LUNG TISSUE MODEL

FIELD OF THE INVENTION

The present invention provides for an engineered three dimensional pulmonary model tissue culture which is free of any artificial scaffold. Three dimensional models of healthy lung tissue as well as disease tissues are available. The product according to the invention can be marketed e.g. in the form of tissue cultures, plates or arrays comprising such cultures or kits. The invention is applicable in medical and scientific research, for testing compounds for their effect on lung tissue, for screening, testing and/or evaluating drugs, and in certain cases in diagnostics of lung diseases.

BACKGROUND ART

Tissue engineering is a rapidly developing field of biomedical research that aims to repair, replace or regenerate damaged tissues. Due to the latest events of disastrous phase II clinical drug trials (e.g. TGN1412), further goals of tissue engineering include generation of human tissue models for safety and efficacy testing of pharmaceutical compounds. Tissue engineering in general and our model in particular exploits biological morphogenesis, which is an example of self-assembly.

Cell and tissue engineering is pursued nowadays from several perspectives. In one aspect, it is used to gain deeper insight into cell biology and physiology.

Two main directions are being developed in tissue engineering research: tissue scaffold based and tissue scaffold free systems. While scaffold based systems use mostly biodegradable scaffold material to provide an artificial 3D structure to facilitate cellular interactions, a scaffold free system allows direct cell to cell interactions, and allows cells to grow on their secreted scaffold material in the model.

In US 2001/0055804 A1 („Three dimensional in vitro model of human preneoplastic breast disease") discloses a three dimensional *in vitro* cell culture system useful as a model of a preneoplastic breast disease for screening drugs. Said model is prepared by co-culturing preneoplastic epithelial cells of breast origin, endothelial cells and breast fibroblasts on a reconstituted base membrane in a medium comprising further additives like growth factors, estrogens etc. Thus, in this solution a base membrane, preferably Matrigel® is used as a tissue scaffold. According to the description a network of branching ductal alveolar units vasculature is formed within about 3-7 days in this system.

The system relates to breast and not lung and there is no suggestion to make a lung culture by that method.

In US 2003/0109920 („Engineered animal tissue") microvascular endothelial cells were obtained from adult lung and placed between two layers of human dermal fibroblasts present in a three dimensional collagen gel. Thus, a sandwich structure was formed.

Though the vascular endothelial cells were obtained from human lung, the artificial tissue prepared by this method was similar to human skin, therefore, it can not be actually considered as a pulmonary tissue model.

In US 2008/0112890 („Fetal pulmonary cells and uses thereof") a 3D tissue-like preparation is taught which is based on fetal mouse epithelial, endothelial and mesenchymal cells. The authors used mouse embryonic lung cells to the preparation in order to obtain lung prosthesis and perform screening on the 3D tissue-like preparation. As a matrix, a hydrogel, MATRIGEL™ was used to establish appropriate cell-cell interactions. It appears from the description that a fibroblast overgrowth was experienced upon coculturing epithelial cells and fibroblasts.

WO2008/100555 („Engineered lung tissue construction for high throughput toxicity screening and drug discovery")

relates to a lung tissue model preparation comprising fetal pulmonary cells and a tissue scaffold made of a biocompatible material and preferably a fibroblast growth factor. Fetal pulmonary cells comprise epithelial, endothelial and mesenchymal cells. A number of applicable biocompatible materials are listed.

WO2004/046322 ("Replication of biological tissue") preparation of an artificial 3-D tissue is proposed under microgravity environment. The tissue is based on human breast cancer cells and is useful as a breast cancer model. In a rotating bioreactor chamber at first connective tissue cells are cultured till the formation of a 3-D spheroid structure, then sequentially endothelial and epithelial cells are added to the culture. It is to be noted that the teaching is theoretical and while protocols are provided to culture the cells and to handle to cultures, no actual results of the experiments are disclosed.

Vertrees, RA, Zwischenberger, JB et al. (2008) used a well documented normal immortalized lung cell line to grow as a traditional monolayer (ML) in culture flasks and as 3-D cultures in rotating walled vessels. Comparison for presence of differentiation and marker expression between these cultures and control tissue collected from surgical patient specimens was studied. The purpose of the authors was to develop both traditional monolayer and 3-D cell cultures of a known cell line.

Recently, Kelly BéruBé and her co-workers developed a 3D tissue engineering model of the human lung epithelium for safety testing. They used normal human bronchial epithelial cells [NHBE]. Primary cells, obtained from humans, were grown on a microporous membrane to form a 3-dimensional (3D) cell culture. The 3D cultures formed tight junctions between cells, cells with active cilia, and others producing and secreting mucus. These characteristics closely resemble those found in the native human respiratory epithelial tissue and were supposed to accurately mimic the human responses to tissue damage. The culture in fact forms a thick layer of cells modeling the mucous membrane surface [Hughes Tracy et al. 2007].

Despite the extensive literature of lung models, it appears that the prior art discloses only tissue scaffold based three dimensional models and no simple, tissue scaffold free lung tissue model, comprising at least epithelial cells and fibroblasts, is disclosed in the prior art.

The present Inventors have surprisingly found that by simple biochemical methods a tissue scaffold free lung tissue model system can be rapidly created which is in several aspects more favorable than two dimensional systems or systems based on a matrix. The present invention provides a model tissue which is ready for use in various tests. The model is suitable to study cell-cell interactions in various lung tissues to mimic normal function and disease development.

BRIEF DESCRIPTION OF THE INVENTION

The invention provides for an engineered three dimensional pulmonary model tissue culture, said model tissue culture

a) being free of any artificial tissue scaffold,

b) being composed of cultured cells or having a cultured cellular material wherein the cells are in direct cell-cell interaction with cells of one or more other cell types of the tissue material,

c) comprising at least pulmonary epithelial and mesenchymal cells, preferably pulmonary mesenchymal cells, preferably fibroblasts. Wherein preferably the ratio of the pulmonary epithelial cells and the mesenchymal cells in the model tissue is at least 1:6, preferably at least 1:3, and at most 6:1, preferably at most 3:1,

d) having a morphology of one ore more cellular aggregate(s) wherein the surface of the aggregates is enriched in the pulmonary epithelial cells, or wherein the pulmonary epithelial cells and the mesenchymal cells, preferably fibroblasts are at least partially segregated in said aggregates, and

e) wherein the epithelial cells express epithelial differentiation markers.

a) In a preferred embodiment said model tissue culture is free of an artificial matrix material for providing a three dimensional environment to the cells. In an embodiment said model tissue culture is free of any artificial tissue scaffold material, either biodegradable or non-biodegradable tissue scaffold material, e.g. a porous three dimensional matrix; a three dimensional gel matrix. In an embodiment said model tissue culture is free of or does not comprise a microporous membrane support.

b) In a preferred embodiment said model tissue culture also comprises an extracellular matrix, the extracellular matrix proteins of which are secreted by at least one of the cell types comprising the tissue, preferably by fibroblasts.

c) In further preferred embodiments the pulmonary epithelial cells comprise at least one of the following cell types:

- type I pneumocytes, [alveolar type I cells (ATI)]
- type II pneumocytes, [alveolar type II cells (ATII)].

Preferably, said type II pneumocytes (alveolar epithelial cells with ATII characteristics) express one or more of the following markers: TTF1 transcription factor, surfactant protein A (SFPA), surfactant protein C (SFPC) and aquaporin 3 (AQP 3).

Preferably, said type I pneumocytes (alveolar epithelial cells with ATI characteristics) express one or more of the following markers: TTF1 transcription factor, aquaporin 3 (AQP 3), aquaporin 4 (AQP 4) and aquaporin 5 (AQP 5).

In various further embodiments at least one of pulmonary epithelial cells and pulmonary mesenchymal cells are present in the model.

Preferably, the cells are amphibian, reptilian, avian or, more preferably mammalian cells.

Preferred avian cells are poultry pulmonary cells.

Preferred mammalian cells are cells of herbivorous animals, preferably livestock animals like cells of e.g. sheep, goat, bovine cells, or rodent cells, e.g. rabbit or murine cells. Further preferred mammalian cells are those of omnivorous animals like pig cells. Highly preferred cells are human cells.

In further embodiments the pulmonary epithelial cells and/or the mesenchymal cells are obtained from

- established cell lines, preferably from commercial sources,
- healthy donors
- patient donors.

In a preferred embodiment the cells are primary cells. In a preferred embodiment the cells are not de-differentiated cells or only partially de-differentiated cells.

In a further preferred embodiment the cells are de-differentiated cells or the cells are de-differentiated before culturing them to 3D model tissue culture.

In a preferred embodiment the pulmonary epithelial cells comprise small airways epithelial cells, preferably small airways epithelial cells with ATII characteristics.

In a further preferred embodiment the model tissue culture of the invention also comprises endothelial cells. In a preferred embodiment the endothelial cells are HMVEC or HUVEC cells.

Optionally, the model tissue culture of the invention may further comprise cells of further type selected from macrophages, mast cells, smooth muscle cells.

d) In a preferred embodiment the average diameter or the typical diameter of the aggregate is at least 10 μm, 40 μm, 60 μm. 80 μm, 100 μm or 120 μm and the average diameter or typical diameter of the aggregate is at most 1000 μm, 800 μm, 600 μm, 500 μm, 400 μm or 300 μm.

Highly advantageously the average diameter or typical diameter of the aggregate is 100-300 μm, in a preferred embodiment it is about 200 μm.

Average size of the aggregates can be assessed and calculated or estimated by any experimentally and mathematically correct means. While the aggregates are essentially spherical in shape, it is evident that diameters for each aggregate multiple diameters can be determined due to a deviation from the exact sphere and depending on the position of the aggregate during measurement and on the measurement method. For example, smallest and largest diameter can be measured directly in the microscope measuring the size of several aggregates and averaged. Expediently, a microscope is used for this purpose.

In a preferred embodiment the majority of the aggregates, preferably at least the 60%, 70%, 80% or 90% of the aggregates has a diameter of at least 10 µm, 40 µm, 60 µm. 80 µm, 100 µm or 120 µm and a diameter of at most 1000 µm, 800 µm, 600 µm, 500 µm, 400 µm or 300 µm, highly advantageously the diameter of the above ratio of the aggregates is 100-300 µm, in a preferred embodiment it is about 200 µm.

In a preferred embodiment, the culture samples in each aggregate or each container of a kit comprise cells in an amount of

at least 10^3, preferably at least 10^4, more preferably at least $2*10^4$, $3*10^4$, $4*10^4$, $5*10^4$ cells, and

at most 10^6, more preferably at most $5*10^5$, $4*10^5$, $3*10^5$, $2*10^5$ or at most 10^5 cells.

In a preferred embodiment the pulmonary epithelial cells and the fibroblasts are segregated based on a difference in their surface tension. Preferably, the majority of the pulmonary epithelial cells are located on the surface of the aggregate.

Preferably, the majority of the pulmonary epithelial cells form a pulmonary epithelial cell lining on the surface of the aggregate, preferably said pulmonary epithelial cell lining covering, at least partly, the surface of the aggregate.

In a further preferred embodiment the aggregates also comprise endothelial cells.

In a preferred embodiment the ratio of the endothelial cells, in comparison with the epithelial and fibroblast cells is higher in the center or central region of the aggregates that in the surface of the aggregates, or the ratio of the endothelial cells is increasing from the surface of the aggregates towards the center of the aggregates.

In a preferred embodiment the aggregates have a layered structure wherein the core or central region of the aggregates comprises the maximum ratio of endothelial cells, the intermediate layer or region of the aggregates comprises the maximum ratio of fibroblasts and the outer layer or surface layer of the aggregates comprises the maximum layer of epithelial cells.

e) In a preferred embodiment the epithelial differentiation markers expressed by the tissue cells of the engineered three dimensional pulmonary model tissue are at least one or more markers selected from the following group:

- ATII type differentiation markers, preferably TTF1 transcription factor, cytokeratin 7, (KRT7), surfactant protein A (SFPA), surfactant protein C (SFPC) and aquaporin 3 (AQP 3). and/or markers
- ATI type differentiation markers, preferably aquaporin 4 (AQP 4) and aquaporin 5 (AQP 5).

The markers expressed also depend on the cell type used in the tissue culture.

The level of any of the markers can be detected at mRNA or a protein level. Thus, the level of the marker may be mRNA level and/or protein level.

Preferably, the model tissue culture of the present invention at least one of the level of AQP3 and SFTPA is increased, i.e. they are up-reguated in comparison with a control 2D culture.

Preferably, the model tissue culture of the present invention at least one inflammatory marker selected from IL1b, IL6 and CXCL8 is down-regulated, i.e. their level is decreased in 3D culture conditions in comparison with a control 2D culture.

Preferably, the model tissue culture of the present invention the level of at least one of de-differentiation markers S100A4 and N-cadherin is decreased in comparison with purified primary cells in 2D culture conditions or in a

control 2D tissue culture.

In various further embodiments at least one of pulmonary epithelial cells and pulmonary mesenchymal cells are present in the model and, in analogy with embryonic lung development,

- pulmonary epithelial cells secrete one or more fibroblast growth factors selected from FGF4, FGF8, FGF9.
- pulmonary epithelial cells express on the cell surface FGFR2b receptors.
- pulmonary mesenchymal cells, preferably fibroblasts secrete FGF7 and FGF10 and expresses FGFR1c and FGFR2c receptors.

In a preferred embodiment of the tissue culture of the present invention the ATII type differentiation markers and/or ATI type differentiation markers are expressed at a level higher, more preferably at a level of at least 10%, at least 20%, or at least 30% higher than that measured in a two dimensional tissue culture used as a reference.

Preferably, mRNA level(s) and/or protein level(s) of said epithelial marker(s) in the model tissue is higher than one or more or each of the following reference cultures

- a two dimensional culture of the same cell types,
- a culture of only pulmonary epithelial cells,
- a culture of only primary fibroblast cells, preferably human fibroblast cells.

Preferably, the level of at least two of said markers is elevated.

In a preferred embodiment small airways epithelial cells are applied.

