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Epithelial 3D-spheroids as a tool to study air pollutant-induced lung pathology

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ABSTRACT

Cigarette smoke (CS) and air pollutants (AP) activate pathological processes in bronchial epithelial cells resulting in lung function decline which severely impacts human health. Knowledge about the molecular mechanism(s) by which CS and AP induce pathology is limited. Our previous studies in 2D cultures of human bronchial epithelial (BEAS-2B) cells showed that CS exposure activates transforming growth factor-β1 (TGF-β1) release and signaling. Furthermore, CS exposure reduced the expression of E-cadherin, which was prevented by applying a TGF-β1 neutralizing antibody. Exposure of BEAS-2B cells cultured in 2D to diesel exhaust particles (DEP) increased TGF-β1 protein expression and reduced the expression of epithelial cell markers, whereas mesenchymal markers are upregulated. Conventional 2D cell culture may, however, not fully reflect the physiology of bronchial epithelial cells *in vivo*. To simulate the *in vivo* situation more closely we cultured the bronchial epithelial cells in a 3D environment in the current study. Treatment of epithelial spheroids with TGF-β resulted in reduced E-cadherin and increased collagen I expression, indicating the activation of epithelial-to-mesenchymal transition (EMT). Similarly, exposure of spheroids to DEP induced and EMT-like phenotype. Collectively, our data indicate AP induces an EMT-like phenotype of BEAS-2B cells in 3D spheroid cultures. This opens new avenues for drug development for the treatment of lung diseases induced by AP. The 3D spheroid cell culture is a novel, innovative and physiologically relevant model for culturing a variety of cells. It is a versatile tool for both high-throughput studies and for identifying molecular mechanisms involved in bronchial epithelial cell (patho)physiology.

Introduction

Asthma and chronic obstructive pulmonary disease (COPD) are chronic inflammatory lung disease and leading causes of death worldwide. Both these chronic diseases are characterized by pathological alterations in the airways, referred to as airway remodeling, which encompasses amongst others an increase in smooth muscle mass, epithelial alterations and (subepithelial) fibrosis [1–4]. One of the processes potentially contributing to these structural alterations in the airways is epithelial-to-mesenchymal transition (EMT) in which (bronchial) epithelial cells lose part of their characteristics and markers, while gaining mesenchymal ones [5]. A role for EMT in the pathogenesis of several lung diseases has been widely hypothesized and over the last decade it was demonstrated that EMT is an active process in both the small and large airways of smokers and individuals with COPD [6–8]. Furthermore, several markers indicating EMT showed a correlation with lung function in individuals with chronic airflow limitation, one of the

pathological hallmarks of COPD [6,7]. This once more indicates the relevance of EMT in chronic lung diseases. Currently, no medicinal drugs are available which selectively target EMT in chronic lung diseases.

Several risk factors, like cigarette smoke and air pollution, have been identified that contribute to the development of chronic lung disease. In a recent study, we demonstrated that exposure of bronchial epithelial cells to cigarette smoke induces the release and activation of the profibrotic growth factor transforming growth factor-β1 (TGF-β1) and initiation of the EMT process [9]. The cigarette smoke-induced EMT was prevented by applying a TGF-β1 neutralizing antibody, implying that cigarette smoke requires activation of this growth factor for activation of EMT. The EMT of bronchial epithelial cells was characterized by the loss of the epithelial marker E-cadherin and Zona-occludens-1 (ZO-1, also known as tight junction protein-1) and an increase in the mesenchymal markers collagen 1A1 (a component of the extracellular matrix), and to a lesser extend fibronectin and α-smooth-muscle-actin [9]. Interestingly, a variety of medicinal drugs that elevate the expression

of cyclic AMP (cAMP), an important signaling molecule that alleviates symptoms in chronic lung diseases, could differentially attenuate the TGF-β1-induced expression of the mesenchymal marker collagen 1A1. However, epithelial markers could not be restored by these medicinal drugs [9].

