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Airway ciliated cells in adult lung homeostasis and COPD

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This review describes the diversity of ciliated cells in the airways and highlights their origin, function, regulation and alteration in respiratory diseases such as COPD. https://bit.ly/46O1RO8

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Abstract

Cilia are organelles emanating from the cell surface, consisting of an axoneme of microtubules that extends from a basal body derived from the centrioles. They are either isolated and nonmotile (primary cilia), or grouped and motile (motile cilia). Cilia are at the centre of fundamental sensory processes and are involved in a wide range of human disorders. Pulmonary cilia include motile cilia lining the epithelial cells of the conductive airways to orchestrate mucociliary clearance, and primary cilia found on nondifferentiated epithelial and mesenchymal cells acting as sensors and cell cycle keepers. Whereas cilia are essential along the airways, their regulatory molecular mechanisms remain poorly understood, resulting in a lack of therapeutic strategies targeting their structure or functions. This review summarises the current knowledge on cilia in the context of lung homeostasis and COPD to provide a comprehensive overview of the (patho) biology of cilia in respiratory medicine with a particular emphasis on COPD.

Ciliogenesis in the adult lung

Cilia are conserved from unicellular eukaryotes to animals; therefore, it is not surprising that they were first described in protozoa in the seventeenth century [1]. Four centuries were needed to tackle the ultrastructure of the organelle in the lung to the complex deciphering of its movement *via* progress in physiology and microscopy [2]. An important challenge when addressing the question of cilia in the lung is to discriminate two organelles that may appear very similar in shape and role, but differ tremendously: the primary cilium and the motile cilium. The comparative structural and functional features of primary cilia and motile cilia are extensively discussed in biology, with a particular emphasis on the centrioles constituting the foundations of both cilia [3–8]. However, although cilia are the first line of pulmonary defence, this aspect is frequently neglected in respiratory medicine, particularly from an integrative perspective.

Primary ciliogenesis

Since multiciliated cells (MCCs) are of the utmost importance in the airways as orchestrators of mucociliary clearance in the conducting airways, the primary cilia did not appear to be a priority to explore homeostasis or pathophysiology. In fact, until 10 years ago, only one article documented the presence of primary cilia *in vitro* during epithelial repair and in embryonic lungs, but reported their absence in the adult lungs [9]. Interestingly, the primary cilium is found in nearly all cells in humans, as it transiently appears and disappears at the cell surface, contributing to cell cycle regulation. A primary cilium consists of an antenna that functions as a mechanical and chemical sensor relaying molecular signalling and orientating cell fate. Therefore, it seemed reasonable to assess the presence of primary cilia at the surface of pulmonary cells and investigate their potential roles in lung homeostasis and diseases.

In airway epithelial cells, the pioneer experimental study highlighted that primary ciliogenesis is required for optimal multiciliogenesis in the lung [9]. The primary cilium appears in the course of airway epithelial cell (AEC) differentiation and during repair. Since then, we have demonstrated their presence on nondifferentiated basal cells *in vitro* and *ex vivo* (figure 1) [10]. Considering the other tissues present in the lung where various cell populations may harbour a primary cilium, ciliogenesis orchestrates several crucial functions responsible for lung homeostasis in the stroma. Thus, the primary cilia may be found on nonepithelial cells, as illustrated in figure 1. More specifically, primary cilia were observed on lung endothelial cells, regardless of the area considered [11]. They have also been detected on bronchial muscle cells [12, 13], parenchymal fibroblasts [14] and myofibroblasts [15]. In addition, primary cilia have been detected on chondrocytes outside the respiratory system [16], but have not been described on immune cells.

Multiciliated cells

Motile cilia appear as hundreds of plasma membrane folds, each anchored on a basal body located at the apical pole of MCCs (figure 1). These final products result from a complex and partially described process recently tackled by single-cell RNA-sequencing (seq) approaches. Motile cilia gradually appear during AEC differentiation in the airway epithelium. The precursors are basal cells following a specific lineage leading to terminally differentiated cells: secretory cells or MCCs. The precursors may vary geographically along the airways and are controlled by different signalling pathways (such as Wnt, Notch or Hedgehog)

