultivated under submerged conditions for another day. D: The epithelial cells are afterwards cultivated at the air-liquid interface to promote their differentiation while the fibroblasts remain submerged in the culture medium (1= Undifferentiated epithelial cells; 2= Fibroblasts; 3= Collagen matrix; 4= Ciliated cell; 5= Basal cell; 6= Goblet cell; 7= Mucus).

In initial approaches, the volume of the collagen/fibroblast solution was optimized, in order to form a uniform matrix within the wells while leaving an appropriate space between the matrix and the insert membrane for medium supply. The optimal volume was 270 µL.

The nutrition supply was found to be a critical factor in the CCM *variant II*. The capacity of the wells was reduced due to the collagen matrix, which was especially problematic during the ALI cultivation. A lack of nutrition indicated by a rapid acidification of the medium was often observed in initial approaches, leading to a reduced differentiation ability of the epithelial cells. However, if the medium level was too high, it penetrated the epithelial layer and hindered the ALI cultivation and accordingly also decreased the differentiation ability of these cultures. Therefore, the medium volume was optimized in a stepwise process and was found to be appropriate in a range of 550 – 580 µL. The medium was renewed every 48 h and care was taken to avoid damage of the collagen matrix.

The collagen matrix was stable over several months and no contraction was observed. The fibroblasts, either NHLF or IMR90, showed a continuous growth and normal spindle-shape morphology (Figure 4-56). Due to the 3-dimensional growth, they nevertheless did not form a confluent layer and accordingly contact inhibition was prevented.

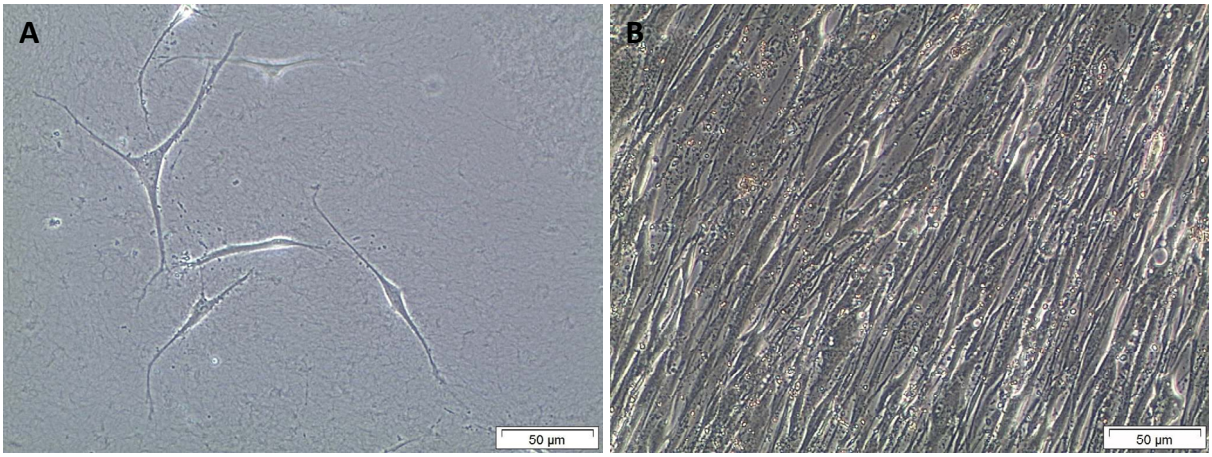

Figure 4-56: NHLF embedded in a collagen matrix and grown on the bottom of 12-well receiver plates. **A**: NHLF in collagen two days after seeding. **B**: NHLF in collagen after a cultivation time of 29 days in a CCM *variant II culture* with NHBE cells.

Similar to the SCM, degradation of the collagen was also observed in these cultures, starting after approx. 3 weeks. While this degradation had no obvious adverse effects on the fibroblasts or epithelial cells, it hindered the observation of the fibroblasts by *in situ* microscopy. Interestingly, the degradation was partially prevented when no additional calcium chloride was added to the differentiation medium. Due to this observation, the basic differentiation medium was modified for all CCM *variant II* cultures by omitting the additional calcium chloride. This modification did not negatively influence the differentiation of the epithelial cells but was highly advantageous for the stability of the collagen matrix.

The CCM *variant II* co-culture system was first evaluated with different lots of NHBE cells and either IMR90 or NHLF cells. A normal differentiation pattern of the NHBE cells was observed in these cultures with a pseudostratified structure and columnar-shaped cells. Moreover, the cell population included ciliated, mucus-producing and basal cells (Figure 4-57).

The cultures were stable for at least three months. Their exact life span has not been investigated; however, since no differences in cell viability and morphology were observed after 12 weeks, it is likely that they can be used for long-term experiments beyond three months.

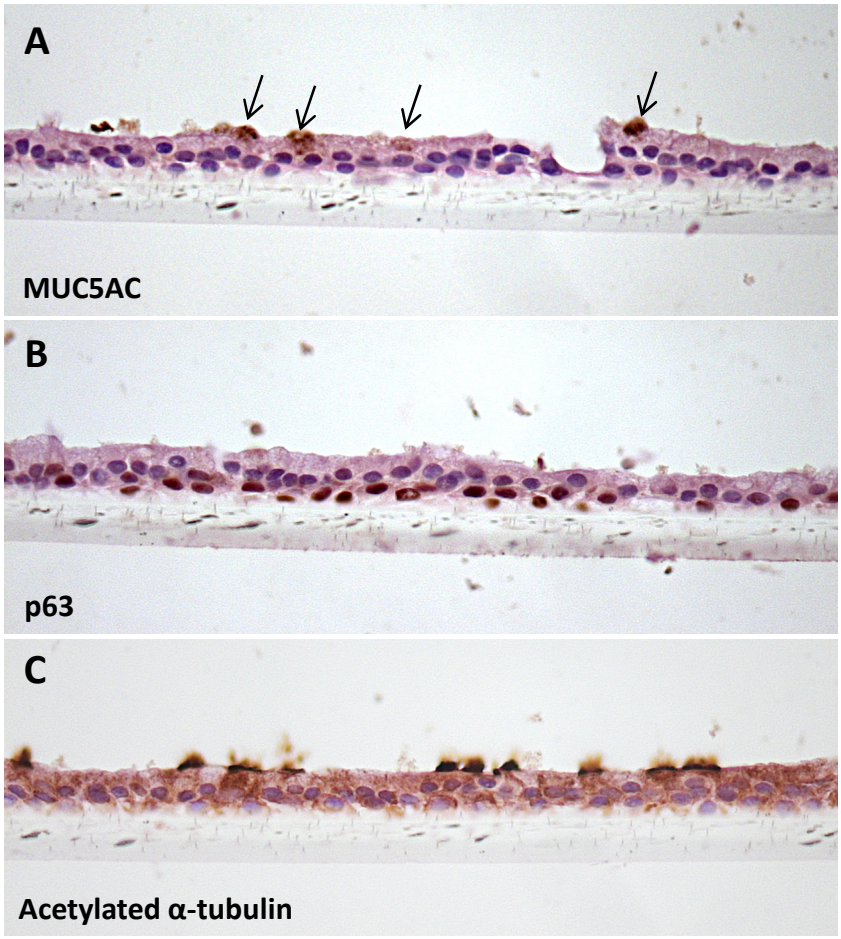

Figure 4-57: NHBE cells (P01) cultivated in CCM *variant II* co-cultures with NHLF after a cultivation time of 84 days (79 days under ALI conditions). The cell population exhibited MUC5AC secreting cells (A; arrows), p63 positive basal cells (B) and ciliated cells (C; cilia stained with acetylated α-tubulin; cell nuclei counterstained with hematoxylin; magnification: 400x)

The cultures were characterized by an extensive secretion and an enhanced development of cilia when compared to the respective monocultures. The epithelial cell layer was rinsed with PBS frequently (once a week) to partially remove the mucus that otherwise accumulated on top of the cultures. This procedure revealed that the viscosity of the secretion products varied between the mono- and co-cultures. The mucus of the co-cultures was characterized by a very high viscosity indicating an enhanced expression of gel-forming mucins, like MUC5AC and 5B. In some cultures the mucus layer could be retained after fixation and paraffin embedding and was thus visible in the histological sections. Immunohistochemical analysis showed a strong staining for MUC5AC in this mucus layer (Figure 4-58).

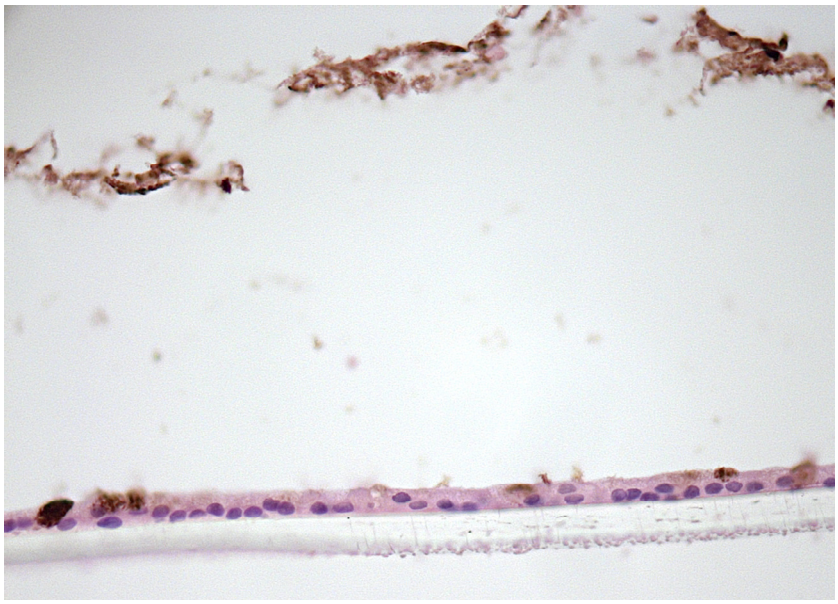

Figure 4-58: NHBE cells cultivated in CCM *variant II* co-culture with NHLF after a cultivation time of 84 days (79 days under ALI conditions). The culture showed an extensive mucus secretion and the mucus accumulated on top of the epithelial cells. This mucus layer could be preserved during fixation and paraffin embedding and was thus still visible in the histological section. Immunohistochemical analyses revealed a strong staining of MUC5AC in this mucus layer (the cell nuclei were counterstained with hematoxylin; magnification: 400x).

As stated in 4.1.2.4, monocultures of NHBE cells showed donor-specific differences in their growth pattern and differentiation properties. In some cultures, a pseudostratified structure with columnar-shaped cells was barely achieved. Moreover, the development of ciliated cells was rare in some lots of NHBE cells. In these cases it was shown, that the differentiation could be highly enhanced by co-culturing these cells with NHLF or IMR90 fibroblasts in CCM *variant II* cultures (Figure 4-59).

The ciliogenesis generally started earlier and was more pronounced in the CCM *variant II* co-cultures than in the corresponding monocultures. Immunofluorescence analysis on these co-cultures revealed the presence of a high number of ciliated cells which were uniformly distributed across the epithelial cell layer (Figure 4-60).

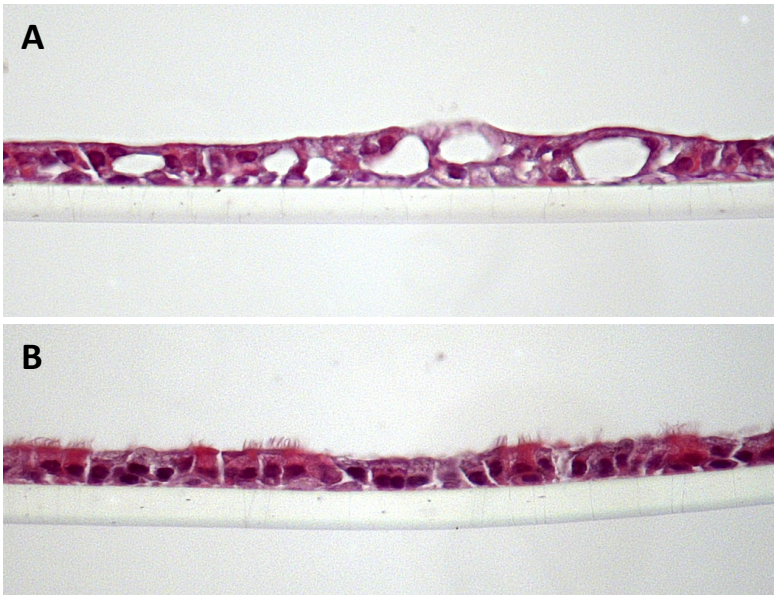

Figure 4-59: NHBE cells cultivated in monoculture (A; 29 days; 25 days under ALI conditions) and CCM *variant II* co-culture with IMR90 fibroblasts (B; 38 days; 33 days under ALI conditions). NHBE cells of the same donor and passage were used and the cultures were cultivated under identical conditions (HE stained histological sections; magnification: 400x).

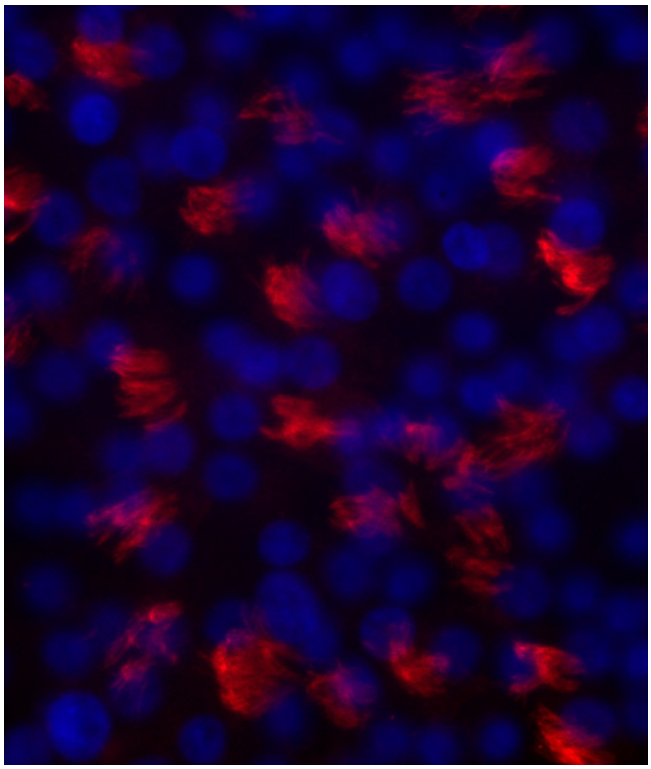

Figure 4-60: Immunofluorescence analysis of NHBE cells (P01) in CCM *variant II* co-cultures with IMR90 after a cultivation time of 55 days. Cilia are stained with acetyl-α-tubulin (red) and cell nuclei with DAPI (blue; magnification: 400x).

The barrier function of the NHBE cell layer through the development of tight junctions was furthermore enhanced in the CCM *variant II* co-culture system. The TEER values were generally significantly higher in the co-cultures than in NHBE monocultures. Examples are given in Table 4-5.

Table 4-5: Comparison of TEER values of NHBE cells either cultivated in CCM *variant II* co-cultures with NHLF fibroblasts or as monoculture (average of three inserts each ±SD).

	NHBE in co-culture with NHLF (n=3)	NHBE monoculture (n=3)	Culture age (days)
TEER value [Ω/cm^2]	2349 (± 191)	896 (± 39)	27
	2016 (± 57)	1058 (± 182)	39

After the comprehensive evaluation of the CCM *variant II* co-culture system for the cultivation of NHBE cells, its suitability was further tested with Calu-3 cells. Here, generally no remarkable differences in cell morphology and differentiation were detected in histological sections of mono- and co-cultures.

The CCM *variant II* prepared with Calu-3 resulted in cultures that were stable for at least four weeks, a life span which is comparable to Calu-3 monocultures. However, when grown as monocultures, Calu-3 cells often showed a flattened morphology when reaching the end of their culture life span. On the contrary, in the CCM *variant II*, the Calu-3 cells still exhibited a normal, columnar morphology after 29 days (Figure 4-61). Although the exact life span of Calu-3 cells in CCM *variant II* cultures was not yet investigated, the results suggest that this co-culture system might prolong the cell viability of these cells.

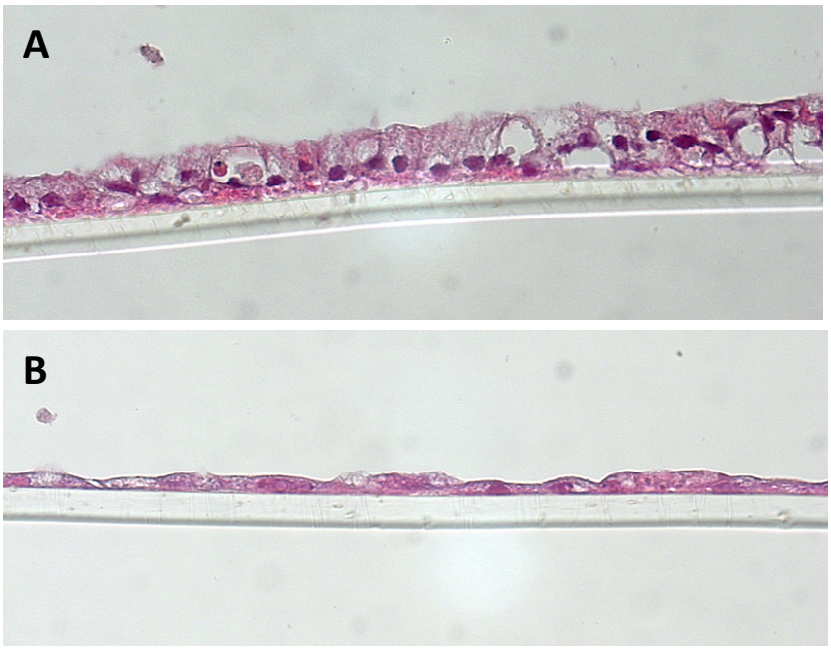

Figure 4-61: Calu-3 cells cultivated in CCM *variant II* cultures with IMR90 (A) or as monoculture (B) after a cultivation time of 29 days (23 days under ALI conditions; HE stained histological sections; magnification: 400x).

A positive effect of the cultivation of Calu-3 cells in CCM *variant II* cultures was furthermore suggested when regarding the TEER values. Higher values were consistently observed in Calu-3 co-cultures than in Calu-3 monocultures, indicating an enhancement of the formation of barrier properties by the fibroblasts. Examples are given in Table 4-6.

Table 4-6: Comparison of TEER values of Calu-3 cells either cultivated in CCM *variant II* co-cultures with IMR90 fibroblasts or as monoculture. One insert each was analyzed.

	Calu-3 in co-culture with IMR90	Calu-3 monoculture	Culture age (days)
TEER value [Ω/cm^2]	1392	2065	29
	790	1590	18
	916	1257	26

Conclusions

The results highlight the suitability and usefulness of the CCM *variant II* co-culture system to serve as a relevant *in vitro* model of the airway epithelium, due to the following reasons:

1. It was clearly demonstrated that interactions between fibroblasts and bronchial epithelial cells - normal cells as well as Calu-3 - were enabled in the CCM *variant II,* leading to an enhanced differentiation of the epithelial cell layer. These results suggest that the presented co-culture model provides a more realistic *in vitro* replica than monocultures by taking into consideration the reciprocal interactions taking place in the EMTU. *In vivo* these complex interactions between fibroblasts, ECM and epithelial cells play a pivotal role in inflammation response and wound healing [(244)]. Accordingly, the CCM *variant II* might provide a valuable model for toxicological testing *in vitro*.