In a further preferred embodiment small airways epithelial cells showing at least some ATII type characteristics are applied. In this case the model tissue shows increased mRNA level(s) and/or protein level(s) of at least one or more markers selected from the following group of ATII type differentiation markers, e.g. those listed above.

The engineered three dimensional pulmonary model tissue of the invention preferably shows a reduced expression of one or more pro-inflammatory cytokine and or one or more EMT markers.

Preferably, mRNA level(s) and/or protein level(s) of said on or more pro-inflammatory cytokine in the model tissue is lower than one or more or each of the following reference cultures

- a two dimensional culture of the same composition of cells,
- a culture of only pulmonary epithelial cells, wherein said pulmonary epithelial cells are treated analogously to the model tissue culture.

Preferably, the pro-inflammatory cytokine(s) are selected from the following group:

CXCL-8 pro-inflammatory cytokine, IL6, IL1a, IL1b, TNFalpha.

In a highly preferred embodiment the pro-inflammatory chemokine is CXCL-8 chemoattractant.

In a preferred embodiment the model tissue culture comprises pulmonary epithelial cells and pulmonary mesenchymal cells and does not comprise endothelial cells, wherein

- the level of one or more of the following markers is increased relative to a control comprising non-cultured cells: E-cad, IL-1b and/or IL6,
- the level of E-cad is increased relative to a control 2 dimensional culture,
- the level of one or more of the following markers is decreased relative to a control 2 dimensional culture: IL-1b, CXCL8, IL6.

In a preferred embodiment the model tissue culture comprises pulmonary epithelial cells, pulmonary mesenchymal cells and endothelial cells, wherein

the level of one or more of the following markers is decreased relative to a control comprising non-cultured cells: E-cad, N-cad.

- the level of E-cad is increased relative to a control 2 dimensional culture,

- the level of one or more of the following markers is decreased relative to a control 2 dimensional culture: N-cad, S100A4.

In a preferred embodiment the three dimensional pulmonary model tissue culture further comprises cells selected from the following group:

- endothelial cells, e.g. to mimic vasculature,
- macrophages,
- mast cells.

At later stages the model can be extended using cell types:

- smooth muscle cells,
- nerve cells.

Disease Models

The invention also relates to an engineered three dimensional pulmonary model tissue culture as defined above wherein

said epithelial and/or fibroblast cells comprise affected cells having a pathologic feature of a diseased lung tissue so that said model tissue culture is a pulmonary disease model tissue culture.

In preferred embodiments the disease involves a condition selected from inflammation, tumor, fibrosis, injury of a tissue and the model tissue culture is to be considered as an inflammatory model, a tumor model, a fibrosis model or a regeneration model, respectively.

Affected cells of the disease model can be but are not limited to cells obtained from patients (patient cells), cell lines which have a disease feature, e.g. tumor cell lines; cells exposed to an environmental effect, e.g. pro-inflammatory material, causing a disease feature; cells exposed to the effect of a mutagen and selected for a disease feature; or genetically modified cells transformed to express a protein or in which a gene is silenced so as to have a diseased feature.

In a preferred embodiment the cells are obtained from healthy subjects and disease state is induced therein. In this embodiment for example signaling of tumor induction or potential drug targets can be determined.

In an other embodiment tumor model tissue is prepared from immortal cells, e.g. from malignously transformed or tumorous cells or cell lines. While in this embodiment no "healthy" control is present, this system is useful in drug testing as a sample contacted with a placebo drug provides a control for drug treatment samples.

In an embodiment tumorous cells are obtained from a patient, and efficiency of a projected therapy can be tested. Thus, the model tissue culture can be used for establishing personalized therapy.

Method for preparation

The invention also provides for a *method for the preparation* of the engineered three dimensional pulmonary model tissue culture as defined herein, said method comprising the steps of

- preparing a mixed suspension of at least primary fibroblast cells and pulmonary epithelial cells,
- placing the mixed suspension or an aliquot thereof in a container suitable for pelleting the cells of the suspension,
- pelleting the cells,
- incubating the pelleted suspension in the presence of CO_2 for a time sufficient to the cells to form a three dimensional pulmonary model tissue comprising cellular aggregate(s),
- optionally assaying the model tissue for

a) expression of one or more epithelial differentiation markers characteristic to lung tissue, and an increased expression level as compared to a suitable reference culture e.g. as disclosed herein, is considered as indicative of the formation of a three dimensional pulmonary model tissue culture; and/or

b) expression of one or more pro-inflammatory cytokine, and a decreased expression level as compared to any suitable reference culture e.g. as disclosed herein, is considered as indicative of the formation of a three dimensional pulmonary model tissue culture.

Preferably, the container is a non-tissue culture treated container.

Preferably, multiple aliquots are placed into multiple containers,

Preferably, the containers are wells of a plate, e.g. a 96 well plate or a 384 well plate,

Preferably, pelleting is carried out at 200g to 600g, 1 to 20 minutes, preferably 2 to 10 minutes.

Preferably, the cells are supplied with a reporter molecule, e.g. are stained with a biocompatible dye to report on cellular features as disclosed herein.

The containers can be V-bottom, flat-bottom or U-bottom containers, depending on the purpose they are used for.

Upon preparation of a mixed suspension one or more type of cells are added to a container within 18 hours, preferably within 16 hours, 14 hours, 12 hours, 10 hours, 8 hours, 6 hours, more preferably within 4 hours, 3 hours, 2 hours, highly preferably within 1 hour or 0.5 hours. Preferably each type of cell used is added within the period defined above.

In a preferred embodiment the pelleted suspension is incubated in the presence of CO_2 for a period not longer than 50 hours, 40 hours, 30 hours, 24 hours, 22 hours, 20 hours, 18 hours, 16 hours, 14 hours, 12 hours or 10 hours. In a preferred embodiment the pelleted suspension is incubated in the presence of CO_2 for a period not less than 2 hours, 4 hours, 6 hours, 8 hours, 10 hours, 12 hours.

In an embodiment of the invention further type of cells are added to the mixed suspension of the cells. In an embodiment of the invention further type of cells are at least endothelial cells. In an embodiment of the invention further type of cells are selected from endothelial cells, smooth muscle cells, nerve cells, granulocytes, dendritic cells, mast cells, T/B lymphocytes, macrophages. Granulocytes, dendritic cells, mast cells, T/B lymphocytes and macrophages can be added to the cultures either in inactive or in immunologically active state.

Optionally, the method according to the invention comprises de-differentiation of one or more type of cells prior to preparation of a mixed suspension.

Optionally, the method according to the invention comprises a propagation of one or more type of cells prior to preparation of a mixed suspension. This step is particularly required if parallel testing of a large number of samples are required, for example in HTS (High Throughput Screening) solutions.

Method for screening

The invention also relates to a *method for screening of a drug for its effect on lung tissue*, said method comprising the steps of

- providing an engineered three dimensional pulmonary model tissue culture as defined herein,
- taking at least a test sample and a reference sample of said model tissue culture,
- contacting the test sample with a drug while maintaining the test sample and the reference sample under the same conditions,
- detecting any alteration or modification of the test sample in comparison with the reference sample

wherein if any alteration or modification of the test sample is detected it is considered as an indication of the effect of the drug.

In certain variant of the method only the direction of an alteration or modification is observed and no physiological values are calculated. In certain variants a predetermined threshold value is defined based on a calibration curve and comparison is made with this value.

In a preferred embodiment the model tissue culture is the *model of a healthy lung tissue* and an adverse effect of a

drug is tested, wherein alteration or modification which is detrimental to the cells of test sample is considered as a toxic or adverse effect of said drug.

In a preferred embodiment the model tissue culture is a *pulmonary disease model tissue culture* comprising affected cells having a pathologic feature and the beneficial effect of a drug is tested, wherein

- an assay to measure or assess said pathologic feature is provided for said model tissue culture to obtain a measure of disease,
- a reference sample of a healthy lung tissue (healthy reference sample) and/or a reference sample of a diseased lung tissue (diseased reference sample) is provided,
- the pathologic feature is measured or assessed in the healthy reference sample and/or in the diseased reference sample and in said at least one test sample before and after contacting it with the drug, wherein

any alteration or modification in the test sample which shifts the measure of disease in the test sample towards the measure of disease in the healthy reference sample and or away from the measure of disease in the diseased reference sample is considered as a beneficial effect of said drug. In other words, it is more similar to the state of the healthy reference sample than to the diseased reference sample.

In a preferred embodiment primary cells obtained from a patient are applied. In a preferred embodiment primary cells from a given patient are not or only partly de-differentiated and used within 5, 4, 3. 2 or 1 day(s) or within 12, 10, 8, 6, 4, 3, 2, 1 hour(s) after obtaining them from said patient to prepare the mixed suspension of the cells. In a model tissue culture made of primary cells, features of the disease state of the given patient can be studied and therapeutic drugs and/or regimens can be tested.

Kits

The invention also relates to an engineered three dimensional pulmonary model tissue kit comprising a test plate having an array of containers wherein at least two containers contain

- samples of one or more types of engineered three dimensional pulmonary model tissue cultures as defined in any of the previous claims, each sample placed in separate containers of said plate,
- an appropriate medium for culturing cells of the model tissue cultures.

Preferably a subset of the containers comprises one or more control samples. Control samples can be pure cultures of certain cell types, e.g. cultures of epithelium and fibroblasts only, and/or two dimensional (2D) cultures. In disease models controls can be cultures of healthy cells.

Preferably, the engineered three dimensional pulmonary model tissue kit has one or more of the following characteristics:

- the plate is a 96 well plate.
- the plate is a V-bottom plate or a flat bottom plate or a plate comprising both V-bottom and flat-bottom wells. U-bottom plates also can be applied.
- the culture samples in each container comprise cells in an amount of

at least 10^3, preferably at least 10^4, more preferably at least $2*10^4$, $3*10^4$, $4*10^4$, $5*10^4$ cells, and

at most 10^6, preferably at most $5*10^5$, $4*10^5$, $3*10^5$, $2*10^5$, or at most 10^5 cells,

- the containers are sealed, either separately or together and contain a CO_2 enriched environment or atmosphere suitable for a lung tissue culture.
- the CO_2 enriched environment or atmosphere comprises

at least 2%, 3%, 4% CO_2 environment,

at most 10%, 9%, 8% or 7% CO_2 environment,

highly preferably about 5% CO_2.

In preferred embodiments the samples comprise test sample(s) and corresponding control sample(s).

In preferred embodiments the test samples are present on a V-bottom plate or in V-bottom wells on a plate and the control samples are present on a flat-bottom plate or in flat-bottom wells on a plate.

DEFINITIONS

The meaning of an *"artificial tissue scaffold"* in the context of the present description is a solid support material having a structure specially designed for and useful for cell attachment and/or for assisting the structural three dimensional arrangement of cells, in tissue or cell culture. Preferably, said artificial tissue scaffold is manufactured prior to culturing of the tissue or cells and contacted with the tissue or the cells before or during culturing said tissue or cells. Thus, an artificial tissue scaffold is typically a cell growth support structure or material which contributes to the structure, e.g. the three dimensional structure of the tissue or cell culture by affecting at least a part of cellular interactions (e.g. the cell-cell interactions) or the cellular environment itself. As a consequence, if a tissue scaffold is removed from the tissue or cell culture, the tissue or cell culture will disintegrate or become disorganized. A skilled person will understand, however, that if the artificial tissue scaffold is made of a biodegradable material and it is degraded gradually, allowing cell-cell interactions to be formed, this is not to be considered as a removal of the tissue scaffold and in this process the tissue or cell culture may not become disintegrated or disorganized.

In a version the artificial tissue scaffold is a three dimensional matrix, preferably a three dimensional gel matrix or a porous three dimensional matrix, said matrix preferably having microspaces or pores in which the cells are located.

In a version the artificial tissue scaffold itself is a support on the surface of which the cells are attached, preferably a porous membrane support. In this version of the scaffold it has a structure specially designed for and useful for cell attachment, e.g. a porous or curved or engrailed or grooved surface to which the cells are attached so that this facilitates the formation of a 3 dimensional structure.

Preferably, an artificial tissue scaffold

- has a defined three dimensional structure
- is a porous, preferably a highly porous material or matrix,
- is a porous membrane,
- is a porous three dimensional matrix
- is made of a biocompatible material, and/or
- is made of a polymer.

Optionally, the *"artificial tissue scaffold"* is a polysaccharide-based matrix, e.g. it is a cellulose-based matrix, e.g. a methyl-cellulose matrix.

Optionally, the *"artificial tissue scaffold"* has a bead structure, e.g. it is a cytodex bead.

A *"three dimensional tissue culture free of any artificial tissue scaffold"* is understood herein as a tissue culture having a three dimensional structure wherein the three dimensional structure of said tissue culture is formed or contributed by inherent cell-cell interactions and is not assisted by an artificial tissue scaffold.

Thus, a three dimensional tissue culture free of any artificial tissue scaffold does not disintegrate or become disorganized in lack of an artificial tissue scaffold but maintains its three dimensional structure. Even if said three dimensional tissue culture free of any artificial tissue scaffold is cultured and formed on a solid support material, the formation of the three dimensional structure is not assisted by and is not due to attachment of cells to this solid support and it can be separated without destruction of the three dimensional structure.

"Segregation of cells" as used herein relates to the spatial separation of at least two types of cells of a tissue or cell culture, whereby after this spatial separation i.e. segregation, a region of the culture, e.g. a (partial) volume or

surface, can be defined or found in which the ratio of the two types of cells is different from both the ratio of the same types of cells in the same region of the culture before segregation and the ratio of the same types of cells in an other region of the culture. Preferably, a difference in the surface tension of at least two types of cells significantly contributes to their segregation in vitro.

"Enrichment" of a region, e.g. a volume or partial volume or surface or partial surface of a culture in a certain cell type is to be understood as a phenomenon when the ratio of a certain cell type is higher in that region than in a reference region, e.g. an other region of said culture. Typically "enrichment" of a region of a culture is the result of segregation of cells.

"Inflammation" is an adaptive response that is triggered by noxious stimuli and conditions, such as infection and tissue injury.

