Title

A composite Gelatin/hyaluronic acid hydrogel as an ECM mimic for developing mesenchymal stem cell derived epithelial tissue patches

Running title: *Composite hydrogels for epithelial patches*

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Summary
Here we report fabrication of Gelatin based biocomposite films and their application in developing epithelial patches. The films were loaded with an epithelial cell growth factor cocktail and used as an extracellular matrix (ECM) mimic for *in vitro* regeneration of organised respiratory epithelium using Calu-3 cell line and mesenchymal stem cells (MSCs). Our data show differentiation of Calu-3 cells on composite films as evidenced by tight junction protein expression and barrier formation. The films also supported attachment, migration and proliferation of alveolar basal epithelial cell line A549. We also show the suitability of the composite films as a biomimetic scaffold and growth factor delivery platform for differentiation of human MSCs to epithelial cells. MSCs differentiation to the epithelial lineage was confirmed by staining for epithelial and stem cell specific markers. Our data show that the MSCs acquire the epithelial characteristics after two weeks with significant reduction in vimentin, increase in pan cytokeratin expression as well as morphological changes. However, despite the expression of epithelial lineage markers these cells did not form fully functional tight junctions as evidenced by low expression of junctional protein ZO1. Further optimisation of culture conditions and growth factor cocktail is required to enhance tight junction formation in MSCs derived epithelial cells on the composite hydrogels. Nevertheless, our data clearly highlight the possibility of using MSCs in epithelial tissue engineering and the applicability of the composite hydrogels as transferrable ECM mimics and delivery platforms with potential applications in regenerative medicine and *in vitro* modelling of barrier tissues.

Keywords
mesenchymal stem cell, Gelatin/HA, hydrogel, growth factors, controlled release, epithelium differentiation

Introduction

Respiratory tissue injuries, infections and degeneration lead to several serious pathologies such as Chronic Obstructive Pulmonary Disease, Asthma, and Chronic Bronchitis. The bronchial epithelium plays a critical role in maintaining the integrity and functionality of the respiratory system. The regeneration of airway epithelium is a complex phenomenon, and several parameters need to be considered to optimise the epithelium like organised tissue regeneration including epithelial migration, induction of differentiation of different cell types of functional epithelium, *in-vivo* epithelium migration, epithelium interaction with other tissues (immune cells), and effects of underlying tissues on epithelium functionality (Soleas, Paz, Marcus, McGuigan, & Waddell, 2012). Thus far the *in-vitro* development of functional epithelium has not been achieved due to its complex nature, limited differentiation ability of epithelial basal cells and formation of a non-organized epithelium even when cells are cultured at air-liquid interface (ALI) (Vrana et al., 2013),. Various studies recommend the potential use of stem cells (embryonic stem cells, induced pluripotency stem cells; and adult stem cells) for epithelium development using biochemical approaches, however these methods need several cumbersome steps and long culture time to differentiate and thus are unattainable for lab-bench to clinical translation or easy to use *in vitro* model systems (Firth et al., 2014; Huang et al., 2014; Ricciardi et al., 2012).

Stem cells are key elements of tissue engineering and offer hope as a therapeutic avenue. However, major consideration when using stem cells for respiratory epithelium development is the identification of the correct stem cells and their capacity for organised epithelial differentiation (Kumar, Vrana, & Ghaemmaghami, 2017). Mesenchymal stromal cells (MSCs) are one of the most widely used cells and their presence also have been reported in lung tissue and have role is tissue regeneration *in-vivo*. However, MSCs have shown limited application *in-vitro* respiratory tissue regeneration. For example, the MSCs support the development of respiratory mucosa-like tissue in co-culture with normal human bronchial epithelial (NHBE) but did not acquire the epithelial characteristics (Visage, Dunham, Flint, & W., 2004).

The interaction of basement membrane ECM with epithelium stem cells/progenitor cells promotes the airway epithelium repair *in-vivo* by modulating epithelial cell migration and proliferation via differentiation of these progenitors cells to the epithelium subtypes (Coraux, Roux, Jolly, & Birembaut, 2008). The comparative study of lung and bone marrow (BM) derived MSCs suggest that the epithelial differentiation of BM-MSCs can be achieved in the presence of retinoic acid, however this effect was minimal compared to lung derived MSCs or epithelium basal cells (Ricciardi et al., 2012). Thus, mesenchymal-to-epithelial-transition is still controversial; however, if successful, this phenomenon can significantly accelerate the respiratory epithelium development using MSCs.