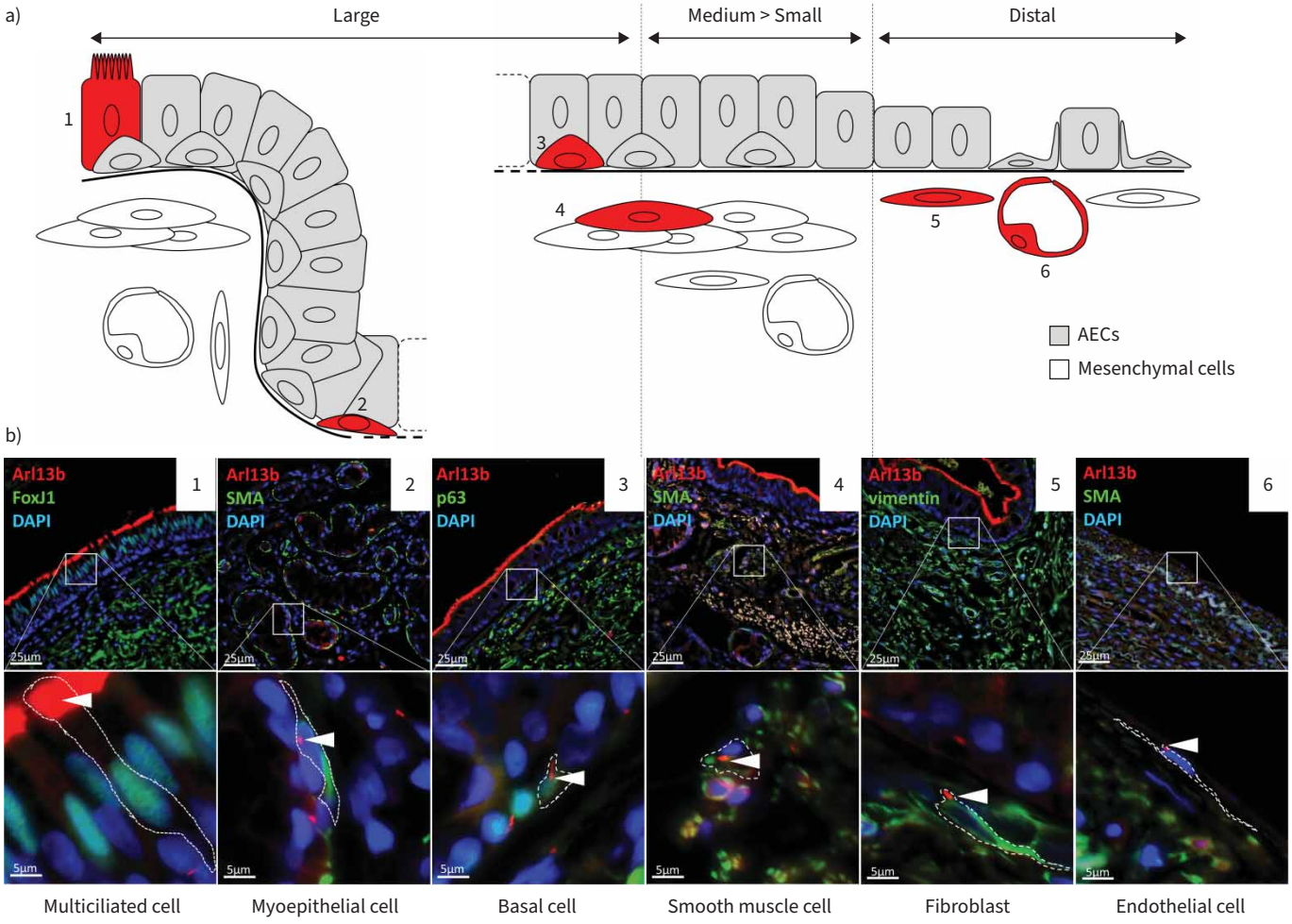

FIGURE 1 Cilia are present in the principal lung cell populations. a) The main pulmonary airway cell populations from bronchi (large airways) to alveoli (distal airways), depicting airway epithelial cells (AECs) and mesenchymal cells. b) Representative micrographs showing the specific cell population (numbered 1–6) analysed by immunostainings on formalin-fixed paraffin-embedded non-COPD smoker lung tissue for identification marker (FoxJ1, smooth muscle actin (SMA), p63, vimentin; all green); cilia (Arl13b; red) and cell nuclei (4′,6-diamidino-2-phenylindole (DAPI); blue) as observed on formalin-fixed paraffin-embedded lung tissues. Arrowheads indicate ciliated cells (either motile cilia or primary cilia); dashed lines trace an example of the cell population. Scale bars=25 µm and 5 µm.

and stimuli driving cell fate. Considering MCC differentiation, the basal cells are precursors of deuterosomal cells, which are intermediates for MCCs featuring signature genes such as Polo-like kinase 4 (PLK4), Cyclin O (CCNO) and Centrosomal protein 78 (CEP78) [17]. At the end of the process, MCCs generally express gene markers responsible for ciliary functions, such as intraflagellar transport (IFT) proteins. Forkhead Box J1 (FOXJ1) is generally the readout of the duplication of centrioles forming deuterosomes that migrate to the apical membrane, while dynein and radial spokes such as DNAH5/LI1 and RSPH4A/9 delineate the formation of functional motile cilia with a central doublet of microtubules and the ability to exploit ATP, enabling ciliary movement (figure 2) [18].