2. The surrounding collagen matrix allows a 3-dimensional growth of the fibroblasts. Thus, a continuous proliferation is enabled without reaching complete confluency. Accordingly, the fibroblasts can also be cultivated for several months and monitored throughout the complete cultivation time of the CCM *variant II* cultures. The use of collagen as scaffold for the 3D cultivation furthermore provides a more realistic environment for the fibroblasts and might support their natural physiological properties.

3. In contrast to the SCM, the CCM *variant II* allows the separate analyses of both cell types - epithelial cells and fibroblasts - with all commonly used analytical methods, such as ELISA, Western Blot, immunofluorescence and cell viability assays. The production of histological sections of the epithelial layer was also straightforward and the determination of TEER values was feasible in these cultures. This point further supports the usefulness of the CCM *variant II* cultures for toxicological assessments.

4.3 Stimulation of NHBE cells by special additives in the culture medium

The most important requirement of suitable *in vitro* models of the airway epithelium for toxicological testing is a realistic response, including natural signaling pathways, after the exposure to selected molecules or stimuli. Moreover, pathologic features, as goblet cell hyperplasia or squamous metaplasia, should ideally be inducible in these cultures in order to provide *in vitro* models of diseased airways which most likely behave differently in exposure studies.

In this thesis, a squamous metaplasia-like phenotype was occasionally observed in mono- or SCM co-cultures prepared with NHBE cells or HBEpC. These cultures showed a stratified

growth with one or more layers of p63 positive basal cells covered by multiple layers of flat, metaplastic epithelial cells which showed strong expression of the squamous cell markers CK6 and CK13 (Figure 4-62). A similar structure was found in human and rat lungs exhibiting squamous metaplasia [245-246].

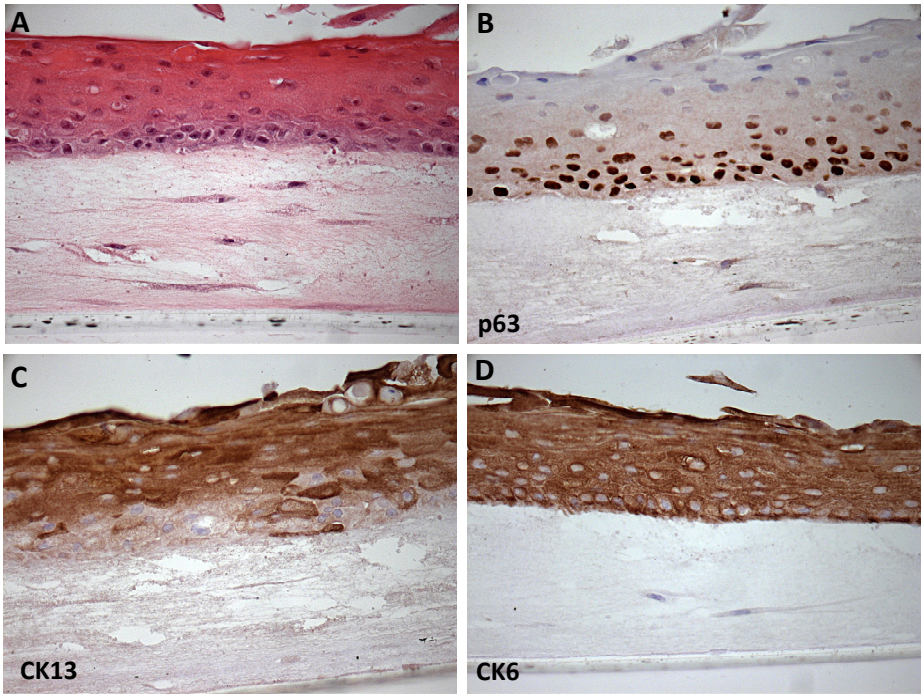

Figure 4-62: SCM culture of HBEpC 908 cells (P03) with IMR90 fibroblasts after a cultivation time of 19 days (14 days under ALI conditions). The epithelial cells show a stratified growth (A; HE stained histological section) with multiple layers of p63 positive basal cells (B). The upper layers are characterized by cells with a squamous morphology which show a high expression of the squamous cell markers CK13 (C) and CK6 (D; the cell nuclei were counterstained with hematoxylin; magnifications: A-C: 630x; D: 400x)

This structural alteration was consistently observed in all cultures prepared with the commercial HBEpC 812 and could thus clearly be attributed to the cell line in this case (see also section 4.1.2.1).

However, in other cases, the altered phenotype was only achieved sporadically while other cultures prepared with the same cells (same donor and passage) that were cultivated under identical conditions showed a normal differentiation with ciliated, mucus-producing and basal cells (Figure 4-63). In these cultures, the factors leading to the development of the stratified growth and squamous morphology of the cells could not be determined and this altered phenotype was thus not specifically inducible.

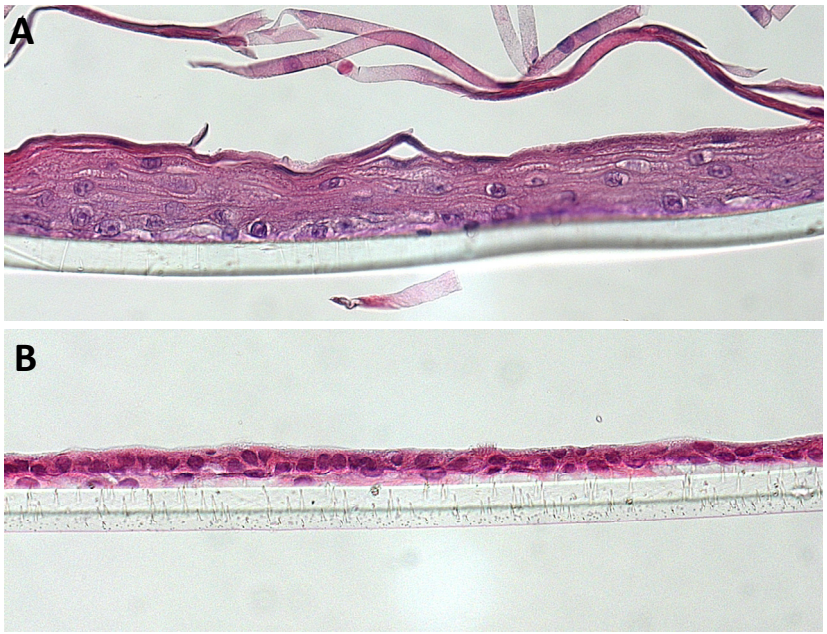

Figure 4-63: Monocultures of NHBE cells, lot 005 (P02) cultivated under identical conditions for 23 days (19 days under ALI conditions). A: In some insert cultures, a stratified growth, squamous morphology and keratinization of the NHBE cells was detected. B: In other insert cultures, the NHBE cells showed a normal growth and differentiation pattern (HE stained histological sections; magnification: 400x)

In order to find conditions that reproducibly lead to the development of the pathological phenotype of squamous metaplasia or goblet/ basal cell hyperplasia, different modifications of the cell culture medium were tested by changing concentrations of single components or by adding special signaling molecules that are associated with airway diseases.

4.3.1 Effects of retinoic acid deficiency in NHBE cultures

Retinoic acid (RA) deficiency is described to induce squamous metaplasia in airway epithelial cells both *in vitro* and *in vivo* (see 3.5.4) [(195)]. In the present study, the lack of RA in the cell culture medium was shown to induce diverse responses in the NHBE cell cultures. While in some cultures a squamous metaplasia-like growth and cell morphology was observed, others did not reveal any differences compared to the control cultures that were supplied with 100 nM RA. These cultures showed a rather normal differentiation into ciliated- and mucus producing cells and did not show a flattened morphology also under RA deficiency (Figure 4-64). These observations suggest that the induction of squamous metaplasia by RA deficiency is strongly dependant on the respective cell line and thus it was rejected as universal factor for the induction of pathologic features of airway diseases in *in vitro* models.

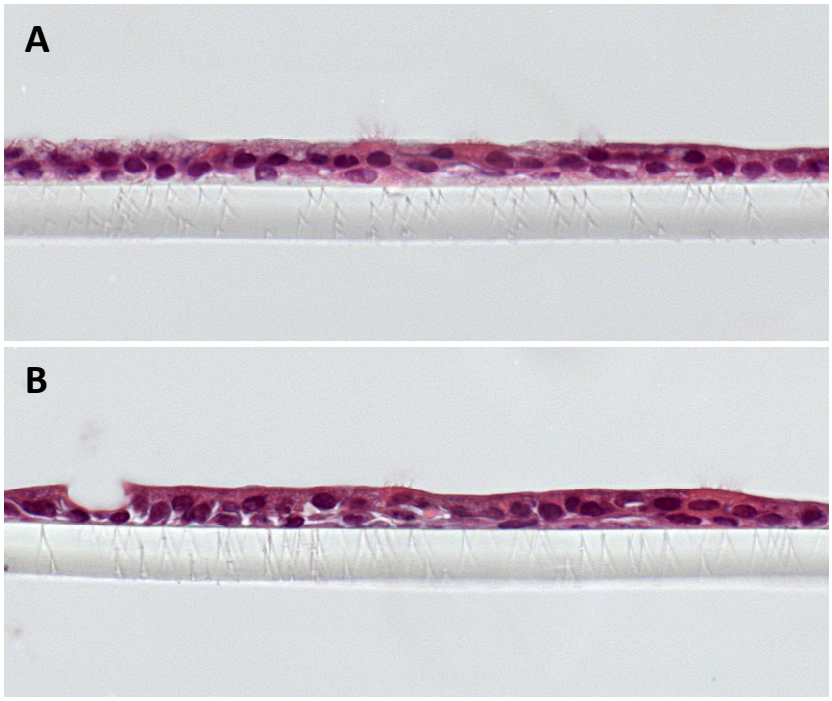

Figure 4-64: NHBE monocultures after a cultivation time of 30 days (26 days under ALI conditions). The cultures were either cultivated under the conventional culture conditions (with basic differentiation medium (**A**) or without retinoic acid in the differentiation medium (**B**). Ciliated cells were detected in both cultures (HE stained histological sections; magnification: 630x)

4.3.2 Effects of different EGF concentrations in NHBE cultures

The Epidermal Growth Factor (EGF) is important for the growth of epithelial cells *in vitro* and is thus an essential component of commercial cell culture complete media for airway epithelial cells, like the BEGM (Lonza). A concentration of 0.5 ng/mL is generally used and higher concentrations, like 10 or 25 ng/mL were shown to impair the normal differentiation of airway epithelial cells [9, 158, 200]. In this thesis, the effect of different EGF concentrations was tested in different lots of NHBE cells. As a starting point, a concentration of 250 ng/mL was evaluated, which is a 100-fold increase of the concentration used in some formulations of cell culture media for airway epithelial cells [9, 158]. In subsequent approaches, EGF concentrations of 50 and 40 ng/mL were tested. The control cultures were supplied with the basic differentiation medium which contained 0.5 ng/mL EGF. All analyses were performed using NHBE monocultures. The effect of 50 ng/mL EGF was additionally tested in NHBE cells that were co-cultivated with IMR90 fibroblasts in CCM *variant II* co-cultures. The stimulation of the cultures by increasing the EGF content in the cell culture medium was started at different time points of the cultivation. Until then, the cells were cultivated under

conventional culture conditions. The duration of the stimulation was also varied in the different approaches. Table 4-7 gives an overview of all experiments conducted in order to analyze the effect of elevated EGF concentrations.

Table 4-7: List of different approaches for the stimulation of NHBE cells with increased EGF concentrations. Two different NHBE cell lots (011 and 015) were stimulated with varying concentrations of EGF. The starting point (culture day) as well as the duration of stimulation (days) was varied. The stimulation of the NHBE015 cells with 50 ng/mL was performed with mono- and CCM *variant II* co-cultures (with IMR90 cells).

NHBE lot no.	EGF concentration [ng/mL]	Starting point (culture day)	Days of stimulation	Co-culture (CCM variant II)
011	250	9	20	N
015	50	13	22	Y
011	40	20	8	N

The results clearly demonstrated that the EGF concentration was a critical factor for the normal differentiation of NHBE cells and structural alterations are inducible by modifying the EGF content in the cell culture medium.

In the first approach, a 500-fold increased EGF concentration was applied. The starting point was set within the early differentiation phase of the NHBE cultures, only five days after the ALI cultivation was initiated. After 20 days of stimulation with 250 ng/mL, the NHBE cells showed a severe impairment of their normal differentiation. Histological sections revealed a stratified growth with flattened cells that started to detach in the upper layers (Figure 4-65). The cell morphology further suggested a strong cornification. In addition, the cultures had no measurable TEER.

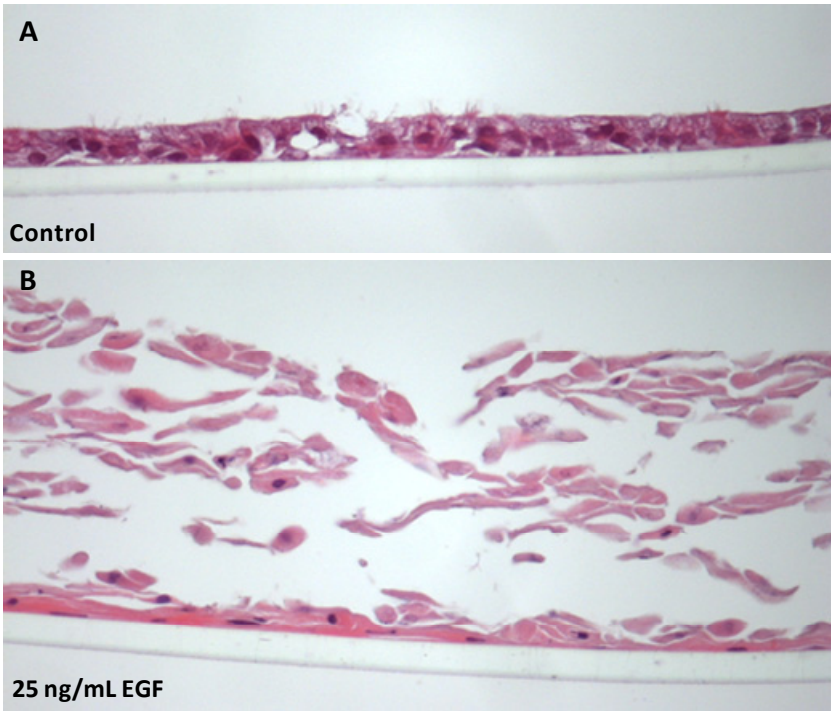

Figure 4-65**:** Differentiated NHBE cultures after a cultivation time of 29 days (25 days under ALI conditions). The cultures were either cultivated under conventional culture conditions (with basic differentiation medium; **A**) or with an increased EGF concentration of 250 ng/mL leading to a severe impairment of the normal differentiation (**B**; HE stained histological sections; magnification: 400x)

In the subsequent approach, the effect of 50 ng/mL EGF, an 100-fold increase of the standard concentration, was tested. Here, another lot of NHBE cells was used and CCM *variant II* co-cultures were additionally included. As a control, monocultures of fibroblasts embedded in a collagen matrix were also provided with the increased EGF concentration. The stimulation started at day 13 after seeding the epithelial cells, which was the ninth day of ALI cultivation. After 22 days of stimulation with the elevated EGF concentration, all NHBE monocultures showed an irregular growth pattern with several areas showing a multilayered (stratified) structure interspersed by areas with only one or two cell layers. The cells in the middle layers of the stratified areas showed a squamous morphology and stained positive for the squamous cell markers CK6 and CK13 (Figure 4-66).

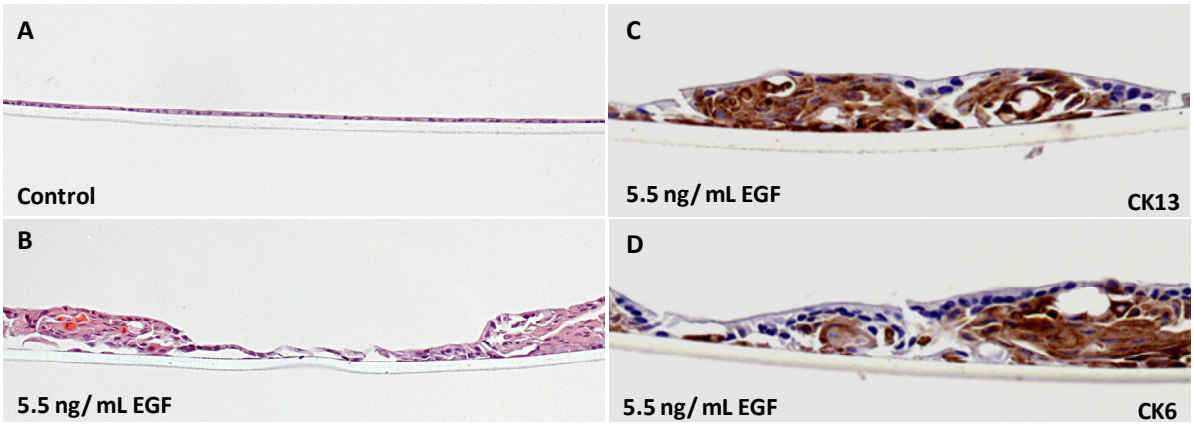

Figure 4-66: Differentiated NHBE cultures after a cultivation time of 35 days. The cultures were either cultivated under conventional culture conditions (with basic differentiation medium; A) or with an increased EGF concentration of 50 ng/mL (B-D). The stimulation with 50 ng/mL EGF resulted in the formation of stratified areas which were irregularly distributed over the culture surface [(B); A-B: HE stained histological sections; magnification: 200x]. The middle layers of these stratified areas were composed of squamous cells which showed a positive staining in immunohistochemical analyses of CK13 and CK6 [(C-D); the cell nuclei were counterstained with hematoxylin; magnification: 400x].

Interestingly, the increased EGF concentration had no obvious effect on the cell growth and morphology when the NHBE cells were co-cultivated with IMR90 in the CCM *variant II* co-culture system. This might be explainable by the fact that fibroblasts also possess EGF receptors (EGFR) that can bind EGF molecules and thus, less EGF is available that can be bound by the EGFR of the epithelial cells. *In vivo*, injured epithelial cells release a variety of factors, including EGF, to which fibroblasts respond by migration, contraction and proliferation. This activation of fibroblasts, in turn, is essential for the wound healing of the epithelium which is associated with structural alterations like hyperplasia or squamous metaplasia. Therefore, it is likely that these alterations will also be detectable in CCM *variant II* cultures, when increasing the time of EGF stimulation.

An impairment of the barrier function of the NHBE cells was already observable in both mono- and co-cultures, indicated by a decrease in the TEER values (Table 4-8).

Moreover, ELISA analysis revealed a slight increase of IL-8 secretion after stimulation with 50 ng/mL EGF in NHBE cells in both mono- and co-culture, as well as in IMR90 monocultures, compared to the respective non-stimulated control cultures (Figure 4-67). These results suggest an inflammatory response in both cell types after the stimulation with 50 ng/mL EGF.

