Chapter 11

Production of Tissue-Engineered Human 3D Bronchi *In Vitro*

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1. Introduction

1.1. Respiratory disorders and goblet cells

Asthma is experienced during the life span in approximately 10% of the population [1]. Chronic obstructive pulmonary disease (COPD) is a complex inflammatory airway disease that results in airflow limitation that is not fully reversible. Many animal model systems have been developed that recapitulate various features of COPD but all present significant limitations [2-3]. The lack of understanding of the underlying mechanisms and mediators that drive the onset and progression of chronic inflammation, emphysema and changes in lung function have limited the development of useful models and subsequently effective treatments. Most of the normal human airway is lined by a pseudostratified epithelium of ciliated cells, secretory cells and 6–30% basal cells, the proportion of which varies along the proximal-distal axis [4]. Among several other bronchial cell types, goblet cells (GC) are important players in the development and maintenance of pulmonary disorders. Disturbance of their distribution and phenotypic features, hyperplasia, metaplasia, dysplasia and mucus hypersecretion are common pathological features of chronic respiratory diseases, including asthma, COPD, lung cancer, and cystic fibrosis. Despite numerous studies, the molecular basis for GC phenotypic changes remain elusive [5-6]. In asthmatic bronchi, the number of GCs is increased, due to the trans-differentiation of ciliated and club cells (Clara) to GCs, resulting in more abundant mucus production [7]. GCs secrete the mucins MUC5B and MUC5AC, which are responsible for the viscoelasticity and hydration of the mucus covering the ciliated escalator [7]. The sputum in

asthmatic bronchi is often so dry that it can lead to airway obstruction. An intratracheal injection of IL-13 in mice models leads also to hyperproliferation of GCs, since this cytokine induces the pathophysiological features of asthma in a manner that is independent of immunoglobulin E and eosinophils [8]. MUC5AC is the predominant mucin in the epithelium, whereas MUC5B is predominant in the submucosal glands [9]. In cystic fibrosis, the primary defect is dehydration of the mucus layer resulting in a failure of mucus clearance leading to plaque accumulation on the airway surface causing persistent airway infections, [10]. Modulation of GCs function could contribute to regulate the symptoms and or the evolution of respiratory diseases. Bronchial models are needed to evaluate new molecules to inhibit or promote selective mucus secretion pathways.

1.2. Animal models

Several animal models have been proposed or used to assess physiopathologic mechanisms leading to pulmonary diseases. The rat [11-12], the mouse [13-14], the guinea pig [15], the dog [16] and the horse [17-20] have been exploited to establish potential mechanisms of control of respiratory diseases. Despite valuable data gathered from animal experimentation, the failure to translate promising drug candidates from animal models to humans has led to demand for more predictive models and tools based on the latest technologies [2]. The current paradigm where animal models of allergen sensitization and challenge are considered the gold standard falls some way short of human asthma [3]. The use of human tissue-engineered bronchial models (BMs) may facilitate the design of studies that shed light on pathogenesis, which in turn can lead to the development of worthwhile therapeutic interventions [21].

1.3. Tissue-engineered airway models *in vitro*

In vitro models are proposed in the literature. One of the *in vitro* approach consists in a model of trachea and bronchi, ventilated through an endotracheal tube during controlled mechanical ventilation, assist control, pressure support, and continuous positive airway pressure, with a dry and humidified ventilator circuit [22]. This model is original and clinically useful, but such experimentation requires specific equipment and is mostly limited to assess conditions of administration of nebulizers and metered-dose inhalers. In terms of cell culture models, several cell populations are commercially available. For example, Clonetics™ human primary airway cells can be useful to produce airway cell monolayers in the study of respiration, mucin production, cilia formation and airway model cell culture [23]. However, two-dimensional cultures exclude the possibility to reproduce intercellular interactions with other cell populations. Three-dimensional models of different degrees of complexity present such advantage, allowing two or more cell types, e.g. epithelial and mesenchymal cells, to interact in culture [24-28]. Some airway models include immune cells [29-30], but ideally, an autologous culture system is required.