A number of cytokines, known collectively as *"pro-inflammatory cytokines"* because they accelerate inflammation, also regulate inflammatory reactions either directly or by their ability to induce the synthesis of cellular adhesion molecules or other cytokines in certain cell types. The major pro-inflammatory cytokines that are responsible for early responses are IL1-alpha, IL1-beta, IL6, and TNF-alpha. Other pro-inflammatory mediators include IFN-gamma, CNTF, TGF-beta, IL12, IL17, IL18, IL8 (CXCL8) and a variety of other chemokines that chemoattract inflammatory cells, and various neuromodulatory factors. The net effect of an inflammatory response is determined by the balance between pro-inflammatory cytokines and anti-inflammatory cytokines (for example IL4, IL10, and IL13, IL16, IFN-alpha, TGF-beta, IL1ra, G-CSF, soluble receptors for TNF or IL6). Activation of IL1-beta by various caspases proceeds in a large multiprotein complex that has been termed inflammasome.

LIF, GM-CSF, IL11 and OSM are further cytokines affecting inflammation processes and which are possibly useful in the preparation of disease models of the invention.

To the contrary, "*anti-inflammatory cytokines*", like IL10, regulate inflammation processes so that they are inhibited or alleviated.

The *"average diameter"* of three dimensional tissues is taken as the aritmetic mean of several measurements of three dimensional tissue diameters generated by the above described method.

The "*typical diameter*" (median diameter) is the diameter which marks the division of a given sediment sample into two equal parts by weight, one part containing all aggregates larger than that diameter and the other part containing all aggregates smaller.

An *"array"* of containers is to be understood as an arrangement of multiple containers of the same size, shape and material. The arrangement can be for example a sequence of container. or a two dimensional matrix of the containers.

Viruses are obligate intra-cellular pathogens that infect cells, often with great specificity to a particular cell type. In *"recombinant virus vectors"* genes that are needed for the replication phase of the viral life cycle are deleted and genes of interest added to the viral genome. The recombinant viral vectors can transduce the cell type it would normally infect. To produce such recombinant viral vectors the non-essential genes are provided in trans, either integrated into the genome of the packaging cell line or on a plasmid. A number of viruses have been developed, interest has centred on four types; retroviruses (including lentiviruses), adenoviruses, adeno-associated viruses & herpes simplex virus type 1.

"Cancer" is a class of diseases in which a group of cells display *uncontrolled growth*, *invasion* (intrusion on and destruction of adjacent tissues), and sometimes metastasis (spread to other locations in the body via lymph or blood). These three malignant properties of cancers differentiate them from benign tumors, which are self-limited, do not invade or metastasize. 95% of lung tumors are bronchogenic carcinoma; also bronchial carcinoids,

mesenchymal, miscellaneous neoplasms.

"Fibrosis" is the formation or development of excess fibrous connective tissue in an organ or tissue as a reparative or reactive process, as opposed to a formation of fibrous tissue as a normal constituent of an organ or tissue. Pulmonary fibrosis is a severe chronic disease characterized by a loss of elasticity and lung epithelial cells, replaced by interstitial myofibroblasts and deposition of extracellular matrix proteins in the lung interstitium leading to pulmonary structural remodelling.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Structure of 3D two-cell type microcultures. SAEC and NHLF cells were stained with the vital dyes of CFSE or DiI, respectively. Cell populations either pure or mixed at various ratios were pelleted and aggregates were formed after 24 hour incubation, then transferred into 24 well cell culture plates for imaging. **Top row:** phase contrast microscopic images; **Middle row:** fluorescent microscopic images; **Bottom row:** confocal images. SAEC: green channel; NHLF: red channel.

Figure 2. Matrigel based culture of 50%SAEC and 50%NHLF mix.

Figure 3. Pelleted micro-tissue cultures containing different ratios of SAEC and NHLF. Due to physiological fluorescent markers SAEC cells are green or light grey on black and white copies whereas NHLF cells are red or dark gray.

Figure 4a. mRNA levels of TTF-1 in 3D human lung micro-tissues. TTF1 transcription factor is a characteristic marker of alveolar epithelial cells. While 3D fibroblast cultures show no TTF1 expression, TTF1 is present in 3D SAEC monocultures and increased in 2D SAEC/NHLF co-cultures indicating the beneficial effect of fibroblasts. The highest level of TT1 expression was reached in 3D SAEC/NHLF tissues.

Figure 4b. mRNA levels of AQP-3 water transporter in 3D human lung micro-tissues. AQ3 is an ATII epithelial type marker in the lung. While 3D fibroblast cultures show no AQ3 expression, AQ3 is present in 3D SAEC monocultures and increased in 2D SAEC/NHLF co-cultures indicating the beneficial effect of fibroblasts, but the highest level of AQ3 was still observed in 3D SAEC/NHLF tissue cultures.

Figure 5. Gene expression changes in SAEC differentiation markers. **Panel A:** Relative mRNA levels of AQP3 water transporter in 2D and 3D human lung micro-tissues. Relative AQP3 expression levels increased in mixed cell cultures to that of SAEC-only cultures while no difference was detectable between 2D and 3D culture conditions. **Panel B:** Relative level of KRT7 mRNA expression was increased in mixed cell cultures compared to SAEC-only cultures. (Independent experiments: n=3) **Panel C:** RT-PCR analysis of SFTPA1 and beta-actin expression in 2D and 3D cultures. SFTPA1 expression was only detected in SAEC+NHLF co-cultures. The expression of SFTPA1 were consistently higher in 3D cultures than in 2D cultures. A representative image of 3 independent experiments is shown. **Panel D:** After 72h 2D or 3D co-culturing with NHLF cells, gene expression changes in FACS-sorted SAEC were examined. The levels of differentiation markers AQP3 and TTF-1 in re-purified SAEC were significantly up-regulated in 3D co-cultures compared to 2D co-cultures. Data shown are means of two independent experiments. (Purified primary lung cells used in all our experiments originated from random donors).

Figure 6. EMT markers in the 3D lung tissue model. **Panel A:** Relative mRNA levels of S100A4 in 2D and 3D co-cultures. The presence of fibroblasts significantly decreased the level of S100A4 in SAEC-NHLF co-cultures compared to SAEC-only cultures while 2D or 3D culture conditions did not alter S100A4 expression significantly. **Panel B:** Relative mRNA levels of E-cadherin (E-cad) is increased in 3D cultures in the presence of NHLF. **Panel C:** Relative mRNA levels of N-cadherin (N-cad) in 2D and 3D human lung micro-tissues. **Panel D:** After 72h 2D or 3D co-culturing with NHLF cells, gene expression changes in FACS-sorted SAEC were examined. The levels of EMT

markers S100A4 and E-cad in sorted lung epithelial cells were increased in 3D two-cell co-cultures compared to 2D co-cultures, while N-cad was expressed at much lower levels in 3D mixed cultures. Purified primary lung cells used in our experiments originated from random donors. Data shown are means of three (Panels A-C) or two (Panel D) independent experiments. Purified primary lung cells used in all our experiments originated from random donors.

Figure 7. Inflammatory cytokine and chemokine secretion in human primary lung cell cultures. **Panel A:** CXCL-8 secretion of 2D and 3D NHLF monocultures was barely detectable in cell culture supernatants. 3D SAEC cultures still produced CXCL8, although to a lesser degree than 2D SAEC cultures. 2D co-cultures didn't significantly alter CXCL-8 expression, indicating, that the presence of fibroblasts cannot influence cytokine expression. CXCL-8 expression levels were significantly reduced in 3D tissue systems in both pure SAEC and SAEC-NHLF co-cultures. **Panel A and B:** Expression levels of IL-1b and IL-6 mRNA in human primary lung cell cultures, respectively. Compared to 2D cultures, inflammatory mRNA levels of inflammatory cytokines IL-1b and IL-6 are consistently lower in 3D cultures. In pure fibroblast cultures IL-1b mRNA expression was not detectable, while IL-6 levels were much lower and the expression changes were also less prominent. (See also Table 2) **Panel D:** Similarly to mixed cell culture samples, inflammatory cytokines IL-1b and IL-6 levels also decreased markedly in SAEC purified from 3D cultures, than that of 2D cultures. Data shown are means of three (Panels A-C) or two (Panel D) independent experiments. Purified primary lung cells used in all our experiments originated from random donors.

Figure 8. Structure of 3D three-cell type microcultures consisting of SAEC, NHLF, and HMVECs. SAEC, NHLF and HMVECs were stained with the vital dyes CFSE, DiI, or DiD, respectively, then aggregated. After 24 hour incubation, the spontaneously rearranged two- or three-cell type microcultures were carefully transferred into 24 well cell culture plates for imaging. **Panel A:** two-cell type cultures; **Panel B:** three-cell type cultures. **Top row:** phase contrast microscopic images; **Middle row:** fluorescent microscopic images; **Bottom row:** confocal images. SAEC: green channel; NHLF: red channel; HMVEC: blue channel.

Figure 9. Gene expression changes in three-cell type cultures. **Panel A:** The expression levels of AQP3 and KRT7 increased, S100A4 and N-cad decreased in 3D cultures compared to 2D cultures. **Panel B:** Comparison of expression changes of molecular markers in 3D SAEC-NHLF two-cell type cultures and SAEC-NHLF-HMVEC three-cell type cultures. AQP3 and E-cad mRNA levels are increased, S100A4 and N-cad are decreased in indicating that differentiation of the tissue was maintained in the three-cell type model. Purified primary lung cells used in all our experiments originated from random donors.

Figure 10. Flow chart of the preparation of a test-ready lung tissue kit delivered in a 96 well plate.

Figrue 11. Adenoviral gene delivery into SAEC in the two-cell type model.

Panel A.: SAEC appear green in the surface of the 3D tissue model due to GFP expression. Fibroblasts were pre-stained red with a physiological dye prior to the aggregation. **Panel B:** RT-PCR reaction proves effective GFP gene delivery into the model. GFP can be detected in adenovirally transduced model cultures.

DETAILED DESCRIPTION OF THE INVENTION

The present inventors created a simple engineered three dimensional pulmonary model tissue culture, useful as a lung tissue model and ready for use in various test methods.

During the generation of the model, several aspects of tissue characteristics including main characteristics of tissue types of the lung, interaction of cell types during embryonic lung development and technological advances in tissue engineering were considered.

Both scaffold based and scaffold free systems were tested as described herein. Analysis of lung tissue specific markers surprisingly showed that the three dimensional scaffold free system showed striking similarities with native

lung tissue.

In the prior art fibroblast overgrowth was usual experience during attempts of utilizing these type cells in model tissues (US2008/0112890). In light of the results presented herein it can be assumed that the cause of such overgrowth could have been a lack of formation of aggregates. Cells left out from the aggregates might have attached to the surface of the vessel, start propagation till contact inhibition is achieved by them.

Hitherto developed lung tissue models used specialized scaffold materials and were kept in culture lengthily [Nichols, J.E. and Cortiella J. 2008]. In contrast, the model presented herein allows easy handling, uses a simple experimental setup and a relatively short culturing time. Moreover, no special laboratory equipment is required. The present system is appropriate for use with human cells, including human primary cells and non-transformed human cells.

It is to be understood that in certain systems the scaffold e.g. a matrix is biodegradable. However, these systems are not be considered as scaffold free systems even after the scaffold is degraded because and if the scaffold affected or defined the structure or shape of the tissue culture. Besides, in *in vitro* systems like in the present invention generally it can not be expected that a degradable membrane will be dissolved.

Moreover, dissolution of a biodegradable scaffold takes a long time, much longer that the time for preparation and usage of the tissue culture of the present invention.

Cell which are not de-differentiated cells can also be applied in the present invention, however, the number of cells will be small. Therefore, this embodiment is useful mainly in cases when a small number of cells is sufficient to a projected test, e.g. when the test is sensitive enough. In a rapid test it is possible to start the preparation of the model tissue culture of the invention from purified, differentiated cells. Such cells can be freshly prepared from a subject. This version of the method is particularly useful e.g. in patient-specific testing of drugs or compounds or if the effect of an active agent is to be tested in a specific disease setting (for example for a potential manufacturer).

Primary cells can be obtained from commercial sources, too. For example Lonza Verviers, S.p.r.l. Parc Industriel de Petit-Rechain B-4800 Verviers, Belgium; Biocenter Ltd., Temesvári krt. 62 H-6726 Szeged, Invitrogen Corporation (part of Life Technologies Corporation 5791 Van Allen Way, Carlsbad, California 92008 USA)

If large number of samples are needed, cells are to be propagated before preparing the model tissue culture samples. During this process de-differentiation may occur. This is the case in screening (e.g. HTS) applications. In patient-specific testing a smaller number of samples is sufficient.

When the cells are differentiated in an aggregate, propagation is slowed down or stopped and thereafter the aggregates do not increase significantly

If a small number of samples are sufficient no preliminary propagation is needed. Typically, this may occur in patient sample testing for a few drugs or if certain phenomena, e.g. signaling processes are to be observed. However, in screening processes a large number of samples are needed and propagation of cells before the preparation of the model tissue culture should be performed.

In preferred embodiment of the invention adult, dedifferentiated epithelial cells are used. It was not known in the art that in adult, dedifferentiated epithelial cells, simply co-cultured in the presence of fibroblasts are capable of effecting differentiation that would further increase in 3D conditions, in particular in conditions appropriate for formation of 3D aggregates. Thus, in a preferred embodiment the model tissue culture preparation is started from de-differentiated cells.

Primary cells, kept in a culture, e.g. in a 2D tissue culture will exhibit certain dedifferentiation markers. Thus, such cell can be applied in the present invention, as well. Dedifferentiation markers include S100A4, N-cadherin and inflammatory markers. Thereby a larger number of cells can be applied.

Pluripotent or undifferentiated cells can be rendered capable of differentiation after addition of tissue component and/or factors.

Cellular interactions within pulmonary tissue

Cellular de-differentiation and re-differentiation

Both stem cells and tissue specific progenitor cells can undergo directed steps of tissue specific differentiation and therefore represent an ideal source for generating organ specific tissue culture material. Unfortunately, both cell types are only available in limited numbers in differentiated tissues.