From primary ciliated cell to multiciliated cell

Primary cilia and motile cilia were observed, but not connected, based on the first experimental evidence [9, 19]. It was initially proposed that the primary cilia may disappear during differentiation to give rise to motile cilia. However, *in vitro* monitoring and histological observations of both types of cilia (figure 3) [10] favour a model where the primary cilium persists during differentiation. The AEC progenitors may cycle (proliferate) or remain in a state of quiescence and display a primary cilium. Upon cell cycle re-entry, the two future daughter cells will adopt different fates: one will remain undifferentiated, either

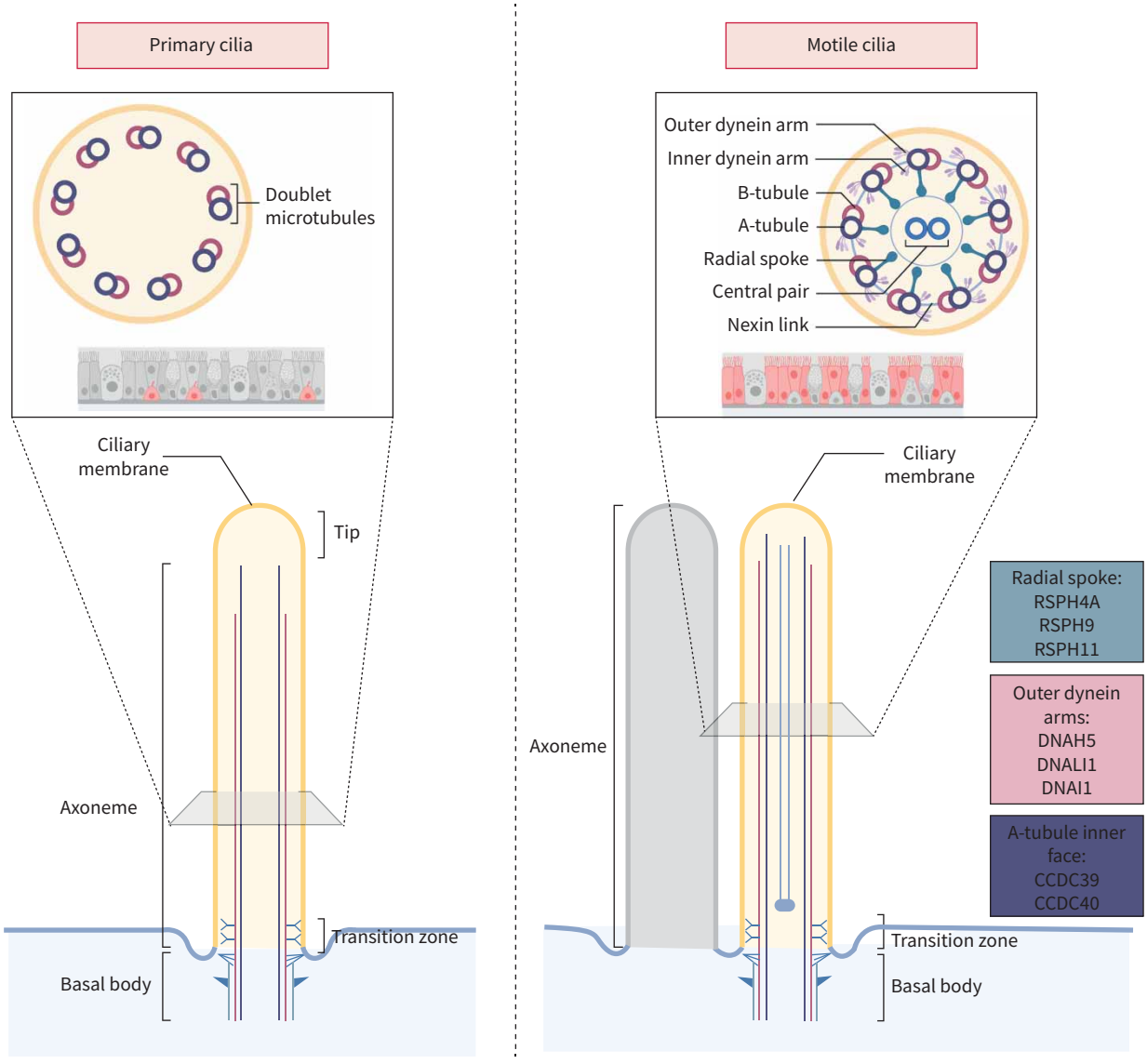

FIGURE 2 Structural aspects of primary cilia and motile cilia. The schematic shows the global structure of primary cilia and motile cilia in the respiratory context with a focus on the axoneme and its molecular composition. Figure created using BioRender.com.