1.4. Human tissue-engineered three-dimensional BM

We were the first to establish the methods for HBEC and HBEF isolation from bronchial biopsies taken during standard bronchoscopy [31]. Using these two cell types and a native

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collagen matrix, we produced the first three-dimensional human normal and asthmatic BMs in culture, using the tissue-engineering approach [27-28]. We produced tubular and disk-shaped BMs. Cell culture is often criticized as it doesn't provide the support for the maintenance of cells phenotype. Freshly isolated HBEC lose their ciliated phenotype and similarly, GCs are not detectable in primary cultures. However, three-dimensional tissue culture systems significantly differ from two-dimensional (monolayer) cell culture, providing an environment that enhances cell differentiation and organization, matrix remodeling, cell-cell and cell-matrix interactions *in vitro* [24,27-28,32-36]. The main challenge remains to define the best and specific conditions that apply to each cell type *in vitro*. In the case of bronchi, some essential factors, e.g. retinoic acid, combined to specific culture conditions, such as the air-liquid interface, stimulate and maintain functional and differentiated properties of HBEC in tissue-engineered BMs in vitro [27-28]. This chapter presents the potential of tissue-engineered BMs, as an alternative to animal use, to study respiratory diseases *in vitro*.

2. Experimental approach

2.1. Collagen matrix and bronchial cell source

Our models are produced using bovine type I collagen as a matrix. It is a natural substrate, so HBEC attach, migrate and proliferate on it. HBFC seeded in this matrix grow, synthesize de novo human collagen and contract microfibers by gathering them into fibers. In the disk-shaped BMs, cells and collagen fibers become aligned horizontally, in the same plan with the tension induced by the peripheral anchorage that prevents circumferential matrix contraction. Human asthmatic and healthy bronchial cells were isolated from biopsies of volunteers taken during standard bronchoscopy [31]. The asthmatic donors (aged from 20 to 50 years-old), had never taken anti-inflammatory drugs, were non-smokers and their asthma required only an inhaled β2 agonist agent on demand. The study was approved by the local Ethics Committee and subjects had given informed written consent.

2.2. How do we produce human tissue-engineered BMs?

Step 1.Preparation of the mesenchymal layer of the BMs

A mixture of bovine Type I collagen (2,0 mg/ml) is prepared by dissolving the powder overnight at 4°C, in sterile 0.017 M acetic acid. A solution of 0.84 ml of DMEM 2.7X pH 7,4, containing 200 IU/ml penicillin G and 50 μg/ml of gentamicin, pH 8,0, is mixed with a second solution containing 1.43 ml of the stock collagen solution and 30 μl of NaOH 0.7 N to stabilize the final pH at 7,4. It is important to mention that collagen polymerization is pH-dependent and that it occurs at a pH above 7.0. However, cells should not be mixed with an acidic collagen solution. Thus, 0,56 ml of fetal calf serum (FCS) is mixed with 0.15 ml of a HBFC suspension (1×10^6 cells/ml; Figure 1B). Non-asthmatic and asthmatic HBFC are seeded individually in corresponding mesenchymal layers to produce non-asthmatic and asthmatic BMs, respectively. The cell suspension is slowly mixed with the neutralized collagen solution. This mixture (pH 7,4) is quickly distributed in a bacteriological Petri dish (35-mm diameter) already

containing the peripheral anchorage (sterile ring of Whatman paper), to produce the mesenchymal layers for each BM. The anchorage method prevents collagen lattice to contract diametrically [27]. The dish must not be shaken until collagen polymerization has occured (20-30 min), at room temperature. The mesenchymal layers of the BMs are covered with 2 ml of DMEM supplemented with 10% FCS, 100 IU/ml penicillin G and 25 ug/ml gentamicin following collagen polymerization and cultured in this medium until their epithelialization. All mesenchymes are kept in an 8% CO_2 atmosphere at 37°C thereafter.

Step 2.Epithelialization of the BMs under submerged culture conditions.

Four days later, the epithelialization is performed by seeding HBEC (8 x 10^5 cells/ BM; Figure 1A), on the mesenchymal layers. Again, non-asthmatic and asthmatic HBEC are seeded on the corresponding mesenchymal layers. The constructs are maintained under submerged culture conditions in medium supplemented with 10% FCS, 1,8 x 10^{-6} M (or 24,3 ug/ml) adenine, 1,6 x 10^{-9} M (or 10 ng/ml) human EGF, 8,6 x 10^{-7} M (or 5 ug/ml) bovine crystallized insulin, 6,2 x 10^{-4} M (or 5 ug/ml) human transferrin, 2 x 10^{-9} M (or ug/ml) 3,3',5', triiodo-L-thyronin, 1,1 x 10^{-6} M (or 0.4 ug/ml) hydrocortisone, until a confluent epithelial cell layer is obtained. The culture media are changed daily, (Figure 1C).