As tissue models need to be set up on a regular basis according to experimental and/or testing requirements, tissue specific differentiated cells represent a better source of primary material, simply as there are more of them. The present model system utilizes, at least in part, the phenomenon that differentiated cells in two dimensional culture conditions can de-differentiate and can be forced to re-differentiate using the right culture conditions.

Cellular interactions

Lung development, as well as epithelial injury repair, is tightly coordinated by a fine balance between stimulatory versus inhibitory genes that appear to co-regulate the function of stem and adult progenitor cells in the lung. For example, FGF receptor tyrosine kinase signaling is essential for respiratory organogenesis and is negatively regulated by a family of inducible FGF pathway inhibitors (Zhang, Stappenbeck et al. 2005). Additionally, FGF signaling is required for formation of new alveoli, protection of alveolar epithelial cells from injury, as well as migration and proliferation of putative alveolar stem/progenitor cells during lung repair. Conversely, TGF beta receptor serine-threonine kinase signaling via Smads 2, 3 and 4 inhibits lung morphogenesis and can inhibit postnatal alveolar development, while excessive TGF beta signaling via Smad3 causes interstitial fibrosis.

Thus, there is a requirement for reciprocal, albeit rather complex system of interactions between the mesenchyme and the epithelium. The present inventors hypothesized that a basic lung model can be created by using a mix of purified alveolar epithelium and fibroblasts.

Therefore, initially only two cell types were used: primary human fibroblasts (NHLF) and small airways epithelial cells (SAEC with ATII characteristics) that are both commercially available (Lonza). It has been surprisingly found that this two cell type sufficiently provides the necessary factors to form a three dimensional pulmonary tissue model. The skilled person will understand that by addition of further cell types, for example of cell types listed herein, the model can be further developed.

Segregation of cell types in mixed culture (sorting)

Our microscopic examinations demonstrate that spontaneous tissue reorganization – "sorting" - occurs in 3D lung primary cell cultures. The present inventors used herein a simple centrifugation method to aggregate cells similarly to that of the preparation of fetal thymic organ cultures. [Hare, K.J. et al. (1999)] Evidence is provided herein that 3D co-culturing of primary pulmonary epithelial cells with fibroblasts is more advantageous for SAEC to maintain a more differentiated status than in either 2D or 3D in vitro monocultures. Inclusion of NHLFs not only facilitated epithelial differentiation but the cohesion and structure of the 3D micro-tissues were much more firm and compact compared to SAEC-only (Figure 3) or SAEC-HMVEC (Figure 8.a) cultures.

The present two-cell type co-culture system, consisting of human small airway epithelial cells (SAEC) and normal human lung fibroblasts (NHLF) did not require the presence of externally added ECM for the formation and maintenance of 3D structure (Figure 3). Pelleting SAEC and NHLF cell suspensions of single cell type and cell mixtures of various ratios revealed that creation of pulmonary micro-tissues require the presence of fibroblasts to maintain a compact and stable 3D structure (Figure 3). Morphologic examinations of 3D micro-tissues revealed that segregation of the two cell types in mixed cultures was a feature of 3D micro-tissues, fibroblasts forming the inner,

core part, while epithelial cells were covering the outer layer (Figure 3). The phenomenon of segregation or "sorting" is based on different adhesive energy characteristics of cell types and has not been described for primary human pulmonary tissue before. The spontaneous cell "sorting" is based upon the disparity of the cohesive forces between different cell types: the most cohesive core or central region of the pulmonary micro-tissue is formed by the NHLF population being surrounded by the less cohesive SAEC. The process of segregation in primary, differentiated human pulmonary tissues is particularly interesting, as underlies the notion that even differentiated, human adult cells maintain their ability to actively explore their own microenvironment. The cells in the 3D co-cultures are capable of exchanging position with adjacent cells thus structurally reorganizing the tissue. This process also requires reorganization of the extracellular matrix. Studies of the models of various SAEC/NHLF ratios in the 3D micro-tissue models revealed that 1:1 ratio is sufficient for the epithelial cells to cover the inner core of fibroblasts therefore further analysis of the model was performed using the 1:1 setting. The model can be useful, however, at other epithelial-mesenchymal cell ratios. The ratio sufficient for full coverage may vary to some extent depending on cell type.

Without being bound by theory, the present Inventors think that segregation of cells in the present model is due to different cohesivity of the cell types in which a difference in their surface tension plays a major role.

Any cell would actively explore its own microenvironment, are able to exchange position with adjacent cells or to reorganize the extracellular matrix in their vicinity. The latter process is known to involve both mechanical traction forces and enzymatic activity by matrix metalloproteases (MMPs). Based on different adhesive energy characteristics, it is a known experimental fact that certain cell compositions of mixed cell types can segregate in an aggregate.

In segregated cell aggregates of hanging drop cultures the most cohesive population occupies the central region, being surrounded by the less cohesive one. A measure of tissue cohesivity is the surface tension of the cells. Thus, surface tension, which is an experimentally detectable quantity, can predict the sorting hierarchy. Therefore there early attempts have been made in the art to by this sorting hierarchy can be predicted to a certain extent if a new cell type is to be involved (Neagu 2006).

However, surface tension factors are not known for specific cell types of the human lung. The prior art was fully silent as to whether the phenomenon of tissue sorting would happen in other cultures or only in hanging drop cultures. The present inventors experimentally determined show herein for the first time that, surprisingly, segregation of epithelial and fibroblast cells happens in pelleted mixed alveolar epithelium and fibroblast cultures.

Size and structure of the microaggregates

It was unexpectedly found by the present inventors that even very small but structured aggregates exhibit tissue features and thus are appropriate for studying interactions, and testing compounds or environmental effects.

Small aggregates have several advantages, for example, no special reaction vessels are needed, their size and ratio of different cell types are reproducible and thereby interactions are more easyly controlled. In small aggregates practically no necrotization of the inner parts of the tissue aggregates can be expected. Furthermore, a surprisingly uniform size distribution can be achieved which renders them quite appropriate for parallel testing.

Thus, preferably, according to the invention the size of the aggregates should be kept small provided that tissue features appear and thereby interactions can be examined.

If the aggregates are too small, a correct morphology as disclosed herein may not take form and the aggregate may not have a tissue like characteristic. If the aggregates are too large, their size may largely deviate from the average. Moreover, necrotization may occur inside the aggregates, due to a longer culturing time and less perfusion of the

aggregates.

In lack of special additives, for example receiving only cell culture media the growth of aggregates is controlled by contact inhibition of the cells.

The size depends, however, on the number of cells in an aggregate. The skilled person will understand that the size and cell number of the aggregates can vary within the limits given herein provided that the above-described requirements are met.

It has been found that addition of endothelial cell did not change significantly the size of the aggregates.

Cell types useful in the present invention

Fibroblast cells

Fibroblasts are the most versatile of the connective tissue cell family and they are in fact the most ubiquitous cell type. Fibroblasts are important structural elements of tissue integrity. They participate in repair and regenerative processes in almost every human tissue and organ, including the lung. Their primary function is to secrete extra cellular matrix (ECM) proteins that provide a tissue scaffold for normal repair events such as epithelial cell migration.

Fibroblasts, or distinct subpopulations thereof, perform tissue-specific functions as immunoregulatory cell, secrete chemokines and cytokines, which are able to trigger immune responses by attracting inflammatory cells and immune cells. Fibroblasts from different anatomical locations show an array of common phenotypic attributes. Fibroblasts, however, show distinct phenotypes in different anatomical locations. Characteristic expression of fibroblast growth factors and receptors are also a feature of pulmonary fibroblasts [De Moerlooze, Spencer-Dene et al (2000)].

The present inventors have found that it is possible to rely on fibroblast physiology to create an artificial tissue scaffold-free tissue system to mimic some aspects of distal pulmonary tissue and an artificial matrix based model not necessarily the only way to create three dimensional pulmonary cultures. Without being bound by theory, the present inventors assume that the fact that fibroblasts in the lung secrete ECM significantly contributes to this result.

Pulmonary epithelial cells (Pneuomocytes)

Pneumocytes (pulmonary or alveolar epithelial cells or AECs) are epithelial cells that line the normal alveolar basement membrane, i.e. the peripheral gas exchange region within the distal airways of the lungs. Pneumocytes or AECs can be subdivided into type I and type II pneumocytes.

Characteristic markers for the two alveolar epithelial cell types are easily traceable and can be monitored during experiments e.g. using RT-PCR reactions or immuno-histochemistry.

Type 1 pneumocytes

Type 1 pneumocytes [alveolar type 1 pneumocytes, type 1 alveolocytes, alveolar type 1 cells (abbr. ATI cells), also called small alveolar cells, squamous alveolar cells, membranous pneumocytes, or type 1 alveolar epithelial cells], are complex branched cells with multiple cytoplasmic plates that represent the gas exchange surface in the alveolus of the lung. These cells are metabolically active and harbour cell surface receptors for a variety of substances, including extracellular matrix (ECM) proteins, growth factors, and cytokines. About ninety-five per cent of the alveolar surface is covered with type I pneumocytes.

Type 2 pneumocytes

Type 2 pneumocytes (alveolar type 2 pneumocytes, alveolar type 2 cells; abbr. ATII cells, T2P) are cuboidal epithelial cells also being referred to as type 2 alveolar epithelial cells (abbr. AEC, also EPII cells), type 2 granular pneumocytes, type 2 cells, type 2 alveolocytes, septal cells, or great alveolar cells, large alveolar cells, or granular pneumocytes. These cells arise from immature epithelial cell progenitors. Alveolar type 2 pneumocytes are thought to be progenitor cells of the alveolar epithelium. They are capable of self-renewal and differentiation into squamous

type 1 pneumocytes. Type II cells are cuboidal cell, which comprise only 4 % of the alveolar surface area, but constitute 60 % of alveolar epithelial cells and 10-15 % of all lung cells (Crapo et al, 1982).

Type 3 alveolar epithelial cells

Type 3 alveolar epithelial cells differ from flat type 1 cells and cuboidal type 2 cells by the presence of an apical tuft of microvilli and the absence of lamellar type secretory granules. These cells are being referred to also as alveolar brush cells.

Endothelial cells

Endothelial cells are oblong shaped cells that line the lumen of all blood vessels as a single squamous epithelial cell layer. They are derived from angioblasts and hemangioblasts.

Macrophages

Macrophages are cells derived from bone marrow-derived monocytes (bone marrow-derived macrophages) that have homed in to tissues. The differentiation of macrophages from uni- and bipotential progenitor cells in the bone marrow is controlled by a variety of cytokines. Further differentiation takes place in tissues and the resulting macrophage populations are being referred to as resident macrophages.

Mast cells

Mast cells arise from a multipotent CD34(+) precursor in the bone marrow (Nakahata and Toru 2002; Austen and Boyce, 2001). Immature mast cells assume their typical granular morphology when they have migrated into tissues. These cells also express Fc-epsilon R1 and stop expressing CD34 and Fc-gamma R2. Most mast cells in the lung and intestinal mucosa produce only tryptase (designated MCT) or only chymase. Mast cells play a central role in immediate allergic reactions by releasing potent mediators.

Smooth muscle cells

Smooth muscle cells are highly specialized multifunctional contractile cells that regulate the lumen of hollow organs transiently (reversible contraction), or chronically (due to fibrosis and muscle hypertrophy). Smooth muscle cells play an important role in vasculogenesis and shape the wall of blood vessels and maintain vascular tone.

Observations with further cells

Addition of endothelial cells resulted in stable aggregates comprising differentiated cells. The degree of differentiation is not reduced if endothelial cells are included into the model tissue culture, as found based on the markers expressed. It appears that these aggregates maintain a layered structure, wherein the endothelial cells are located inside.

EMBODIMENTS

Preparation of a three dimensional model tissue culture

In the method of the present invention at least pulmonary epithelial cells and mesenchymal cells, preferably fibroblasts are used. The cells are cultured separately in order to obtain viable cultures, then mixed in an appropriate ratio and cocultured in the presence of CO_2 under appropriate conditions as will be understood based on the present disclosure and art methods. By setting ratio of the cells and selecting conditions overgrowth of one cell type by another can be avoided.

In a preferred embodiment said cells are obtained from human subject as primary cells and either de-differentiated or used immediately. De-differentiation can be carried out e.g. by known methods (passages, removing other type of cells, addition of growth factors). If the cells are capable of confluence, they are considered as dedifferentiated.

Pelleting the cocultured cell mixture is an important step to establish cell-cell contacts and to result in an appropriate

distance between the cells. The most convenient way to pellet the cells is to apply centrifugation. To select suitable means for pelleting is well within the skills of a skilled person based on the teaching provided herein.

In the present models in principle any of the cells listed above can be used to obtain a lung model tissue close to a native lung tissue. Each cell type applied have to be capable of growth under conditions useful to obtain the three dimensional model tissue as disclosed herein and being capable of association with other cell types of the model. These factors should be tested in preliminary experiments. Expediently a relatively small ratio of further cells should be initially applied then the ratio of the further cell type can be increased, typically till a ratio similar to *in vivo* ratios is achieved.

In preferred embodiments additional cell types that can be included in the model are e.g. endothelial cells and smooth muscle cells.

Disease models

Based on the above model and using various gene delivery methods and variable target genes, the above system is easily adaptable to study genetic changes during pulmonary diseases that can lead to identification of novel drug targets and development of novel therapies:

wherein the disease involves inflammation, the affected cells, preferably the epithelial cells, express inflammatory cytokines (above normal level) and the model is an inflammatory model,

wherein the disease is a tumor, the cells are transformed, e.g. malignantly transformed or immortal cells and the model is a tumor model,

wherein the disease involves fibrosis and the model is a fibrosis model,

wherein the disease involves injury of the tissue and the model is a regeneration model.

Disease models can be utilized in drug testing.

Cells obtained from patients

In an embodiment, pulmonary cells are obtained from patients and cultured in accordance with the present invention. In this embodiment preferably no or only partial de-differentiation is allowed. Thereafter, in a rapid preparation method 3D model tissue culture is formed and drugs proposed for treating said patient are tested or a projected therapeutic regime can be tested. The advantage of this embodiment among others is that pure and parallel sample cultures with uniform composition and size can be prepared. Said samples are also free of any pathogens and may be purified as needed.