Air pollution, including exhaust gases from diesel engine powered vehicles, is a modern world problem with high impact on human health as it increasingly contributes to overall morbidity and mortality. Recent evidence suggest that air pollution in the form of particular matter with an aerodynamic diameter smaller than 2.5 um (PM2.5), which includes diesel exhaust particles (DEP), correlates to initiation of EMT [10–12]. Indeed, long term exposure (i.e. 15 weeks) of bronchial epithelial cells to nontoxic concentrations of diesel exhaust particles (SRM2975) changes the cell morphology to mesenchymal/fibroblast-like cells and caused marked alterations in EMT-associated genes, including the epithelial marker CDH1 (encoding e-cadherin) [12]. Furthermore, short-term exposure of non-pulmonary epithelial cells to diesel exhaust particles promotes EMT via the generation of reactive oxygen species and the upregulation of TGF-β1 and associated signaling pathways [13]. These data were obtained with conventional cell culture methods, in which cells are cultured on an exogenous matrix or plastic. Valuable information can be obtained by these conventional 2D cell culture methods, however they may not fully reflect the physiology of epithelial cells *in vivo*. Over recent years, more advanced 3D cell culture methods have been developed to more closely mimic the *in vivo* situation of various cells present in the lungs. In the current study, we investigated the effects of DEP and TGF-β1 on the expression of EMT markers in 3D spheroid cultures of human bronchial epithelial cells (BEAS-2B) and compared them to current and previous finding in conventional 2D cell cultures of human bronchial epithelial cells.

Material and methods

3D spheroid cultures human bronchial epithelial cells

Human bronchial epithelial cells (BEAS-2B, ATCC CRL-9609) were cultured in RPMI medium supplemented with 10% (v/v) foetal bovine serum (FBS), 100 µg/l streptomycin and 100 U/ml penicillin. For each independent experiment applies that a single spheroid culture of human bronchial epithelial (BEAS-2B) cells was composed of 50.000 cells in total. The spheroids were manufactured using the 3D bioprinting technique using biocompatible NanoShuttle^{TM} to magnetize the cells (Greiner Bio-one). In order to obtain 3D spheroids cultures, BEAS-2B cells were first grown to confluence in RPMI medium supplemented with 10% (v/v) foetal bovine serum (FBS), 100 µg/l streptomycin and 100 U/ml penicillin. Once confluent, the cells were washed with warm phosphate buffered saline and subsequently detached from cell culture flask using trypsin. Cells were resuspended in RPMI medium supplemented with 10% (v/v) foetal bovine serum (FBS), 100 µg/l streptomycin and 100 U/ml penicillin. BEAS-2B cells were counted and the required amount of cells for a specific experiment were seeded. Cells were allowed to attach to the cell culture plate and incubated overnight with NanoShuttle^{TM} (Greiner Bio-one: 1 µl per 20.000 cells) at 37°C and 5% CO$_2$. The next day, cells containing NanoShuttle^{TM} were trypsinized and resuspended in RPMI medium supplemented with 10% (v/v) foetal bovine serum (FBS), 100 µg/l streptomycin and 100 U/ml penicillin. Subsequently, the cells were seeded in a cell-repellent 96-wells plate (CELLSTAR, Greiner Bio-one) at a density of 50.000 cells per well; corresponding to 1 spheroid per well. The cell repellent plate was placed on top of the plate with 96 small magnets (outer radius of 6mm) and placed at 37°C and 5% CO2. The specific plate with magnets used for this essay applies a magnetic field of approximately 700 G on the cells [14]. After 3 hours the 3D spheroids are formed, the magnet was removed and the cells were cultured overnight at 37°C and 5% CO2. The 3D spheroid cultures were stimulated with transforming growth factor-β_1 (TGF-β_1),

diesel exhaust particle (DEP; SRM2975) or respective vehicle control conditions for 24 hours or 5 days.