Biomaterial based scaffolds have been used for the development of various tissues(Beckstead et al., 2005; Calejo et al., 2017; Grolik et al., 2012; Tan et al., 2017). However, there is still a need to develop application specific biomaterials with appropriate mechanical properties and capacity to support cell growth, migration and proliferation. Novel Extracellular matrix (ECM)-based delivery platforms carry and deliver the therapeutic agents (e.g. growth factor) in a controlled manner, and simultaneously protect them from fast degradation (Geckil, Xu, Zhang, Moon, & Demirci, 2010). The mechanical properties of *in-vitro* microenvironment could also direct the stem cell differentiation to a specific cell-lineage (Baeza-Squiban et al., 1994; Engler, Sen, Sweeney, & Discher, 2006; Mendez, Ghaedi, Steinbacher, & Niklason, 2014; Wen et al., 2014). Moreover, the extracellular matrix (ECM)-based scaffolds are potential tools for *in vitro* tissue development due to their ability to mimic the native microenvironment, e.g. collagen type I, collagen IV, laminin, and glycoproteins (Yen, Chan, & Lin, 2010). These ECM components induce epithelial

migration via integrin signalling and are through to play a key role in directing epithelial repair. Thus, the airway epithelial regeneration in the presence of thin biomaterial substrates is considered as one of the possible methods to induce airway epithelium formation for developing robust models which have the basement membrane component (Vrana et al., 2011). In this context, Gelatin based biomaterials have been used in supporting the growth of liver(Yan et al., 2005), bone (Yang, Hsu, Wang, Hou, & Lin, 2005), cardiac (Pok, Myers, Madihally, & Jacot, 2013) and skin (Lu, Oh, Kawazoe, Yamagishi, & Chen, 2012) tissues. The mechanical properties of Gelatin can be further manipulated using cross linking and/or encapsulating nanoparticles (Rao, 2007) . Controlled cross linking of Gelatin-based scaffolds also induces the porous structures, conductive to enhanced cell attachment and differentiation(Yan et al., 2005). The advantage of patch-based delivery is to ensure the positioning of the epithelial cells. We envision an endoscopic delivery with a releasable clamp that holds the patch in place; with fibroscopy the positioning of the patch can be ensured and once in the correct location the clamp is released to apply the patch to the target surface. As both gelatin and HA are adhesive by their nature, the establishment of the interface would not be problematic. If necessary, a layer of wet adhesive can be added as we have described previously (Barthes et al., 2017). Another advantage of the patch system is that the MSCs can continue their differentiation with the right polarity (as the substrate defines their positioning) in a microenvironment that is particularly suitable for respiratory epithelium differentiation.

In this study, we describe fabrication of Gelatin based biocomposite films loaded with an epithelial cell growth factor cocktail for developing epithelial patches using BM-MSCs. It was hypothesised that growth factor loaded composite films can act as an ECM mimic that is able to facilitate the BM-MSCs differentiation to multiple respiratory epithelium. First, we demonstrated the feasibility of supporting an epithelial layer with a respiratory epithelial cell line (Calu3 cells). Calu-3 is a highly studied respiratory epithelial cell line with the ability to form tight junctions and an efficient barrier *in vitro* making it suitable for modelling the airway epithelial barrier in respiratory tract research (Grainger, Greenwell, Lockley, Martin, & Forbes, 2006). Moreover, the system was used to assess whether it is capable to support the differentiation of the BM-MSCs towards respiratory epithelium lineage.

Materials and methods

All tissue culture plastics were purchased from Sarstedt and Nunc. Tissue culture inserts were purchased from Costar Corning. The FGF- 7 & FGF 10- were purchased from Peprotech UK. The bronchial epithelium media and stem cell media were purchased from Promocell. The bronchial differentiation media was prepared using epithelium growth medium from Lonza UK without adding triiodothyronine. All primary and secondary antibodies were purchased from Abcam UK and Thermo Fisher Scientific UK respectively. Gelatin type B (Mw =2-2.5×104 Da, pI = 4.7-5.2) from bovine skin, Fluorescein isothiocyanate labeled bovine Albumin (BSAFITC, Mw = 6.6×104 Da) were purchased from Sigma Aldrich (St. Quentin Fallavier, France). Microbial Transglutaminase (M-TG) (Mw =3.8×104 Da, pI = 9) was a kind gift of Ajinomoto (Japan). Hyaluronic acid (HA, Mw = 3 x105Da) and HA-Tyramine (HA-Tyr, Mw = 3.1 x105 Da), were produced and characterized by CONTIPRO (Czechia). All other chemicals, cell culture media and reagents were purchased from Sigma Aldrich unless otherwise stated.