Models prepared from healthy cells

In a preferred embodiment, disease models are prepared by starting from healthy cells and factors effecting disease features (symptoms) in the cells are added later.

For example, tumor models are prepared from healthy cells and factors effecting malignous transformation are added and/or genes causing malignous transformation are expressed therein. It has been observed that the level of Wnt proteins, e.g. Wnt5 has increased in a pulmonary tumor tissue. It is thus contemplated that tumor models can be prepared by addition of tumorogenic factors, like EGF (epithelial growth factor), IGF (insulin-like growth factor), insulin, Wnt factors e.g. Wnt5 or a cocktail thereof to the cell mixture or culture of the invention.

In an alternative of this method tumorous cells are added to the medium in which the model culture according to the invention is present but are separated by a semi-permeable membrane. Thereby the factors produced by the tumorous cell induce tumorous (malignus) transition of the cultured cells of the invention.

Lung tumor models made of lung tumor cell lines

Lung tumor models can be prepared from lung tumor cell lines. Such cell lines are readily available at the American Type Culture Collection (ATCC; Rockville, MD), upon searching for tumor cell lines.

Advisably, experiments are to be performed to find appropriate conditions for culturing the cells and optimize the ratio of the cell types used in a cell mixture.

Inflammation models

For inflammation models monocytes and/or macrophages can be added to the model culture of the invention preferably during the preparation process.

In this model pretreatment with LPS or WNT5A is advisable.

Cytokine production of activated macrophages as well as production of other factors like Wnt5 affects the tissue culture and enable an inflammation model.

If an inflammation model system is to be examined, neutrophyl cells can be provided separated from the pulmonary aggregates by a membrane in an appropriate chamber. In this case neutrophyl migration and MMP production can be measured as well.

In an alternative embodiment disease model pulmonary cell lines a cultured in accordance with the invention. In this embodiment drugs can be tested for efficiency against said disease.

In the disease models use of an overexpressing gene is to be avoided, rather an inducible promoter is to be applied.

Inflammatory models from native three dimensional pulmonary cell aggregates

To mimic inflammatory conditions, native three dimensional pulmonary cell aggregates can be treated with various materials eliciting inflammatory reactions.

Such materials are for example:

chemical substances causing acute inflammation, such as vasoactive amines, eicosanoids, etc.

proinflammatory polypeptides, such as growth factors, hydrolytic enzymes etc.

reactive oxygen species,

proinflammatory cytokines, e.g. IFN-γ and other cytokines,

bacterial cell wall extracts.

Inflammatory conditions are tested by detecting cytokine expression e.g. by biochemical assays, immunological assays, such as ELISA, by a PCR-based method, e.g. real time PCR, or by expression analysis e.g. by applying a gene chip.

Genetic modification of primary cells

Both epithelial and mesenchymal cells can be genetically modified using recombinant viral delivery vectors (rAdenoviral and rLentiviral vectors) and these gene delivery methods do not harm the ability of cells to aggregate. Characteristic genes for inflammation, tumor, fibrosis and regeneration can be constitutively or inducibly overexpressed or silenced and tissue morphology, cellular responses, gene and protein expression changes can be studied in a three dimensional microenvironment.

For example, one or more genes known to promote tumor formation can be introduced into a pulmonary cell line, e.g. an alveolar type I or type II cell line, preferably type II cell line or into a fibroblast cell line. Such a gene can be e.g. an oncogene, e.g. a *ras* gene or a gene or a set of genes typical of expression pattern of a tumor, e.g. a COX-2 gene It may happen that the expression of a *ras* gene alone is insufficient to transform the cells, preferably immortal cells, but proliferation is likely to be increased [Wang, XQ, Li, H et al. (2009)], which may provide a disease feature for the model.

Modification of secreted factor composition in primary cell aggregates using genetically modified and sub-lethally irradiated cell lines

In this embodiment all cell types are left non-infected, gene expressions therefore are as normal as in any given three dimensional lung tissue model. Cellular composition of the aggregates however contains sub-lethally irradiated cells (5-10% of total cell number of the aggregate), either fibroblast (WI-38) or alveolar epithelial (A549) cell lines or both, that are genetically modified and produce secreted factors (Wnt-s, Bone Morphogenic Protein (BMP)-s, inflammatory and pro-inflammatory cytokines, growth factors, etc) that modify the cellular microenvironment within the aggregates. Sub-lethal irradiation can reduce propagation of cells and prevent overgrowth of one cell type by the other.

Products

The invention also provides for a kit comprising multiple samples of a 3D model tissue culture.

Preferably, the containers are wells of a plate, e.g. a 96 well plate or a 384 well plate.

The 3D model tissue can be a model of a healthy tissue or a disease model (disease model kit).

The plate expediently comprises an array of containers or wells wherein a multiplicity of containers contain samples of one or more types of engineered three dimensional pulmonary model tissue cultures in an appropriate medium.

The container can be e.g. flat bottom, an U-bottom or, preferably, a V-bottom container, on a plate allowing parallel testing of multiple samples.

Preferably, the containers are non-tissue culture treated containers so as to avoid sticking of the cells to the container wall.

In a preferred embodiment, each container comprises a single aggregate. In a preferred embodiment, the culture samples in each container comprise cells in an amount as defined in the brief description of the invention.

Preferably, the containers are sealed, either separately or together and contain a CO_2 enriched environment or atmosphere suitable for a lung tissue culture as defined in the brief description of the invention.

Typically, disease models require the same environment.

Preferably, the cells are stained with a biocompatible dye suitable to report on one or more of the following cellular features: cellular state for example cell phase, cellular viability, apoptosis or moribund state of the cell; cell type; cell location; malignous transformation; inflammation.

Controls

As control samples the kit contains cultures of epithelium and fibroblasts only. On a plate, preferably at least 3-3 wells of controls (epithelium and fibroblasts, respectively) are present.

Preferably, a further control which is a 2D lung tissue is used to identify or assess features specific to the 3D tissue.

Thus, on request, a 2D control plate (preferably a flat-bottom, adhesive tissue culture plate) can be included to accompany the 3D tissue. Alternatively, the plate may also contains wells of 2D lung tissue as a control, preferably in a flat bottom wells.

Thus, in an embodiment of the invention a plate is used which contains both V-bottom wells for 3D tissue and flat or U-bottom wells for 2D tissue.

EXAMPLES

Example 1 – *Materials and methods*

Primary SAEC, NHLF and pulmonary HMVEC cells were purchased from Lonza. All cell types were isolated from the lungs of multiple random donors of different sexes and ages. We used SAGM, FGM or EGM-2 medium for the

initial expansion of SAEC, NHLF or pulmonary HMVEC, respectively, as recommended by the manufacturer. All types of cell cultures were incubated in an atmosphere containing 5% CO_2 at 37°C. For 2D and 3D culturing, pure or mixed cell populations were cultured in a 50-50% mixture of SAGM (Small Airway Growth Medium, Lonza) and complete DMEM. For two and three-cell cultures containing HMVEC cells, the appropriate growth factor supplements for HMVEC cells were added to the 50-50% mixture of SAGM and DMEM. The compositions of cell culture media were prepared in accordance with instructions of the manufacturer. For 2D and 3D culturing, cells were mixed at the indicated ratios and dispensed onto flat-bottom 6 well plates or 96-well V-bottom plates (Sarstedt), respectively. V-bottom plates were immediately centrifuged after cell seeding at 600xg for 10 minutes at room temperature.

SAECs and NHLFs were stained with the following fluorescent physiological dyes: DiI [Honig, M. G. and R. I. Hume (1989)] and CFSE [Wang, X. Q., X. M. Duan, et al. (2005)] to be able to follow cellular movements in culture. Cells with or without matrigel were pipetted into V-bottom, 96-well, non-tissue culture treated plates and were incubated for one hour in a CO_2 incubator at 37°C. Following incubation, cells were pelleted with 2000 rpm, 5 min, room temperature, then the resulting cell pellets were incubated overnight (5% CO_2, 37°C).

The A549 line was initiated in 1972 by D. J. Giard *et al.* (1973) through explant culture of lung carcinomatous tissue from a 58-year-old male. A549 cells are adenocarcinomic human alveolar basal epithelial cells. A549 cells fall under the squamous subdivision of epithelial cells. Cells seeded at a concentration of $2x10^4$ cells/cm^2 in the above culture medium will be 100% confluent in 5 days.

A549 cells are available at the American Type Culture Collection (ATCC; Rockville, MD) as CCL-185 and can be grown in Ham's F-12 medium (GIBCO BRL, Grand Island, NY) with 10% fetal calf serum (FCS; GIBCO BRL) or according to recommendations of the supplier.

The WI-38 cell line was developed in 1962 from lung tissue taken from a therapeutically aborted fetus of about 3 months gestational age. Cells released by trypsin digestion of the lung tissue were used for the primary culture. The cell morphology is fibroblast-like. The karyotype is 46,XX; normal diploid female. A maximum lifespan of 50 population doublings for this culture was obtained at the Repository. A thymidine labelling index of 86% was obtained after recovery. G6PD is isoenzyme type B. This culture of WI-38 is an expansion from passage 9 frozen cells obtained from the submitter.

WI-38 cells available at http://www.atcc.org/ATCCAdvancedCatalogSearch/tabid/112/Default.aspx American Type Culture Collection (ATCC; Rockville, MD) as CCL-75 are grown according to recommendations of the supplier.

CL13 or CL30 cells (Wardlaw et al., 2002) were cultured in Dulbecco's modified Eagles/F12 medium containing 5% fetal calf serum and 25% microg/ml gentamicin.

Human cells are preferably maintained at 37°C in a humid atmosphere containing CO_2 as needed.

Fluorescent and confocal microscopy

Prior to 2D and 3D culturing, SAECs, NHLFs and HMVECs were stained with fluorescent physiological dyes CFSE, DiI and DiD, respectively (all from Molecular Probes). Cells were washed twice in PBS and incubated with CFSE, DiI or DiD at the concentration of 0,5 µg/ml at 37°C for 10 minutes. The excess dyes were removed by washing the cells with DMEM+10%FCS. 2D and 3D cultures were prepared using the fluorescent-labeled cells, as indicated before. After overnight incubation, 3D cell cultures were removed carefully from the V-bottom plates and transferred to coverslip-bottom dishes (MatTek). Lung tissue microcultures were investigated by fluorescent microscopy (Olympus IX-81 microscope) or confocal microscopy (Olympus FV1000 confocal imaging system)

Cell sorting

SAEC and NHLF were stained with CFSE and DiI according to manufacturers instructions (Molecular probes). Cells were mixed and cultured for 72 hours in 2D and 3D systems. Stained cells were dissociated by mild trypsin treatment followed by PBS+EDTA treatment. Dissociated cells were sorted using a BD FACSVantage cell sorter into tubes with lysis buffer for mRNA preparation (Miltenyi Biotech).

cDNA synthesis and quantitative RT-PCR

Total RNA was prepared from 2D and 3D cell cultures using NucleoSpin RNAII kit (Machery-Nagel) with on-column DNase digestion. Messenger RNA was prepared from sorted SAEC samples with μMACS mRNA isolation system (Miltenyi Biotech). cDNA was prepared from RNA samples with a MMuLV reverse transcriptase kit (Thermo Scientific). Real-time quantitative PCR examinations were carried out using ABsolute QPCR SYBR Green Low ROX master mix (ABGene) and an Applied Biosystems 7500 thermal cycler system. Primers are listed in Table 1.

Recombinant adenoviral vectors

The full gene-of-interest or GFP only sequence was amplified by PCR reaction using Forward (5'): 5'- -3', Reverse (3'): 5'- -3' primer sequences and cloned into the Shuttle vector, then by homologous recombination into the adenoviral vector. Adenovirus was produced by transfecting the linearised plasmid DNA into the 293 packaging cell line (American Type Culture Collection, Rockville, MD) using Lipofectamine 2000 (Invitrogen). The resulting plaques were amplified, the adenovirus purified and concentrated using the adenoviral purification kit (BD Biosciences).

Adenoviral Infection of epithelial cells

Adenovirus containing GFP or gene-of-interest-GFP were added to SAEC in 2D or 3D. $1x10^6$ cells were resuspended in 250 μl of cell culture medium and 50μl of virus for 90 minutes at 37°C.

Table 1

Abbrevi-ation	Official gene name	Accesion number	Forward primer sequence	Reverse primer sequence	Product length
AQP3	Homo sapiens aquaporin 3	NM_004925	AGCCCCTTCAGGATTTCCA	GACCCAAATTCCGGTTCCA	86
AQP4	Homo sapiens aquaporin 4	NM_001650	GCGAGGACAGCTCCTATGAT	ACTGGTGCCAGCATGAATC	110
AQP5	Homo sapiens aquaporin 5	NM_001651	CCTTGCGGTGGTCATGA	ATGGGGCCCTACCCAGAAAAC	61
E-cad	Homo sapiens cadherin 1	NM_004360	GACCGGTGCAATCTTCAAA	TTGACGCCGAGAGCTACAC	93
IL-1b	Homo sapiens interleukin 1, beta	NM_000576	TCAGCCAATCTTCATTGCTCAA	TGGCGAGCTCAGGTACTTCTG	62
IL-6	Homo sapiens interleukin 6	NM_000600	AGGGCTCTTCGGCAAATGTA	GAAGGAATGCCCATTAACAACAA	62
KRT7	Homo sapiens keratin 7	NM_005556	CCACCCACAATCACAAGAAGATT	TCACTTTCCAGACTGTCTCACTGTCT	78
N-cad	Homo sapiens cadherin 2	NM_001792	AGCTTCTCACGGCATACACC	GTGCATGAAGGACAGCCTCT	133
S100A4	Homo sapiens S100 calcium binding protein A4	NM_002961	TGGAGAAGGCCCTG	CCCTCTTTGCCCGAGTACTTG	58
SFTPA1	Homo sapiens surfactant protein A1	NM_005411	CCCCTTGTCTGCAGGATTT	ATCCCTGGAGAGTGTGGAGA	128
SFTPB	Homo sapiens surfactant protein B	NM_198843	GCACTTTAAAGGACGGTGTCTT	GATGCCCACACCACCTG	128
SFTPC	Homo sapiens surfactant protein C	NM_003018	AAAGTCCACAACTTCCAGATGGA	CCTGGCCCAGCTTAGACGTA	73
TTF-1	Homo sapiens NK2 homeobox 1 (NKX2.1),	NM_003317	CATGTCGATGAGTCCAAAGCA	GCCCACTTTCTTGTAGCTTTCC	85

CXCL-8 assay

CXCL-8 (IL-8) content of 2D and 3D cell culture supernatants was measured with Quantikine CXCL-8/IL-8 ELISA kit (R&D Systems). The sandwich ELISA assay was performed according to the manufacturer's instructions. Briefly, identically diluted cell culture supernatant samples and CXCL-8 standards were dispensed onto the wells pre-coated with anti-CXCL-8 monoclonal antibody. After 2 hours incubation at room temperature, the plate was washed 4 times with the provided washing buffer. Then HRPO-conjugated polyclonal anti-CXCL-8 antibody was added for one hour. After a final washing step, TMB substrate solution was added to the wells. The optical density was determined with an iEMS Reader MF (Thermo Labsystems) at 450 and 570nm and data were analyzed using Ascent software.

