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In vitro culturing of ciliary respiratory cells—a model for studies of genetic diseases

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Abstract Primary ciliary dyskinesia (PCD) is a rare genetic disorder caused by the impaired functioning of ciliated cells. Its diagnosis is based on the analysis of the structure and functioning of cilia present in the respiratory epithelium (RE) of the patient. Abnormalities of cilia caused by hereditary mutations closely resemble and often overlap with defects induced by the environmental factors. As a result, proper diagnosis of PCD is difficult and may require repeated sampling of patients' tissue, which is not always possible. The culturing of differentiated cells and tissues derived from the human RE seems to be the best way to diagnose PCD, to study genotype–phenotype relations of genes involved in ciliary dysfunction, as well as other aspects related to the functioning of the RE. In this review, different methods of culturing differentiated cells and tissues derived from the human RE, along with their potential and limitations, are summarized. Several considerations with respect to the factors influencing the process of in vitro differentiation (cell-to-cell interactions, medium composition, cell-support substrate) are also discussed.

Abbreviations

AA	Antibiotic-antimycotic solution
BEGM	Bronchial epithelial cell growth medium
CT	Cholera toxin
DMEM	Dulbecco's modified Eagle's medium
EGF	Epithelial growth factor
F12	Ham's F12 medium
HC	Hydrocortisone
IDA	Inner dynein arms
INS	Insulin
MT	Microtubule
ODA	Outer dynein arms
PCD	Primary ciliary dyskinesia
pen	Penicillin
RA	Retinoic acid
RE	Respiratory epithelium
SCD	Secondary ciliary dyskinesia
strep	Streptomycin
TEM	Transmission electron microscopy
TR	Transferrin

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Primary ciliary dyskinesia

Impaired functioning of cilia has been implicated in numerous human hereditary diseases, collectively referred to as ciliopathies. Their classification reflects the physiological role of different types of cilia involved, with the two main classes being motile cilia and sensory cilia (Badano et al. 2006). The human disorders due to the dysfunction of motile cilia are best exemplified by primary ciliary dyskinesia (PCD).

PCD is a rare genetic disease (with a prevalence of 1:16,000 to 1:20,000 live births), characterized by recurrent respiratory airways infections, sinusitis, otitis media, bronchiectasis, and male subfertility (Afzelius 1998; Schidlow 1994; Escudier et al. 2009). In about 50% of cases, PCD is

also associated with situs inversus—a mirror image arrangement of the internal body organs (Kartagener's syndrome [KS]) (Afzelius 1998). The respiratory symptoms, which are the primary cause for PCD patients' presentation to the physician, reflect the impairment of mucociliary clearance caused by the dysfunction of motile cilia in the respiratory epithelium (RE). Subfertility in PCD patients is due to the dysfunction of flagella in spermatozoa and of cilia in the epithelium lining of the oviducts. Randomization of the organ symmetry in KS is caused by the impaired motility of the cilia present in the embryonic node, during the early phase of development (Geremek and Witt 2004).

Proper diagnosis of PCD, especially in patients without situs inversus, is often impeded by the fact that aberrant ciliary function (with clinical manifestation similar to PCD) can also result from non-hereditary mucosal injuries, due to inflammation, bacterial or viral infections, allergies, and smoking (Escudier et al. 2009). Such acquired cilia alterations are transient and collectively named secondary ciliary dyskinesia (SCD) (Jorissen et al. 1997). Clinically, SCD symptoms are similar or even indistinguishable from those of PCD; furthermore, PCD-caused inflammation leads to further, secondary changes of the ciliary function. This superimposition of the genetic and environmental defects can heavily perturb and obstruct the differential diagnosis of PCD versus SCD.

In the majority of cases, the inherited dysfunction of cilia in PCD is caused by aberrations in the ultrastructure of these organelles. A large diversity of ultrastructural defects reflects the complicated architecture of the cilium (Fig. 1).