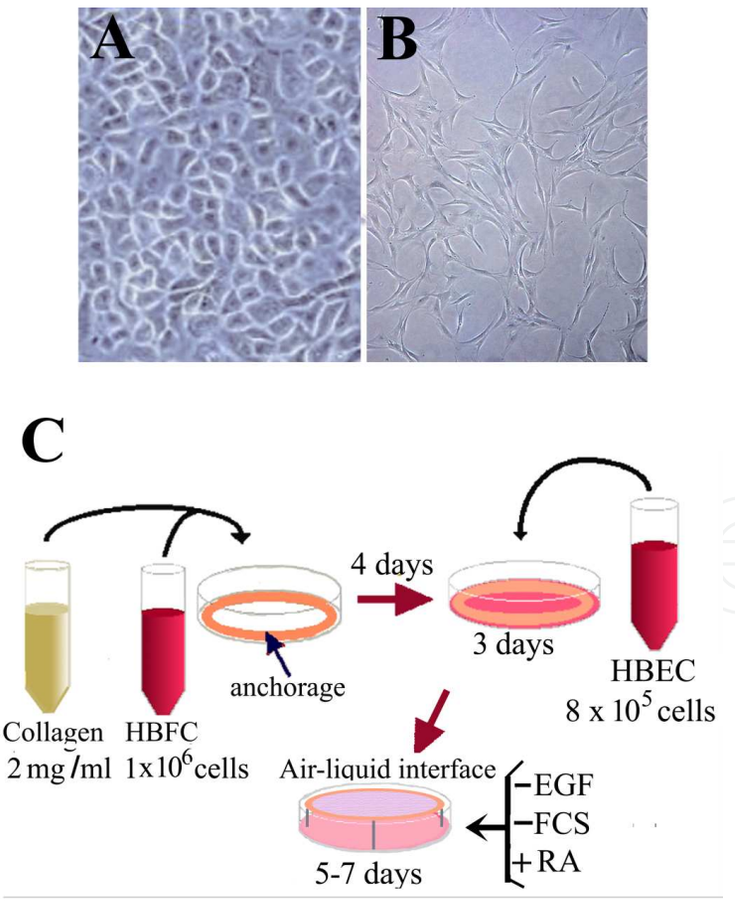

Figure 1. Photomicrographs taken under phase contrast microscope of non-asthmatic HBEC (A) and HBFC (B), in primary culture. (C): Steps of production of human tissue-engineered BMs *in vitro*.

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Step 3.Culture at the air-liquid interface.

On the fourth day after epithelialization, the BMs are raised at the air-liquid interface in serum-free medium supplemented with 1,8 x 10^{-6}M adenine, 5 ug/ml bovine crystallized insulin, 6,2 x 10^{-4} M human transferrin, 2 x 10^{-9} M 3,3',5', triiodo-L-thyronin, 1,1 x 10^{-6} M hydrocortisone, and 5 x 10^{-8} M retinoic acid (RA). EGF and FCS are not added in the medium since these growth factors enhance the production of gelatinases by the cells, which degrade the collagen matrix of the mesenchymal layer in culture at the air-liquid interface. The BMs are placed on Petri dishes containing an internal elevated support (Falcon No 3037), or on any other type of air-liquid support. They are cultured at the air liquid interface for 5 to 7 days or more, according to each experiment. The culture media must be changed daily. Each experiment is always done 3 times on at least 3 samples per group of BMs tested.

2.3. Histological analyses

Human bronchial models were fixed with formol 10% and then embedded in paraffin. The 4 μm thick sections were stained using the hematoxylin-eosin staining.

2.4. Scanning electron microscope analyses

The samples were fixed with a solution of 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.5 for 24 hrs, post-fixed with 1% osmium tetroxyde, dehydrated with ethanol and coated with gold (Sputtercoater, Nanotech, England). Photographs were performed using a JEOL JSM-35CF scanning electron microscope (Université Laval).

2.5. Technical tips to isolate pure populations of HBEC and HBFC to produce various BMs

The methods established to produce non-and asthmatic BMs could be applied to develop other BMs, using bronchial cells isolated from 5-10 small biopsies (≤ 1 mm^2) taken from tumoral, cystic fibrosis, MPOC or other tissue types. HBEC and HBFC can be isolated from the same bronchial biopsies [31]. They are initially plated in the same dishes in primary culture, and colonies of both cell types can be observed under phase contrast microscope after 7-10 days. To purifie HBEC, some culture dishes containing at least one colony of HBEC were selected. The addition of lethally irradiated but living 3T3 cells in the selected dishes, allowed the progressive elimination of the HBFC. Then, the colonies of HBEC slowly reached confluence, while the 3T3 cells progressively detached from the dishes. The lethally irradiated 3T3 play three roles; 1) they still secrete some growth factors as feeder layer, 2) they increase cell density to support small colonies of HBEC in primary culture and 3) they contribute to eliminate HBFC that would overgrow HBEC. This methodological approach leads to reproducible and excellent results in obtaining pure HBEC and HBFC populations. This approach has great potential to isolate HBEC and HBFC from various bronchial tissues. The best medium to maintain bronchial cells viability after long-term storage in liquid nitrogen is composed of 90% FCS and 10% DMSO.