Data quantitation

Quantitative real-time RT-PCR data were analyzed by the delta Ct (dCt) and Relative Quantity (RQ) methods as suggested by Applied Biosystems using 7500 System SDS Software. All samples were set up in duplicates. Briefly, Ct values were determined for each sample using an automatic threshold level determined by the 7500 System SDS Software. Delta Ct (dCt) values were determined according to the following formula: dCt(target gene) = Ct(target gene)– Ct(housekeeping gene). Changes in gene expression are shown as RQ values calculated using the next formula:

$RQ = 2^{-ddCt}$, where ddCt values were calculated as ddCt = dCt(sample) – dCt(reference sample).

CXCL-8 content of the cell culture supernatants were determinded by comparing the OD to a standard curve calculated from 7 different concentrations in the range of 31.2 – 2000 pg/ml CXCL-8. Samples were dispensed in duplicates and the means were used for further data analysis.

EXAMPLE 2 – *Experiments for development of a three dimensional lung tissue model*

Hanging drop model

To simulate human lung structure, we started with a 3D cell aggregate of 100 000 cells, in roughly equal amounts of distinct fibroblast (NHLF) and small airway epithelial cell populations (SAEC), randomly intermixed. Within a day of incubation in a hanging drop assay, the cells generated loose tissue structures. On Figure 1 a hanging drop culture of 50%SAEC and 50% NHLF mix is shown.

The formation, however, was not stable and was not possible to transfer the generated micro-tissues from the initial culture conditions to another test plate without irrecoverable damage to the tissue structure.

Pelleted, matrigel containing model

To improve the stability of mixed lung micro-tissues, 1:1 ratio of SAEC and NHLF were pelleted and grown in the presence of matrigel. Many 3D lung and other tissue models use matrigel to create a three dimensional structure where various cell types can grow and interact with one another. SAECs and NHLFs were stained with a fluorescent physiological dyes DiI (Honig and Hume 1989) and CFSE (Wang, Duan et al. 2005) to be able to follow cellular movements in culture. Cells and matrigel were pipetted into V-bottom, 96-well, non-tissue culture treated plates and left for one hour in a CO_2 incubator at 37°C. Following incubation, cells were pelleted with 2000 rpm, 5 min, room temperature, then the resulting cell pellets were incubated overnight (5% CO_2, 37°C).

On Figure 2 a matrigel based culture of 50%SAEC and 50%NHLF mix is shown. It is apparent that, despite the presence of matrigel, SAEC and NHLF were unable to form stable 3D structures. Furthermore, it appeared that small spherical tissue structures containing mostly epithelial cells were leaving the tissue/matrigel mass.

EXAMPLE 3 - *Pelleted, artificial tissue scaffold free model*

As a next step in simulating 3D culture conditions of the human lung, the random mix of equal number of epithelial cells (SAEC) and fibroblasts (NHLF) were pelleted without matrigel in two stages. Cells were

pipetted into V-bottom, 96-well, non-tissue culture treated plates and left for one hour in a CO_2 incubator at 37°C. Following incubation, cells were pelleted with 2000 rpm, 5 min, room temperature, then the resulting cell pellets were incubated overnight (5% CO_2, 37°C). Cells prior to mixed culturing were stained using 1:1000 dilutions in PBS (phosphate buffer saline pH 7.2) of physiological fluorescent dyes of Dil (1mg/ml stock in DMSO) and CFSE (1mg/ml stock in DMSO). In these culture conditions cells formed a stable aggregate, where the most adhesive fibroblast (red or dark gray) were surrounded by those of the less adhesive epithelial cell type (green or light grey) (Figure 3). The aggregate diameter is about 200 μm.

In a further experiment, the ratio of SAEC and NHLF cells was systematically changed and cultures were prepared using the following SAEC:NHLF ratios, respectively: 0/100%; 25/75%, 50/50%, 75/25, 100/0%, otherwise as described above. On Figure 3 the pelleted micro-tissue cultures containing different ratios of SAEC and NHLF are shown. As evidenced by the figure, the most pronounced 3D structure aggregates with a surface epithelial cell lining were formed when equal amount of epithelial and fibroblasts cells were applied, aggregates have a fair epithelial cell lining albeit are somewhat smaller and less convincing in morphology at a ratio of 25/75%, whereas a much more even epithelial lining is formed at an excess of SAEC cells, i.e. when the ratio of cell epithelial cells and fibroblasts is 75/25%. Pure cultures either do not form aggregates of 3D structure (epithelial cells) or the aggregates are much smaller in size (fibroblast cells).

EXAMPLE 4 - *Characterization of the two cell type tissue scaffold-free 3D pulmonary tissue model*

Differentiation markers

Molecular characterization of the model was based on epithelial differentiation markers using real-time PCR analysis. mRNA was purified from the cell aggregates and cDNA was generated. Using TTF1 (Figure 4a), AQ3 (Figure 4b) and AQ5 specific primers, results were analysed relative to beta-actin as internal control.

On Figure 4 a. mRNA levels of TTF-1 in 3D human lung micro-tissues are indicated. TTF1 transcription factor is a characteristic marker of alveolar epithelial cells. While 3D fibroblast cultures show no TTF1 expression, TTF1 is present in 3D SAEC monocultures and increased in 2D SAEC/NHLF co-cultures indicating the beneficial effect of fibroblasts. The highest level of TT1 expression was reached in 3D SAEC/NHLF tissues.

Figure 4b. shows mRNA levels of AQP-3 water transporter in 3D human lung micro-tissues. AQ3 is an ATII epithelial type marker in the lung. While 3D fibroblast cultures show no AQ3 expression, AQ3 is present in 3D SAEC monocultures and increased in 2D SAEC/NHLF co-cultures indicating the beneficial effect of fibroblasts, but the highest level of AQ3 was still observed in 3D SAEC/NHLF tissue cultures.

Thus, the above markers indicated an inducible increase in ATII type differentiation that was further supported by no increase in ATI type marker expressions.

The purified differentiated cell types we used in the experiments were obtained from commercial sources. Although these cell types originated from differentiated tissues, once they were purified and kept in 2D culture conditions the cells have shown almost immediate signs of dedifferentiation indicated by increased level of S100A4 (Figure 6 and Table 2). Once SAEC was co-cultured with NHLF, S100A4 and N-cadherin levels decreased significantly, while the E-cadherin levels increased. The "cadherin-switch" [Zeisberg M and E.G. Neilson (2009)]was more prominent in 3D than in 2D culture conditions (Figure 6) indicating that apart from the presence of NHLF, the 3D structure was also necessary to decrease dedifferentiation of SAEC.

These changes are also a feature of epithelial-mesenchymal transition (EMT), characteristic for the epithelial dedifferentiation process.

Pro-inflammatory cytokine expression

As triggered by pulmonary infection or alveolar epithelial injury (disruption of continuous epithelial cell layer) pro-inflammatory cytokines are produced by the alveolar epithelium to attract inflammatory cells, including neutrophils. To test CXCL-8 pro-inflammatory cytokine expression, CXCL-8 protein levels were tested from

cellular supernatants of 2D mono and co-cultures and 3D tissue co-cultures, set up as seen in Figure 3. Using a commercially available ELISA test kit (R&D Laboratories) it has become apparent that CXCL-8 expression levels were significantly reduced in 3D tissue systems compared to conventional 2D tissue cultures (Figure 7.a) and the lowest levels were detected where epithelial cell ratios were the highest, implicating that CXCL-8 expression is triggered by discontinuation in epithelial cell layers.

On the diagram on Figure 7.a CXCL-8 secretion is shown in a human, *in vitro,* 3D lung model. It was found that 3D monocultures of fibroblasts secreted no CXCL-8, while 3D SAEC still produced the cytokine, (although to a lesser degree than 2D SAEC cultures – data not shown). 2D co-cultures didn't significantly alter CXCL-8 expression, indicating, that the presence of fibroblasts cannot influence cytokine expression. CXCL-8 expression levels were significantly reduced in 3D tissue system where 75/25 % was the epithelial-fibroblast ratio, where epithelial cells essentially fully covered the fibroblast sphere, implicating that CXCL-8 expression can be triggered by discontinuation in the alveolar epithelial cell layer. This reduction in CXCL-8 expression was somewhat less pronounced at a ration of 50/50% and 25/75%.

Inflammatory cytokines IL-1beta and IL-6 mRNA levels were also investigated using quantitative real time RT-PCR analysis. In 3D cultures compared to the respective 2D cultures, both IL-1beta and IL-6 mRNA levels were significantly down-regulated (Figure 7B and 7C, respectively), when the PCR was performed from the mix of SAEC-NHLF mRNA. Once SAEC cells were sorted out from 2D and 3D NHLF co-cultures, decreased expression of IL-1beta and IL-6 in SAEC cultured in 3D conditions was even more striking (Figure 7D).

EXAMPLE 5 - Three-cell type model with epithelial, endothelial and fibroblast components

To further improve the complexity our lung tissue culture model we added primary human lung-derived microvascular endothelial cells (HMVEC) to SAEC and NHLF cells and set up 2D and 3D tissue micro-cultures similarly to the SAEC and NHLF co-cultures.

Morphological examination of the micro-tissues by fluorescent and confocal microscopy revealed, that HMVECs could successfully be co-cultured with both SAEC and NHLF cells in 3D conditions (Figure 8.a). Interestingly, in co-cultures with either SAEC or NHLF, HMVECs formed the inner, compact core of the micro-cultures. When the three cell types (1:1:1) were cultured together in 3D conditions, they adhered together and formed a markedly compact and stable 3D structure (Figure 8.b).

Molecular characterization of three-cell cultures revealed that the level of AQP3 and KRT7 expression increased remarkably in 3D cultures compared to that of measured in 2D culture conditions (Figure 9.a and Table 2). The level of EMT markers S100A4 and N-cad were increasing when cells were kept in 2D cultures, but stabilized in 3D cultures. The slight decrease of E-cad in 3D cultures was less than detected in 2D culture conditions (Figure 9.a and Table 2). As the quantitative RT-PCR was performed from the mixed mRNA of the three cell types, further analysis of the three cell type 3D cultures is required, however, to discover the optimal proportions of the three cell types that would aid tissue building from purified tissue elements.

Table 2 shows gene expression changes in human primary lung cell cultures. Numbers are RQ values, calculated according to the formula $RQ = 2^{-ddCt}$, where ddCt values were calculated as ddCt = dCt(sample) – dCt(reference sample). Except for Sorted SAEC**, where data are presented as dCt values, calculated as follows: dCt= Ct(target gene) – Ct(housekeeping gene). A part of collected cells before the set-up of the various cultures were used always as reference samples. Delta Ct (dCt) values were calculated as follows: dCt(target gene) = Ct(target gene)– Ct(housekeeping gene). 18S ribosomal RNA was used as housekeeping gene, except in case of Sorted SAEC** saples, where actin was used as housekeeping gene.

Gene	2 dimensional cultures					3 dimensional cultures				
	SAEC only	SAEC-NHLF	SAEC-NHLF-HMVEC	NHLF only	Sorted SAEC**	SAEC only	SAEC-NHLF	SAEC-NHLF-HMVEC	NHLF only	Sorted SAEC**
AQP3	6,890182077	11,40487803	0,271602751	0,674274063	9,771933	5,78456172	13,31255896	3,62800556	2,854211675	8,571433
AQP4	N.D.	N.D.	N.A.	N.D.	N.C.	N.C.	N.C.	N.A.	N.D.	N.C.
AQP5	N.C.	N.C.	N.A.	N.C.	N.C.	N.C.	N.C.	N.A.	N.C.	N.C.
E-cad	2,409657267	1,772261436	0,215508996	N.D.	7,976	0,644138229	3,016491578	0,296617715	N.D.	6,4226
IL-1b	5,728006854	29,60546927	N.A.	N.D.	4,6346	0,667615932	2,016755678	N.A.	N.D.	5,7802
IL-6	5,189026016	28,09596712	N.A.	0,807285548	7,425867	0,114404135	8,323721329	N.A.	0,498089943	10,67543
KRT7	4,851098336	7,462934362	2,181196885	N.D.	-1,46075	1,34235123	3,684848466	3,765852951	N.D.	0,4287
N-cad	0,527520842	1,111301286	9,374683602	0,132931047	6,17955	0,05583254	0,095682573	1,244810959	0,056255969	11,4096
S100A4	2,536012377	0,421634905	1,860860619	0,380836664	8,929833	2,03353463	0,415210465	1,050573185	0,157182738	6,653067
SFTPA1	N.D.*	Low*	N.D.*	N.D.*	N.A.	N.D.*	High*	N.D.*	N.D.*	N.A.
SFTPB	N.D.	N.C.	N.D.	N.D.	N.A.	N.C.	N.C.	N.D.	N.D.	N.A.
SFTPC	N.D.	N.C.	N.D.	N.D.	N.A.	N.C.	N.C.	N.D.	N.D.	N.A.
TTF-1	1,023345722	0,863026227	N.A.	N.D.	10,351	0,926426355	1,059201358	N.A.	N.D.	8,034067

Abbreviations: N.A.: data are not available, expression level was not determined. **N.D.:** No specific PCR product was detected with real-time QPCR. **N.D.*:** No specific PCR product was detected with conventional PCR. **N.C.:** Specific expression levels were not consequent in parallel wells or samples with real-time QPCR. **Low*:** Relatively low expression levels were detected by conventional PCR. **High*:** Relatively high expression levels were detected with conventional PCR.