1. Development of Gelatin based film

a. Spin coating of Gelatin and Gelatin-HA-tyr films

Gelatin and Gelatin-Hyaluronic Acid-Tyramine films (Gelatin; Gelatin-HA-Tyr), were made using the spin coating method (spin coater WS-650Mz-23NPP; Laurell). Powders of Gelatin

(15% w/v) or Gelatin mixed with or HA-Tyr (1% w/v) were dissolved in 0.15 M NaCl/10 mM Tris solutions (pH = 7.4). The solution was heated to 50°C with constant stirring. Then 200µL of Gelatin solution was dropped on a glass slide (previously installed in the spin coater) and the spin coating program was started. To obtain the self-standing films 60µL of cellulose acetate (1 % w/v in acetone) was deposited on top of the glass slide prior to spin coating process and the sample were dried for one day at 4°C. The parameters for spin coating were 2500 rpm with an acceleration of 1250rpm for 2 minutes time. Afterwards, the films were kept dry at 4°C for at least 3 hours before cross linking. All solutions were prepared using ultrapure water (Milli Q-plus system, Millipore) with a resistivity of 18.2 MΩ.cm and filtered using 0.22µm filter before use.

b. Crosslinking of Gelatin and Gelatin-HA-Tyr films

A solution of 10% (w/v) of transglutaminase (TGA) in PBS was prepared. The Gelatin and Gelatin-HA-Tyr (Gelatin-HA) films were incubated in 100µL of this solution for 30 minutes. The non-crosslinked ingredients were washed out using two rinsing steps with 100µL of PBS each time for 5 minutes. The Gelatin-HA-Tyramine film an additional crosslinking step was performed for the dimerization of tyramine to make dityramine using horseradish peroxidase (HRP) mediated reaction. For this, the solution of H_2O_2: HRP (10:1) was prepared with 0.24mg/mL of HRP in PBS and 0.1M H_2O_2. 100µL of this solution were than incubated on Gelatin/HA films for further 30 minutes. Again, the non-crosslinked ingredients were washed out using two rinsing steps by incubating with 100µL of PBS each time for 5 minutes.

c. Thickness determination

The thickness of Gelatin and Gelatin/HA films were estimated with confocal microscope (ZEIS LSM 710). To visualize the film and estimate the thickness, BSA^{FITC} solution (green fluorescent probe) was used to label the films. By reconstructing the whole film thickness using multiple z-stacks can be visualized, hence allowing the determination of the thickness of the film with a 20x objective.

d. Film transfer on transwell and growth factors loading

For the biocomposite delivery system, the films were prepared according the protocol described above. A 6% w/v solution of Gelatin type A was prepared in MilliQ water. Gelatin solution was put in water bath at 50°C until complete dissolution. At the same time a 20% w/v TGA solution was prepared in PBS. Then 5 µL of TGA solution and 25 µL of Gelatin solution was added on top of transwell (Costar Transwell®, 0.4µm) just before the transfer of crosslinked film in order to attach the film on transwell insert. The transwell with attached film was then put in incubator at 37°C for at least 15minutes. The non-crosslinked Gelatin washed out using 100mL PBS washing (2X) under aseptic conditions. The growth factors; Recombinant Human FGF-10; FGF-10 (10µL, [25µg/mL]) and Recombinant Human KGF; FGF7 (10µL, [10µg/mL]) solutions both prepared in MilliQ water and were incubated on top of each film overnight at 4°C (previously sterilized 15 minutes under UV).

e. Release profile of the film

Release experiment from Gelatin-HA-tyramine (Gelatin-HA) was performed using a fluorescently labeled protein (BSA^{FITC}: Bovine serum albumin labelled with fluorescein isothiocyanate). BSA^{FITC} solution was prepared in PBS solution at 1mg/mL. Then 100µL BSA^{FITC} solution was incubated on top of Gelatin-HA film crosslinked with TGA and HRP overnight at 4°C. The release experiments were performed at 37 °C in a PBS solution (1mL). The supernatant was analyzed with a spectrofluorimeter (SAFAS Genius XC, Monaco). A

new PBS solution (1 mL) was added after each analysis. For BSA^{FITC} the wavelength parameters were λex/λem = 495 nm/520 nm.

2. A549 cells experiment & cellular migration (Time lapse microscopy)

A549 human lung carcinoma epithelial cells were used as model of respiratory epithelial cells. They were cultured in RPMI 1640 basal medium supplemented with 10% v/v Fetal bovine serum, 1% v/v Pen-Strep and 1% v/v Fungizone. After trypsinization, 50,000 cells prepared in 30µL of medium were deposited on top of the Gelatin-HA film previously crosslinked with TGA and HRP and then UV treated for 15 minutes. After seeding, samples were then left to incubate at 37 °C with 5% CO2 for 7 days. Culture medium was changed every 48 h. Metabolic activity was determined with a resazurin-based test (Sigma Aldrich) and checked at day 1, 3 and 7 to evaluate the proliferation. DAPI/Phalloidin (F-actin) staining was performed after 7 days of culture. After fixation with paraformaldehyde (3.7% v/v in PBS for 15 minutes), cells were incubated with Triton X-solution (0.1% in PBS for 5 minutes) and BSA solution (1% v/v in PBS for 20 minutes). Then samples were incubated for 1 hour with phalloidin (Alexa Fluor 568 phalloidin [6.6µm], Molecular Probes Life Technologies) at a dilution of 1/40 in BSA solution (1% v/v in PBS). After that, two rinsing steps in PBS were performed and the samples were incubated 5 minutes in Hoechst 33342 solution (20µg/mL). Finally, samples were visualized with confocal microscope.