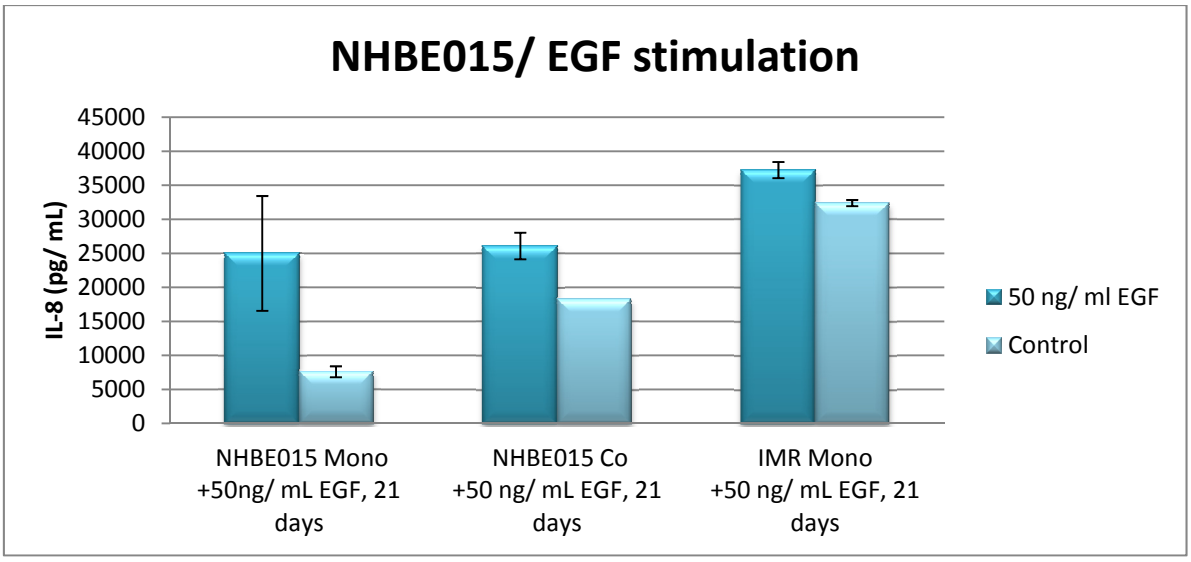

Figure 4-67: Results of ELISA analysis of IL-8 secretion (pg/mL) in NHBE015 mono- or CCM *variant II* co-cultures and IMR90 monocultures (embedded in collagen) after stimulation with 50 ng/mL compared to control cultures.

In a third approach, the EGF concentration was raised to 40 ng/mL, which is an 80-fold increase of the normal concentration. In contrast to the previous approaches, the cells were almost fully differentiated before the stimulation started, indicated by cilia beating and mucus secretion as observed by *in situ* microscopy. Moreover, the duration of the stimulation was reduced to eight days. Histological sections of the stimulated cultures revealed an altered phenotype with a stratified structure, which was uniformly distributed over the entire cultures. These alterations were found in all four inserts of the test cultures, suggesting a high reproducibility of the results (Figure 4-68).

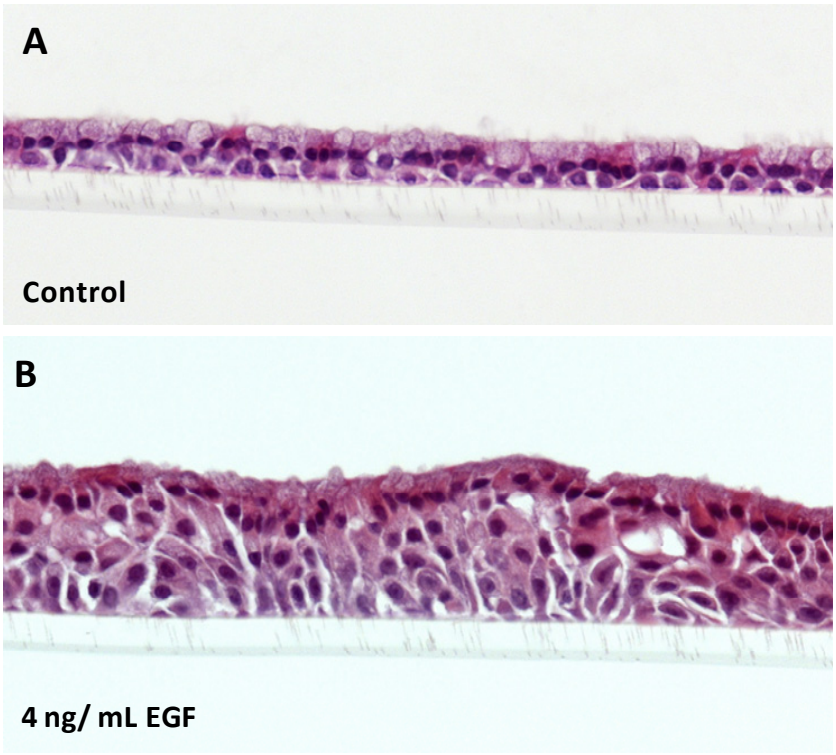

Figure 4-68: NHBE monocultures after a cultivation time of 28 days (24 days under ALI conditions). The cultures were either cultivated under conventional culture conditions (with basic differentiation medium; A) or with an increased EGF concentration of 40 ng/mL leading to basal cell hyperplasia (B; HE stained histological sections; magnification: 400x)

Immunohistochemical analyses with several markers consistently confirmed the existence of basal cell hyperplasia in the EGF-stimulated cultures (Figure 4-69). The basal cell marker p63 revealed the presence of multiple layers of basal cells (Figure 4-69 A-B). The superficial layer showed no positive staining of p63 but here a normal differentiation pattern with mucus-producing and ciliated cells was verified (Figure 4-69 C-F). This structure is typical for basal cell hyperplasia *in vivo*. Interestingly, immunohistochemical analysis of the proliferation marker Ki-67 showed positive signals only in a few basal cells, although the basal cell hyperplasia is generally induced by an increased proliferation of the basal cells (Figure 4-69 G-H). The fact that the Ki-67 marker was expressed only in a low number of basal cells might indicate that the development of the basal cell hyperplasia was completed in these cultures. *In vivo*, basal cell hyperplasia is believed to be an irreversible alteration but also a transitory state for the development of squamous metaplasia. Therefore there were three possible explanations for the low proliferative activity in the culture: (1) a steady state of basal cell hyperplasia was entered; (2) reversion of the basal cell hyperplasia was initiated; (3) a transition from basal cell hyperplasia to squamous metaplasia was in progress. The second

hypothesis is not likely, due to the fact that the EGF stimulation was still proceeding at the time point when the analyses were conducted. The induction of squamous metaplasia was also not indicated, since the squamous cell marker CK13 was only sporadically expressed in a few cells, which was similar to the CK13 staining pattern in the control cultures (Figure 4-69 I-J). However, considering the structural alterations found in the previous approach with 50 ng/mL, the later transition to squamous metaplasia is conceivable, when increasing the stimulation time.

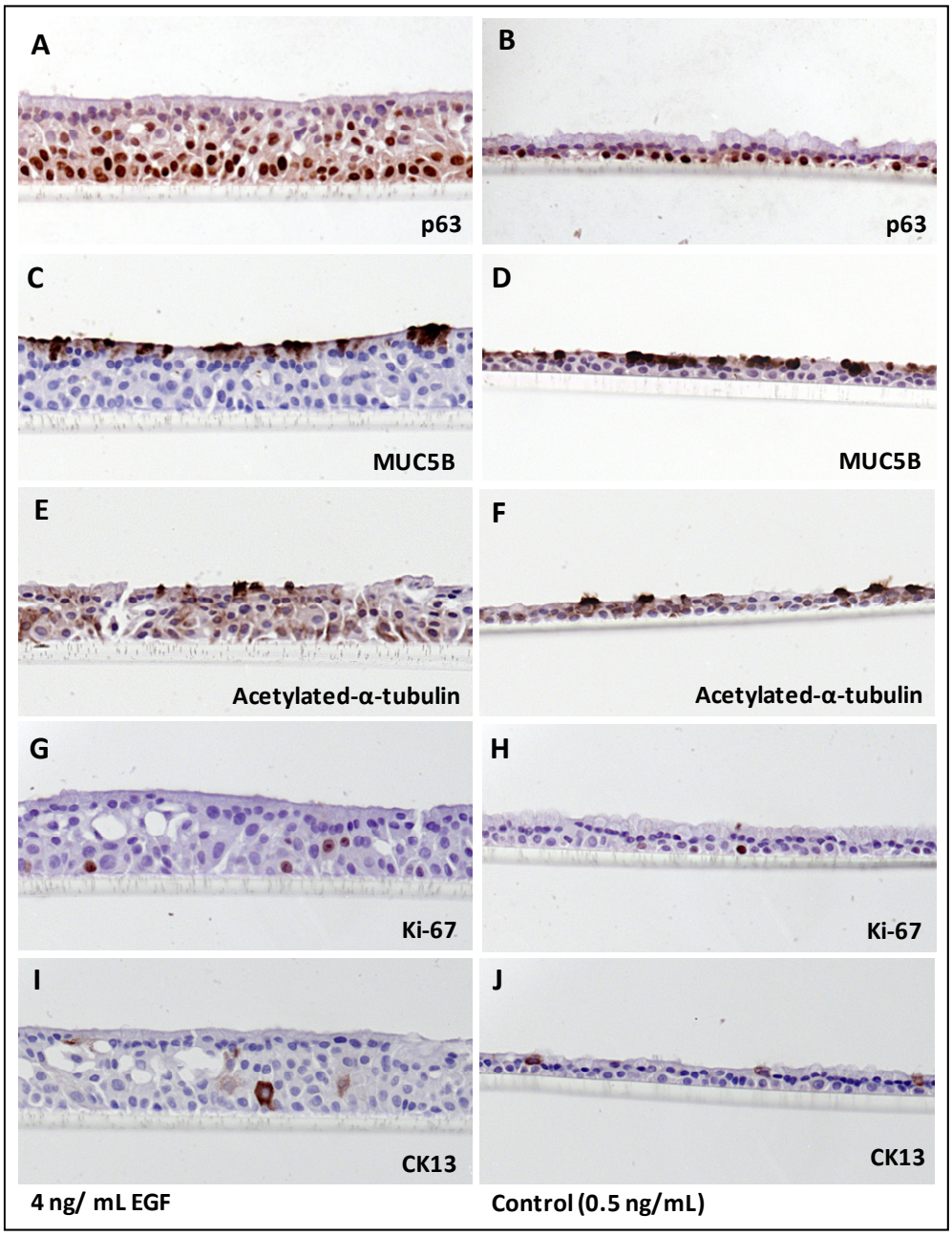

Figure 4-69: Immunohistochemical analyses of various markers in NHBE monocultures stimulated with 40 ng/mL and control cultures. Further information are provided in the main text (the cell nuclei were counterstained with hematoxylin; magnification: 400x).

The TEER values revealed a severe impairment of the barrier function properties of the NHBE cells after stimulation with 40 ng/mL EGF (Table 4-8).

Table 4-8: TEER values of NHBE cultures (cultivated as mono- or CCM *variant II* co-cultures) after stimulation with increased EGF concentrations compared to control cultures. The TEER values of the test cultures are listed for each insert individually, while those of the respective control cultures are shown as mean values with standard deviation (±).

Culture Type	EGF conc. [ng/mL]	Days of EGF stimulation	Culture age	TEER [Ω/cm2]	Control (Mean TEER; [Ω/cm2])
Mono (NHBE015)	50	22	35	1585 1317	2001 (± 268)
CCM variant II (IMR90, NHBE015)	50	22	35	1155 1395	1835
Mono (NHBE011)	40	8	28	487 528 518 496	1201 (± 16)

ELISA analyses showed again an increased secretion of IL-8 in the EGF-stimulated NHBE cultures, which was in congruence with the results of the previous experiment. However, both ELISA and TEER analyses also clearly demonstrated the existence of extensive donor-specific differences. The average TEER value of the non-stimulated monocultures of NHBE015 was 2001 Ω/ cm^2, while that of NHBE011 monocultures was 1201 Ω/ cm^2 (Table 4-8). The average IL-8 secretion of non-stimulated NHBE015 monocultures was 7597 pg/mL at a total cultivation time of 35 days and it was elevated to 24995 pg/mL after stimulation with 50 ng/mL EGF for 21 days (Figure 4-70). In comparison, non-stimulated NHBE011 monocultures revealed a low IL-8 secretion, in average of 214 pg/mL, which was increased to 1125 pg/mL after eight days of stimulation with 40 ng/mL EGF. These donor-specific variations might lead to varying effects of certain stimuli in different lots of NHBE cells and it is thus recommendable to test the reproducibility of the results by using different NHBE lots for each experiment in the future.

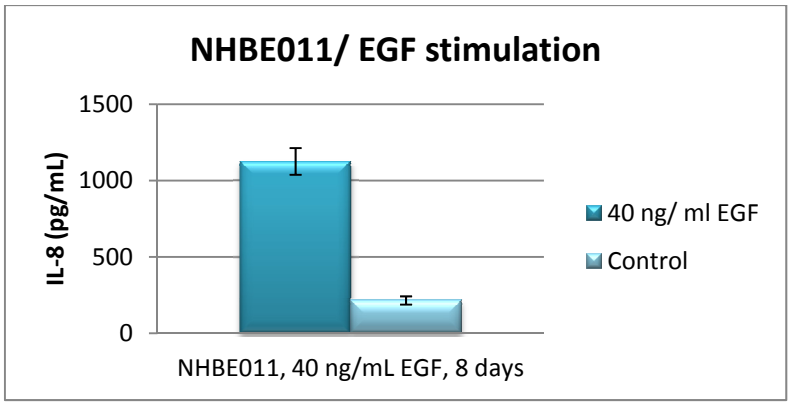

Figure 4-70: Results of ELISA analysis of IL-8 secretion (pg/mL) in NHBE011 monocultures after stimulation with 40 ng/mL compared to control cultures.

In summary, dependent on the concentration and/ or the donor, EGF either induced basal cell hyperplasia or metaplasia-like changes in NHBE cultures. The latter was only found in some distinct areas of the culture surface and here squamous cells were observed. The secretion of the proinflammatory cytokine IL-8 was shown to be increased and the barrier function property was impaired in both cases, suggesting a general effect of EGF on these two parameters.

4.3.3 Effects of TGF-ß1 stimulation in NHBE cultures

The Transforming Growth Factor-ß1 (TGF-ß1) has repeatedly been reported to cause squamous metaplasia-like alterations in several lung cell lines [117, 131]. In order to develop *in vitro* models showing such pathologic features, NHBE cultures were stimulated with different concentrations of TGF-ß1 and for varying time periods in this thesis. Based on the findings of Doerner and Zuraw (2009), which described morphological changes in NHBE cells after the exposure to 2 ng/mL for 3 days, this concentration was initial adopted in this study and the stimulation was performed for four days [128]. However, in these initial studies, no obvious morphological alterations could be observed between the cultures that were exposed to TGF-ß1 and the control cultures by histology and *in situ* microscopy. Moreover, the TEER values were also similar in both cultures.

Therefore, the concentration as well as the duration of exposure was increased in later approaches. Table 4-9 gives an overview of all stimulation experiments on NHBE cells with TGF-ß1.

Table 4-9: List of different approaches for the stimulation of NHBE cells with TGF-ß1. Four different NHBE lots (008, 011, 015 and 023) were stimulated with varying concentrations of TGF-ß1. The starting point (culture day) as well as the duration of stimulation (days) was varied. The stimulation of the NHBE008 and NHBE015 cells with 3 ng/mL was performed with mono- and CCM variant II co-cultures (with IMR90 cells).

NHBE lot no.	TGF-ß1 concentration [ng/mL]	Starting point (culture day)	Days of stimulation	Co-culture (CCM variant II)
008	2	22	4	N
011	4	20	8	N
023	4	22	7	N
008	3	13	13	Y
015	3	20	22	Y

It was shown that the TGF-ß1 stimulation of NHBE cells with all the tested variations of concentration and duration did not induce a squamous metaplasia-like change. Although alterations of the cell morphology from columnar to squamous were observed after stimulation with 40 ng/mL for seven or eight days in cultures of two different NHBE lots, no stratified structure resembling the squamous metaplasia *in vivo* was developed (Figure 4-71).

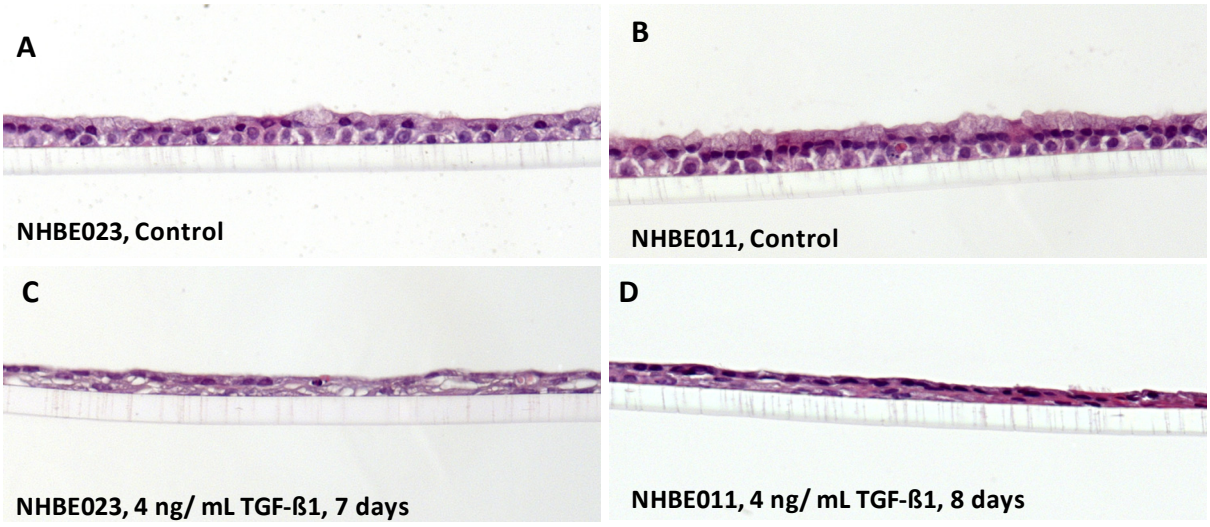

Figure 4-71: NHBE monocultures of two different lots (023 and 011) after a cultivation time of 28 (NHBE011) and 29 (NHBE023) days, respectively. The cultures were either cultivated under conventional culture conditions (with basic differentiation medium; A-B) or with the addition of 40 ng/mL TGF-ß1 for 7 (NHBE023; C) or 8 (NHBE011, D) days leading to alterations of the cell morphology from columnar to squamous (HE stained histological sections; magnification: 400x)

The stimulation with 3 ng/mL TGF-ß1 for 13 and 22 days in two different lots of NHBE cells resulted in the formation of stratified areas which were irregularly distributed over the culture surface. These areas also did not show the typical structure of squamous metaplasia, since they were composed of two to three layers of squamous cells and an upper layer with columnar cells (Figure 4-72). *In vivo*, the squamous metaplasia is characterized by an upper layer showing squamous cells and multiple underlying layers of proliferating cells which appear less flattened.