Summary of results with cellular markers and factors secreted by the cells

Here we provide evidence that 3D co-culturing of primary pulmonary epithelial cells with fibroblasts is more advantageous for SAEC to maintain a more differentiated status than in either 2D or 3D in vitro monocultures. Inclusion of NHLFs not only facilitated epithelial differentiation but the cohesion and structure of the 3D micro-tissues were much more firm and compact compared to SAEC-only (Figure 3) or SAEC-HMVEC (Figure 8.a) cultures.

TTF1 transcription factor is a characteristic general marker of alveolar epithelial cells during embryonic development and after birth in ATII cells. Cytokeratins are components of the intermediate filaments of the cytoskeleton and their expression patterns are important in cell lineage identification. In our experiments, lung epithelial markers TTF-1 and cytokeratin 7 KRT7 showed elevated expression levels in 3D co-cultures.

Type II pneumocytes facilitate transepithelial movement of water (via members of the aquaporin protein (AQP) family). ATII marker Aquaporin 3 show elevated levels in the presence of fibroblasts (Figure 5) Secretion of surfactant proteins is a unique feature of ATII lung epithelial cells. [Dobbs, L.G. (1989), Alcorn, J.L., et al., (1997)] In our experiments, SAEC cells in monocultures failed to express surfactant proteins, while surfactant protein A1 mRNA was consequently expressed in 3D and at a lesser amount in 2D co-cultures.(Figure 5.c) However, surfactant proteins B and C were not consequently detectable in our 3D co-culture systems (data not shown). We also examined the expression of ATI markers Aquaporin 4 and 5 in both 2-cell and 3-cell cultures, but the expression of these molecules was not consequently detectable, either, presumably because the cells (SAEC) used herein are rather of the ATII type. Moreover, a considerable amount of time is needed for ATI differentiation (data not shown). During a somewhat longer culturing time, if cells with ATI type characteristics are used, ATI markers would appear.

Thus, except of SFPC, differentiation markers AQP3, KRT7, TTF1 and SFPA were up-regulated in the presence of fibroblasts. Levels of AQP3 and SFTPA but not KRT7 or TTF1 differentiation markers were further increased in 3D culture conditions.

S100A4 is a well-known molecular marker for epithelial-mesenchymal transition and the level of its expression is often high in metastatic carcinomas [Sherbet, G.V et al. 2009] as well as in lung fibrosis [Guarino, M. et al., 2009]. Up-regulation of S100A4 and N-cadherin and parallel down-regulation of E-cadherin [Zeisberg, M. and E.G. Neilson, (2009), Seike, M., et al., (2009)] are also features of epithelial-mesenchymal transition (EMT), that is characteristic for the epithelial dedifferentiation process and is characterized by loss of cell adhesion, repression of E-cadherin expression, and increased cell mobility.

De-differentiation markers S100A4 and N-cadherin appeared in purified primary cells in 2D culture conditions. The above de-differentiation markers decreased in the presence of fibroblasts and further decreased in 3D conditions.

Decrease of inflammatory markers including IL1b, IL6 and CXCL8 could be observed in 3D cultures in comparison with 2D cultures. Said markers were significantly down-regulated in 3D culture conditions; the mere presence of fibroblasts, e.g. in 2D cultures, were not sufficient to decrease their levels.

SFTPA1 expression was observed in 2-cell but not in 3-cell cultures. (Figure 5.c and data not shown).

EXAMPLE 6 – *Disease models*

Lung tumor models from lung tumor cell lines.

Artificial three dimensional lung tissue culture is prepared as described in Example 3 with the following modification.

Instead of epithelial cells (SAEC) a combination of type II alveolar epithelial cells (A549) and 5-20% of CL13 or CL30 cells (Wardlaw et al., Molecular Pharmacology, 62, 326–333 2002), derived from NNK [4-(methylnitrosamino)-1-(3-pyridal)-1-butanone] treated A/J mouse, a model of lung adenocarcinoma [Belinsky et al. (1992)] is used. CL13 or CL30 cells carry mutations of the Ki-ras gene.

A series of experiment is performed to find appropriate ratio of A549 cells and CL13 or CL30 cells. A ratio when tumor is spontaneously formed is used to prepare a 3D lung model tissue, which is used as a lung tumor disease model.

In an alternative of the above method patient tumor cells are used.

Modification of secreted factor composition in primary cell aggregates using genetically modified and sub-lethally irradiated cell lines

All cell types are left non-infected, gene expressions therefore are as normal as in any given three dimensional lung tissue model. Cellular composition of the aggregates however contains sub-lethally irradiated cells (5-10% of total cell number of the aggregate) –either fibroblast (WI-38) or alveolar epithelial (A549) cell lines that are genetically modified and produce secreted factors (Wnt-s, Bone Morphogenic Protein (BMP)-s, inflammatory and pro-inflammatory cytokines, growth factors, etc) that modify the cellular microenvironment within the aggregates.

Native three dimensional pulmonary cell aggregates

To mimic inflammatory conditions, native three dimensional pulmonary cell aggregates are treated with various concentrations of bacterial cell wall extracts. Cytokine production is determined using cytokine specific ELISA techniques from tissue culture media, gene expression changes both in epithelial and fibroblasts can be quantified by real-time PCR reactions.

EXAMPLE 7 - *3D Lung tissue kit*

In this example a test-ready 3C lung tissue kit of the following features is prepared:

1. Test-ready lung tissue is delivered in 96 well plates.
2. Small (80 000 cells/well) samples of lung model tissue, ready for experiments or tests, are present in the wells.
3. Each tissue consists of a mixed culture of human primary alveolar epithelium and fibroblasts (25, 50 and 75 % epithelium respectively).
4. The plate contains 3-3 wells of controls (epithelium and fibroblasts only).
5. The plates are sealed with a transparent, preferably adhesive, plastic foil, e.g. with Saranrap.

The quality of tissue is guaranteed for three days, including delivery.

The plate itself is a 96, V-bottom well, non-adhesive tissue culture plate.

The model tissue is prepared as described in EXAMPLE 3. Each tissue is submerged in 200 l of tissue culture medium, optimal for lung culture in 5% CO_2 environment, sealed and delivered at room temperature or on ice.

Quality control: one tissue is taken from each well and viability is tested. The differentiation markers are tested by real-time PCR.

On request, a 2D control plate (tissue grown in 96-well, flat-bottom, adhesive tissue culture plate) can be included to accompany the 3D tissue.

INDUSTRIAL APPLICABILITY

Above, basic parameters and culture conditions are established for an ATII-type tissue scaffold free lung model, where spontaneous self-assembly of cells and cellular interactions can be studied. The model allows easy

handling and genetic manipulation of complex tissue systems in both theoretical and applied research and in pharmaceutical testing. The model is also easily expanded by additional cell types to include endothelial cell for vascularization and even smooth muscle cells, where further reciprocal tissue and cellular interactions can be studied.

The scaffold-free 3D culturing allows trouble-free genetic manipulation of simple or more complex tissue systems in either theoretical or applied research and in pharmaceutical testing. This pulmonary tissue model is especially suitable for studying spontaneous self-assembly of cells and cellular interactions. Both biomedical research and pharmaceutical corporations are in need for in vitro models to enhance the effectiveness of the prediction for toxicity or efficacy of drug candidate molecules in the preclinical stage. [Kramer, J.A. et al. 2007] The newly set guidelines also put emphasis on the replacement of animal models and urge the development of new preclinical testing methods. [*Innovative Medicine Research Initiative Strategic Research Agenda*. 2008, European Technology Platform.]

Based on the above model and using various gene delivery methods and variable target genes, our 3D human pulmonary micro-tissue model system is easily adaptable to study genetic changes during pulmonary diseases that can lead to identification of novel drug targets and development of novel therapies. These disease models may include inflammatory models, tumor model, lung fibrosis model, or a regeneration model.

Three dimensional models of healthy lung tissue as well as disease tissues are available. The product according to the invention can be marketed e.g. in the form of tissue cultures, plates or arrays comprising such cultures or kits.

REFERENCES

Alcorn, J.L., et al., (1997). Primary Cell Culture of Human Type II Pneumonocytes: Maintenance of a Differentiated Phenotype and Transfection with Recombinant Adenoviruses. Am. J. Respir. Cell Mol. Biol., 17(6): p. 672-682.]
Belinsky et al., (1992). Role of the alveolar type II cell in the development and progression of pulmonary tumors induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in the A/J mouse. Cancer Res. 52 3164-3173
Bellusci, S., J. Grindley, et al. (1997). "Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung." Development. 124(23): 4867-4878.
Bruno, M. D., R. J. Bohinski, et al. (1995). "Lung cell-specific expression of the murine surfactant protein A (SP-A) gene is mediated by interactions between the SP-A promoter and thyroid transcription factor-1." J.Biol.Chem. 270: 6531-6536.
Cardoso, W. V. (2001). "Molecular regulation of lung development." Annu Rev Physiol. 63: 471-494.
Colvin, J. S., A. C. White, et al. (2001). "Lung hypoplasia and neonatal death in Fgf9-null mice identify this gene as an essential regulator of lung mesenchyme." Development 128(11): 2095-2106.
Colvin, J. S., B. Feldman, et al. (1999). "Genomic organization and embryonic expression of the mouse fibroblast growth factor 9 gene." Dev Dyn. 216(1): 72-88.
De Moerlooze, L., B. Spencer-Dene, et al. (2000). "An important role for the IIIb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during mouse organogenesis." Development 127(3): 483-492.
Dobbs, L.G., (1989). Pulmonary Surfactant. Annual Review of Medicine, 40(1): p. 431-446.;

Giard D. J. et al. (1973). In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J. Natl. Cancer Inst. [Bethesda] 51: 1417-1423
Guarino, M., A. Tosoni, and M. Nebuloni, (2009). Direct contribution of epithelium to organ fibrosis: epithelial-mesenchymal transition. Human Pathology, 40(10): p. 1365-1376.
Hare, K.J., E.J. Jenkinson, and G. Anderson, (1999). In vitromodels of T cell development. Seminars in Immunology, 11(1): p. 3-12.
Hare, K.J., E.J. Jenkinson, and G. Anderson, (1999). In vitromodels of T cell development. Seminars in Immunology, 11(1): p. 3-12.
Honig, M. G. and R. I. Hume (1989). "Dil and diO: versatile fluorescent dyes for neuronal labelling and pathway tracing." Trends Neurosci. 12(9): 333-335.
Honig, M. G. and R. I. Hume (1989). Trends Neurosci. 12(9): 333-335.
Hughes Tracy et al., a poster presented at National Center for Replacement, Refinement and Reduction of animals in Research, Showcasing the 3Rs Portcullis House, 28 February 2007
Ikeda, A., J. C. Clark, et al. (1995). "Gene structure and expression of human thyroid transcription factor-1 in respiratory epithelial cells." J. Biol.Chem. 270: 8108-8114.
Innovative Medicine Research Initiative Strategic Research Agenda. 2008, European Technology Platform.
Königshoff, M., et al., (2008). Functional Wnt Signaling Is Increased in Idiopathic Pulmonary Fibrosis. PLoS ONE, 3(5): p. e2142.
Kramer, J.A., J.E. Sagartz, and D.L. Morris, (2007). The application of discovery toxicology and pathology towards the design of safer pharmaceutical lead candidates. Nat Rev Drug Discov, 6(8): p. 636-649.
Kreda, S. M., M. C. Gynn, et al. (2001). "Expression and localization of epithelial aquaporins in the adult human lung." Am. J. Respir. Cell. Mol. Biol. 24: 224-234.
Lazzaro, D., M. Price, et al. (1991). "The transcription factor, TTF-1, is expressed at the onset of thyroid and lung morphogenesis and in restricted regions of the foetal brain." Development 113: 1093-1104.
Min, H., D. M. Danilenko, et al. (1998). "Fgf-10 is required for both limb and lung development and exhibits striking functional similarity to Drosophila branchless." Genes Dev. 12(20): 3156-3161.
Napolitano, AP, Chai, P et al. (2007). "Dynamics of the self-assembly of complex cellular aggregates on micromolded nonadhesive hydrogels." Tissue Eng. 13(8): 2087-94.
Neagu, A. (2006). "COMPUTATIONAL MODELING OF TISSUE SELF-ASSEMBLY." Modern Physics Letters B 20: 1207-1231.
Nichols, J.E. and J. Cortiella, (2008). Engineering of a Complex Organ: Progress Toward Development of a Tissue-engineered Lung. Proc Am Thorac Soc,. 5(6): p. 723-730.
Ornitz, D. M. and N. Itoh (2001). "Fibroblast growth factors." Genome Biol. 2(3): 3005.
Seike, M., et al., (2009). Epithelial to Mesenchymal Transition of Lung Cancer Cells. Journal of Nippon Medical Scool, 76(4): p. 181-181.
Sekine, K., H. Ohuchi, et al. (1999). "Fgf10 is essential for limb and lung formation." Nat Genet. 21(1): 138-141.
Shannon, J. M. and B. A. Hyatt (2004). "Epithelial-Mesenchymal Interactions in the Developing Lung." Annual Review of Physiology 66: 625-645.
Sherbet, G.V., (2009). Metastasis promoter S100A4 is a potentially valuable molecular target for cancer therapy. Cancer Letters, 280(1): p. 15-30.