Exposure of cells to diesel exhaust particles

For the experiments, we used diesel exhaust particles (DEP; SRM2975; National Standards of Technology) derived from an industrial diesel-powered forklift. Prior to each set of experiments, a fresh DEP stock solution of 1 mg/ml was prepared in RPMI medium containing 1% FBS, 100 µg/l streptomycin, 100 U/ml penicillin and 0,5% dimethyl sulfoxide (DMSO). For the cell stimulations the DEP stock solution was diluted to 100 µg/ml RPMI medium containing 1% FBS, 100 µg/l streptomycin, 100 U/ml penicillin and 0,5% dimethyl sulfoxide (DMSO). Cells were exposed to 100 µg/ml or vehicle control conditions for 24h.
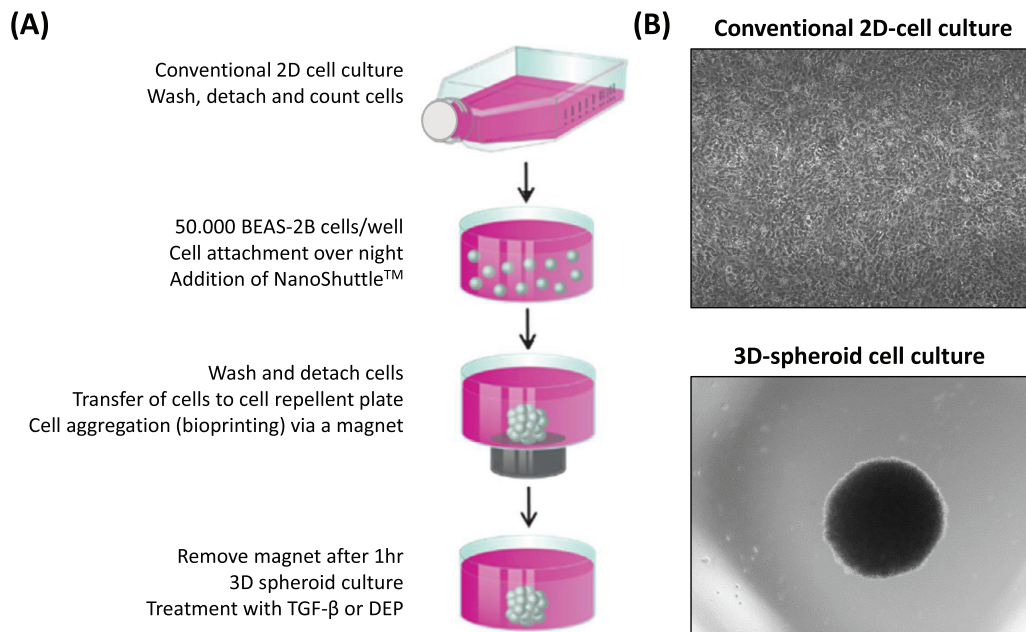
Immunofluorescence stainings