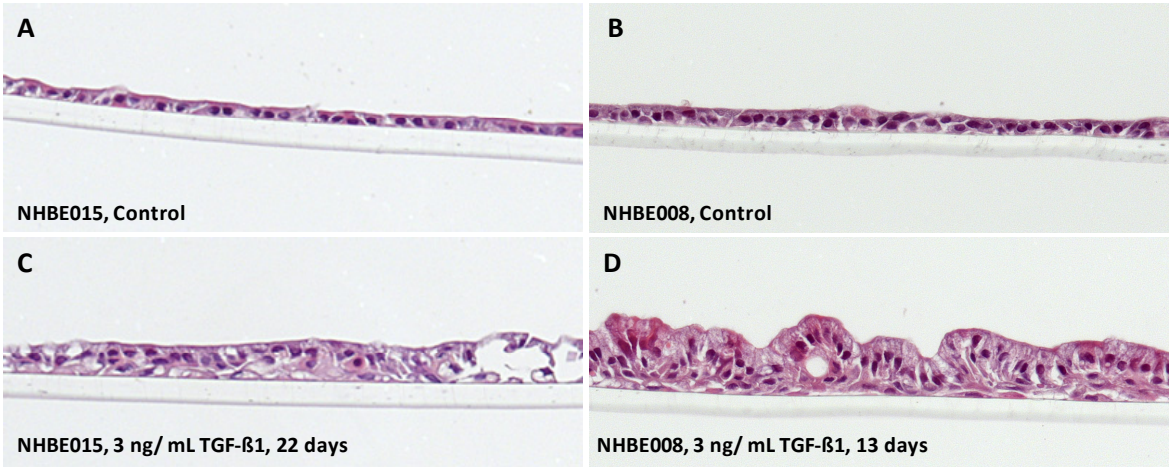

Figure 4-72: NHBE monocultures of two different lots (015 and 008) after a cultivation time of 26 (NHBE008) and 42 (NHBE015) days, respectively. The cultures were either cultivated under the conventional culture conditions (with basic differentiation medium; A-B) or with the addition of 3 ng/mL TGF-ß1 for 22 (NHBE015; C) or 13 (NHBE008, D) days leading to the formation of stratified areas with an upper layer composed of columnar cells and 2 – 3 underlying layers with squamous cells (HE stained histological sections; magnification: 400x)

The TEER values as well as the IL-6 and IL-8 secretion showed high donor-specific variations but did not deliver any concluding results and were thus not considered further.

The TGF-ß1 stimulation of NHBE cells co-cultivated with fibroblasts in CCM *variant II* cultures did not induce obvious morphological changes under the tested conditions. Comparable to the EGF stimulation, a potential explanation is that both cell types, NHBE and fibroblasts, have TGF beta receptors 1 which bind competitively the free TGF-ß1 molecules in the cell culture medium.

In conclusion, the stimulation of NHBE cells with TGF-ß1 resulted in morphological alterations in all different approaches. However, the cultures did not show characteristics of squamous metaplasia, as it was expected. The potential of TGF-ß1 to induce such metaplastic changes when modifying the conditions, with regard to the concentration and duration of the stimulation, needs further investigation.

4.3.4 Effects of IL-13 stimulation in NHBE cultures

Interleukin-13 (IL-13) is described to induce goblet cell hyperplasia *in vitro* [132-133, 247]. In the literature, there is different information on the IL-13 concentration leading to an increase of the number of goblet cells. In the present study, a concentration of 10 ng/mL IL-13 was tested for the stimulation of NHBE cells of two different lots (Table 4-10). In both experiments, the cultures were in the late differentiation stage (day 22), showing already some ciliated cells and mucus secretion, when starting the IL-13 stimulation.

Table 4-10: List of different approaches for the stimulation of NHBE cells with IL-13. Two different NHBE lots (008 and 023) were stimulated with 10 ng/mL IL-13. The starting point (culture day) as well as the duration of stimulation (days) was varied.

NHBE lot no.	IL-13 concentration [ng/mL]	Starting point (culture day)	Days of stimulation	Co-culture (CCM variant II)
008	10	22	15	N
023	10	22	7	N

In the first approach the cultures were prepared for histology after seven days of stimulation and the histological sections revealed that an increase of mucus-producing cells was already detectable at that time (Figure 4-73).

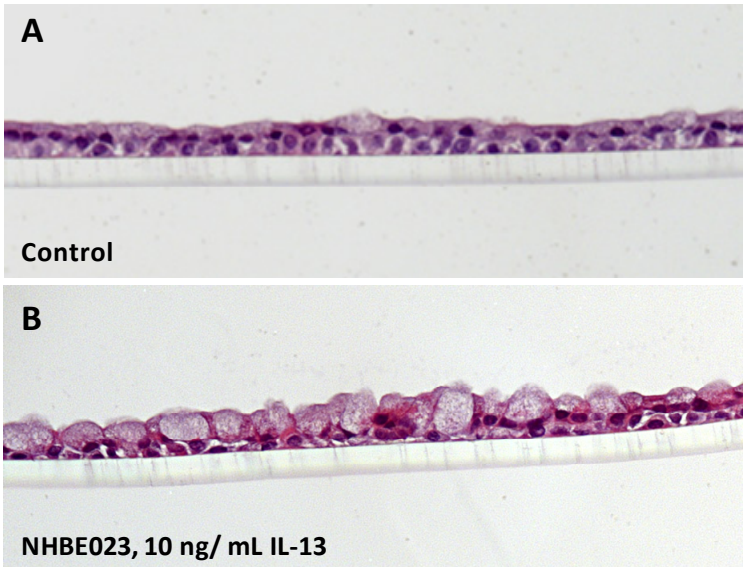

Figure 4-73: NHBE monocultures after a cultivation time of 29 days (24 days under ALI conditions). The cultures were either cultivated under conventional culture conditions (with basic differentiation medium; A) or with the addition of 10 ng/mL IL-13 for 7 days leading to goblet cell hyperplasia (B; HE stained histological sections; magnification: 400x)

In the second approach, the duration of the stimulation was prolonged to 15 days and here it was shown, that an extensive goblet cell hyperplasia was induced and a strong expression of mucin 5AC was observed by immunohistochemistry (Figure 4-74).

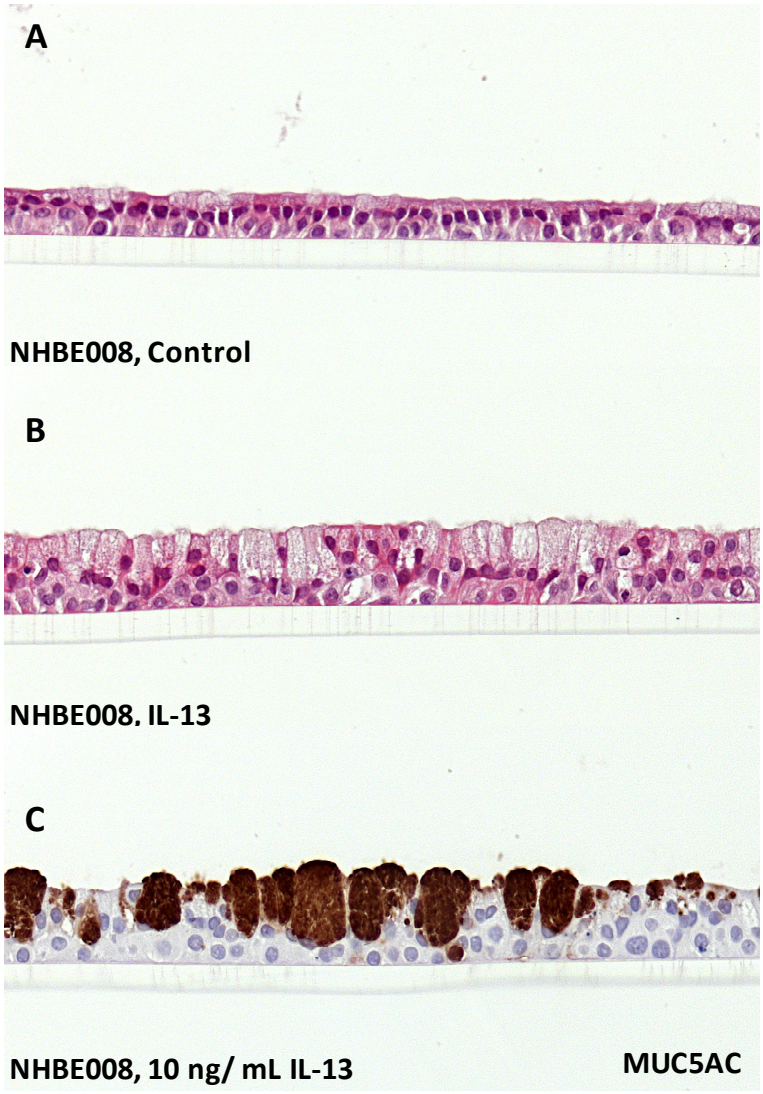

Figure 4-74: NHBE monocultures after a cultivation time of 37 days (32 days under ALI conditions). The cultures were either cultivated under conventional culture conditions (with basic differentiation medium; A) or with the addition of 10 ng/mL IL-13 for 15 days leading to goblet cell hyperplasia (B; A-B: HE stained histological sections; magnification: 400x). A strong staining of the mucin 5AC was verified in the IL-13 stimulated cultures by immunohistochemistry (C; the cell nuclei were counterstained with hematoxylin; magnification: 400x)

ELISA analyses revealed that there was no significant change in IL-6 and IL-8 secretion in the IL-13 stimulated cultures compared to the control cultures. The TEER values again showed donor-specific variations and did not deliver concluding results.

In summary, it was shown that the stimulation with 10 ng/mL IL-13 induces goblet cell hyperplasia in NHBE cells and that the extent can potentially be influenced by the duration of the stimulation. Since both tested lots of NHBE cells developed the hyperplastic change using the same concentration of IL-13, the reproducibility of the goblet cell hyperplasia in other lots under the same conditions is suspected.

Conclusions

Due to the fact that the prevalence of chronic airway diseases has increased in recent decades, studies on the function of inflamed airways has become more important. Inflammation of the airway epithelium impairs the barrier properties, including the mucociliary clearance and the formation of tight junctions, and thus alters the effects of inhaled substances. Accordingly, *in vitro* models simulating certain aspects of the airway epithelium under pathologic conditions could provide an important contribution for toxicological risk assessment.

Basal and goblet cell hyperplasia as well as squamous metaplasia are common pathologic features in chronic airway diseases and these lesions are described to play a critical role in the bronchial carcinogenesis [(248)].

In this thesis, the development of protocols for the induction of basal cell hyperplasia as well as goblet cell hyperplasia in NHBE cultures was achieved by applying special culture conditions. Thus, *in vitro* models simulating these pathologic conditions can be easily generated from “normal” NHBE cultures and used as a complement for toxicological evaluations of substances. Moreover, it was shown that squamous metaplasia might be inducible by the stimulation of the cells with appropriate concentrations of TGF-β1.

4.4 The CULTEX® Long Term Cultivation (LTC) System

The CULTEX® LTC system was recently developed by Cultex Laboratories GmbH in order to automate the cultivation of cell cultures grown on insert membranes at the air-liquid interface. The automated cultivation is a time-saving method and furthermore enables the standardization of cell culture conditions and accordingly the reproducibility of experiments. During this thesis, the suitability and usefulness of the CULTEX® LTC was tested with different cell lines of the human airways.

4.4.1 Material testing

In preliminary experiments, 16HBE14o- and A549 cells were cultivated in the prototype of the CULTEX® LTC. The cultivation system allows the control of the three insert positions individually, which means that the medium levels as well as the time intervals for the medium renewal and mixing can be adjusted in each chamber separately. For initial evaluation of the system and for determination of appropriate cell-specific conditions, the three parameters were varied arbitrarily for each insert position. For comparison, all runs were accompanied by control cultures of the respective cell line cultivated under conventional culture conditions (in receiver plates).

These preliminary approaches showed that none of the tested conditions produced viable cell cultures that were comparable to the control cultures. In situ microscopic analysis revealed that all A549 and 16HBE14o- cultures that were cultivated in the CULTEX® LTC prototype for 7 - 20 days exhibited an uneven cell layer with large gaps between single cell clusters (Figure 4-75).

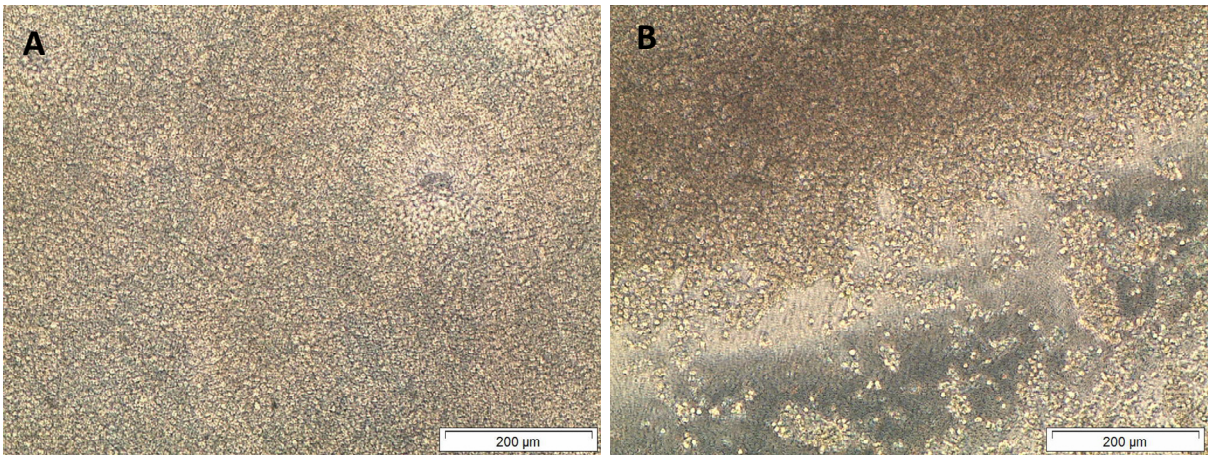

Figure 4-75: Inverse microscopical images of 16HBE14o- monocultures after a total cultivation time of 15 days. The cultures were either cultivated under conventional conditions with manual medium renewal (A) or in the CULTEX® LTC for 7 days with a computer-controlled medium renewal (B) every 48 h. The cultures that were cultivated in the LTC showed large gaps in the cell layer (B) while the control cultures had a normal, uniform cell layer (A).

Histological sections verified these observations. Here, it was shown that only few cells remained attached to the membranes while the control cultures showed a normal growth pattern and cell morphology. These results were frequently obtained with both A549 and

16HBE14o- cultures, even when the conditions in the LTC chambers were adjusted to those of the control cultures, with a medium renewal frequency of 48 h, a similar medium level and a suppression of the teflon membrane movement (see section 3.6.1). This led to the assumption that single material components might be the cause for the failure of the system rather than the cultivation process itself. To examine this hypothesis, all materials of the CULTEX® LTC that came into contact with the cell cultures or the medium during the cultivation were tested for cytotoxicity. For that purpose, an easy and efficient method for material testing was developed. In principal, this method is based on the "test by direct contact" which is described in the International Standard (ISO) 10993-5 for testing *in vitro* cytotoxicity of medical devices. Here, the cells are cultivated in direct contact to the test material. The main drawback of the original "test by direct contact" is that the test materials might have a physical impact on the cell cultures through their movement in the culture vessel. To overcome this, the samples were physically separated from the cell cultures in the newly developed method. This was enabled by placing the material samples onto the microporous membrane of a standard cell culture insert (6-well format) while the cells were grown in the wells of the receiver plate. In order to guarantee an unrestricted diffusion of all substances leaching from the material, the insert membranes were further perforated with a dissecting needle. The cell cultures were thus in contact with the test materials via the medium and their cytotoxic potential could be analyzed both qualitatively and quantitatively.

The qualitative analysis was performed by *in situ* microscopy and the quantitative by a commercial cell viability assay - either the CellTiter Blue Cell Viability Assay (Promega GmbH) or the WST-1 (Roche Diagnostics). The protocol for the material testing procedure is described in detail in section 6.1.4 and was published recently [249].

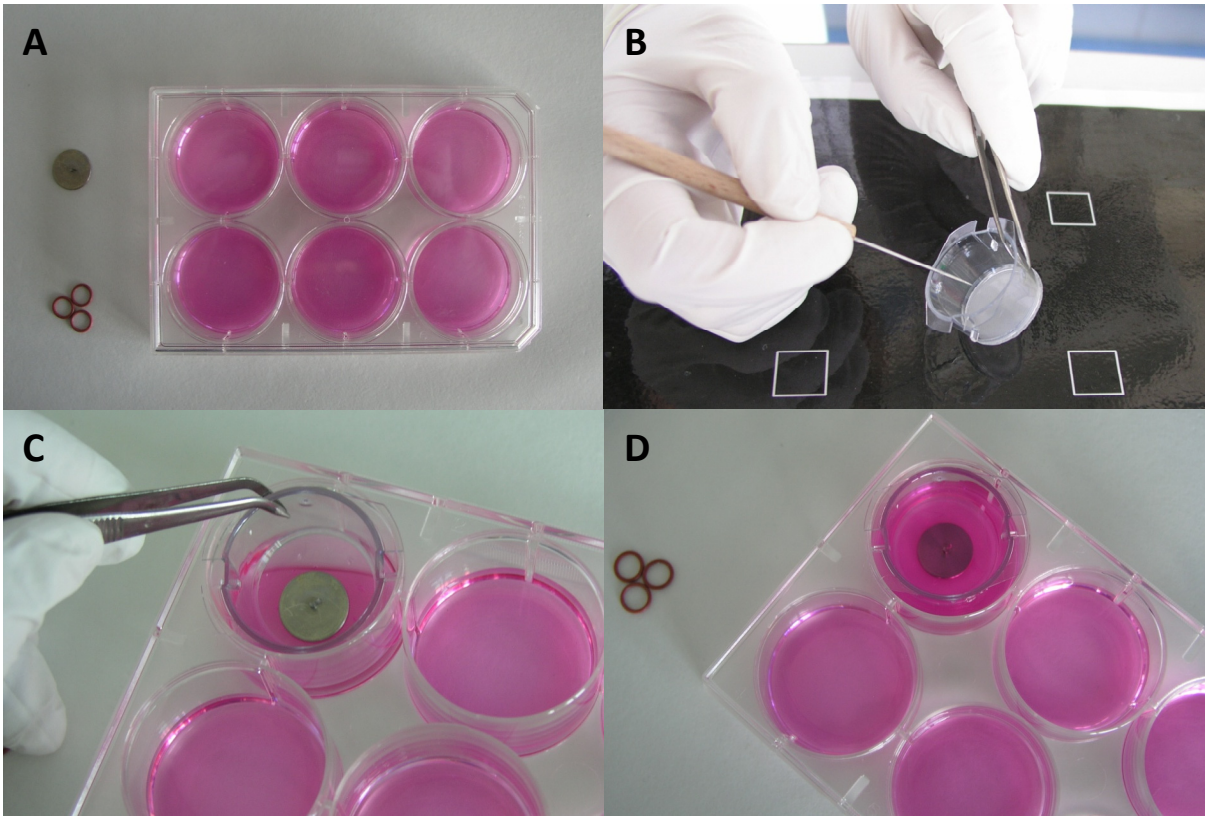

Figure 4-76: Procedure of biomaterial testing. A. The cells are seeded onto the bottom of 6-well receiver plates and cultivated for 24 h in their cell specific growth medium prior to the experiment. B. The membranes of commercial cell culture inserts (6-well format) are dissected with a needle. C. The test specimens are placed onto the perforated membrane and the cell culture inserts are applied into the wells of the receiver plate. D. The specimen is covered with cell culture medium and the cell cultures are thus cultivated in presence of the test sample for several days.

All experiments were accompanied by positive-, negative-, and blank controls. As positive control, a brass alloy (CuZn39Pb3) was used since brass, in general, is described to be cytotoxic [(218)]. Its cytotoxic potential was further verified in a preliminary test. The negative control consisted of silicone O-rings (peroxidically cross-linked; dimension: 3 mm x 1 mm) which are FDA (US Food and Drug Administration) approved and which have been proven to be non-cytotoxic in previous experiments. The wells of the blank controls were also equipped with a cell culture insert but without a test sample.