For cellular migration analysis, A549 cells were stained with Hoechst 33358 solution (20µg/mL) for 30 minutes in a T75 cm^2 for 30 minutes. Then they were trypsinized and seeded on the different Gelatin based films (15000 cells/films) for 15 minutes and mounted in a Ludin Chamber (Life Imaging Services, Basel, Switzerland) at 37°C, 5% CO2. Then time-lapse experiment was performed on a Nikon Ti-E microscope equipped with a 10x objective and with an Andor Zyla sCMOS camera and driven by the Nikon NIS-Elements Ar software. Images were acquired every 10 min for 24 h simultaneously by phase contrast and by fluorescence microscopy with nucleus staining by Hoechst 33342. Cell tracking by the software "NIS-Elements Ar 3D tracking" (Nikon) was carried out in different fields of the substrates. First, the software detected objects "nuclei" by thresholding and they were afterwards tracked over the 24 hours. Phase contrast images were used to check the viability of the followed cells. Cells that died during the experiment were eliminated.

3. MSCs cell culture & epithelium differentiation on Gelatin based film

To get enough number of cells the MSCs (derived from a single donor; Promocell, Germany) were expanded in MSCs medium (Promocell Germany). The cells were routinely cultured at 37 °C and 5% CO_2 in stem cell media (consist of basal media and medium supplement) as per the manufacturer's protocol. The medium was changed routinely in every 2-3 days up to the confluence. For all experiments the lower passage MSCs (p4-p5) were used.

The MSCs were seeded on Gelatin based films (100,000 cells per inserts) on a transwell insert in epithelium media (Promocell). The cells were left for 2-3 weeks in submerged culture followed by two weeks culture at air-liquid interface (ALI) in epithelium differentiation media (Lonza). For this, the medium from upper chamber was removed and 500µl differentiation medium was used in lower chamber. The experiment was also repeated using DMEM-F12 media to assess the impact of the Gelatin films in the absence of growth factor in the differentiation media. Moreover, to assess the impact of Rho-associated protein kinase (ROCK) inhibition on MSCs's differentiation towards respiratory epithelium; 1µL/ml of Rho kinase inhibitor Y-27632 was added throughout the culture in a separate experiment. In each case, the medium was changed every 2-3 days.

4. Calu-3 cell seeding and differentiation on gelatin based films

Calu-3 cells were used as a model epithelial cell to assess barrier formation. Briefly, Calu-3 cells were seeded on Gel-HA film (within transwell) at 200,000 cells/CM^2 in DMEM-F12 medium with 10% FBS, 1% P/S, 100 mM non-essential amino acid and 100mM of L glutamine supplement. The cells were transferred to ALI after growing in submerged conditions for a week.

5. Evaluation of MSCs and Calu 3 differentiation on film

a. Immunofluorescence for epithelium differentiation

After submerged culture of MSCs for 2-3 weeks the expression of epithelium and MSCs markers was assessed using various epithelium markers; wide spectrum cytokeratin, pan cytokeratin and cytokeratin 18 (1:100 dilution) and mesenchymal marker; vimentin (1:200 dilution) antibodies (all antibodies from abcam). At the end of each culture, the cells were fixed with 3% formaldehyde in PBS (pH 7.4) for 30 minutes and were permeabilised using the 0.25% Triton X 100 solution for 20 minutes. After each step, the samples were washed three times in PBS (5 minutes). The samples were further incubated with 3% BSA for 1 hour to stop the non-specific binding of proteins. Cell layers were incubated with primary antibodies diluted in PBS for 90 minutes at room temperature. After primary incubation, the samples were incubated with secondary antibody for 45minutes at room temperature. The secondary antibodies used were Alexa Fluor® 488, chicken anti rabbit 1:250 dilution in PBS or rhodamine red anti mouse 1:250 dilution in PBS). Each antibody incubation was followed by three washes in PBS for 5 minutes. For nuclear staining, DAPI (4' 6-diamidino-2-phenylindole) was used at 1: 4000 dilution (Invitrogen, UK) for 15 minutes and followed by 3 PBS washing for 5 minutes. Finally, the samples were mounted on glass slides with VectaShield (Vector Laboratories, UK) for direct observation. The images were taken using Leica DMRB fluorescence microscope using 10X or 40X objective. The intensity of the fluorescence images was evaluated using the image J software.