Immunofluorescence was performed on 3D spheroid cell cultures. After cell stimulation, the spheroid cultures were fixed with ice-cold 4% paraformaldehyde at -20 °C. The 3D spheroid cultures were washed 3 times with PBS and then blocked with 1% (w/v) bovine serum albumin (BSA) in PBS. Primary antibodies for ZO-1 (1:200, Invitrogen), collagen-1 (1:200, Southern Biotech), and beta-catenin (1:200, BD Biosciences)) were applied overnight at 4 °C. After washing, cultures were subjected to secondary antibody (Alexa Fluor 488 nm donkey anti-goat (1:2000) and Cy^{TM}3 AffiniPure donkey anti-mouse (1:2000), Jackson, Cambridgeshire, UK)) for 2 h. Slides were mounted with mounting medium containing DAPI (Abcam, Cambridge, UK). Images were captured with a Leica DM4000b Fluorescence microscope (Leica Microsystems, Germany) equipped with a Leica DFC 345 FX camera.
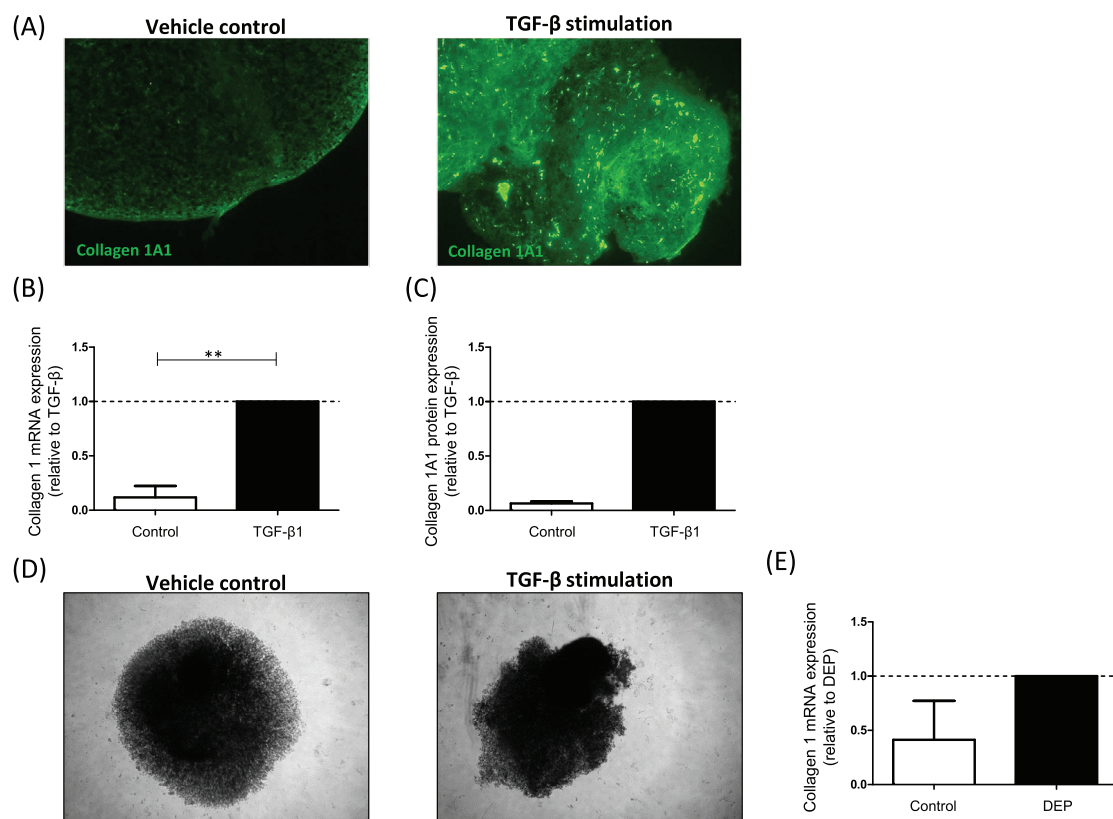
Western blotting