For test purposes, different cell lines were initially used (A549, IMR90 and 16HBE14o-). In case of a strong cytoxic potential of a test material, morphological alterations or a decrease in the number of cells were easily detectable in all tested cell lines. However, a moderate cytotoxic potential was only observable in IMR90 cells by a reduction of the cell size but not by a visible decrease of the cell number. Fibroblasts in general are characterized by a large cell size, a spindle-shaped morphology with long cell elongations and an orientated growth pattern. Small alterations of these typical characteristics are easily detectable by *in situ*

microscopy. Therefore, they are suitable cell models for the material testing and accordingly, the IMR90 cells were later further used for evaluating the materials for the CULTEX® LTC.

The *in situ* microscopic observations, in complement with the results obtained from the commercial cell viability assays, clearly demonstrated that some of the materials used in the CULTEX® LTC prototype had a cytotoxic potential, which was presumably the cause for the failure of the cultivation system. In order to select or preclude materials for the renewed construction of the CULTEX® LTC, all potential material candidates were thus tested for cytotoxicity according to the here described method before they were used.

4.4.2 Application of the CULTEX® Long Term Cultivation (LTC) System

The newly constructed CULTEX® LTC cultivation system was afterwards initially tested with Calu-3 and A549 cells. Here again, different variations of the medium level and time intervals for the medium renewal and mixing were tested. All runs were accompanied by control cultures of the respective cell line that were cultivated under the conventional conditions with a manual medium renewal every 48 h.

After seeding, all cultures were first cultivated in receiver plates under submerged conditions until they formed a confluent cell layer. Subsequently, the test cultures were placed into the CULTEX® LTC where they were cultivated at the air-liquid interface and supplied with basic differentiation medium according to the adjusted medium renewal frequencies. The basic experimental set-up is shown in Figure 4-77: The CULTEX® LTC modules were placed into an incubator with standard cultivation conditions (37 °C, 5 % CO_2, 95 % rH). The modules were connected to the medium supply flask and medium waste flask via silicone hoses and to the power supply via cables. The power supply was connected to the computer as well as to the pumps for the medium circulation. In order to guarantee the stability of the light and temperature sensitive ingredients of the culture medium, the medium bottle was placed into a lightproof thermo cover.

Figure 4-77: Experimental setup for the cultivation of cell cultures grown on insert membranes at the air-liquid interface in the computer-controlled CULTEX® Long Term Cultivation (LTC) system

The initial experiments with A549 and Calu-3 cells aimed on the evaluation of the CULTEX® LTC with regard to the practicability for the contamination-free cultivation of cell cultures under stable culture conditions. For that purpose the cells were cultivated for 7 to 18 days in the CULTEX® LTC.

It was shown that the cultivation under sterile conditions was enabled in the system. Moreover, the adjusted culture conditions could be maintained by the computer software during the entire cultivation time and small deviations from the original medium level were balanced immediately by pumping medium from or to the respective chamber.

Histological analysis of the cell cultures further demonstrated that the cultivation in the CULTEX® LTC in general enhanced the stability of the cell cultures. Furthermore, the growth pattern was greatly influenced by the adjusted conditions in the system. For example, the frequency of the teflon membrane movement, which enabled the mixing of the cell culture medium in the chambers and provided mechanical stress, was found to affect the

proliferation activity in both, A549 and Calu-3 cells. In A549, an increase in the number of cell layers was observed in correlation with an increase of the frequency of the teflon membrane movement (Figure 4-78).

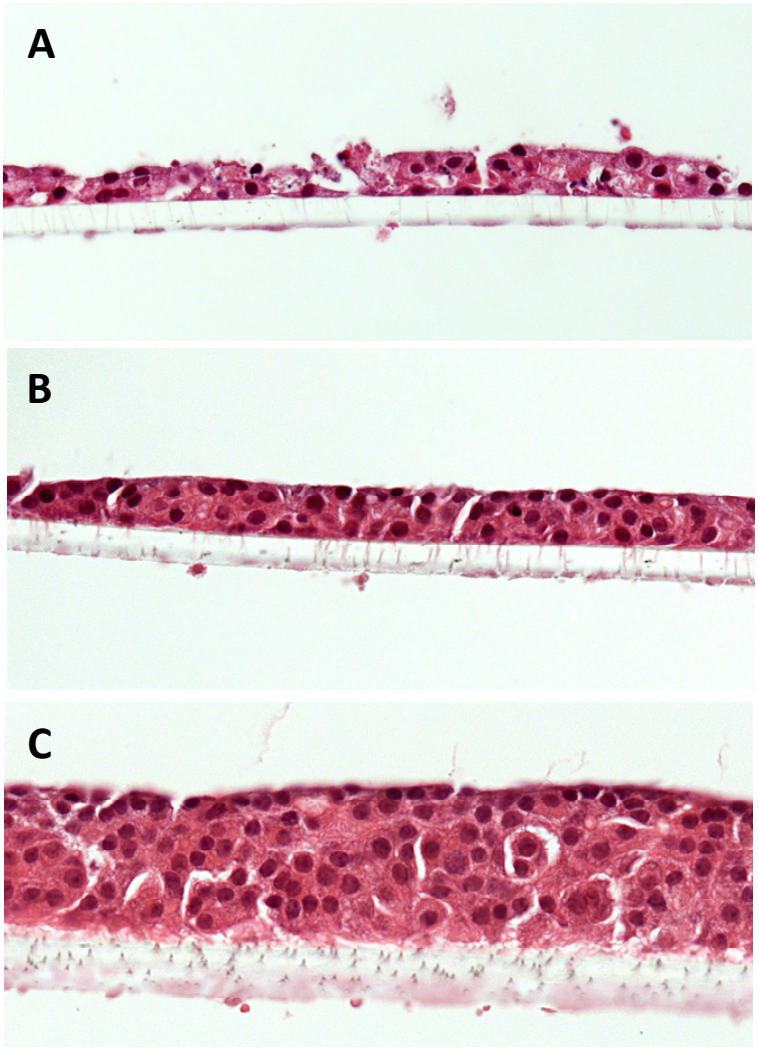

Figure 4-78: A549 monocultures after a total cultivation time of 14 days. The cultures were either cultivated under conventional conditions with manual medium renewal every 48 h (A) or in the CULTEX® LTC with varying computer-controlled conditions (B-C). An increase of the mixing frequency from 60 min (B) to 24 min (C), while maintaining the medium renewal frequency at 36 h and the medium renewal volume at 1 mL, resulted in an enhanced proliferation and the formation of a stratified structure in the A549 cultures [HE stained histological sections; magnification: 400x (A-B) or 1000x (C)].

In Calu-3, the combination of both, mixing frequency and medium exchange was found to greatly influence the growth pattern in the cultures. Here, a normal phenotype with a monolayered growth was observed when the medium was exchanged every 24 h. The

columnar shape of the Calu-3 cells was more pronounced when the frequency of the teflon membrane movement was set to every 60 min compared to every 24 min or when no movement was applied. An increase of the time period between the medium exchange cycles from 24 to 48 h in combination with either omission of the movement or a frequency of 24 min resulted in an unordered “tumorous” cell growth with a multi-layered structure. Interestingly, an almost normal growth pattern was observed when the movement frequency was set to 60 min while maintaining the medium exchange rate at 48 h (Figure 4-79).

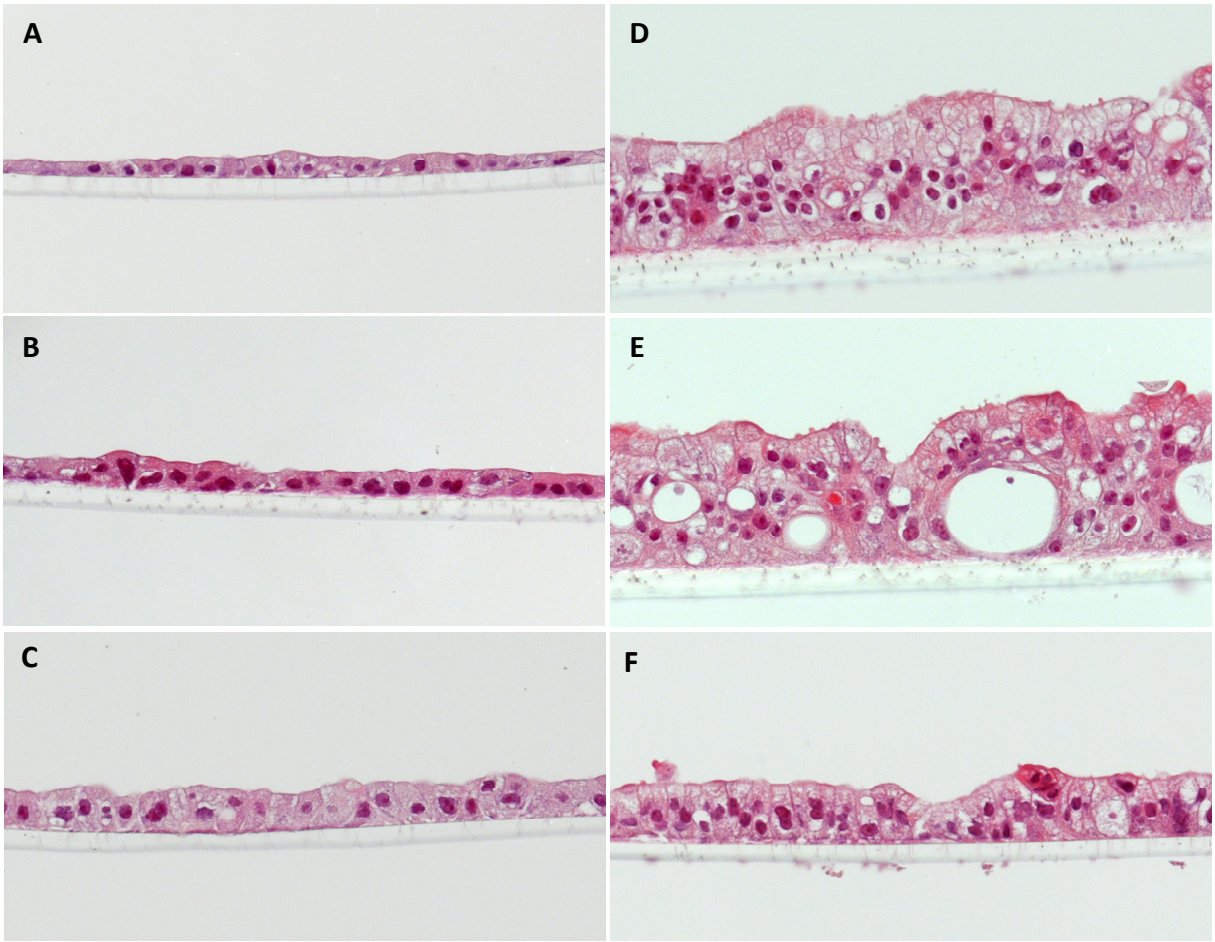

Figure 4-79: Calu-3 cells cultivated in two CULTEX® LTC systems. In each system, the medium renewal frequency was constant in all three insert positions: A - C: every 24 h; D - F: every 48 h. The stirring frequency was varied in all chambers: A + D: no stirring; B + E: every 30 min; C - F: every 60 min. The increase of the medium renewal frequency from 24 h to 48 h resulted in an enhanced proliferation. A stirring frequency of 60 min was found to promote a normal monolayered growth and the columnar shape of the Calu-3 cells (C + F).

Conclusions

The practicability of cultivating cell cultures on insert membranes at the air-liquid interface under sterile and stable conditions using the computer-controlled CULTEX® LTC was proven. Moreover, it was shown that the dynamic medium flow, in general, had a positive effect on the cells and that the phenotype of the cultures could be influenced by modifying the frequencies of the medium exchange and teflon membrane movement. The desired phenotype can thus be selected after testing different modifications and reproduced by applying the same conditions. However, it was shown, that there are cell line-specific responses to different alterations and therefore, the optimal conditions have to be adjusted to the respective cell line individually.

The presented computer-controlled system might be of great value especially for the cultivation of NHBE cells, since high variances between inserts of the same culture were observed with the conventional handling procedure. These variations are potentially related to small deviations in the culture conditions, like the medium level during the ALI cultivation. Since the reliability and reproducibility of data is a major requirement for toxicological studies, the observed variances are highly disadvantages.

The developed techniques enabling the regulation of the adjusted culture conditions in the CULTEX® LTC, like the ultrasonic sensor and the special software, could later also be used for the development of more sophisticated systems that allow the cultivation of a higher number of cultures under identical and stable conditions. Such systems would be highly useful in order to increase the throughput of toxicological studies while ensuring the reproducibility of the data.

5 Summary and Final Conclusions

The overall aim of this thesis was the development of suitable *in vitro* models of the bronchial epithelium that can universally be used for toxicological studies. Critical factors for appropriate cell culture models are the presence of important differentiation characteristics of bronchial epithelial cells, including the secretion of the main airway mucins 5AC and 5B, the development of cilia and the formation of functional tight junctions.

Long-term cultivation is often required for toxicological studies, since certain questions require the long-term or repeated exposure to the test substances in order to provide "normal" exposure conditions. For example, to mimic the cigarette smoke exposure of the airways in smokers in a near-realistic manner, a repeated exposure of the *in vitro* models over a certain time is required. Therefore, the stability and viability of the cultures during the study period are additional requirements for suitable cell models.

Normal bronchial epithelial cells isolated from human lung tissue (NHBE cells) were shown to provide the most reliable cell model of the airway epithelium since they generally retained the ability to develop all important differentiation characteristics of the bronchial epithelium *in vivo*. In addition, the long shelf life of the differentiated cultures also recommends them for long-term studies. A protocol for the isolation of epithelial cells from cryopreserved bronchial tissue was developed in this thesis which enables the flexible on-demand provision of NHBE cells. However, due to the fact that only cells of the passages one and two are generally able to show the desired differentiation patterns and because their storage in liquid nitrogen was found to be unfavorable, the availability of these cells is highly limited. The occurrence of donor-specific variations further restricts their use for large-scale and repeated experiments.

The shortage of available cells might be overcome by using continuous cell lines, since they can be cultivated, in general, over many passages without impairment of their cell viability and differentiation ability. However, the studies presented here revealed that these cell lines mostly lack natural differentiation patterns and are thus not comparable to their normal *in vivo*-counterparts. Only the Calu-3 cells shared many important features with differentiated normal bronchial epithelial cells, but here limitations in the shelf life of the cultures were observed, restricting their suitability for the long-term cultivation.

Cancer cell lines generally bear the risk of showing tumor-specific characteristics but they are nevertheless mostly used in current toxicological studies since those cell lines derived from normal cells are not widely available. To overcome this situation, a few cell lines have been developed by genetic manipulation (immortalization) of normal human epithelial cells (e.g. 16HBE14o-). Unfortunately, these cell lines are often licensed and can thus only be used with certain restrictions. Moreover, comparable to cancer-derived cell lines, they mostly lack some important features of the normal bronchial epithelium. The development of new immortalized cell lines exhibiting at least the most important characteristics for target-orientated toxicological assays is therefore highly desirable. In this thesis it was shown that the immortalization of normal bronchial epithelial cells can be achieved by transformation with the viral gene HPV E6/E7. However, the differentiation ability of these cells was limited. Besides the selected gene and vector for the immortalization, there are other possible reasons for the failure of the cells to develop natural differentiation characteristics, like the use of an inappropriate cell source. For example, cryopreservation was often shown to cause damage to cultured cells and thus, in general, it is recommended to only use freshly isolated, non-cryopreserved cells of the first two passages for the immortalization procedure. In summary, no final conclusions on the suitability of the transformation of normal human bronchial epithelial cells with the HPV E6/E7 gene for the generation of stable, continuous cell lines with natural differentiation patterns could be drawn in this thesis. But, it is worth making more efforts to develop suitable strategies for the immortalization of normal human airway epithelial cells, in order to be able to overcome the shortage of appropriate continuous cell lines and available primary cells for routine toxicological assessments *in vitro.*

In vivo, bronchial epithelial cells and fibroblasts form a unit - the *Epithelial Mesenchymal Trophic Unit* - which is characterized by complex interactions between the two cell types during growth, differentiation, repair and remodeling of the epithelium. To better reflect these conditions, *in vitro* models of the lung epithelium should ideally include both cell types. However, the establishment of such models is still a major challenge. In this work two different 3D *in vitro* models of the bronchial epithelium, including epithelial cells and fibroblasts were established - the Sandwich Cell Model (SCM) and Compartment Cell Model (CCM). It was shown that both co-culture models have certain advantages and disadvantages.

The SCM provides a good reflection of the structure of the lining epithelium in the bronchi and bronchioles and it might be valuable for studying remodeling aspects of the epithelium after inflammation processes. However, contraction and degradation of the collagen matrix as well as limitations in the differentiation ability of NHBE cells in this co-culture model restrict its suitability for toxicological assessments.

In the CCM, the epithelial cells and fibroblasts are spatially separated but interactions between the two cell types are enabled through secreted factors that are distributed via the cell culture medium. The original CCM, based on the protocol developed by Pohl and colleagues [174], was shown to be unsuited for the long-term cultivation and was thus excluded from further studies. As a substitute, two different alternatives, the CCM *variant I* and *variant II,* were developed. In these models, the fibroblasts are grown in wells of receiver plates which are equipped with inserts carrying the epithelial cells. In contrast to the CCM *variant I*, the CCM *variant II* additionally includes an ECM component (collagen type I matrix) and therefore provides a more natural environment for the cells. The practicability of this co-culture model was proven in this study and moreover, clear interactions of the two cell types were verified in these cultures. In summary, the development of a realistic reproduction of the bronchial epithelium, which further includes natural interactions of epithelial cells and fibroblasts, was achieved in this thesis. The epithelial component can be represented by various cell lines, depending on the respective requirements. The easy generation and handling recommends the CCM *variant II* as universally applicable *in vitro* model of the bronchial epithelium for a variety of studies.

The increasing prevalence of chronic lung diseases worldwide demands the inclusions of *in vitro* models showing common types of pathologic conditions for toxicological risk assessment. In this thesis, it was shown that certain pathologic features that are often found in inflamed airways can be induced in cultures of NHBE cells by stimulation with certain growth factors or cytokines. For example, the development of protocols for the induction of goblet cell and basal cell hyperplasia was achieved. These cultures could provide valuable *in vitro* models for studying the effects of various substances in the airways of patients with chronic airway diseases, like asthma or COPD.

Besides the selection of suitable cell models, another important requirement for toxicological risk assessment is the reliability of the data. For that purpose an appropriate

number of samples and repetitions of the experiments is necessary. However, it was shown that especially in cultures of NHBE cells, high variations between individual inserts were observed with regard to cell morphology, TEER values and IL-6 or IL-8 secretion under conventional culture conditions. One possible reason for this variability might be the occurrence of slight differences in the medium level caused by inaccurate pipetting. In order to provide stable and reproducible culture conditions, automated cultivation systems are valuable. The CULTEX® LTC was specifically designed for the computer-controlled cultivation of cell cultures grown on inserts at the air-liquid interface. The here presented data revealed that the cultivation in the CULTEX® LTC under sterile and stable conditions was successful and, moreover, that the frequency of medium renewal and mixing greatly influenced the cell culture phenotype. The special technical features of the CULTEX® LTC which enable the computer-controlled regulation of the adjusted culture conditions and the detection of slight deviations of the medium level, like the ultrasonic sensor or the special software, could be adopted for the design of future generations of such automated systems that allow the cultivation of high numbers of cell culture inserts at the same time. Thus, toxicological studies could be greatly enhanced with regard to throughput and reproducibility of the data.