2.6. Ciliogenesis in bronchial epithelia grown on three-dimensional mesenchymes

To screen and target mechanisms that initiate and promote structural and functional disorders in bronchi, tissue-engineered three-dimensional BMs are excellent tools. Ciliogenesis, mucus secretion, pseudo-stratified epithelium and collagen remodeling have been observed in all human non-asthmatic and asthmatic BMs, produced under defined culture conditions [27-28,31]. Non-asthmatic BMs show well-differentiated pseudo-stratified epithelia (Figure 2A). In asthmatic BMs, partial desquamation occurs after 7-10 days in culture at the air-liquid interface, in a fashion that mimics clinical histopathological observations [28]. In addition, the asthmatic cells show an irregular distribution of cilia, with variable lengths, and some asthmatic cells don't become ciliated (Figure 2B).

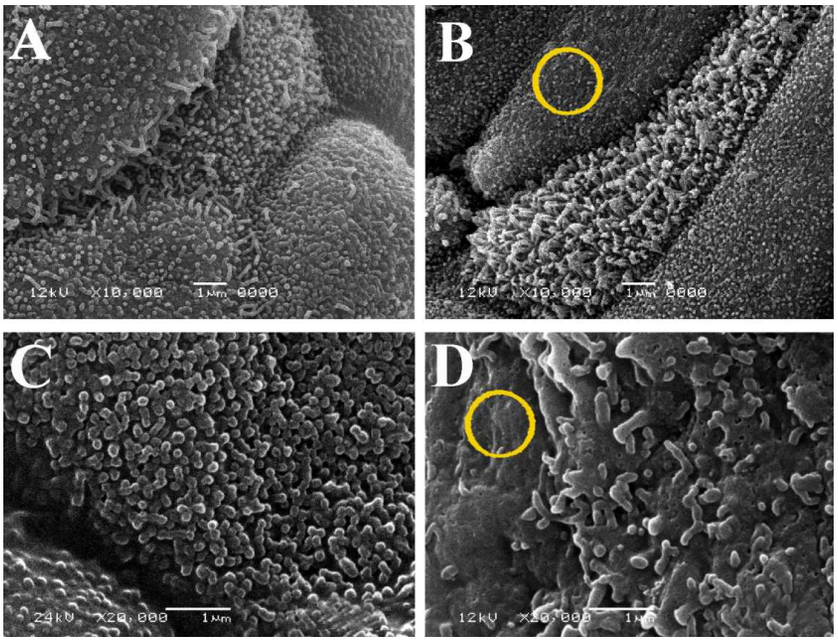

Figure 2. Photomicrographs taken under scanning electron microscopy, of BM's epithelia produced with non-asthmatic (A,C) and asthmatic (B,D) HBEC after 10 days of culture at the air-liquid interface. Ciliogenesis occurred in all BMs, but some asthmatic HBEC were devoided of cilia (yellow circles) and showed sparsely distributed shorter cilia, compared to normal HBEC. (Magnification: A-B: X 10,000; C-D: X 20,000).

Mucus secretion was previously shown in GCs observed on histological sections of BMs stained with periodic-acid-Schiff (PAS) [28]. The PAS staining reveals mucus-secreting cells on histological sections (Figure 3C-D, G-H). To eliminate cross-reaction with endogenous glycogen in situ, some sections were digested before PAS staining using a solution of 0.5% maltase (Fisher) in phosphate-buffered saline (PBS) for 30 min at 37°C and washed 10 min with distilled water. At least 10 tissue sections were analyzed for each BM tested. We observed the entire sections to take representative pictures. GCs can be seen in both, non-and asthmatic BMs cultured at the air-liquid interface (Figure 3B-D and E-H respectively). GCs were observed under scanning electron microscope in non-and asthmatic epithelia of BMs, cultured at the air-liquid interface for at least a week (Figure 4).

2.7. Mucus secretion GCs in human bronchial models *in vitro* in response to IL-13

The effects of cytokines, such as 10 ng/ml of IL-13 (PeproTec Inc., Rocky Hill, NJ, USA) [37], was assessed on the induction of GC metaplasia in non-asthmatic and asthmatic BMs (Figure 3B,D,F,H). PAS staining was performed to determine the percentage of mucine-positive cells on histological sections (Figure 3D,H).