Cellular protein was collected with radioimmunoprecipitation (RIPA) buffer composed of 65 mM Tris, 155mM NaCl, 1% Igepal CA-630, 0.25% sodium deoxycholate, 1 mM EDTA, pH 7.4 and a mixture of protease and phosphatase inhibitors: 1 mM Na3VO4, 1 mM NaF, 10 µg/mL leupetin, 10 µg/mL pepstatin A, 10 µg/mL aprotinin. Total protein content was quantified by BCA protein assay (Pierce) according to the manufacturer's instructions. Equal amounts of total protein were separated by 10% SDS–polyacrylamide gel electrophoresis and subsequently transferred to nitrocellulose membranes. After blocking the membranes with Roti-Block (Carl Roth, Karlsruhe, Germany), primary antibodies for E-cadherin (1:1000, Biosciences), β-catenin (1:2000, BD Biosciences), and GAPDH (1:3000, Sigma) were applied overnight at 4 °C. After thorough washing with tris-buffered saline containing 0.1% tween (TBST), the membranes were incubated with secondary antibody at room temperature (RT) for 2 h. The antigen-antibody complexes were detected using a Western detection ECL-plus kit (PerkinElmer, Waltman, MA). ImageJ software was used for densitometric analysis of the bands; expression was normalized to GAPDH.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using TRIZOL reagent (TRI Reagent Solution, Applied Biosystems, the Netherlands) according to manufacturer's instructions. The total RNA yield was determined by a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Equal amounts of RNA were used to synthesize cDNA and qRT-PCR was carried out in the presence of SYBR Green using an Illumina Eco Real-Time PCR system. PCR cycling was performed by denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, and extension at 72 °C for 30 s for 45 cycles. The data was analyzed with LinReg-PCR software. To analyze RT-qPCR data, the amount of target gene was normalized to the reference genes 18S ribosomal RNA, SDHA, and/or RPL13A.

(A)

Conventional 2D cell culture
Wash, detach and count cells

50.000 BEAS-2B cells/well
Cell attachment over night
Addition of NanoShuttle™

Wash and detach cells
Transfer of cells to cell repellent plate
Cell aggregation (bioprinting) via a magnet

Remove magnet after 1hr
3D spheroid culture
Treatment with TGF-β or DEP

(B) **Conventional 2D-cell culture**

3D-spheroid cell culture

Fig. 1. Generation of 3D spheroid cell cultures of human bronchial epithelial cells. (A) Experimental procedure to generate 3D cell cultures from human bronchial epithelial cells (BEAS-2B) cultured in 2D. The *materials and methods section* contains a comprehensive and more detailed description of the experimental procedure. (B) Photographs of human bronchial epithelial cells (BEAS-2B) cultured conventionally in 2D or in 3D spheroid cultures.

(A)   **Vehicle control**   **TGF-β stimulation**

Collagen 1A1   Collagen 1A1

(B)   **(C)**

Collagen 1 mRNA expression (relative to TGF-β)   Collagen 1A1 protein expression (relative to TGF-β)

Control   TGF-β1   Control   TGF-β1

(D)   **Vehicle control**   **TGF-β stimulation**   **(E)**

Collagen 1 mRNA expression (relative to DEP)