In conclusion, the results presented in this thesis demonstrated that there is currently no ideal cell model of the bronchial epithelium available. Thus, the necessity of developing new continuous lung epithelial cell lines was highlighted, in order to overcome the disadvantages of the existing ones. At present, cultures of isolated normal human bronchial epithelial cells of the first two passages provide the most realistic models with regard to natural differentiation patterns and the ability to develop common types of pathologic conditions *in vitro*. For studies that require high throughput, the Calu-3 cells might be a good alternative, since they share many features of normal bronchial epithelial cells and, moreover, they can be subcultivated over many passages and are thus permanently available. Unfortunately, they are not suitable for long-term exposure studies and cancer-specific characteristics cannot be precluded. Investigations on repair and remodeling processes in the airway epithelium *in vitro* ideally require the inclusion of natural interactions of the EMTU. For that purpose, the here developed co-culture model - CCM *variant II* – is highly suitable.

The here provided information and protocols are useful for the design and implementation of *in vitro* studies on lung toxicity and can therefore make a valuable contribution for the reduction of animal experiments in this field.

6 Appendix

6.1 Methods

6.1.1 Cultivation and handling of cell cultures

In general, the different cell lines were handled separately in the laminar flow hood to avoid cross-contamination. All cell culture works were performed according to the principles of sterile working procedures until fixation of the cultures with formaldehyde.

6.1.1.1 Cell culture media

Table 6-1 provides an overview of the cell line specific growth media and special additional ingredients or supplements. The compositions of the basic differentiation medium and the media used for the isolation of NHBE cells from lung tissue are listed below the table.

For the stimulation experiments with NHBE cultures, the basic differentiation medium was modified by adding the respective growth factors or cytokines in the mentioned concentrations (see section 4.3).

Table 6-1: Overview of cell line-specific growth media, including special ingredients or additional supplements

Cell line	Basal cell culture medium	Order information	Additional ingredients included	Supplements
A549	DMEM	Biochrom AG, FG 0415	stable L-glutamine, 3,7 g/L $NaHCO_3$	10 % FBS
NHLF	RPMI 1640	Life Techologies Inc, 31870025	2 g/L $NaHCO_3$	10 % FBS 2mM L-glutamine 1mM sodium pyruvate
IMR90, Calu-3	MEM Eagle with Earle´s salts (EMEM)	Lonza, BE12-125F		10 % FBS 2mM L-glutamine 1mM sodium pyruvate
16HBE14o-	MEM Earle´s (MEM)	Biochrom AG, FG 0325	stable L-glutamine, 3,7 g/L $NaHCO_3$	10 % FBS
NHBE	BEGM (BEBM + *SingleQuots*®)	Lonza, CC-4175	*SingleQuots*, including: Hydrocortisone Insulin Transferrin Epinephrin Triiodothyronin Human epidermal growth factor (hEGF) Bovine pituitary extract (BPE) Gentamicin/amphotericin B (GA) Retinoic acid (RA)	
HBEpC	Airway Epithelial Cell Growth Medium Kit	Promocell GmbH, C-21160	Insulin Transferrin Epinephrin Triiodothyronin Human epidermal growth factor (hEGF) Bovine pituitary extract (BPE) Retinoic acid (RA)	

Order information for supplements:

- Fetal bovine serum (Lot 0310L; Biochrom AG, S0210)
- L-glutamine (200 mM, Gibco; Life Technologies Inc., 25030-024)
- Sodium pyruvate (100 mM, Gibco; Life Technologies Inc., 11360-039)

Basic differentiation medium:

Ingredients:

- BEGM™ Bulletkit™ (Lonza, order no.: CC-3170)
- DMEM (Gibco®, Life Technologies Corp., order no.: 22320)
- $CaCl_2$ (Merck KGaA, order no.: 1.02382)
- Retinyl acetate-water soluble (Sigma-Aldrich; order no.: R0635)

- Ultroser® G (Cytogen GmbH, order no.: 15950-017)

500 mL of the basic differentiation medium were prepared as follows:

BEBM/DMEM (Gibco) **1:1**	
BEBM	240 mL
DMEM	240 mL
+ BEBM Single Quots® (w/o RA)	
+ 2 % Ultroser® G	10 mL
+ 1 mM $CaCl_2$ (10 % solution)	0.735 mL

The retinoic acid (RA) „SingleQuot" was not added but replaced by a homemade retinol stock solution with a concentration 100 nM, prepared from water soluble retinyl acetate.
For CCM *variant II* co-cultures, the addition of $CaCl_2$ was omitted, since thus the turbidity of the collagen matrix could be prevented.

Incubation Medium for Isolation of Bronchial Epithelial Cells from human tissue

Ingredients:

- MEM Eagle (Joklik Modification; Sigma-Aldrich, M8028)
- Amphotericin B solution (250 µg/ml; Sigma-Aldrich, A2942)
- Ceftazidim (Sigma-Aldrich, C3809)
- DL-Dithiothreitol (DTT; Sigma-Aldrich, D0632)
- DNase I (from bovine pancreas; Sigma-Aldrich, DN25)
- Gentamycin (10 mg/ml; Biochrom AG, A2712)
- Penicillin/streptomycin (10000 U/10000 µg/ml; Biochrom AG, A2213)
- Tobramycin (Sigma-Aldrich, T4014)
- Vancomycin (Sigma-Aldrich, V2002)

100 mL were prepared as follows:

Tobramycin	4 mg
Ceftazidim	5 mg
Vancomycin	5 mg
DNase	1 mg
DTT	50 mg

Each powder was solved in 5 - 10 mL of MEM Eagle (Joklik Modification). The solutions were added to 50 – 75 mL of MEM Eagle (Joklik Modification; final volume: 100 ml) and sterilized by filtration through a 0.2 µm filter. The medium was stored at 4 °C up to six weeks.

The following antibiotics were added shortly before use:

Penicillin/streptomycin solution	0.5 mL
Amphotericin B solution	1 mL

Digestion Medium for Isolation of Bronchial Epithelial Cells from human tissue

Ingredients:

- Same as for incubation medium
- Pronase XIV (Sigma-Aldrich, P5147)

50 mL were prepared as follows:

Incubation medium (see above)	50 mL
Pronase XIV	50 mg

The solution was vortexed thoroughly and sterilized by filtration through a 0.2 µm filter. The medium was prepared shortly before use.

Dissociation Medium for rescuing isolated Bronchial Epithelial Cells from clumps

Ingredients:

- DL-Dithiothreitol (DTT; Sigma-Aldrich, D0632)
- DNase I (from bovine pancreas; Sigma-Aldrich, DN25)
- Collagenase (Life Technologies Inc., 17018-029)
- Ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, ED4SS)

20 mL were prepared as follows:

Collagenase	5 mg
DNase	0.2 mg
DTT	10 mg
EDTA	11.7 mg

Each powder was solved in 5 ml of MEM Eagle (Joklik Modification). The solutions were mixed and the final solution was sterilized by filtration through a 0.2 µm filter. The medium was prepared shortly before use.

6.1.1.2 Subcultivation (passaging) of cells

Adherent growing cells, like epithelial cells and fibroblasts, are normally in close contact with their substrate. For subcultivation or for the preparation of cells for experiments (e.g. seeding on cell culture inserts), the cells have to be detached from their substrate. This can be realized by means of a trypsin solution, complemented with EDTA. The enzyme trypsin breaks connections of adhesion proteins between cells, and between cells and the contact substrate. Bivalent ions inhibit trypsin. For this reason, the trypsin solution also contains EDTA, a chelator for bivalent ions (especially Ca^{2+}).

Long trypsin incubation times can cause irreversible damage to the cells. To avoid this, the cells should be monitored carefully during the incubation with trypsin and the inhibition of the enzyme activity should be performed immediately after the majority of the cells was detached from the culture vessel. The enzyme can be inhibited by serum-containing

medium, since sera (like FBS) contain trypsin inhibitors. For serum-free cultivation, a trypsin inhibitor isolated from soybean can be used for stopping the activity of trypsin.

Before starting, all solutions and media in appropriate volumes as well as cultureware products (cell culture flasks or inserts) that were required for the subcultivation or for the preparation of experiments were prewarmed at 37 °C in a waterbath or incubator.

The passaging was performed according to the following procedure:

After removal of the cell culture medium from the culture flask, the cell layer was rinsed thoroughly with sterile PBS (w/o Mg2+ and Ca2+; 8 mL for 75 cm2 and 4 mL for 25 cm2 cell culture flasks) and the PBS was afterwards removed. Subsequently, the trypsin/ EDTA solution was applied onto the cells and either incubated at 37 °C (epithelial cells) or at room temperature (fibroblasts). The trypsin concentration as well as the duration of the incubation was varied according to the requirements of the respective cell line. The detachment of the cells was monitored carefully by means of an inverted microscope. The trypsin activity was stopped when the majority of cells was detached from the culture vessel by adding an appropriate volume of either serum-containing or trypsin inhibitor-containing medium. Table 6-2 gives an overview of the concentrations and volumes of the trypsin/ EDTA solution as well as the medium or solution used for stopping the trypsin activity for each cell line.

The cell suspension was transferred to a 50 mL centrifugation tube and 100 µL were used for the determination of the number of viable cells per mL with the CASY Cell Counter and Analyzer System. The CASY Model TT Cell Counter and Analyzer system (Roche Diagnostics GmbH) uses the “Electrical Current Exclusion” method for measuring cell viability of mammalian cell lines. This method depends on the membrane integrity of the cells. The electrical barrier of the intact cell membrane is higher than in dead cells and therefore, viable cells cause an increased level of resistance in the measuring pore of the system. Thus, the number of viable cells in a defined volume of the cell suspension can be determined.

Depending on the number of viable cells per mL, the cell suspension was either directly used for inoculation of new culture flasks or cell culture inserts or resuspended in a smaller medium volume after centrifugation, in order to increase the cell concentration per mL. In general, it is recommended to use only small volumes of the cell suspension which still contains residual trypsin and to dilute it in a ratio of at least 1:10 with cell culture medium.

Centrifugation was performed for 7 min at 220 x g and after removing the supernatant, the cell pellet was resuspended in an appropriate volume of cell culture medium to obtain the calculated cell concentration per mL. The number of viable cells was determined by the CASY system. The prewarmed cell culture flasks containing an appropriate volume of cell culture medium were inoculated with the desired number of cells by pipetting the respective volume of the cell suspension into the flasks. Before seeding the cells on cell culture inserts, a volume of 1200 µL of cell culture medium was pipetted into the wells of the receiver plate. The appropriate volume of the cell suspension containing the desired number of cells was subsequently applied onto the membrane and diluted with cell culture medium to a final volume of 500 - 600 µL.

Table 6-2: Information on the trypsin/ EDTA concentrations and volumes as well as the tryspin stopping solutions used for the subcultivation of different cell lines

Cell line	**Cell culture flask [cm^2]**	**Trysin/ EDTA (concentration, volume)**	**Trypsin reaction stopped with**
NHBE/ HBEpC	75	0.05 %/ 0.02 % (w/v), 2 mL	2 mL trypsin inhibitor from soy bean (1x) with 4 mL BEGM
NHBE/ HBEpC	25	0.05 %/ 0.02 % (w/v), 1 mL	1 mL trypsin inhibitor from soy bean (1x) with 4 mL BEGM
Calu-3	75	0.25 %/ 0.02 % (w/v), 4mL	8 mL EMEM incl. 10 % FBS
16HBE14o-	75	0.05 %/ 0.02 % (w/v), 3 mL	8 mL MEM incl. 10 % FBS
ChaGo-K1	75	0.05 %/ 0.02 % (w/v), 2 mL	8 mL RPMI incl. 10 % FBS
IMR90	75	0.05 %/ 0.02 % (w/v), 2 mL	8 mL EMEM incl. 10 % FBS
NHLF	75	0.05 %/ 0.02 % (w/v), 2 mL	8 mL RPMI incl. 10 % FBS

6.1.1.3 Cryopreservation of cells and tissue

For the storage of cells in the gaseous phase of liquid nitrogen, for example to guarantee the availability of viable cells for future experiments, they have to be transferred to a specialized medium which contains a cryoprotectant agent. Mostly, DMSO is used for this purpose. DMSO is cytotoxic at room temperature and therefore all steps have to be performed on ice

and the freezing should be started as soon as possible after the addition of Dimethyl sulphoxide (DMSO). Mostly the ready-to-use "Recovery Cell Culture Freezing Medium" (Gibco) obtained from Life Technologies Corp. (12648-010) was used. Occasionally, the freezing medium was self-prepared as follows: 80 % DMEM (including L-glutamine, sodium pyruvate and HEPES) was mixed with 10 % DMSO (cell culture tested; Sigma-Aldrich) and 10 % FBS.

To avoid lethal intracellular freezing, the freezing down of cells was performed stepwise, in defined rates, by using a freezing container ("Mr. Frosty", Nalgene), which provides a repeatable cooling rate of 1 °C/ min. As soon as a temperature of -80 °C was achieved, the frozen cells were transferred to a cryogenic tank.

Before starting, the required number of cryovials was labelled (description of cells, passage number, date). The freezing container (Mr Frosty) was filled up with isopropanol to the filling mark. The cryovials were placed into the "Mr Frosty" and stored at 4 °C for 1 h before use.

The adherent cells were trypsinized according to the cell-specific protocol (see 6.1.1.2) and the number of viable cells was determined (by the CASY™ Cell Counter + Analyzer). If required, a new culture flask was inoculated with the appropriate cell number before continuing with the next step.

The cell suspension was centrifuged for 7 min at 220 x g and 18 °C and meanwhile, the cooled and labeled cryovials were place on the clean bench. After centrifugation, the culture medium was discarded and the cell pellet was resuspended in cell culture freezing medium.

The volume of freezing medium was adjusted to obtain the desired cell density (optimally between 2 - 3 × 10^6 cells/mL).

Each cryovial was filled with 0.5 mL of the cell suspension (if a large number of vials was prepared, the cell suspension was occasionally swirled to guarantee a uniform distribution of the cells within the suspension). The vials were quickly placed into the Freezing Container and stored at -80 °C overnight, before they were transferred to the cryogenic tank.

6.1.1.4 Coating of cell culture insert membranes or culture vessels

In order to provide a realistic environment for cells *in vitro*, the surfaces on which they are grown are often coated with proteins of the ECM. The nature of the surface coating plays an important role in their ability to attach, proliferate and differentiate [(250)]. Commonly used

ECM components for surface treatment are collagen type IV, collagen type I and human fibronectin.

Type IV collagen is a ubiquitous component in basement membranes and provides the major structural support for this matrix. Especially epithelial cells show an enhanced attachment and growth when cultivated on surfaces coated with this ECM component.

Collagen type I is found in most tissues and organs. The surfaces coating with collagen I is most commonly used and it was shown that it promotes the growth and differentiation of many different cell types.

Human fibronectin is a glycoprotein that binds to integrins or ECM proteins, like collagens. It plays a major role in the attachment, growth and differentiation of cells *in vivo* and is therefore often used as a substrate for cell cultures.

In this thesis, varying surface coatings were used for the different epithelial cell lines. For the Calu-3, ChaGo-K1 and occasionally NHBE cells, the cell culture insert membranes were coated with 6.5 µg/ cm^2 collagen type I. For that purpose, collagen type I from bovine skin with a concentration of 3 mg/mL (aqueous solution in 0.01 M HCl) was diluted in a ratio of 1:60 with 0.01 HCl and 130 µL of this solution were applied on each insert membrane (12-well format; membrane diameter: approx. 1 cm^2). The inserts were incubated at 37 °C for at least 1 h (preferably overnight). Afterwards, the membranes were rinsed twice with 200 µL of sterile PBS and treated with UV for 2 h. If not immediately used, the inserts were stored at 2 - 8 °C for up to six weeks.

The collagen type IV coating (10 µg/ cm^2) was used for the cultivation of NHBE cells and HBEpC on insert membranes. A stock solution of 1 mg/mL was prepared with 0.5 M HCl. For each insert, 10 µl of this stock solution were mixed with 140 µL of ultrapure water (ddH_2O) and applied on the membrane. The incubation and storage was performed according to the same protocol as for the collagen type I coating.

A coating made up of a mixture of collagen type I and fibronectin was used for the following purposes: (1) Coating of culture flasks (75 cm^2) and insert membranes for the cultivation of 16HBE14o- cells, (2) coating of 6-well plates for the cultivation of NHBE cells directly after the isolation from tissue and (3) coating of cell culture flasks (25 cm^2) for the cultivation of primary NHBE cells after the first passaging.

The coating solution was prepared as follows:

For 100 mL:

10 mL of a BSA solution with a concentration of 1 mg/mL in PBS was prepared, filtered through a sterile filter (0.2 µm pores) and added to 88 mL of cell culture medium [optimally LHC Basal Medium is used (Life Technologies Inc., order no. 12677-019)]. Each 1 mL of collagen type I (3 mg/mL) and fibronectin (1 mg/mL) were added. The solution was gently mixed and applied in the following volumes:

- 150 µL on insert membranes (12-well format; approx. 1 cm^2)
- 3 mL per 75 cm^2 culture flask
- 2 mL per 25 cm^2 culture flask
- 1 mL per well (6-well plate)

The incubation and storage is performed as for collagen I and IV coated culture vessels.

6.1.2 Preparation of co- cultures according to the Sandwich Cell Model (SCM)

In the SCM, airway epithelial cells are co-cultivated with lung fibroblasts to enable natural interactions between the two cell types comparable to the *in vivo* conditions. The SCM cultures are basically prepared as follows: the epithelial cells (e.g. NHBE or Calu-3) are seeded on top of a collagen type I matrix in which fibroblasts are embedded.
For the polymerization of the collagen type I matrix, the collagen solution had to be brought to a neutral pH. This was achieved by adding a NaOH-containing buffer solution which was prepared as follows:

100 mL of 0.05N NaOH (e.g. 5 mL of 1N NaOH diluted to 100 mL with dH_2O)
\+ 2.2 g $NaHCO_3$
\+ 200mM HEPES

The solution was autoclaved and stored at 4 °C for up to 6 months.

All solutions and media for the trypsinization and – if necessary – passaging of the fibroblasts as well as the required materials for cell seeding, including the appropriate number of cell culture inserts and culture flasks, were prewarmed at 37 °C. The trypsinization was

performed as described in section 6.1.1.2. After estimating the number of viable cells per mL by means of the CASY system, the fibroblast solution was centrifuged for 7 min at 220 x g. The culture medium was afterwards removed and the cell pellet was resuspended in a calculated volume of cell culture medium, to obtain a concentration of approximately 1.0 or 2.0 x 10^6 cells/mL. The new culture flask was inoculated with the desired cell density.

The collagen matrix in which the fibroblasts are embedded was prepared shortly before use and stored on ice. Only small volumes of the collagen solution (up to 2.5 mL) were prepared. If higher volumes were required, the procedure was repeated.

The collagen/fibroblast layer was prepared as follows:

	4 parts Cellmatrix I-A	(e.g. 400 µL)
+	4 parts Cellmatrix I-P	(e.g. 400 µL)
+	1 part 10x MEM (or 10x DMEM)	(e.g. 100 µL)

The solution was mixed thouroughly.

+	1 part of the NaOH buffer solution	(e.g. 100 µL)

The solution was mixed thouroughly and bubbling was avoided. A shift of the pH towards neutral was indicated by a change of the phenol red indicator in the 10x MEM from orange to pink colour.

Subsequently 100 – 150 µL of the fibroblast suspension were added to the collagen solution. For 1 mL of the collagen solution, 100 µL of the fibroblasts suspension containing 1.0 x 10^6 cells/mL were used. For 2 mL, the cell concentration was increased to approximately 2.0 x 10^6 by using a reduced amount of the culture medium for resuspension of the cell pellet after centrifugation. Of this cell suspension, 100 µL were added to 2 mL collagen solution

A final volume of 200 – 250 µL of this collagen/fibroblast solution was quickly applied onto the insert membrane (12-well; 0.4 µm pores) and incubated at 37 °C for 3 - 5 h.