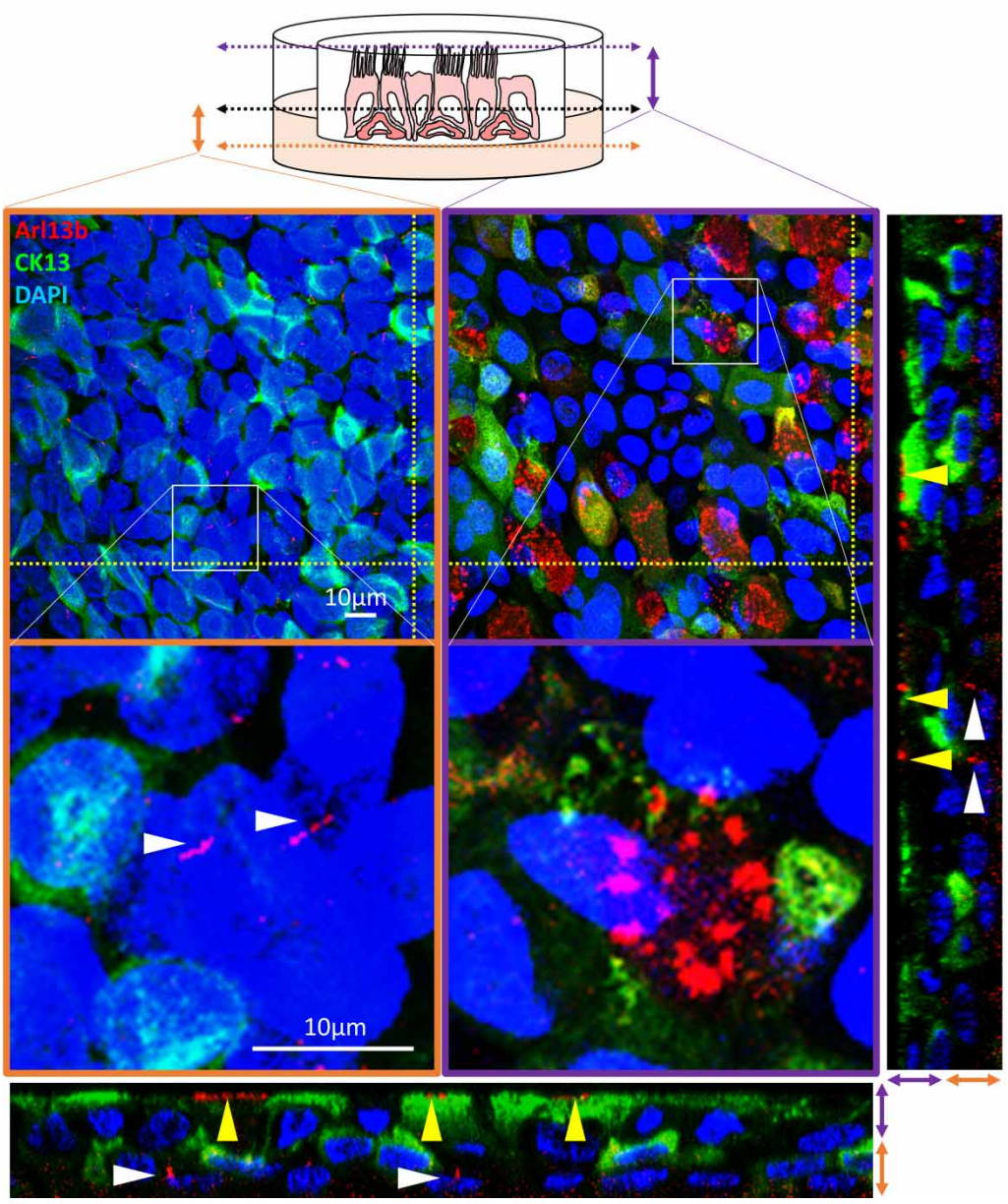

FIGURE 3 Primary cilia and motile cilia appear during airway epithelial cell differentiation *in vitro*. Representative confocal acquisitions from large airway epithelial cell cultures from a non-COPD smoker at ALI-d14 for CK13 (green), cilia (Arl13b; red) and cell nuclei (4′,6-diamidino-2-phenylindole (DAPI); blue). Merged z-projections on two selected areas depicted by orange or purple frames are shown with orthogonal projections (dashed lines). Arrowheads indicate primary cilia (white) and motile cilia (yellow).