Control   DEP

Fig. 2. Effect of either TGF-β1 or diesel exhaust particle (DEP) treatment on collagen expression in 3D spheroid cultures of human bronchial epithelial cells. (A) Immunofluorescence staining of collagen 1A1 3D spheroid cultures of human bronchial epithelial (BEAS-2B) cells treated with TGF-β1 (3ng/ml; 24h) or vehicle control. (B-C) Collagen 1 mRNA (B) and protein (C) expression in 3D spheroid cultures of human bronchial epithelial (BEAS-2B) cells treated with TGF-β1 (3ng/ml; 24h) or vehicle control. Data shows mean $\pm$ standard deviation of n = 2-3 independent experiments and ** represents $p < 0.01$. (D) Representative images of long term exposure of 3D spheroid cultures of human bronchial epithelial (BEAS-2B) cells with TGF-β1 (3ng/ml; 5 days) or vehicle control (5 days). (E) Collagen 1 mRNA expression in 3D spheroid cultures of human bronchial epithelial cells treated with DEP (100 ug/ml; 24h) or vehicle control. Data shows mean $\pm$ standard deviation of n = 2 independent experiments.
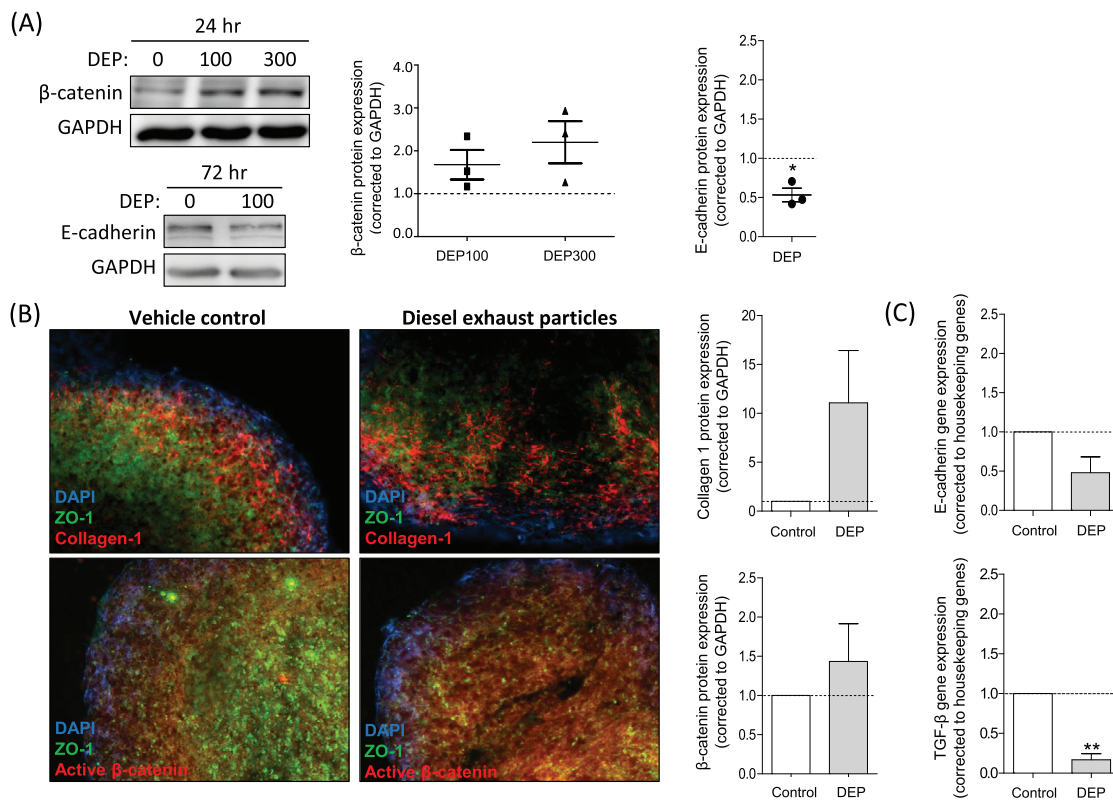
Fig. 3. Effect of diesel exhaust particle (DEP) treatment on epithelial-to-mesenchymal transition (EMT) marker expression in human bronchial epithelial cells. (A) Expression of β-catenin and E-cadherin in 2D cell cultures of human bronchial epithelial (BEAS-2B) cells exposed to either DEP (in μg/ml) or vehicle control. Data shows mean $\pm$ standard deviation of $n = 3$ independent experiments. β-Catenin protein expression: $p = 0.19$ for DEP100 (24hr) and $p = 0.13$ for DEP300 (24hr). E-cadherin protein expression; * represents $p < 0.05$ (B) Immunofluorescence staining and quantification of protein expression of EMT markers collagen 1A1, ZO-1 and β-catenin in 3D spheroid cell cultures of human bronchial epithelial (BEAS-2B) cells exposed to either DEP (100 ug/ml; 24h) or vehicle control. Data shows mean $\pm$ standard deviation of $n = 3$ independent experiments. Collagen 1A1 and β-catenin expression relative to vehicle control; $p = 0.20$ and $p = 0.44$, respectively. (C) mRNA expression of epithelial cell marker E-cadherin and TGF-β1 in 3D spheroid cell cultures of human bronchial epithelial cells exposed to either DEP (100 ug/ml; 24h) or vehicle control. Data shows mean $\pm$ standard deviation of $n = 3$ independent experiments. E-cadherin mRNA expression: $p = 0.12$ and TGF-β1 mRNA expression; ** represents $p < 0.01$.