b. Trans-Epithelial Electrical Resistance (TEER) Measurements

The trans-epithelial electrical resistance (TEER) measurements across the cell monolayer cultured at the ALI were performed according to the method described elsewhere (Harrington et al., 2014). Briefly; the measurements were performed using an EVOM volt-ohm-meter and STX2 chopstick electrodes (World Precision Instruments, U.K.). The cell culture media was added to the upper chamber (500 µL) and lower chamber (1.5 mL total volume) and allowed to equilibrate for 30 minutes prior to measurements (37 °C, 5% CO_2). The TEER values were measured several times in several samples. The chopstick electrodes were sterilized using 70% v/v ethanol in distilled water. Control measurements were performed using Calu 3 cell lines cultured in a similar way.

c. ZO-1 and Mucin staining

At the end of ALI culture, the samples were fixed with 3% formaldehyde in PBS (pH 7.4) for 30 minutes and were permeabilized using the 0.25% Triton X 100 solution for 20 minutes. The samples were further incubated with 3% BSA for 30 minutes to stop the non-specific binding of proteins. Cell layers were incubated with primary antibodies diluted in PBS for 90 minutes at room temperature. After each step, the samples were washed three times in PBS (5 minutes). The primary antibody used was rabbit anti ZO1 and Mucin5A/C from Abcam (1:100 dilution in PBS). After primary incubation, the samples were incubated with secondary antibody for 45 minutes at room temperature. The secondary antibodies used

were Alexa Fluor® 488, chicken anti rabbit 1:250 dilution or anti mouse Rhodamine red. For nuclear staining, DAPI (4' 6-diamidino-2-phenylindole) was used at 1: 4000 dilution (Invitrogen, UK). Finally, the samples were transferred on glass slide and the cover slips were mounted on samples with VectaShield (Vector Laboratories, UK) for direct observation. The images were taken using a Leica DMRB fluorescence microscope using 40X objective.

6. Statistical analysis

The statistical analysis was performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA (www.graphpad.com). All results are shown as mean ± standard deviation (SD) from three independent experiments. Statistical differences were determined using the student t-test or one-way ANOVA method with Tukey post-hoc testing. A p-value <0.05 was considered statistically significant.

Results and discussion

Composite film fabrication and their protein release profile

The epithelium stem cells are scarce and have limited regenerative capacity for epithelium tissue regeneration thus there is an imperative need for alternative cell sources which can potentially be used for airway epithelium development (Vrana et al., 2013). The current literature suggests the significant contribution of various stem cells in epithelium regeneration e.g. MSCs, ESCs and iPSCs. The MSCs have been widely used for the development of bone, cartilage and adipose tissue, originated from the mesodermal layer of embryo. Recent literature also provides some evidence for MSCs differentiation towards non-mesodermal cell and tissue development e.g. neuronal, liver, epithelium and pancreatic tissue. Some recent studies also suggested the differentiation capability of BM-MSCs to epithelial cells *in vitro* (Păunescu et al., 2007) and *in vivo* (Kotton et al., 2001). They acquire phenotypic and functional epithelial characteristics when co-cultured with airway epithelial cells (Ma et al., 2011; Spees et al., 2003). Providing appropriate growth factors and mechanical and biochemical cues that simulate extracellular matrix in the ECM membrane could potentially support MSCs differentiation towards epithelial cells, removing the need for complex co-cultures. Thus, we proposed to use a Gelatin based bio-composite film capable of controlled release of epithelium inducing growth factors (FGF-7 + FGF10) as a ECM mimic for developing a respiratory epithelium patch using BM-MSCs.

Gelatin, as a natural biomaterial, has been used in various tissue regeneration applications in two dimensional and three dimensional cross-linked form (Sell et al., 2010). The physiochemical properties of the Gelatin can be controlled easily for development of substrates for cells, e.g. the controlled cross linking of Gelatin based scaffold induces porous structure, conductive to enhanced cell attachment and differentiation (Yan et al., 2005). The hyaluronic acid (HA) is an important component of extra cellular matrix in various tissues in human body, such as connective, epithelial, and neural tissues Due to its high water retention capacity, the presence of HA provides the advantage of increased volume for the substrate which substantially increase its ability to be loaded with growth factors. Moreover, as a component of ECM, HA has intrinsic interactions with most of the growth factors together with its polyanionic nature under physiological conditions which provides additional electrostatic retention for particularly positively charged growth factors. Thus, Gelatin (15%) and Gelatin-HA (14%+1%) films were developed using the spin coating methods for the *in vitro* development of respiratory epithelium.