All solutions and media for the trypsinization of the epithelial cells and - if required – new cell culture flasks were prewarmed at 37 °C. The trypsinization was performed as described in section 6.1.1.2. The number of viable cells was determined with the CASY system and the appropriate volume of the cell suspension containing the desired cell number was applied

onto the polymerized collagen matrix. In case of low cell densities in the suspension, the number of cells per mL was increased by centrifugation, removal of the supernatant and resuspension in a reduced volume of cell culture medium. To reach a final volume of 550 µL on the apical side of the membrane, an appropriate volume of cell culture medium was added. The final medium volume in the wells was 1200 µL. The cultures were provided with the specific growth medium of the respective epithelial cells line and cultivated under submerged conditions. As soon as the epithelial cells formed a confluent layer, the cultures were provided with differentiation medium and cultivated at the air-liquid interface.

6.1.3 Preparation of co-cultures according to the Compartment Cell Model (CCM) *variant II*

In the co-culture model CCM *variant II*, the fibroblasts are embedded into a matrix composed of collagen type I and grown in the wells of insert receiver plates. The epithelial cells are cultivated on conventional cell culture inserts (12-well; 0.4 µm pores) whose membranes were previously coated with collagen type I or IV (see 6.1.1.4). After a short growth phase, the insert were placed into the fibroblast-containing wells and thus a direct contact between the two cell types was provided through the distribution of secretion products via the culture medium.

The passaging of the epithelial cells and fibroblasts was performed according to the procedure described in section 6.1.1.2. The epithelial cells were seeded on the insert membranes with the desired seeding density. The inserts were prewarmed before use. The final medium volume for the submerged cultivation was 550 µL on the apical side and 1200 µL on the basal side (in the wells).

The collagen type I matrix with embedded fibroblasts was prepared according to the same protocol as for SCM cultures (section 6.1.2) and 270 µL of the solution was applied in wells of a new 12-well receiver plate. After an incubation of 3 h, 500 µL of the fibroblast-specific growth medium was applied onto the hardened collagen matrix.

The epithelial cells and fibroblasts were cultivated separately until the epithelial cells nearly formed a confluent cell layer. The inserts were subsequently placed into the fibroblast-containing wells and the co-cultures were supplied with differentiation medium under submerged conditions for one day. The inserts were then raised to the air-liquid interface

and supplied with 580 µL of differentiation medium that was applied onto the collagen/fibroblast layer in the wells. Care was taken during the renewal of the medium to avoid damage of the collagen matrix.
Histological section were only prepared from the epithelial cells. The determination of the TEER values in the epithelial cell layers was performed in the absence of the fibroblasts by placing the inserts in new receiver plates.

6.1.4 Material testing

The test materials were provided by Halter Engineering GmbH (Herisau, Switzerland), who was the constructor of the CULTEX® LTC. The size of the test materials was adjusted to fit the 6-well cell culture inserts. The samples were rinsed in 70 % ethanol, placed into glass Petri dishes and autoclaved (121 °C, 20 min). Afterwards they were placed on the membranes of cell culture inserts which had previously been perforated with a dissecting needle (approx. 1 mm in diameter), in order to enable the uniform mixing of the cell culture medium on the apical and basal side of the membrane. The cell culture inserts were then set in the wells of the companion plate carrying the cells that had been seeded the day before. The seeding density was 1 x 10^5 (A549) or 1.5 x 10^5 (IMR90, 16HBE14o-), respectively, per well (6-well format). The cultures were supplied with 3 mL of their cell line- specific growth medium which was renewed before the inserts with the test samples were placed into the wells. The samples were covered with 1 mL medium and thus, the total volume of medium per well was 4 mL. All experiments were accompanied by control cultures which were prepared in the same way as the test cultures but without a test sample on the insert membrane.

The cells were cultivated in presence of the test materials for 3 days or until the control cultures reached approx. 80 % confluency. After removing the inserts with the samples, the *in situ* microscopic analysis was performed immediately. Then, the qualitative analysis of cytotoxicity was conducted by either using the resazurin-based CellTiter-Blue® Cell Viability Assay (Promega Corp.) or the formazan-based WST-1 (Roche Diagnostics).

The fluorometric CellTiter-Blue® Cell Viability Assay uses the indicator dye resazurin to measure the metabolic capacity of the cells. Viable cells retain the ability to reduce the non-fluorescent resazurin into the highly fluorescent resorufin. The cell culture medium was replaced by 240 µL CellTiter-Blue solution diluted in 1.28 mL fresh serum-free medium per

well. After an incubation time of 2 h, the optical density was read on a multiwell microtiter plate photospectrometer (Appliskan, Thermo Scientific) at a wavelength of 570 nm.

The colorimetric WST-1 assay is used to determine the ratio of proliferating cells through reduction of a water-soluble tetrazolium salt (WST) to formazan by electron transport across the plasma membrane of dividing cells. Thus, the absorbance of the formazan dye is proportional to the number of metabolically active cells. The cell culture medium in the wells was replaced by 125 µL of the WST-1 solution diluted in 1.375 mL fresh serum-free medium. After an incubation time of 1 h at 37 °C, the absorbance was determined by means of a microplate spectrometer (Spectra Max 340 PC, Molecular Devices) at 450 nm.

6.1.5 Analytical methods

6.1.5.1 Histology

The paraffin embedding of cell cultures that were grown on microporous insert membranes enables the generation of histological sections. Thus, the growth pattern and differentiation status of the cells can be analyzed by staining the sections, for example with haematoxylin-eosin (HE) staining, or by immunohistochemistry.

The cells were first fixed with 10 % formaldehyde (each 1 mL on the basal and apical side) for 1 h at room temperature. The samples were then rinsed in tap water for 30 min (each 1 mL on the apical and basal side).

The inserts were subsequently dehydrated by applying increasing alcohol concentrations (each 1 mL on the apical and basal side) in the following order:

(1) 10 min 30 % ethanol
(2) 10 min 50 % ethanol
(3) 10 min 70 % ethanol
(4) 10 min 95 % ethanol
(5) 10 min absolute ethanol
(6) 10 min absolute ethanol
(7) 10 min Roticlear® (Carl Roth)
(8) 10 min Roticlear®

Subsequently the paraffin infiltration was performed as follows: The inserts were transferred to a receiver plate, covered with liquid paraffin from the apical and basal side and incubated for 2 h at 60 °C in a heating cabinet.

The paraffin was renewed after 1 h and the inserts were incubated for another hour at 60 °C. The excessive paraffin was afterwards removed and the paraffin-coating of the inserts was allowed to cool down by incubating the inserts upside down at room temperature.
The membrane was then isolated carefully from the plastic housing of the insert with a scalpel.
Prior to the paraffin embedding, the following preparations were made: Each two braces of conventional embedding cassettes were removed with a cable cutter. The cassettes were labelled with the respective culture name or number with a pencil and afterwards placed into metal embedding moulds.

The paraffin embedding was performed as follows: The embedding moulds were filled with liquid paraffin and the membranes were positioned into the prepared gap of the embedding cassette by means of a pair of self-spanning tweezers. The tweezers were fixed, for example by placing them on a staple of receiver plate lids, in order to keep the membrane in the right position within the paraffin during hardening. The embedding moulds were left on the heating plate until the thin paraffin-coating of the membranes was melted and mixed with the liquid paraffin in the moulds. Subsequently, the embedding moulds were placed on a small cooling plate for a minimum of 20 min for cooling down, before they were transferred to another cooling plate which reaches temperatures of -5 °C, for final hardening. During the hardening, the membranes remained fixed in their position by the tweezers.

Prior to the generation of histological sections, the tweezers were removed and the paraffin blocks were detached from the embedding mould. The paraffin blocks were trimmed with a microtome in 50 µm steps until reaching the desired cutting level of the membrane. Sections of each 5 – 7 µm were then generated from the paraffin block containing cross sections of the embedded cell culture. These sections were transferred to a 40 °C tissue floating waterbath for stretching and placed on silanized glass slides for HE staining or poly-l-lysine coated glass slides for immunohistochemistry.

The sections on glass slides were allowed to dry by placing them on the warm edge of the tissue floating waterbath for a minimum of 1 h. Afterwards they were either stored at room temperature overnight or incubated for 30 min in a heating cabinet at 60 °C before use.

The hematoxylin-eosin staining was routinely used for visualization of the cells in the histological sections. Hematoxylin stains basophilic structures bluish-purple. The nucleic acids are usually basophilic and therefore cell compartments and organelles which are rich in DNA or RNA, like the cell nucleus, the ribosomes and some cytoplasmic regions are stained by hematoxylin. The alcohol-based acidic eosin Y, on the other hand, colors eosinophilic structures bright pink. These eosinophilic structures generally include intracellular or extracellular proteins and most of the cytoplasm.

The HE staining was performed according to a standard protocol. The sections were de-paraffinized by applying decreasing concentrations of alcohol in the following order:

(1) 5 min Roticlear®
(2) 5 min Roticlear®
(3) 5 min absolute ethanol
(4) 5 min 95 % ethanol
(5) 5 min 70 % ethanol
(6) 5 min 50 % ethanol
(7) 5 min 30 % ethanol

Afterwards the slides were stored in distilled water for at least 5 min before staining.

Before use, the eosin Y and the mayers haematoxylin solutions were gently shaked and subsequently filtered. The eosin Y solution was supplemented with 0.01 % acetic acid (e.g. 10 µL of concentrated acetic acid are added to 100 mL eosin Y). The sections were first incubated in haematoxylin for 5 min. The blue color was developed by rinsing the sections in warm tap water for up to 20 min (the water was replaced occasionally). Afterwards the slides were incubated in eosin Y for 5 min and shortly rinsed in distilled water.

After staining, the sections are dehydrated as follows: Short elution in 95 % ethanol followed by incubation in 99 % ethanol for 3 min and in xylene twice for each 5 min. The mounting medium (Roti®-Histokitt, Carl Roth) was subsequently applied onto the slides and the sections were covered with a cover slip and dried in the fume hood.

The microscopic evaluation was performed with a light microscope (Axiophot, Carl Zeiss) and a microscope camera (Axiocam, Carl Zeiss)

6.1.5.2 Immunohistochemistry

Indirect immunohistochemistry utilizes a specific primary antibody raised against a certain protein and a labeled secondary antibody that binds to the primary antibody. Thus, proteins of interest in histological sections of cell cultures or tissues can be visualized.

There are several methods for the detection of this cascade. The method described here is the most common one. The secondary antibody is conjugated to a horseradish peroxidase which forms a brown precipitate with the 3.3‘-diaminobenzidine (DAB) substrate that can be detected by light microscopy. To prevent unspecific bindings of the antibodies and undesirable reactions, potential binding sites as well as endogenous peroxidases have to be blocked. All necessary reagents for immunohistochemical analyses can be obtained separately or as a kit. In commercially available kits, all reagents are optimally diluted and harmonized resulting in an optimized protocol.

Due to cross-links often caused by formaldehyde fixation, the antigenic sites of the proteins (antibody binding sites) can be masked. There are several antigen retrieval procedures for breaking these cross-links. **H**eat **I**nduced antigen (**E**pitope) **R**etrieval (HIER) is a common procedure. Mostly, the sections are boiled in sodium citrate buffer at pH 6.0 or alternatively in Tris/EDTA buffer at pH 9.0.

The buffers were prepared as follows:

Sodium citrate buffer (pH 6.0):

2.94 g tri-sodium citrate dihydrate ($C_6H_5Na_3O_7 + 2H_2O$) were dissolved in 1000 mL distilled water on a magnetic stirrer. The pH was adjusted to 6.0 with 5 M hydrochloric acid (HCl). Tween 20 was added to a final concentration of 0.05 % (50 µL of 10 % Tween 20 solution).

Tris/EDTA buffer (pH 9.0):

1.21 g Tris(hydroxymethyl)aminomethane ($NH_2C(CH_2OH)_3$ and 0.37 g EDTA **(**Ethylenediaminetetraacetic acid) tetrasodium salt were dissolved in 1000 mL distilled water

on a magnetic stirrer. The pH was adjusted to 9.0 with 5 M hydrochloric acid (HCl). Tween 20 was added to a final concentration of 0.05 % (50 µL of 10 % Tween 20 solution).

Heat induced antigen retrieval /HIER)

The HIER was performed according to the following procedure:

Histological sections of the paraffin embedded cell cultures were prepared and deparaffinized as described in section 6.1.5.1. For the immunohistochemistry, poly-L-lysine coated microscope slides were used.

Before starting, a water bath was heated to 97 °C and an appropriate number of coplin staining jars were filled with 55 – 60 mL of the respective buffer. One to two slides were placed into each coplin staining jar and as much space as possible was left between the two sections and between the sections and the housing of the coplin staining jar to guarantee a uniform heating of the sections. The coplin staining jars were placed into the preheated water bath (97 °C) and incubated for 10 min. Afterwards, the caps of the coplin staining jars were removed and the buffer was cooled down to room temperature by placing the coplin staining jars into a cold water bath (incubation for approximately 30 min). The buffer was discarded and the slides were rinsed in running tap water for 1 min and stored in distilled water until starting the following procedure.

Immunohistochemical staining

The NovoLink™ Polymer Detection System provided by Leica Microsystems, UK, was used for immunohistochemistry. This kit was specifically designed for immunostaining analyses on formalin-fixed, paraffin-embedded histological sections. The kit contains the following components:

- Peroxidase blocking solution (neutralized endogenous peroxidase activity)
- Protein blocking solution (reduces non-specific bindings of primary antibody and polymers)
- Post primary blocking solution (enhances penetration of the polymer reagent)
- Polymer (anti-mouse/rabbit IgG linked to poly-HRP (horseradish peroxidase); detects any tissue-bound mouse or rabbit IgG primary antibody)

- DAB chromogen and DAB substrate buffer (produces a visible brown precipitate when reacting with the peroxidase)
- Hematoxylin solution (0.02 % ,for counterstaining of cells)

The sections on the microscope slides were encircled with a PAP Pen. This hydrophobic circle helps to prevent wasting the reagents. Thus, small volumes of the antibody solutions and other reagents could be dropped directly on top of the sections. The immunohistochemistry with the NovoLink™ Polymer Detection System was performed according to the protocol provided by the manufacturer. All reagents were provided in dropper bottles which allow easy and economical application.

The kit could be used for mouse and rabbit IgG primary antibodies. Almost each step was followed by a washing step in TBS (recommended by manufacturer) or unsterile PBS (mostly used) for 2 x 5 min. The washing was performed in staining boxes (according to Hellendahl) which were filled with approx. 80 mL PBS. After several washing steps, the PBS was partially renewed. After the staining procedure, the sections were dehydrated and mounted using Roti®-Histokitt as described in section 6.1.5.1.

6.1.5.3 Immunofluorescence

The immunoflurescence (IF) is a technique that visualizes the distribution of biomolecules of interest in cell cultures or tissues by means of antibodies that are conjugated to fluorescent dyes. The analyses are conducted through the fluorescence microscopy. In contrast to the IHC technique described above, the IF is often used for cell cultures that are not paraffin-embedded and cross-sectioned.

In this thesis, the indirect IF method was used which employs two sets of antibodies, a primary and a secondary antibody. The primary antibody binds specifically to the respective antigen and the secondary antibody that is conjugated to the fluorescent dye binds to the primary. The complex of antigen, primary and secondary antibody can be detected through a fluorescence microscope that is equipped with an UV lamp and a suitable filter for the respective wavelength of the fluorescent dye. To avoid unspecific bindings of the antibodies, potential binding sites are blocked by incubating the samples in normal serum that has been

isolated from the same organism as the secondary antibody. To enhance the penetration of the antibodies the cell membrane are permeablized by e.g. Triton X.

The immunofluorescence analyses of the cilia marker acetylated-α-tubulin in fresh, non-paraffin embedded NHBE cultures (section 4.2.2) was performed according to the following protocol:

After rinsing the surface of the cell cultures on insert membranes with PBS, the cells were fixed with 3.7 % formaldehyde solution for 20 min at room temperature, in the dark. The formaldehyde was then removed and the cells were washed three times with PBS for each 5 min. Triton-X-100 was diluted with PBS to obtain a 0.25 % solution, and the cells were incubated in this solution for 10 min at room temperature. After removing the Triton X-solution the cells were washed three times for each 5 min in PBS. In order to block unspecific binding sites, the cells were subsequently incubated in 10 % rabbit serum (the secondary antibody used was raised in rabbit). The serum was diluted with PBS and the incubation was performed overnight at 4 °C. The membranes were then isolated from the plastic insert housing with a scalpel and placed in the wells of the receiver plate. The primary antibody was diluted 1:500 (dilution according to suppliers' recommendation) in a solution of PBS with 1 % BSA (PBS/ 1 % BSA) and applied onto the cells. Incubation was performed for 2 h at room temperature. The cells were subsequently rinsed three times with PBS for each 3 min. All following steps were performed in the dark. The secondary antibody "rabbit anti mouse Igg" (labeled with the rhodamine derivateTRITC) was diluted 1:200 with PBS/ 1 % BSA and applied onto the cells. Incubation was performed for 45 min. After washing three times with PBS for each 3 min, the cell nuclei were stained with 1 µg/mL DAPI for 1 min. The DAPI stock solution of 1 mg/mL was prepared in methanol and for the working solution the DAPI was further diluted with PBS. Subsequently, the cells were rinsed with PBS two times for each 2 min. The membranes were then placed on uncoated glass slides and the coverslips were mounted with Roti®-Mount FluorCare (Carl Roth). The microscopic analysis was performed using the Axiophot and Axiocam MRc (Carl Zeiss).

The immunofluorescence analyses of the Ki-67 marker in cross sections of paraffin-embedded 16HBE14o- cultures (section 4.1.1.3) were performed according to the following protocol:

The sections were placed on poly-l-lysine coated glass slides, dried and deparaffinised according to the standard procedures described in section 6.1.5.1. Heat antigen retrieval in sodium citrate buffer was performed as described in section 6.1.5.2. The sections were afterwards encircled with the Pap Pen (see section 6.1.5.2), enabling the use of small amounts (approx. 100 µL) of all reagents. The reagents were directly applied onto the sections using a pipette (10 - 200 µL). The washing was performed in staining boxes (according to Hellendahl) which were filled with approx. 80 mL PBS. After several washing steps, the PBS was partially renewed. Permeablization was performed with 0.25 % Triton-X (in PBS) for 3 min followed by washing in PBS for three times for each 3 min. To block unspecific binding sites, the sections were afterwards incubated with 10 % rabbit serum (in PBS) for 30 min. Subsequently, the antibody against the Ki-67 antigen was applied in appropriate dilution (1:100 diluted in PBS/ 1 % BSA, as recommended by supplier). The sections were washed in PBS three times for each 3 min before the secondary antibody was applied. All subsequent steps were performed in the dark. The anti-mouse IgG –TRITC antibody was used in a dilution of 1:200 (in PBS/ 1% BSA) and incubated for 25 min. The sections were afterwards washed in PBS three times for each 3 min. For visualization of the cell nuclei, DAPI was applied in a concentration of 1 µg/mL (in PBS) and incubated for 1 min. After rinsing the sections with PBS, the coverslips was mounted using Roti®-Mount FluorCare (Carl Roth). The microscopic analysis was performed using the Axio Observer Z.1 (equipped for fluorescence analyses with the VivaTome technique) and Axiocam MRm (Carl Zeiss).