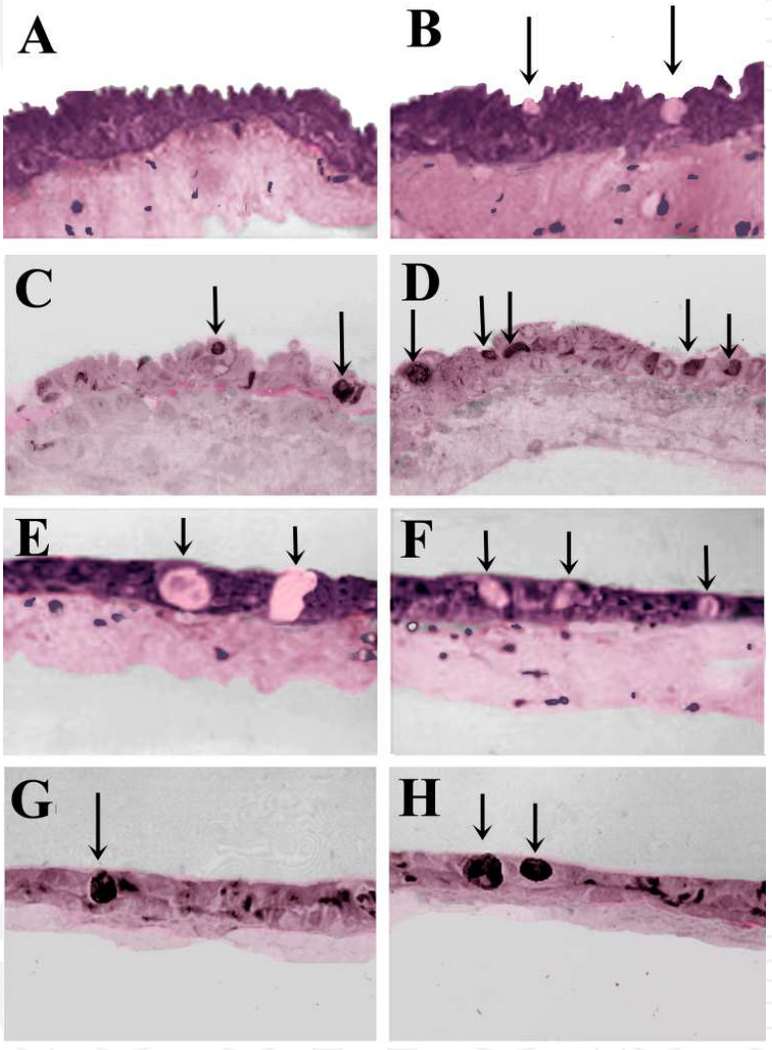

Figure 3. Photomicrographs of histological sections of non-asthmatic (A-D) and asthmatic (E-H) cultured for 10 days at the air-liquid interface without (A,C,E,G) and with (B,D,F,H) IL-13. Sections A-B, E-F were stained with hematoxylin-eosin and sections C-D,G-H were stained using the PAS method. On all BM sections, GCs are pointed with arrows. Magnification: 20 X

The isolation and growth of several populations of asthmatic HBEC (n > 30) have confirmed that they retain their morphological properties in culture. Asthmatic epithelia (Figure 3 E-H) are not as well organized as non-asthmatic controls (Figure 3 A-D) and partial desquamation occurs after 12 days of culture at the air-liquid interface (Figure 2B, D and Figure 4). Similarly, mesenchymal layers populated with asthmatic HBFC (Figure 3 E-H) are thinner than non-

asthmatic mesenchymes (Figure 3 A-D), notably due to increased levels of collagenases secretion [28].