Statistics

Data are expressed as means $\pm$ standard deviation of n individual experiments. Statistical significance of differences was evaluated using Prism 5.0 software by performing One-sample *T*-tests. Differences were considered to be statistically significant when $p < 0.05$.

Results

Conventional 2D cell cultures have been used for several decades to study the (patho)physiology of various cells types and have been of great value to investigate robust cell biological processes. However, these conventional 2D cell cultures (Fig 1A and B) might not fully reflect the physiological environment of bronchial epithelial cells and 3D spheroid cultures (Fig. 1A and B) might be a better model to study (patho)physiological processes in these cells. Hence, we generated 3D spheroids of human bronchial epithelial cells (Fig. 1A and B) which could be cultured over a period of several days.

We and a wide variety of other laboratories have demonstrated that the profibrotic growth factor TGF-β1 induces EMT in BEAS-2B cells cultured in 2D [9,15]. In the current study, we investigated the effect of TGF-β1 and the air pollutant DEP on epithelial-to-mesenchymal (EMT) transition in 3D spheroid cultures. We particularly investigated the expression of the epithelial marker Zona occludens-1 (ZO-1; also known as Tight junction protein-1), E-cadherin and the mesenchymal markers collagen 1A1 and β-catenin. Both ZO-1 and E-cadherin are highly expressed

in (bronchial) epithelial cells and located at the cytoplasmic membrane surface of intercellular (tight) junctions. Collagen 1A1 is an extracellular matrix protein secreted by cells in response to profibrotic stimuli and a marker for mesenchymal cells, whereas β-catenin is both an integral part of the cell membrane of epithelial cells but it also a transcriptional co-activator of a variety of (mesenchymal) genes [16,17]. Both TGF-β1 and the transcriptional co-activator β-catenin have been indicated to be involved EMT in smokers and COPD patients [16,17].

Stimulation of 3D spheroid cultures of BEAS-2B cells for 24h with TGF-β1 (3 ng/ml) resulted in a marked upregulation of collagen expression on both the gene and protein level (Fig. 2A–C). Furthermore, the growth factor reduced mRNA expression of E-cadherin (data not shown). Interestingly, prolonged exposure of 3D spheroid cultures to TGF-β1 induced morphological alterations of the spheroids compared to vehicle control conditions (Fig. 2D). Similarly to short term TGF-β1 treatment, DEP (100 ug/ml, 24h) exposure of 3D spheroid cultures of BEAS-2B cells resulted in enhanced collagen 1A1 gene expression, although this was not significant (Fig. 2E). As DEP is a relevant harmful substance for the development of chronic lung diseases, we investigated its effect on EMT in BEAS-2B cells in more detail. Stimulation of BEAS-2B cells (2D cell culture) with DEP (100 ug/ml; 24h) resulted in increased release of TGF-β1, reduced expression of the epithelial marker E-cadherin and an increase in β-catenin expression (Fig. 3A and [18]). These findings collectively indicate that DEP induces EMT-like phenotype in these cells.

This was further recapitulated in 3D spheroid cell cultures of BEAS-2B cells (Fig. 3B and C). Exposure of 3D spheroid cell cultures of BEAS-

2B cells to DEP (100 ug/ml; 24h) resulted in a marked increase in the expression of the mesenchymal markers collagen 1A1 and β-catenin and a clear decrease in the expression of the epithelial markers ZO-1 and E-cadherin (Fig. 3B and C). Remarkably, the mRNA expression of TGF-β1 was significantly reduced by DEP (Fig. 3D). Collectively, these findings indicate that DEP induces an EMT-like phenotype in human bronchial epithelial cells (BEAS-2B) and that this can be recapitulated in a physiological relevant 3D cell model (i.e. 3D spheroid cell cultures).