Vertrees, RA, Zwischenberger, JB et al. (2008) "Cellular differentiation in three-dimensional lung cell cultures." Cancer Biol Ther. Mar; 7(3): 404-12.
Wang, X. Q., H. Li, et al. (2009) "Oncogenic K-Ras Regulates Proliferation and Cell Junctions in Lung Epithelial Cells through Induction of Cyclooxygenase-2 and Activation of Metalloproteinase-9" Molecular Biology of the Cell 20, 791–800.
Wang, X. Q., X. M. Duan, et al. (2005). "Carboxyfluorescein Diacetate Succinimidyl Ester Fluorescent Dye for Cell Labeling." Acta Biochimica Biophysica Sinica 37(6): 379-385.
Wang, X. Q., X. M. Duan, et al. (2005). Acta Biochimica Biophysica Sinica 37(6): 379-385.)
Wardlaw et al., (2002). Transcriptional regulation of basal cyclooxygenase-2 expression... Molecular Pharmacology, 62, 326–333
Yang, M. C., Y. Guo, et al. (2006). "The TTF-1/TAP26 complex differentially modulates surfactant protein-B (SP-B) and -C (SP-C) promoters in lung cells." Biochem Biophys Res Commun. 344(2): 484-490.
Zeisberg, M. and E.G. Neilson, (2009). Biomarkers for epithelial-mesenchymal transitions. The Journal of Clinical Investigation, 119(6): p. 1429-1437.
Zhang, X., T. S. Stappenbeck, et al. (2005). "Reciprocal epithelial-mesenchymal FGF signaling is required for cecal development." Development. 133: 173-180.

CLAIMS

1) An engineered three dimensional pulmonary model tissue culture, wherein said model tissue culture

a) is free of an artificial tissue scaffold wherein said artificial tissue scaffold is a porous three dimensional matrix; a three dimensional gel matrix or a porous membrane support,

b) is composed of cultured cells,

c) comprises at least pulmonary epithelial cells and mesenchymal cells preferably fibroblasts,

d) has a morphology of one or more cellular aggregate(s), wherein the surface of the aggregate(s) is enriched in the pulmonary epithelial cells,

and

e) wherein the epithelial cells express epithelial differentiation markers.

2) The engineered three dimensional pulmonary model tissue culture according to claim 1) wherein

- the size or the average size of the one or more aggregate(s) is at least 80 μm and at most 600 μm, preferably the size or the average size of the aggregate(s) is between 100 μm and 300 μm and/or,

- the one or more aggregates comprise cells in an amount of

at least $2*10^4$ cells, preferably at least $4*10^4$ cells, and

at most $5*10^5$ cells, preferably at most $2*10^5$ or at most 10^5 cells.

3) The engineered three dimensional pulmonary model tissue culture according to claim 1) or 2) wherein

- said model tissue culture is free of any artificial tissue scaffold and/or

- said model tissue culture further comprises an extracellular matrix the extracellular matrix proteins of which are secreted by at least one of the cell types comprised in the tissue culture; preferably by the fibroblast cells, and/or

- epithelial cells form a pulmonary epithelial cell lining on at least a part of the surface of the aggregate, preferably said pulmonary epithelial cell lining covering, at least partly, the surface of the aggregate.

4) The engineered three dimensional pulmonary model tissue culture according to any of claims 1) to 3), wherein

- said model tissue culture comprises cultured primary cells obtained from a subject, and/or

- said model tissue culture comprises cells de-differentiated before culturing and re-differentiated upon culturing, and/or

- said model tissue culture comprises cells of established cell lines.

5) The engineered three dimensional pulmonary model tissue culture according to any of the previous claims additionally comprising endothelial cells.

6) The engineered three dimensional pulmonary model tissue culture according to any of the previous claims, wherein

- the levels of the following markers are increased relative to a control comprising non-cultured cells, preferably

purified primary cells: TTF1, AQP3, SFTPA, SFTPC, KRT7,

- the levels of the following markers are decreased relative to a control comprising non-cultured cells: CXCL8 IL1b, S100A4,
- the levels of the following markers are increased relative to a control 2 dimensional culture: AQP3, SFTPA,
- the levels of the following markers are decreased relative to a control 2 dimensional culture: CXCL8, IL1b, IL6, S100A4, N-cadherin.

7) The engineered three dimensional pulmonary model tissue culture as defined in any of the previous claims wherein

said cultured cells, preferably said pulmonary epithelial and/or mesenchymal cells, comprise affected cells having a pathologic feature of a diseased lung tissue so that said model tissue culture is a pulmonary disease model tissue culture,

preferably

- wherein the disease involves inflammation and the affected cells express inflammatory cytokines above normal level and the model is an inflammatory model,
- wherein the disease is a tumor and the cells are transformed, e.g. malignantly transformed or immortal cells and the model is a tumor model,
- wherein the disease involves fibrosis and the model is a fibrosis model,
- wherein the disease involves injury of the tissue and the model is a regeneration model.

8) A method for the preparation of an engineered three dimensional pulmonary model tissue culture,

said method comprising the steps of

- preparing a mixed suspension of at least pulmonary epithelial cells and mesenchymal cells, preferably fibroblast cells, more preferably primary fibroblast cells,
- pelleting the cells of the suspension,
- pelleting the cells,
- incubating the pelleted suspension in the presence of CO_2, for a time sufficient to the cells to form a three dimensional pulmonary model tissue comprising one or more cellular aggregate(s)
- optionally assaying the model tissue for

a) expression of one or more epithelial differentiation markers characteristic to model tissue culture, and an increased expression level as compared to a reference culture is considered as indicative of the formation of a three dimensional pulmonary model tissue culture; and/or

b) expression of one or more pro-inflammatory cytokine as defined above, and a decreased expression level as compared to a suitable reference culture (as defined above) is considered as indicative of the formation of a three dimensional pulmonary model tissue culture

c) size and morphology of the one or more aggregate(s).

9) The method according to claim 8) wherein

- the average size of the cellular aggregates is at least 80 μm and at most 600 μm, preferably is between 100 μm

and 300 μm and/or,

- the one or more aggregates comprise cells in an amount of

at least $2*10^4$ cells, preferably at least $4*10^4$ cells, and

at most $5*10^5$ cells, preferably at most $2*10^5$ or at most 10^5 cells.

10) The method according to claim 8) or 9) wherein

the pelleted suspension is incubated in the presence of CO_2

for a time-period not more than two weeks, preferably not more than 12, 10, 8, 7, 6 or 5 days

and for a period not less than 10 hours, preferably not less than 12 or 14 hours.

11) The method according to any of claims 8) to 10) wherein upon preparation of a mixed suspension one or more type of cells are added to a container within 18 hours, preferably within, 12 hours, more preferably within 4 hours, highly preferably within 1 hour or simultaneously, highly preferably each type of cell used is added within said time-period.

12) The method according to any of claims 8) to 11),

- wherein the container is a V-bottom, non-tissue culture treated container and/or
- wherein pelleting is carried out at 200g to 600g for 1 to 20 minutes, preferably 2 to 10 minutes, and/or
- wherein the cells are stained with a biocompatible dye suitable to report on one or more of the following cellular features: cellular state for example cell phase, cellular viability, apoptosis or moribund state of the cell; cell type; cell location; malignous transformation; inflammation.

13) The method according to any of claims 8) to 12), wherein

- said mixed suspension of at least pulmonary epithelial cells and pulmonary mesenchymal cells comprises cultured primary cells obtained from a subject, and/or
- said mixed suspension of at least pulmonary epithelial cells and pulmonary mesenchymal cells comprises cells de-differentiated before culturing and the cells are re-differentiated upon culturing, and/or
- said mixed suspension of at least pulmonary epithelial cells and pulmonary mesenchymal cells comprises cells of established cell lines.

14) The method according to any of claims 8) to 13), wherein

said cultured cells, preferably said pulmonary epithelial and/or mesenchymal cells comprise affected cells having a pathologic feature of a diseased lung tissue so that said model tissue culture is a pulmonary disease model tissue culture.

15) A method for screening a drug for its effect on lung tissue, said method comprising the steps of

- providing an engineered three dimensional pulmonary model tissue culture as defined in any of claims 1 to 7,
- taking at least a test sample and a reference sample of said model tissue culture,
- contacting the test sample with a drug while maintaining the test sample and the reference sample under the same conditions,

- detecting any alteration or modification of the test sample in comparison with the reference sample

wherein if any alteration or modification of the test sample is detected it is considered as an indication of the effect of the drug.

16) The method of claim 15) wherein the model tissue culture is the model of a healthy lung tissue and an adverse effect of a drug is tested, wherein alteration or modification which is detrimental to the cells of test sample is considered as a toxic or adverse effect of said drug.

17) The method of claim 15) wherein the model tissue culture is a pulmonary disease model tissue culture comprising affected cells having a pathologic feature and the beneficial effect of a drug is tested, wherein

- an assay to measure or assess said pathologic feature is provided for said model tissue culture to obtain a measure of disease,
- a reference sample of a healthy lung tissue (healthy reference sample) and/or a reference sample of a diseased lung tissue (diseased reference sample) is provided,
- the pathologic feature is measured or assessed in the healthy reference sample and/or in the diseased reference sample and in said at least one test sample before and after contacting it with the drug, wherein

any alteration or modification in the test sample which shifts the measure of disease in the test sample towards the measure of disease in the healthy reference sample and or away from the measure of disease in the diseased reference sample is considered as a beneficial effect of said drug.

18) An engineered three dimensional pulmonary model tissue kit comprising a test plate having an array of containers wherein at least two containers contain

- samples of one or more types of engineered three dimensional pulmonary model tissue cultures as defined in any of claims 1 to 7, each sample placed in separate containers of said plate,
- an appropriate medium for culturing cells of the model tissue cultures.

19) The engineered three dimensional pulmonary model tissue kit according to claim 18 having one or more of the following characteristics:

- wherein the plate is a 96 well plate.
- wherein the plate is a V-bottom plate.
- the culture samples in each container comprise cells in an amount of

  at least 10^3, preferably at least 10^4, more preferably at least $2*10^4$, $3*10^4$, $4*10^4$, $5*10^4$ cells, and

  at most 10^7, preferably at most 10^6, more preferably at most cells to $5*10^5$, $4*10^5$, $3*10^5$, $2*10^5$, or at most 10^5.
- wherein the containers are sealed, either separately or together and contain a CO_2 enriched environment or atmosphere suitable for a lung tissue culture.
- wherein, the CO_2 enriched environment or atmosphere comprises

  at least 2%, 3%, 4% CO_2 environment,

  at most 10%, 9%, 8% or 7% CO_2 environment,

  highly preferably about 5% CO_2.

20) The use of a three dimensional pulmonary model tissue culture as defined in any of claims 1 to **7** for

- parallel screening of compounds for their effect on pulmonary tissue, e.g. toxicity testing, searching for candidate compounds, etc.
- patient specific testing of compounds for their therapeutic potential, provided that the model tissue culture is obtained from primary patient pulmonary cells,
- studying cellular interaction in the pulmonary tissue,
- studying genetic changes during pulmonary diseases etc.

SEQUENCE LISTING

<110> UNIVERSITY OF PÉCS

<120> LUNG TISSUE MODEL

<130> P107314 SG

<150> HU P0900819
<151> 2009-05-05

<160> 26

<170> PatentIn version 3.5

<210> 1
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Forward primer for AQP3

<400> 1
agcccttca ggatttcca 19

<210> 2
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Reverse primer for AQP3

<400> 2
gacccaaatt ccggttcca 19

<210> 3
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Forward primer for AQP4

<400> 3
gcgaggacag ctcctatgat 20

<210> 4
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Reverse primer for AQP4

<400> 4
actggtgcca gcatgaatc 19

<210> 5
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Forward primer for AQP5

<400> 5
ccttgcggtg gtcatga 17

<210> 6
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Reverse primer for AQP5

<400> 6
atggggccct acccagaaaa c 21

<210> 7
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Forward primer for E-cad

<400> 7
gaccggtgca atcttcaaa 19

<210> 8
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Reverse primer for E-cad

<400> 8
ttgacgccga gagctacac 19

<210> 9
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Forward primer for IL-1b

<400> 9
tcagccaatc ttcattgctc aa 22

<210> 10
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Reverse primer for IL-1b

<400> 10
tggcgagctc aggtacttct g 21

<210> 11

<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Forward primer for IL-6

<400> 11
agggctcttc ggcaaatgta 20

<210> 12
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Reverse primer for IL-6

<400> 12
gaaggaatgc ccattaacaa caa 23

<210> 13
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Forward primer for KRT7

<400> 13
ccacccacaa tcacaagaag att 23

<210> 14
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Reverse primer for KRT7

<400> 14
tcactttcca gactgtctca ctgtct 26

<210> 15
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Forward primer for N-cad

<400> 15
agcttctcac ggcatacacc 20

<210> 16
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Reverse primer for N-cad

<400> 16

gtgcatgaag gacagcctct 20

<210> 17
<211> 14
<212> DNA
<213> Artificial Sequence

<220>
<223> Forward primer for S100A4

<400> 17
tggagaaggc cctg 14

<210> 18
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Reverse primer for S100A4

<400> 18
ccctctttgc ccgagtactt g 21

<210> 19
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Forward primer for SFTPA1

<400> 19
cccttgtct gcaggattt 19

<210> 20
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Reverse primer for SFTPA1

<400> 20
atccctggag agtgtggaga 20

<210> 21
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Forward primer for SFTPB

<400> 21
gcactttaaa ggacggtgtc tt 22

<210> 22
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Reverse primer for SFTPB

<400> 22
gatgcccaca ccacctg 17

<210> 23
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Forward primer for SFTPC

<400> 23
aaagtccaca acttccagat gga 23

<210> 24
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Reverse primer for SFTPC

<400> 24
cctggcccag cttagacgta 20

<210> 25
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Forward primer for TTF-1

<400> 25
catgtcgatg agtccaaagc a 21

<210> 26
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Reverse primer for TTF-1

<400> 26
gcccactttc ttgtagcttt cc 22