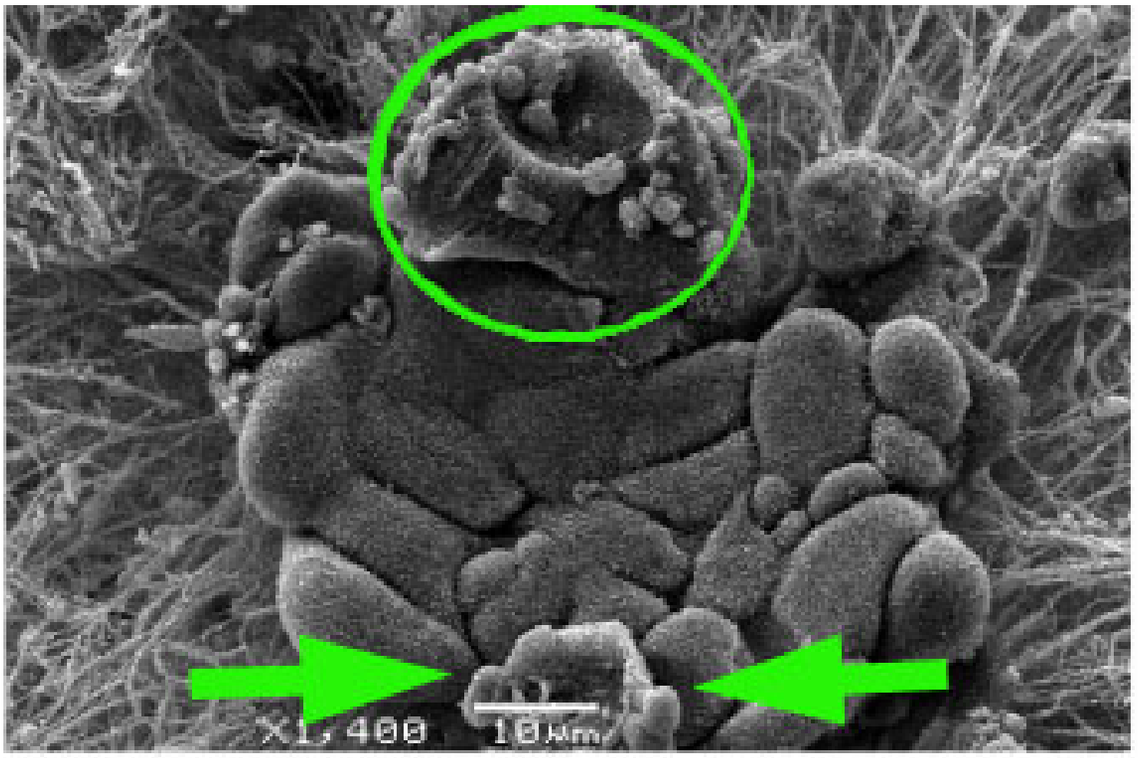

Figure 4. Photomicrograph taken under scanning electron microscopy, of BM's epithelia produced with asthmatic HBEC, after 10 days of culture at the air-liquid interface, showing an islet of HBEC including two GCs (green circle and arrowheads). (Magnification: X 1,400).

Our experiments confirmed that HBEC grown at the air-liquid interface and exposed to IL-13 generate GCs metaplasia *in vitro* [36-38]. After 7 days of culture at the air-liquid interface, IL-13 stimulates growth of normal and asthmatic GCs in tissue-engineered human BMs *in vitro* (10 ± 0,9% and 23 ± 1,3 % more GCs respectively, compared to corresponding controls grown without IL-13). The experiment was stopped before epithelial cell desquamation occurred in the asthmatic BMs, while non-and asthmatic HBEC were confluent on all BMs, to ensure that mucus quantification was not overestimated in normal BMs, due to higher cell number.

ELISA assays were also performed to quantify MUC5AC in the culture (enzyme-linked immunosorbent assay (ELISA) kit cat no: E90756Hu, Biomatik Cambridge, Ontario, Canada). MUC5AC synthesis increased significantly in non-and asthmatic BMs exposed to IL-13, as soon as after 3 days of culture at the air-liquid interface (Figure 5). The mucin synthesis increased up to 30% more in asthmatic than in non-asthmatic epithelia. Thus, the intrinsic properties of the cells can be preserved and expressed in the three-dimensional BMs.

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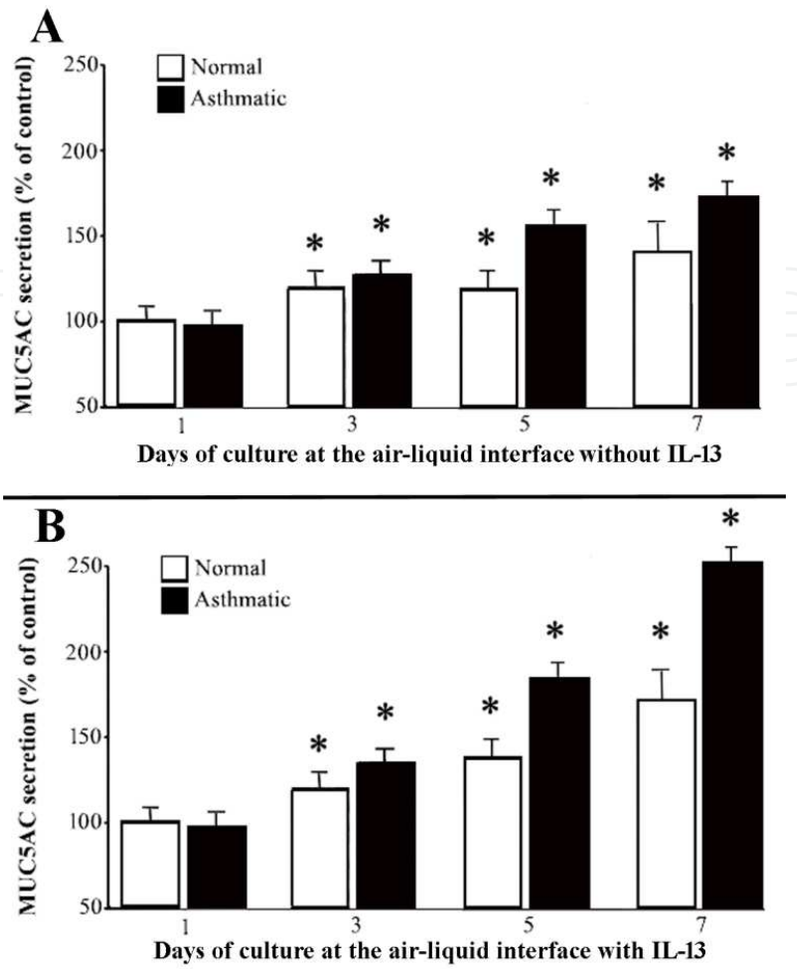