Discussion

In the current study, we demonstrate that we are able to generate 3D spheroid cultures of human bronchial epithelial (BEAS-2B) cells in a short period of time (Fig. 1), which can kept in culture for several days. Treatment of these 3D spheroid cultures with either the profibrotic growth factor TGF-β1 or components of air pollution, i.e. diesel exhaust particles (DEP), results in the initiation of epithelial-to-mesenchymal transition, characterized by a loss of epithelial markers (ZO1 and E-cadherin) and an increase in mesenchymal markers (β-catenin and Collagen 1A1). These findings are in agreement with our previous findings in BEAS-2B using conventional 2D cell culturing methods [9, 18]. In 2D cell culture we were able to demonstrate that activation of EMT in human bronchial epithelial cells (BEAS-2B) by cigarette smoke, another risk factor for developing chronic lung diseases, is depend on the increased release of TGF-β1 by the cells and subsequent activation of associated signaling pathways [9]. This might also be the case for DEP-induced initiation of EMT in these cells, as we observed, in conventional 2D cell cultures, increased release of TGF-β1 by the cells in response to DEP treatment without an effect mRNA expression of the growth factor [18]. In the current study, we observed in 3D spheroid cultures of BEAS-2B cells a decreased mRNA expression of TGF-β1 in response to DEP, although DEP clearly induced an EMT-like phenotype. The potential involvement of TGF-β1 in DEP-induced EMT in bronchial epithelial cells is beyond the scope of this article and will be investigated in more detail in future studies.

The process of EMT has been hypothesized to contribute to a variety of human diseases, including various cancer and chronic lung diseases such as asthma and COPD. Recent evidence suggests that airway remodeling, a pathological hallmark in various lung diseases, is orchestrated from the airway epithelium [5]. This may be highly interesting for the development of effective therapies to halt the progression of airway remodelling in chronic lung diseases. Currently no specific medicinal drugs are available that selectively target EMT in lung diseases. However, medicinal drugs that elevate cAMP, and which are used to alleviate symptoms of lung diseases, can differentially reduce EMT in bronchial epithelial cells *in vitro* [9]. To better understand epithelial plasticity and to potentially interfere in this process with therapy, we need more suitable cell models that closely represent the (patho)physiology of epithelial cells *in vivo*.

Over recent years, advanced 3D cell culture methods have been developed to more closely mimic the *in vivo* situation of the human body. Organoid cultures and precision cut tissue slices are increasingly making their way into biomedical research. These culture methods have been successfully applied to study developmental processes, tissue repair and remodelling processes in the lung [19–23]. In the current study we used 3D bioprinting, a rather novel technique to culture cells in a 3D microenvironment [14,24]. Each of the aforementioned 3D cell culture methods have their specific characteristics and their strengths and weaknesses. Organoid cultures of (primary) human cells have a number of benefits over animal models and traditional 2D cell cultures [25,26]. These organoid cultures provide faster and robust outcomes, mimic the human tissue more representative and are have high potential to model human development and disease [25,26]. However, this assay is dependent on the availability of human tissue to isolate (stem) cells from, is more expensive and more complex than conventional 2D cell culturing methods. Precision cut tissue slices preserve the natural 3D environment

of the cells in an organ and can be derived from animal as well as human tissue. The latter very closely mimicking the (patho)physiological (micro)environment seen in human *in vivo* and can therefore be used as a translational model for an array of diseases. Precision cut tissue slices derived from human tissue relies on the accessibility and availability of human tissue of interest. Non-human derived precision cut tissue slices requires the use of animal experiments and corresponding models of disease, which might not fully represent the (patho)physiology observed in human. The 3D bioprinting technique enables rapid formation of 3D spheroid cultures, with high reproducibility and is scalable for high-throughput formats for a wide variety of cells. This method together with standardized biochemical assay methods allows for a wide array of continuous assessment of cellular functions. In the current study, we generated 3D spheroid cultures using one specific cell type of interest (i.e. bronchial epithelial cells), however the 3D bioprinting method allows the generation of 3D spheroid cultures of various cell types alone or together which we will deploy in future studies.

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Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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