proliferative or nonproliferative, harbouring a primary cilium, and the other one will progress towards the acquisition of cellular and molecular features to turn its role into secretion or mucus sweeper. A phenotypic switch from mucus-secreting cells to MCCs has also been suggested [17]. The inhibition of primary ciliogenesis during AEC differentiation induced an extreme decrease of MCCs, partially through the transcriptional alteration of genes associated with ciliogenesis, such as FOXJ1 [20].

Roles of cilia in homeostasis

Airway primary cilia

Since the airway primary cilium has not attracted much attention in respiratory research, little is known about its roles in the lung epithelia. The primary cilium is formed during the G1 phase of the cell cycle and its disassembly takes place during the G2/M phase, which regulates the cell cycle and the balance

between quiescence and proliferation [21]. Considering its demonstrated functions, the primary cilium may assume an important role in lung homeostasis or repair after injury. It may be involved during lung development and organ regeneration, acting as signalling hubs for several receptors, channels and signalling pathways such as Hedgehog, Wnt and Notch. The primary cilia are distributed throughout the airways, and exhibited on basal cells in bronchi, bronchioles and on type I and II alveolar epithelial cells in the distal compartment [14]. The functions of primary cilia in these different compartments, which could differ, have not been explored and may not be the same.

Primary cilia in the stroma

Primary cilia may participate in and orchestrate many functions depending on the cell type from which they emanate. In the lung, in addition to epithelial tissues, primary cilia are also found in the connective and smooth muscle tissues.

They are thought to be essential in the establishment of the vascular barrier [22], by detecting shear forces during vasculogenesis [23], and acting as a fluid mechanosensor in the blood [24]. In addition, they may relay cytokine stimulation response [25], and maintain the endothelial phenotype preventing endothelial-to-mesenchymal transition [26]. They are present on fibroblasts where they contribute to the fibrogenesis and the maintenance of the fibroblastic identity [27]. The primary cilia relay chemical (*i.e.* parathyroid hormone or flavonoids), electric (*i.e.* pulsed electromagnetic fields) and mechanic signals (*i.e.* microgravity) to direct chondrocyte and osteoblast differentiation [28–30]. Human bronchial smooth muscle cells project a primary cilium enriched in mechanochemical sensors into the extracellular matrix to initiate migration and wound repair and to mediate cell contraction [12, 13].

Mucociliary clearance

The pseudostratified respiratory epithelium (from the nasal cavity to the bronchi) is covered with mucus produced by secretory cells in order to trap inhaled particles, microorganisms and pathogens. The mucociliary clearance establishes a frontline lung defence mechanism: motile cilia remove the mucus *via* ciliary beating into the airway surfactant layer. The ciliary beat efficiency is ensured by ion transport across the channels expressed at the membrane of the epithelial cells, allowing the rehydration of the airway surfactant layer [31–33]. The ciliary beating pattern provides efficient defence, with cilia moving forward and coordinating the recovery sweep in the same plane [34].

The ciliary beat frequency (CBF) is influenced by environmental signalling, especially the redox balance. Cilia contain several regulatory redox systems, such as oxidant-generating (*i.e.* nitric oxide synthase and NADPH oxidase) or antioxidant systems (*i.e.* redoxin family proteins) to modulate the physics of the beating movement [35]. Temperature and intracellular calcium levels are the main regulators of the CBF. A large panel of human biological products such as ADP/ATP, toxins (from *Pseudomonas aeruginosa* or *Staphylococcus aureus*) or natural compounds (curcumin, menthol, vitamins) positively or negatively modulate the CBF depending on the biological context [36]. Antimicrobials, antivirals, drug excipients and medications typically decrease the CBF. However, a few agents, such as long-acting muscarinic antagonists and β-agonists seem to increase the CBF.

Cilia in COPD

The understanding of COPD pathogenesis has often been restricted to the inflammatory component. This has prompted the use of corticosteroids and phosphodiesterase inhibitors, among other therapeutics, for the clinical management of the symptoms and to reduce exacerbations. Nonetheless, there is currently no cure, partially because the role of the epithelium is not fully elucidated, even though the ciliated cells are the guardians of the respiratory functions. Therefore, investigating cilia in chronic airway diseases is of the utmost importance. It is particularly relevant in the context of COPD considering its association with cancer: the basal cell is at the centre of molecular transformations that may lead to epithelial transdifferentiation towards COPD or dedifferentiation towards carcinogenesis. Research on COPD patients provides an ideal model, with readily available lung tissues to investigate the connections between primary cilia and motile cilia.