6.1.5.4 TEER measurement

In an intact epithelial cell layer, the cells are linked to each other by cell-to-cell contacts. These tight junctions occlude the extracellular space between the cells, forming a tight, belt-like adhesive seal, that selectively limits the diffusion of water, ions and larger solutes between the apical and the basolateral sides of the cell layer [(251)].

This feature, called barrier function, leads to a high electrical resistance between the apical and the basolateral regions of the cell. The functionality of the barrier can be determined by measuring the electrical resistance between the two compartments. It is called transepithelial electrical resistance (TEER) and can be measured non-invasively using a

special electrode system. If the tight junctions are disrupted, ions can cross the cell barrier more easily, resulting in loss of the high electrical resistance [252].

In this thesis, the TEER value of the cell cultures that were cultivated on insert membranes was determined by means of the Millicell® ERS-2 system (Millipore Inc.). For that purpose, the cell culture medium was removed and replaced by fresh, serum-free culture medium that was previously prewarmed at room temperature. The culture medium was applied on both sides of the insert membranes with a volume of 1000 µL at the basal side (in the wells) and 500 µL at the apical side (in the inserts). For TEER measurement, the shorter electrode tip was dipped in the apical medium and the longer tip in the basal medium. Both silver chloride pellets on the tips had to be completely immersed in the medium. To ensure stable and reproducible results, the electrode was held steady and at an angle of 90° to the membrane. Contact between the electrode tips and the cell layer or the bottom of the plate was avoided.

The resistance was inversely proportional to the growth area (resistance [Ω] x effective membrane area [cm^2]). A blank value was determined by measuring the TEER value of coated inserts without cells and this blank value was subtracted from the TEER values of the cell cultures.

6.1.5.5 ELISA

The Enzyme-linked immunosorbent assay (ELISA) was performed to analyze the secretion of IL-6 and IL-8, two cytokines that are associated with inflammation processes in the lung. For that purpose the waste medium of the cell cultures was sampled at different time points and used as template for the analyses.

In general, the ELISA technique enables the detection of substances of interest in liquid samples by means of specific antibodies. The procedure, which is carried out in 96-well plates, is divided into five sequential steps.

1. Coating: The wells are coated with an antibody which is directed specifically against the desired antigen.
2. Blocking: Free non-specific binding sites are blocked on the surface of the wells with bovine serum albumin.

3. Application of the sample: The respective antigen in the sample binds specifically to the antibody on the well.
4. Application of Detection Antibody: An enzyme-linked antibody binds to the antigen.
5. Detection: A substrate is added which is converted by the enzyme that is linked to the detection antibody into a colored reaction product. The amount of converted substrate is proportional to the amount of the respective antigen present in the sample and can be measured in a plate reader (Appliskan, Thermo Scientific).

For the determination of IL-6 and IL-8 secretion, two commercial ELISA kits were used:

- BD OptEIA™ Set Human IL-6 (BD Biosciences; order no.: 555220)
- BD OptEIA™ Set Human IL-8 (BD Biosciences; order no.: 555244)

The analyses were performed according to the manufacturer instructions. The optimal dilutions of the samples were determined by trial and error.

The following solutions were prepared for ELISA:

Coating buffer:

3.56 g $NaHCO_3$

0.795 g Na_2CO_3

Dissolved in 500 ml ddH_2O

pH adjsuted to 9.5 with 10 N NaOH

Assay Diluent (for dilution of detection antibody and samples)**:**

10 % FBS in PBS (pH 7)

Wash buffer:

0.05 % Tween in PBS

Substrate solution:

1 mg TMB (Tetramethyl Benzidine) in 100 µl DMSO

+ 9.9 ml 100 mM NaOAc (in H_2O; pH 6)

+ 1 µl 30 % H_2O_2

Stop solution:

1M H_2SO_4

Each ELISA analysis included a standard of 8 samples with defined concentrations of the protein of interest. Thus, quantification of the IL-6 and IL-8 secretion in the test cultures was enabled. The data were analyzed using Microsoft Excel and displayed in a bar chart.

6.2 Abbreviations

Table 6-3. List of abbreviations

Abbreviation	Meaning
AEC	Alveolar epithelial cells
ALI	Air-liquid interface
ATCC	American Type Cell Collection
approx.	Approximately
BPE	Bovine pituitary extract
BEGM	Bronchial Epithelium Growth Medium
cm	Centimeters
COPD	Chronic obstructive pulmonary diseases
CCM	Compartment Cell Model
CULTEX® LTC	CULTEX® Long Term Cultivation system
CK	Cytokeratin
°C	Degree celsius
DMSO	Dimethyl sulphoxide
DMEM	Dulbecco´s Modified Eagle Medium
DSMZ	Deutsche Sammlung von Mikroorgiansmen und Zellkulturen
EMEM	Eagle´s Minimum Essential Medium
ELISA	Enzyme-Linked Immunosorbent Assay
EGF	Epidermal growth factor
EMTU	Epithelial-Mesenchymal Trophic Unit
ECVAM	European Centre for the Validation of Alternative Methods
ECM	Extracellular matrix
FBS	Fetal bovine serum
e.g.	For example
GA	Gentamycin/amphotericin B
g	Gram
HTS	High throughput screening
h	Hour
HBEpC	Human bronchial epithelial cells
HE	Hematoxylin-eosin
HPV	Human papiloma virus
ICCVAM	Inter-agency Coordinating Center for the Validation of Alternative Methods
IL	Interleukin
ISO	International Organization for Standardization
KRH	Klinikum Region Hannover
L	Liter
µg	Microgram
mg	Milligram
MEM	Minimum Essential Medium
M	Molar
MUC5AC	Mucin 5AC

Abbreviation	Meaning
MUC5B	Mucin 5B
nM	Nanomolar
ng	Nanogram
NHBE	Normal human bronchial epithelial cells
NHLF	Normal human lung fibroblasts
OECD	Organisation for Economic Co-operation and Development
Pen/Strep	Penicillin/Streptomycin
PBS	Phosphate buffered saline
RA	Retinoic acid
rH	Relative Humidity
RPMI	Roswell Park Memorial Institute medium
SCM	Sandwich Cell Model
cm^2	Square centimeters
m^2	Square meters
3D	Three-dimensional
TJ	Tight junction
TEER	Transepithelial electrical resistance
TGF	Transforming growth factor
TNF	Tumor necrosis factor
2D	Two-dimensional
ZEBET	Zentralstelle zur Erfassung und Bewertung von Ersatz- und Ergänzungsmethoden zum Tierversuch

6.3 Laboratory devices

Table 6-4: List of laboratory devices

Device	Supplier
Appliskan®	Thermo Fisher Scientific Inc.
Axio Observer.Z1 (inverted microscope equipped for fluorescence analyses, incl. VivaTome technology)	Carl Zeiss MicroImaging GmbH
AxioCam MRc	Carl Zeiss MicroImaging GmbH
AxioCam MRm	Carl Zeiss MicroImaging GmbH
Axiophot	Carl Zeiss MicroImaging GmbH
CO_2 Incubator, Heraeus®, BBD6220	Thermo Fisher Scientific Inc.
Fully Automated Rotary Microtome Leica RM2255	Leika Mikrosysteme Vertrieb GmbH
Heating cabinet TDO66	Medite GmbH
Modular tissue embedding center Leica EG 1150	Leika Mikrosysteme Vertrieb GmbH
Multifuge 1S-R, Heraeus®	Thermo Fisher Scientific Inc.
Olympus CKX41	Olympus Deutschland GmbH
Olympus SC30	Olympus Deutschland GmbH
Paraffin tissue floating bath 25900	MEDAX GmbH & CO.
Safety cabinet, Heraeus® Safe 2020	Thermo Fisher Scientific Inc.
Spectramax® 340 PC	Molecular Devices
Water bath GFL 1006	GFL Gesellschaft für Labortechnik mbH
Water bath W16	Harry Gestigkeit GmbH

6.4 Antibodies

Table 6-5: List of primary and secondary antibodies

Gene/Protein	Organsim	Isotype	Company	Order no.	Dilutions
Acetyl-α-tubulin	Mouse	IgG2b	Abcam	ab24610	1:500
Anti-Cytokeratin-6	Mouse	IgG1	Abcam	ab18586	1:10
Anti-Cytokeratin-13	Mouse	IgG	Abcam	ab16112	1:150
Anti-MMP9	Mouse	IgG2a	Abcam	ab119906	1:300
Anti-MUC5AC	Rabbit	IgG	SantaCruz Biotechnology Inc.	sc-20118	1:300
Anti-MUC5B	Rabbit	IgG	SantaCruz Biotechnology Inc.	sc-20119	1:300
Anti-p63	Mouse	IgG2	Abcam	ab3239	1:200
Anti-MKI67	Mouse	IgG1	Leica Biosystems	NCL-Ki67-MM1	1:100
Name	**Label**	**Organism**	**Company**	**Order no.**	**Dilutions**
Anti-Mouse IgG (whole molecule)	TRITC	Rabbit	Sigma	T2402	1:200

6.5 Laboratory equipment

Table 6-6: List of laboratory equipment

Product	Supplier	Order no.
CASY Model TT	Roche Diagnostics Deutschland GmbH	40017
Coplin staining jars with screw cap	Sigma-Aldrich	S5641-12EA
Mechanical pipette, 0.5 - 10 µL; mLINE	Sartorius Weighing Technology GmbH	12595585
Mechanical pipette, 10 - 100 µL; mLINE	Sartorius Weighing Technology GmbH	12595546
Mechanical pipette, 100 - 1000 µL; mLINE	Sartorius Weighing Technology GmbH	12606243
Mechanical pipette, 20 - 200 µL; mLINE	Sartorius Weighing Technology GmbH	12596853
Mini Vac Eco (vaccum pump)	Peqlab Biotechnologie GmbH	90-6030
PAP pen	Kisker Biotech GmbH & Co. KG	MKP1
Scalpel	Carl Roth GmbH & Co. KG	AH88.1
Scissors	Carl Roth GmbH & Co. KG	3569.1
Serological pipette supporter, CellMate® II	Thermo Fisher Scientific Inc.	1036-7302
Staining box	Carl Roth GmbH & Co. KG	H554.1
Staining boxes according to Hellendahl	Carl Roth GmbH & Co. KG	H550.1
Staining rack	Carl Roth GmbH & Co. KG	H552.1
Transferpette® S-12, 20 - 200 µL	Brand GmbH & Co. KG	703730
Tweezers, self-spanning	Carl Roth GmbH & Co. KG	LH73.1
Vortex Genie® 2	Carl Roth GmbH & Co. KG	P505.1
Wire handle for staining rack	Carl Roth GmbH & Co. KG	H553.1

6.6 Laboratory consumables

Table 6-7: List of laboratory consumables

Product	Supplier	Order no.
Cap mats for deepwell plate	Carl Roth GmbH & Co. KG	EN10.1
CASY clean	Roche Diagnostics Deutschland GmbH	43052
CASY cups	Roche Diagnostics Deutschland GmbH	43003
CASY ton	Roche Diagnostics Deutschland GmbH	43001
Cell culture flask (vented), 25 cm^2	BD Biosciences	353109
Cell culture flask (vented), 75 cm^2	BD Biosciences	353136
Cell culture inserts, 12-well, 0.4 µm pores	BD Biosciences	353180
Cell culture inserts, 12-well, 1.0 µm pores	BD Biosciences	353103
Cell culture petri dishes, 35 mm (Nunc)	Thermo Fisher Scientific Inc.	150318
Centrifuge tubes, 15 mL	BD Biosciences	352096
Centrifuge tubes, 50 mL	BD Biosciences	352070
Centrifuge tubes, self-standing, 50 mL	Corning Inc.	430921
Companion plate, 6-well	BD Biosciences	353502
Companion plate, 12-well	BD Biosciences	353503
Cover slips, 18 x 18 mm	Carl Roth GmbH & Co. KG	0657.2
Cover slips, 24 x 60 mm	Carl Roth GmbH & Co. KG	H878
Deepwell plate	Eppendorf AG	0030505301
Disposable Scalpel Cutfix®	Carl Roth GmbH & Co. KG	T998.1
Disposable syringe, 60 mL	B.Braun Melsungen AG	4616502F
Eppendorf tubes, Safe lock, 1.5 mL	Eppendorf AG	30.120.086
Immuno plates, MaxiSorp surface (Nunc)	Thermo Fisher Scientific Inc.	460984
Microscope slides Polysine® (Menzel-Gläser)	Thermo Fisher Scientific Inc.	J2800AMNZ
Microscope slides, HISTOBOND®	Medite GmbH	46-8101-00
Pasteur pipettes, 150 mm	Carl Roth GmbH & Co. KG	4518
Petri dishes, SureGrip, 100 mm	Sarstedt AG & Co.	831802003
Pipette tips (with filter), 0.1 - 10 µL	Sartorius AG	790301F
Pipette tips (with filter), 5 - 300 µL	Sartorius AG	790011F
Pipette tips (with filter), 50 - 1000 µL	Sartorius AG	790001F
Reagent reservoir	Carl Roth GmbH & Co. KG	HT66.1
riplate® - deepwell plate, 2 mL	Carl Roth GmbH & Co. KG	EN07.1
Scalpel blades	Carl Roth GmbH & Co. KG	AH89.1
Serological pipettes, 10 mL	Sarstedt AG & Co.	86.1254.001
Serological pipettes, 25 mL	Sarstedt AG & Co.	86.1685.001
Serological pipettes, 5 mL	Sarstedt AG & Co.	86.1253.001
Serological pipettes, 50 mL	Sarstedt AG & Co.	86.1689.001
Syringe Filter, 0.2 µm (Nalgene)	Thermo Fisher Scientific Inc.	190-2520
Transwell® Inserts, 12-well, 0.4 µm pores	Corning Inc.	3460

6.7 Chemicals and solutions

Table 6-8: List of chemicals and solutions

Product	Supplier	Order no.
Acetic acid 100%	AppliChem GmbH	A0820
BD OptEIA™ Set Human IL-6	BD Biosciences	555220
BD OptEIA™ Set Human IL-8	BD Biosciences	555244
Bovine serum albumin (BSA)	Sigma-Aldrich	A9418
Bovine serum albumin (BSA, cell culture grade)	Sigma-Aldrich	A2153
Collagen type I (from bovine skin, 3mg/ml)	Sigma-Aldrich	C4243
Collagen type IV (human placenta, Bornstein and Traub)	Sigma-Aldrich	C5533
DAPI *BioChemica*	AppliChem GmbH	A1001
Disodium hydrogen phosphate (Na2HPO4)	AppliChem GmbH	A3567
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich	D2650
EDTA (Ethylenediaminetetraacetic acid) tetrasodium salt dihydrate	Sigma-Aldrich-Aldrich	ED4SS
Eosin G solution	Carl Roth GmbH & Co. KG	X883.2
Ethanol, absolute	AppliChem GmbH	A4930
Fibronectin (human, 1mg/mL)	Biochrom AG	L7117
Formaldehyde solution, 37 %	AppliChem GmbH	A3592
G418 (Genetiticin)	AppliChem GmbH	A6798
H_2SO_4 Sulfuric acid (2N)	Carl Roth GmbH & Co. KG	X873.1
Hemalum solution (hemalum acc. to Mayer)	Carl Roth GmbH & Co. KG	T865.2
Hydrochloric acid (HCl), 5M	AppliChem GmbH	A2668
Immersion Oil	AppliChem GmbH	A3494
Isopropyl alcohol (absolute)	AppliChem GmbH	A0995
LHC Basal Medium	Life Technologies Corp.	12677-019
NovoLink® Polymer Detection System	Leica Microsystems	RE7140-K
Paraplast® (Leica)	Carl Roth GmbH & Co. KG	X880.1
PBS (phosphate buffered saline) tablets	Life Technologies Corp.	518912-014
PBS (phosphate buffered saline, sterile; without Mg^{2+} and Ca^{2+})	Biochrom AG	L1825
Recovery Cell Culture Freezing Medium	Life Technologies Corp.	12648-010
Roti®-Histokitt	Carl Roth GmbH & Co. KG	6638.1
Roti®-Mount FluorCare	Carl Roth GmbH & Co. KG	HP19.1

Product	Supplier	Order no.
Sodium acetate (CH_3COONa)	Carl Roth GmbH & Co. KG	6773.1
Sodium carbonate (Na_2CO_3)	Sigma-Aldrich	57795-500G
Sodium hydrogen carbonate ($NaHCO_3$)	Carl Roth GmbH & Co. KG	HNO1.1
Sodium hydroxide pellets (Na_2CO_3)	AppliChem GmbH	A1551
Sodiumhydrogenphosphate ($NaH_2PO_{4)}$	AppliChem GmbH	A3559
3,3',5,5'-Tetramethylbenzidin (TMB)	Carl Roth GmbH & Co. KG	6350.1
Tris(hydroxymethyl)aminomethane (Sigma-Aldrich 7-9)	Sigma-Aldrich-Aldrich	T1378
Trisodium citrate dihydrate	AppliChem GmbH	A3901
Triton®-X-100	AppliChem GmbH	A1388
Trypsin inhibitor from soybean (50X)	AppliChem GmbH	A8339
Trypsin/EDTA solution (0.05%/0.02% (w/v))	Biochrom AG	L 2143
Trypsin/EDTA solution (0.25%/0.02% (w/v))	Biochrom AG	L 2163
Tween 20 (10% solution)	BioRad Laboratories Inc.	161-0781
Water, bidistilled (ddH_2O)	AppliChem GmbH	A4042
Xylene, 98,5%	Carl Roth GmbH & Co. KG	CN80.2

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Curriculum vitae

Jessica Rach

PERSONAL DATA:

Date of Birth: January, 8, 1977
Place of Birth: Gehrden, Germany
Gender: Female
Family Status: unmarried, no childs

WORK EXPERIENCE:

03/2009 – present *Cultex Laboratories GmbH*, Hannover, Germany
- Research Assistant, Focus: Cell culture

11/2005 – 02/2009 *Stiftung Tierärztliche Hochschule Hannover*, Germany
- Graduate research in Biological Science, Topic: "Development of character-based DNA barcodes for Odonata"

01/1999 – 02/2009 *Agility Logistics GmbH (form. Geologistics GmbH)*, Hannover, Germany
- Employee (11/2000 – 02/2009 temporary worker)

UNIVERSITY EDUCATION:

10/2012 – 06/2013 *Gottfried Wilhelm Leibniz Universität Hannover, Germany*
- Dissertation; Title: "Development of suitable *in vitro* models of the bronchial epithelium for inhalation toxicology"
- Graduation with doctoral degree (Dr. *rer. nat.*)

01/2005 – 10/2005 *Stiftung Tierärztliche Hochschule Hannover*, Germany
- Diploma thesis: "Character-based DNA barcoding: Development of an DNA-based identification system for Odonata"
- Graduation with University-Diploma in Biological Science

2000 – 2005 *Gottfried Wilhelm Leibniz Universität Hannover*, Germany
- Undergraduate studies in Biological Science, concentration in Molecular Biology, Zoology and Ethology

PROFESSIONAL EDUCATION:

01/1999 – 10/2000 *Geologistics GmbH*, Hannover, Germany
- Training as freight forwarding agent
- Certified by Industrie und Handelskammer Hannover, Germany

SCHOOL EDUCATION:

1983 – 1989 *Grundschule Am Castrum*, Gehrden, Germany

1989 – 1991 *Orientierungsstufe*, Gehrden, Germany

1991 – 1996 *Matthias-Claudius-Gymnasium*, Gehrden, Germany
- Higher education entrance qualification

List of publications and posters

Publications:

Rach J, Budde J, Mohle N, and Aufderheide M. 2013. Direct exposure at the air-liquid interface: evaluation of an in vitro approach for simulating inhalation of airborne substances. *J Appl Toxicol.*

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Posters:

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