Figure 5. Synthesis of MUC5AC was assessed after 1, 3, 5 and 7 days of culture without (A) and with (B) IL-13, at the air-liquid interface. Values are relative to control ($p < 0.05$, control or non-exposed versus exposed to IL-13). We performed Student's t-test or one-way ANOVA and Tukey post hoc test considering significant $p < 0.05$. Data are presented as mean±s.d.

2.8. What advantages can be exploited using human tissue-engineered BMs?

The culture of human tissue-engineered BMs allows several advantages. First, histological analyses quality control and the quantification of any cell type can be performed on several groups of healthy, asthmatic or other pathological tissue-engineered BMs, compared to animal experimentation that is complex and expensive. The tissue-engineering approach offers an alternative to animal use, to study physiological pathways, outside the complex microenvironment that prevails *in vivo*. Cell layers organization and the quality of the tissues (ciliogenesis, cell morphology and functional parameters) can be monitored on BMs produced with cells of several donors. The tissue-engineered BMs can be produced in batches of many samples and it allows testing of several conditions, while lowering inter-individual differences. In addition, the models can be modified in various ways. For instance, the interactions epithelium-mesenchyme could be studied in hybrid constructs, using normal HBEC combined to pathological HBFC, and inversely. Isolating collagen can be a laborious task, but native type I

collagen-based scaffolds remain the best matrix to develop viable tissue-engineered tissues and grafts. Many arguments support this affirmation; 1) it is the major component of all connective tissues, 2) it can be recognized and consequently colonized by mesenchymal cells, 3) cells synthesize and remodel collagen scaffolds (Figure 6), including collagens of other species (e.g. bovine collagen), since only a few amino acids differ from one source to another, 4) collagen network of fibers can trap growth factors and bioactive molecules that are then released in the culture medium, for the benefit of the surrounding cells, 5) interestingly, normal epithelial cells attach, migrate and adopt proper organization on collagen gels, but remain on its surface, 6) this type of matrix allows direct visualization under microscope of the cells that populate it, or proliferate on its surface in culture and 7) collagen gels can be produced in various shapes (disk, tubular, spherical) and several collagen layers can be superimposed, knitted or lyophilized and rehydrated, to reproduce the form of almost any type of organ. All these advantages can be fully exploited to establish new biomaterials for fundamental and clinical applications. Finally, airway smooth muscle is a major target in asthma management. A better understanding of the mechanisms underlying airway smooth muscle contraction and airway hyperresponsiveness is a priority, a prerequisite for advances in therapeutic control of asthma. Therefore, we are currently developing a construct containing smooth muscle cell layer (SMCs) as a third cell layer, to evaluate the effects of various bronchodilators on the contractile properties of reconstructed bronchi *in vitro* (Dr. J.-P. Lavoie and collaborators). SMCs frequently lose their typical features, including their contractile properties, in monolayer grown on plastic, but their phenotype is maintained in three-dimensional matrix. Then, exploiting animal models, such as the pseudostratified epithelium of the genetically tractable mouse trachea, could confirm observations made on BMs, and enable crucial discoveries regarding the pathogenesis of airway diseases [1]. Thus, the outcomes of experiments performed *in vitro* can be confirmed in animal models thereafter.