Alteration of primary cilia

In the airways

Epithelial remodelling is a hallmark of COPD. The main histological features are basal and secretory cell hyperplasia and metaplasia. An alteration of the primary cilia could initiate epithelial remodelling. For example, the loss of primary cilia induces a decrease in the integrity of the epithelial barrier with changes in tight-junction components. This is accompanied with a decrease in nondifferentiated cells and an increase in secretory cells [20].

Although the primary cilia were sparse on nondifferentiated cells in morphohistologic normal epithelia, there was an increase of cells presenting primary cilia in remodelled epithelia, and more so in the context of COPD (table 1) [10]. Moreover, the number of primary cilia and their length increased in the AECs of COPD patients [41]. This raised the question of the role of primary cilia in the genesis and maintenance of epithelial remodelling in COPD. The capacity of the nondifferentiated AECs from COPD patients to renew the epithelium is not elucidated. In addition, the maintenance of the primary cilia on nondifferentiated cells may be either the result or the cause of the epithelial remodelling.

In the parenchyma

The alteration of the primary cilia in the parenchyma has not been studied in the context of COPD. Data are available for other respiratory diseases, such as idiopathic pulmonary fibrosis and asthma, which can be extrapolated to propose a novel area of investigation in COPD. The frequency of primary cilia was increased in fibroblasts and alveolar epithelial cells in the context of idiopathic pulmonary fibrosis in association with the activation of the Hedgehog pathway causing the development of fibrosis [14]. The endothelial cells from pulmonary arterial hypertension patients harboured more elongated primary cilia which could not regulate their length following inflammatory stimuli, ultimately leading to endothelial dysfunction [25]. In the context of asthma, primary cilia were more abundant on airway smooth muscle cells and functionally linked to the contractility regulation [13].

Alteration of motile cilia

Cilia dysfunction is long thought to represent a main contributor to mucociliary clearance abnormalities (see [16] for a complete historical perspective).

Dysfunction of motile cilia was evaluated in the airways of COPD patients using a wide range of experimental tools from genetic studies to live imaging (table 1, [10, 37–52]). The main findings commonly included decreased CBF, shortening of motile cilia, and ciliary dyskinesia from the nasal epithelium to the bronchioles (table 1). Recently, autophagy was presented as being involved in pathological processes that are linked to cilia shortening. Cigarette smoke-induced autophagy triggered the degradation of ciliary proteins (such as IFT) mediated by HDAC6 and induced alteration of ciliary growth and function in the context of COPD. In addition, ciliary proteins were found inside autophagosomes sequestered after cigarette smoke-induced autophagy [53–55].

The structure of motile cilia can be altered, like in primary ciliary dyskinesia. These abnormalities include defects of dynein arms (outer and/or inner) or radial spokes, as well as global disorganisation induced by genetic mutations of ciliary component genes such as DNAH5/I1, RSPH9/11 and CCDC39/40. All of these abnormalities are responsible for a decrease in CBF or an absence of cilia [56]. In addition, a few genetic studies pertaining to structural defects of cilia, including our meta-analysis, highlighted cilium-associated gene alterations in COPD patients [42, 46, 48, 57].

Smoking and ageing are the two prime modifiers of mucociliary clearance in the context of COPD [58, 59]. As they may directly affect cilia structure, function and molecular composition, the selection of the population (healthy never-smokers *versus* (ex-)smokers *versus* COPD (ex-)smokers *versus* young/middle-aged/older patients) is crucial to distinguishing COPD-specific or ageing-/smoking-related alteration of cilia. This is particularly important since cilia alterations may not concern all COPD patients, but only a subgroup. Finally, dysfunctions of the motile cilia can affect the cells localised in the parenchyma, especially immune cells. In the COPD context, the phagocytic ability of alveolar macrophages is impaired. This alteration is partially explained by a modification of the crosstalk between epithelial cells and alveolar macrophages. The main culprit is the decrease of Sphingolipid Transporter 2 (SPNS2), an epithelial ciliary protein causing a reduction of sphingosine-1-phosphate (S1P) secretion involved in the phagocytic function of alveolar macrophages [60].