In the first part of this study, the fabrication method of Gelatin film has been optimized to determine the main parameters that will influence the thickness of the film. To do that, different Gelatin concentrations and rotation speeds have been tested to spin coat and the

thickness of the resulting film after crosslinking with transglutaminase has been estimated with confocal microscope after the loading of BSA^{FITC} to visualize the cross section (**Figure 1A**). Our data clearly show that the main parameter that influences the thickness of the film is Gelatin concentration. The difference in gel thickness between three different rotation speeds for the same concentration was not significant. The only trend observed was the increase in the thickness with Gelatin concentration. To have better stability and thicker film, we worked with 15% w/v Gelatin concentration and used a rotation speed of 2500 rpm for the rest of the study. To better simulate the composition of the basement membrane *in vivo*, we incorporate hyaluronic acid in the film formulation. In a previous work from our lab (Knopf-Marques et al., 2017), we have demonstrated that the stability of Gelatin/HA membrane films can be improved using HA derivative such as HA-tyramine by creating an interpenetrated network through a double crosslinking step. Gelatin is crosslinked with transglutaminase to create amide bond between amine groups on lysine residues and carboxamide groups on glutamine residues and HA-tyramine is crosslinked through the formation of dityramine groups in the presence of HRP (Horseradish peroxidase). HA-tyramine was incorporated in the film formulation with the following ratio (Gelatin 14%/ HA-tyramine 1% w/v) and referred to as Gelatin-HA. The addition of hyaluronic acid in the structure resulted in an increase in the film thickness, from about 10µm for Gelatin to 15.5µm for Gelatin/HA (**Figure 1B**). This difference in thickness can be attributed to the intrinsic capacity of hyaluronic acid to absorb large amount of water.

As these Gelatin based membranes are supposed to release growth factors for the differentiation of MSC to epithelial cells, the release property of these materials was tested using a fluorescently labelled model protein BSA^{FITC}. The release property of Gelatin membrane was tested in a previous work from our group (Barthes et al., 2015) and we reproduced this experiment with Gelatin/HA film and the cumulative release of BSA^{FITC} was followed at 37°C in PBS solution by quantifying the fluorescence in the supernatant using a spectrofluorimeter (**Figure 1C**). Both materials have shown the ability for the loading and the release of bioactive molecules for at least two weeks after an initial burst release.

The composite bio-film supports migration, growth and differentiation of alveolar epithelial cells

The next step was to compare the behaviour of both film formulations (Gelatin vs Gelatin-HA) on A549 epithelial cells in terms of migration distance and migration speed to see if the addition of HA in the formulation had an effect. A549 cells were selected due to their aggressive migratory nature (Ciftci et al., 2016) with the hypothesis that with a more migratory cell type the effect of the substrate will be accentuated and easier to quantify. This experiment was carried out using Time Lapse microscopy for 24 hours. It was shown that the addition of HA in the formulation did not have an effect on both cell migration distance and cell migration speed. Both materials exhibited the same response toward A549 epithelial cells (**Figure 2A and B**). Moreover EGF (Epidermal Growth Factor) was also loaded in Gelatin-HA film and the same experiment of migration was repeated. The presence of this growth factor also did not affect cell migration and cell migration distance when we compare to both Gelatin and Gelatin-HA film. The proliferation of A549 epithelial cells on Gelatin material has been studied in a previous work from our group (Ciftci et al., 2016) and we have repeated the same experiment with Gelatin-HA film (**Figure 2C**). The metabolic activity was followed for 7 days and a significant increase was seen between day 1, day 3 and day 7 meaning that cells were proliferating on the film. DAPI/F-actin staining was performed at day 7 to check the morphology and the confluence of the cells on top of the film (**Figure 2D**). After 7 days of culture a confluent layer of epithelial cells was observed in top the film.

These experiments have shown that Gelatin-HA film did not have any negative effects on epithelial cell attachment, migration and proliferation compared to pure Gelatin film while having advantages in having a HA component, higher thickness for increased volume for growth factor loading and improved stability. Moreover, as the films are detachable, it enables to perform ALI cultures in the presence of an ECM mimicking structure.
Based on the application of Gelatin and hyaluronic acid in respiratory epithelium development, the growth factor loaded films were developed for the controlled BM-MSCs differentiation towards respiratory epithelium lineage.

MSCs growth and differentiation to epithelial like cells on gelatin based films
The MSCs were seeded on Gelatin and Gelatin-HA films and cultured in submerged cultures for up to three weeks to adapt to epithelium environment using epithelium media. It is evident that the MSCs seeded on various films adhered well on films and were viable even up to 3-4 weeks of culture. The epithelium cells are cobblestone shaped cell smaller in size than the mesenchymal cells. The morphology of BM-MSCs seeded on transwell or Gelatin based films changed into round shape morphology, similar to epithelium. However, the stem cells in standard medium still demonstrate the spindle morphology (**Figure 3a**). The cells on Gelatin based film without the growth factors also demonstrate the smaller size due to the emulation of softer substrate similar to native epithelium ECM and thus the physicochemical and mechanical properties of culture microenvironment could control the MSCs cell behaviour (Li et al., 2010). The stiffness of dense transwell membranes are significantly higher than the mechanical properties of the described films that have been previously reported to be between 30-50 kPas (Knopf-Marques et al., 2017). Previous work on uncrosslinked Matrigel showed a Young's modulus of 440±250 Pa (Soofi, Last, Liliensiek, Nealey, & Murphy, 2009) at low concentrations, which would prevent the transferability of the patches described here.