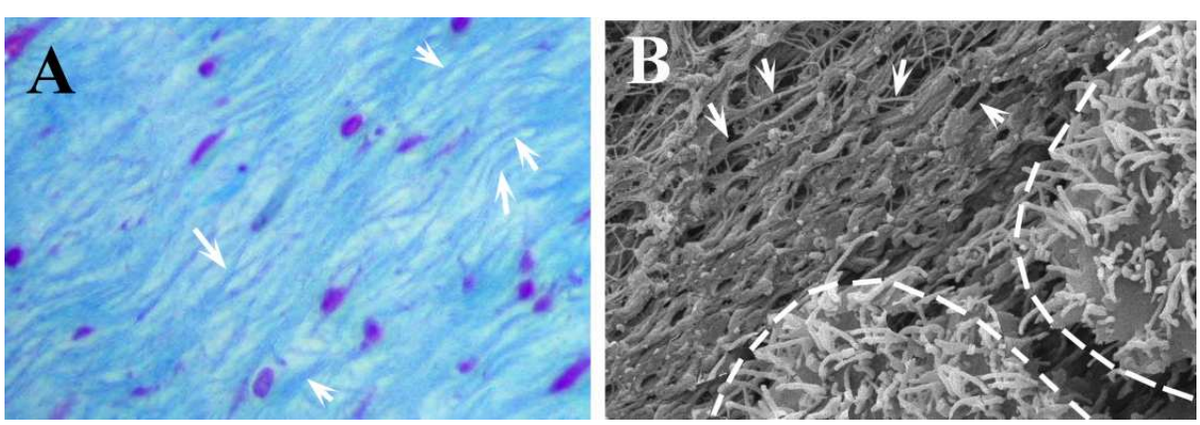

Figure 6. Photomicrographs of the collagen fibers (arrows) that compose the mesenchymal layer of the BMs, cultured for 12 days at the air-liquid interface. (A): Histological section of the mesenchymal layer of a BM stained with hematoxylin-eosin (HBFC in purple surrounded by blue fibers) and (B): View under scanning electron microscopy of the surface of the mesenchyme showing the collagen fibers network that supports epithelial cells (two asthmatic ciliated HBEC are circled with dashed lines). Note the high density and the alignment of the matrix fibers in response to the tension induced by the peripheral anchorage in the tissues, and the partial desquamation of the asthmatic epithelium that allowed mesenchyme visualization. (Magnification: A: 20 X, B: X 6,000)

2.9. What are the limits of this approach?

The quality of the tissues that can be developed using our approach cannot be denied. However, the stability of BMs in culture, especially asthmatic BMs, limits the duration of experiments. In addition, restrained availability of human bronchial tissue donors, combined to limited size of bronchial biopsies, can delay experimentation. Animal experimentation is very expensive, time consuming, complex, and sometimes ethically questionable, depending on the type of study. Tissue-engineered BMs offer a valuable alternative. Animal cells are more easily accessible and can be used to establish useful three-dimensional models and compare them with human materials.

Another challenge associated with tissue engineered BMs is the development of more complex tissues, including more than two types of cells. For instance, the addition of a layer of bronchial SMCs would certainly add functional attributes to our normal and asthmatic bilayered BMs. Many teams have made attempts to isolate and purify bronchial SMCs. It is very difficult to obtain SMCs populations that are not mixed with other mesenchymal cell types such as fibroblasts. But is it necessary to obtain 100% pure SMCs? This question and many others need to be assessed to understand the signaling pathways that support epithelium-mesenchymal interactions. Tissue-engineered three-dimensional BMs might provide answers to some of them, since they favor growth factors exchanges and intercellular crosstalk.

Pleural mesothelial cells are pluripotent cells [41], which could influence the morphology and the state of differentiation of bronchial epithelia and the functional properties of mesenchymes. They have been shown to clonally generate fibroblasts and smooth muscle cells in murine models. This supports the possibility that they may also modulate lung injury-repair by re-activation of developmental programs in the adult, reflecting an altered recapitulation of development, with implications for regenerative biology of the lung [41]. These cells could be included in some normal and pathologic BMs, to study their capacity to affect the phenotypic features and the functional state of HBEC and other cell populations. However, such experiments would involve the development of more complex multilayered BMs, which may not be easily maintained in culture.

3. Conclusion

We propose a tissue-engineering approach to reconstruct bronchi *in vitro*. Using type I bovine collagen, we have characterized human BMs that share several important features with native non-and asthmatic human bronchi. Other models are under development to target key molecules involved and new discoveries could be validated *in vivo* thereafter, as it can be achieved for other bioengineered tissues [38-40]. The creation of new human BMs can be performed with cells isolated from various donors, or pathologies.

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The authors declare no conflict of interest.

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