Future directions

In recent years, fine-tuning of genetic cilia alterations has been investigated using high-throughput methods, such as whole-exome sequencing, RNAseq and single-cell RNAseq to correlate cilia-associated genes and COPD [46, 61]. If whole-exome sequencing identified an altered cilia-associated genetic print in COPD patients [46], interestingly, heavy smokers without airflow obstruction also presented an enrichment of variants in genes related to cilia structure and function [57]. Based on transcriptomics analysis on whole lung tissues and isolated small AECs, we suggested that a broad alteration of cilia-associated genes in COPD patients defines an endotype featuring ciliopathy, named CiliOPD [42]. The investigation of the spatial distribution of ciliated cells throughout the respiratory system from the upper to the lower airways will provide fine-tuned differential aspects of mucociliary regulation in the context of respiratory diseases such as COPD, while it is often admitted that human bronchial epithelia mimic nasal epithelia [62].

TABLE 1 Translational investigations on ciliogenesis homeostasis alterations in COPD and COPD experimental models (2000–2022)

Airways sublocalisation (UA, T, B, b, A, WL)	Models (AEC cultures, animals, *etc*.)	Smoking status (non-COPD: never/ex/current *versus* COPD: never/ex/current)	Experimental tools (EM, VM, IF, *etc*.)	Main findings in COPD	References
b	SAEC ALI cultures (7 non-COPD/9 COPD) FFPE tissues (8 non-COPD/6COPD)	0/6/1 *versus* 0/6/3	IF, PCR	↓MCC ↓E2F4, FOXJ1, RFX2, RFX3, HEATR2 ↓primary cilia	[37]
B	Animal model (miR449$^{-/-}$ mice) Tissues (57 COPD)	0/35/22	RNA/miRNA/scRNAseq meta-analysis, ISH, IF, TEM	miR449 expression correlated with ciliogenesis-associated genes	[38]
B	FFPE tissues: remodelled and non-remodelled epithelial (4 non-COPD/3 COPD)	0/1/3 *versus* 0/3/0	Whole-exome sequencing	Cilia-related pathways	[39]
B	LAECs (12 non-COPD/16 COPD)	0/6/6 *versus* 0/5/11	CBF, TEM	↓CBF ↓cilia in MCC ↑cells with blebbing and projections Ciliary dyskinesia	[40]
B	LAECs (15 non-COPD/17 COPD) LAEC ALI cultures	4/4/7 *versus* 0/12/5	CBF, IF	↓CBF ↓MCC ↑primary cilia	[41]
WL, b	Tissues (238 non-COPD/391 COPD) SAECs (300 non-COPD/117 COPD)	NA	RNAseq meta-analysis	Alteration of cilia transcriptome	[42]
b	SAEC ALI cultures (3 non-COPD/3 COPD)	3/0/0 *versus* 0/0/3	CS exposure, TEER, IHC, IF, VM, PCR, NGS	CS alters differentiation of SAECs: ↓FOXJ1, DNAI1 ↓MCC ↓CBF	[43]
B, b	FFPE tissues (61 non-COPD/81 COPD) LAEC ALI	24/16/21 *versus* 0/49/32	IHC, PCR, WB	TGF-β1 participates in AEC remodelling in COPD: ↓FoxJ1, β-tubulin IV ↓FOXJ1, DNAI2 ↓MCC	[44]
B	LAEC ALI cultures (14 non-COPD/14 COPD)	6/0/8 *versus* 0/0/14	CS exposure, PCR, CBF, calcium imaging	No effect on CBF	[45]
B	FFPE lung tissues (17 non-COPD/19 COPD)	0/4/13 *versus* 0/2/17	IHC	↑primary cilia	[10]
WL	Tissues (774 non-COPD/1769 COPD)	0/0/774 *versus* 0/NA/NA	Whole-exome sequencing	Cilia-related pathways	[46]
B	Animal models (guinea pig, rat) LAEC ALI cultures	NA	CS exposure, Ussing chamber, airway surfactant layer height, CBF	CS alters LAEC airway surfactant layer and CBF: ↓ airway surfactant layer dehydration ↓CBF	[47]
B, b	LAECs (50 non-COPD/70 COPD) SAECs (52 non-COPD/56 COPD)	45/0/57 *versus* 0/0/126	IF, RNAseq	↓cilia length in smokers and COPD smokers ↓IFT transcripts associated with smoking, COPD and cilia shortening	[48]
T, WL	Animal model: mouse MTEC culture Cell-line culture Tissues (LGRC, 43 non-COPD/124 COPD)	18/25/0 *versus* 0/124/0	CS exposure, IF, TEM, SEM, WB, cell viability and cytotoxicity assays, CHARM methylation array	HDAC6 involved in ciliated AEC responses to CS ↓HDAC6 methylation in COPD ↑HDAC6 in smokers and COPD patients	[49]
UA	Nasal brushing (6 non-COPD/19 COPD)	6/0/0 *versus* 0/11/8	CBF	↓CBF in moderate/severe COPD	[50]