The MSCs grown under submerged culture in epithelium medium on Gelatin films also expressed lower vimentin and there was a further reduction in vimentin expression on stem cells grown in growth factors loaded films (**Figure 3b**). The expression pattern of pan cytokeratin and wide spectrum cytokeratin is opposite to the vimentin (**Figure 3c, 4d**).

There is an upregulation of the epithelial markers expression in stem cells grown on growth factors loaded Gelatin based film, which suggested the differentiation of MSCs towards epithelium like cells in response to growth factor release over time. These experiments were also performed using the DMEM-F12 media to assess the role of GFs loaded Gelatin films in the absence of defined epithelium media. Data from these experiments indicated that even in the absence of the epithelium-defined media the composite films support epithelium differentiation of MSCs as evidenced by upregulation of pan cytokeratin and downregulation of vimentin in cultured MSCs over the time. However, the morphological changes (spindle-round shape) were not as prominent as seen in the presence of epithelium medium (**Figure S2**) and the presence of tight junction protein ZO-1 was not observed (**Figure S3**).

More importantly, the expression of pan cytokeratin increases with time in culture. However, in all experiments the MSCs still express the vimentin markers and thus complete epithelial differentiation of MSCs was not achieved using the controlled growth factors delivery approach from the Gelatin based film. Further, two week of ALI culture of these MSCs derived epithelium do not help in the formation of tight junctions and no expression of ZO1 was observed in all samples other than control Calu 3 control cells (**Figure 4a**). The TEER

measurement of MSCs was significantly lower compared to the Calu 3 cells. However, higher TEER was detected in the MSCs derived epithelium cultured on films compared to the MSCs on tissue culture insert alone or films without cells (**Figure 4b**). The TEER value results shown were obtained after reaching the plateau phase (n=3).
These data suggest that despite slight increase in TEER reading, that could indicate the beginning of barrier formation, after 2 weeks of ALI culture the MSCs differentiated to the epithelium still show very low expression of tight junction protein ZO-1. These results also verify the previous study, showing that human bone marrow-derived MSCs are able to differentiate into epithelial-like cells *in vitro* in the presence of growth factor enriched medium (Păunescu et al., 2007). However, the authors did not report the formation of intercellular tight junctions.

Impact of Rho-associated protein kinase (ROCK) inhibition on MSCs full differentiation to epithelial cells

In order to enhance the differentiation of MSCs to respiratory epithelium we investigated the impact of ROCK inhibition. ROCK inhibition using Rho kinase inhibitors like Y-27632 has been shown to promote the induction efficiency, self-renewal and differentiation of MSCs towards the keratinocytes like cell with good expression of cytokeratin 14 and cytokeratin 5 in the presence keratinocyte-conditioned medium (Li et al., 2015). Thus, it was envisaged that this approach can also further push the epithelial differentiation of MSCs in the presence of biocomposite films. Accordingly, we performed the above experiments in the presence of Y-27632, a well-established Rho kinase inhibitor. Data from these experiments indicated better epithelium differentiation with adequate epithelial morphology and better expression of cytokeratin markers; cytokeratin 18 and pan cytokeratin. However, this effect was not statistically significant (**Figure 5a and S4**). Despite changes in linage marker expression in the presence of the ROCK inhibitor, suggesting more efficient differentiation to epithelial cells, the MSCs derived epithelial like cells still did not show barrier function as evidenced by low or no Mucin5A/C or ZO1 expression (**Figure 5b**).

MSCs as a potential cell sourced for developing personalised epithelia patches

Given their more accessible nature and availability in larger numbers (e.g. compared to epithelial cells from nasal turbinate), MSCs could be potentially used for development of personalised epithelial models. The provision of an ECM like membrane layer with appropriate mechanical properties further provides a means to mimic the effects of ECM membrane on the behaviour of healthy and diseased epithelial cells. However, for a more robust model further development that would induce better barrier function and cell-cell contact is required. In order to see if the developed composite ECM membrane can be used for more general respiratory epithelium models, we also cultured a well-established epithelial cell line, Calu 3 (**Figure 6**) on these films. Staining for Mucin and ZO-1 demonstrated mucin secretion and tight junction formation by the cells on the surface of ECM mimics. This further highlights the potential application of the biocomposite films for developing respiratory epithelium models.

Conclusion

Using MSC derived epithelial cells would provide a larger cell pool (compared to primary respiratory epithelium) and patient specificity (compared to respiratory epithelium cell lines) that could enable development of more personalised tissue models with clear benefits for

disease modelling or testing new drug leads. Such endeavour also requires ECM mimicking substrates that are adaptable to air liquid interface culture conditions to provide more physiological relevance. Herein, we describe composite film that mimics the ECM membrane with high stay stability for long term culture periods and the capacity to release growth factors under ALI conditions. MSCs cultured on these substrates in the presence of growth factors showed substantial decrease in mesenchymal marker expression and increased epithelial marker expression. ROCK inhibition provided a more advanced differentiation. This study demonstrates the feasibility of using growth factor loaded biocomposite films and MSCs for development of *in vitro* respiratory epithelial models. Our future work will focus on optimisation of culture conditions including modifying the composition of the films and GF delivery conditions to induce better mucin secretion and epithelial barrier function.

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Authors' contribution: PK and SC carried out the experiments and analysed the data. PK, SC, JB, HK and JB wrote the manuscript and helped with data analyses. HK fabricated the gelatin and gelatin-HA film samples. CBM, CD and NEV helped experimental design and data analyses. NEV and AMG conceived the original idea. AMG supervised the project. All authors read and approved the manuscript.

Conflict of interest statement
The authors declare no conflict of interest in this study.

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Figure legends

Figure 1. A) Thickness of Gelatin film made by spin coating process using different Gelatin concentrations and different rotation speeds. The thickness was characterized with confocal microscope after incubation of BSA^{FITC} in the film to observe the xy section. B) Confocal pictures (xy section) of Gelatin film loaded with BSA^{FITC} to estimate film thickness (20x magnification). C) Release experiment of BSA^{FITC} performed at 37°C from Gelatin-HA film with spectrofluorimeter.

Figure 2. A and B) Comparison of Gelatin and Gelatin-HA on the migration of A549 cells. The effect of the loading of EGF in the film was also tested. These experiments were carried out with Time lapse microscopy and the migration was followed for 24 hours. C) Metabolic activity of A549 cells cultivated on Gelatin-HA film for 7 days. D) DAPI/F-Actin staining of A549 cells cultivated on Gelatin-HA after 7 days of culture. The images are representative of 3 independent experiments (n=3)

Figure 3. The MSCs cultured on Gelatin and Gelatin-HA film. The effect of growth factors released from Gelatin based film on morphology (a) clearly suggest the change in spindle shaped MSCs morphology to epithelial morphology in the presence of growth factors encapsulated film. The effect of growth factors released from Gelatin based film on MSCs marker; vimentin (b), and epithelial markers; pan cytokeratin (c) and wide spectrum cytokeratin (d) expression in the presence of epithelium medium. The corresponding fluorescent intensity (**Figure S1**) suggest the reduction in vimentin and increase in pan cytokeratin as well as wide spectrum epithelium (n=3).

Figure 4. No expression of ZO1 was observed after two weeks of ALI culture of MSCs, however the Calu 3 cell line strongly demonstrated the tight junction formation as expressed by ZO1 expression (5a) and significantly higher TEER measurement (5b) (n=3). The bar represent average of 3 experiments ±SD

Figure 5. ROCK inhibition of MSCs further induces the cytokeratin and pan cytokeratin expression but could not induce the tight junctional barrier formation, as evidenced by absence of ZO1 and Mucin5A/C after 2 weeks of ALI (n=3).

Figure 6: Application of Gel-HA films for the Calu-3 cell differentiation confirmed the supportive role of Gelatin based films for the successful differentiation (n=3) with high expression of mucin, ZO1 and increase in TEER value with time. The error bars represent average value of 3 independent experiment± SD

Supporting information

Figure S1: The fluorescence intensity of stem cells and epithelium markers suggest the reduction in mesenchymal characteristics (Vimentin) and up regulation in pan cytokeratin (PanCK) and wide spectrum cytokeratin epithelium markers.

Figure S2: The biocomposite film encapsulated with GFs also favour the MSCs- epithelium differentiation in the presence of DMEM-F12 medium. The expression of MSCs marker vimentin is downregulated with the time (a & b; 2 & 3 weeks respectively) and increase in pan cytokeratin markers was reported (c & d; 2 & 3 weeks respectively).

Figure S3: There was no formation of tight junctions after 2 weeks ALI culture as no change in TEER measurement and no expression of ZO-1 observed in MSCs derived epithelial like cells in the presence of DMEM-F12 medium.

Figure S4: The fluorescence intensity of BM-MSC for mesenchymal marker (vimentin) and epithelium markers Pancytokeratin and cytokeratin 18) in the presence of Rho kinase inhibitor Y-27632 on GFs loaded Gelatin-HA film. The TEER measurement further confirmed the formation of intercellular tight junctions. However the effects were not significant more than GFs loaded film.