Continued

TABLE 1 Continued

Airways sublocalisation (UA, T, B, b, A, WL)	Models (AEC cultures, animals, *etc*.)	Smoking status (non-COPD: never/ex/current *versus* COPD: never/ex/current)	Experimental tools (EM, VM, IF, *etc*.)	Main findings in COPD	References
UA	Nasal brushing (39 non-COPD/98 COPD)	39/0/0 *versus* 0/98/0	CBF, NMCCt	↑NMCCt in COPD ↓CBF in COPD	[51]
UA	Nasal brushing (8 non-COPD/10 COPD)	NA	CBF	↓salmeterol induced-CBF in COPD	[52]

Data are presented as n. UA: upper airways; T: trachea; B: bronchi; b: bronchioles; A: alveoli; WL: whole lung; AEC: airway epithelial cell; EM: electron microscopy; VM: videomicroscopy; IF: immunofluorescent staining; SAEC: small airway epithelial cell; FFPE: formalin-fixed paraffin-embedded; miRNA: micro RNA; sc: single-cell; RNAseq: RNA sequencing; ISH: *in situ* hybridisation; TEM: transmission electron microscopy; LAEC: large airway epithelial cell; CBF: ciliary beat frequency; MCC: mucociliary clearance; ALI: air–liquid interface; NA: not available; CS: cigarette smoke; TEER: transepithelial electrical resistance; IHC: immunohistochemistry; NGS: next-generation sequencing; WB: Western blot; TGF: transforming growth factor; IFT: intraflagellar transport; MTEC: mouse tracheal epithelial cell; LGRC: Lung Genomics Research Consortium; SEM: scanning electron microscopy; CHARM: comprehensive high-throughput arrays for relative methylation; NMCCt: nasal mucociliary clearance time.

Through advances in imaging modalities and standardised analysis methods (electron tomography) [32, 63–66] or live-cell imaging modalities for motile cilia and primary cilia [67], it will be possible to precisely analyse cilia structure and movement to better phenotype respiratory diseases [63]. Animal models developing COPD-like lesions could also be used to study cilia dysfunctions. Mice, rats and guinea pigs were exposed to cigarette smoke extract to successfully mimic the pulmonary damage of COPD smoker patients with an alteration of the ciliary component [68]. In addition, genetically modified organisms were generated, such as the IFT88- or Dnah9-mutant mice, to study the role of cilia-associated proteins in human development [69, 70]. Notably, they featured epithelial remodelling and a decrease in CBF.

The identification of primary cilia alterations on stromal cells and signalling pathways associated with cilia dysfunctions (Hedgehog, Wnt, Notch) is crucial to decipher the mechanisms of respiratory disease pathogenesis [71–73]. In addition, the characterisation of the intriguing link between immune cells and cilia, such as impaired neutrophil chemotaxis in primary ciliary dyskinesia [74] or cellular inflammatory responses induced by cilia proteins independently of primary cilia [75], would be a complementary approach to developing therapies targeting the ciliary proteome.

Considering that the design of novel drugs directly targeting cilia alterations may require profound research developments, several pharmacological agents have been found to improve mucociliary clearance. For example, CBF could be increased by the addition of inhibitors of phosphodiesterase, such as roflumilast [76], or long-acting muscarinic antagonists/β-agonists, such as tiotropium or glycopyrronium [77].

Conclusions

The current knowledge on cilia in the context of lung homeostasis and pulmonary diseases, such as COPD, places these organelles at the heart of experimental investigations to identify a new endotype of patients, which include the development of related biomarkers and therapies. The complex characteristics of the cilia at structural and functional levels highlight their importance for the pathophysiology and thus accurate diagnosis of respiratory diseases. The complete understanding of early pathogenesis events that may originate from the analysis of lung primary cilia and their crosstalk with mesenchymal cells could pave the way for innovation in complex, multifactorial chronic airway and pulmonary diseases.

Questions for future research

- What are the complex relationships between primary ciliated and multiciliated cell lineages in the respiratory system?
- Is there a dysregulation of primary ciliated stromal cells in COPD and asthma?
- How can the alteration of cilia define an endotype spanning all respiratory diseases?
- Can ciliated cells be identified in the developing lung and take part in homeostasis?
- Will the analysis of molecular, structural and functional features of cilia provide clinical readouts for better patient management?

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