Ultrastructural and functional defects in PCD

Ultrastructural defects of the ciliary architecture can be identified by transmission electron microscopy (TEM). The most frequently observed defects (70–80%) involve the lack or shortening of outer (ODA) and/or inner (IDA) dynein arms (Noone et al. 2004) (Fig. 1, Table 1). Aberrant number and/or localization of the central or peripheral microtubules (MTs) form the other major class of ciliary defects (Table 1). While several different aberrations can be found in a single patient, the defects are often not present in all of the examined cilia; furthermore, some patients do not have any recognizable ultrastructural defects at all (Jorissen et al. 1997; Herzon and Murphy 1980; Greenstone et al. 1983; Escudier et al. 2009; Conraads et al. 1992).

It is important to realize that not all of the structural changes observed in TEM represent generic defects. Some of them may result from the inappropriate preparation of a specimen (for example, dynein arms, especially IDA, are hard to observe in TEM specimens of low quality). Other changes, like the absence of a central MT pair, may not reflect any abnormality, but just the normal architecture of the proximal part of the cilium (at the level of the transition zone), where the central pair is not present (Fig. 1). What is more, abnormalities of the ciliary ultrastructure are not solely restricted to PCD patients. MT defects or swollen cilia are frequently found in patients with different respiratory-tract pathologies (such as cystic fibrosis, bronchial asthma, and bronchitis) (Afzelius 1981). Similarly, discordant orientation of neighboring cilia can occur not only as a primary (genetic) defect, but also secondary to an infection (Rutland and de Iongh 1990). In addition, even in non-PCD individuals, up to 10%
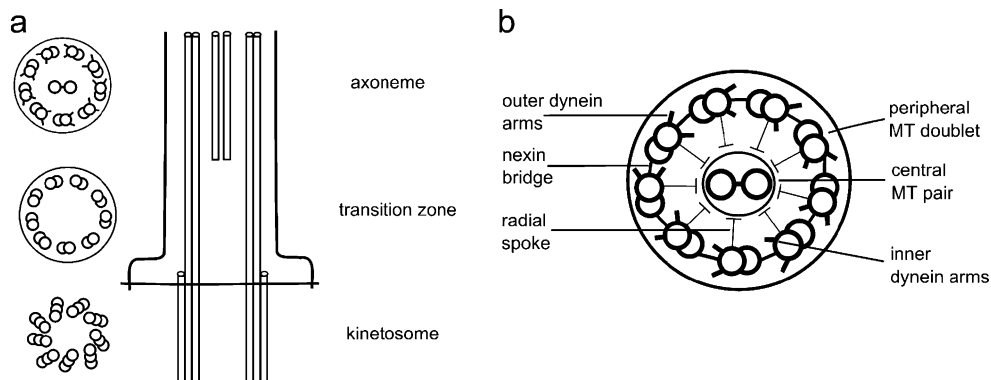
Fig. 1 Schematic representation of the ciliary architecture. **a** Arrangement of microtubules (MTs) in different sections of the cilium. The main body of the organelle, the axoneme, is built of nine peripheral microtubular doublets, organized symmetrically around the central pair of MTs. The arrangement of MTs is different in the proximal parts of the cilium, with the central pair missing in the transition zone and in the anchoring basal body (kinetosome); in addition, in the basal body, peripheral MT doublets are replaced with triplets. **b** MTs and associated elements in the section of axoneme.

Axonemal MTs are associated with a variety of proteins, which form specific elements of the ciliary ultrastructure, periodically arranged along the axoneme length. Dynein arms, outer (ODA) and inner (IDA), are composed of several types of axonemal dynein chains—light, intermediate, and heavy. The heavy dynein chains act as ATP-dependent molecular motor complexes, which generate the ciliary movement. Nexin bridges and radial spokes, each composed of a large number of different protein chains, connect the neighboring peripheral doublets to the central MT pair, stabilizing the ciliary ultrastructure

Table 1 Spectrum of the ultrastructural ciliary defects found in primary ciliary dyskinesia (PCD)

Ultrastructural element	Defect
Dynein arms	Lack or reduction of outer dynein arms
	Lack or reduction of inner dynein arms
	Lack or reduction of outer and inner dynein arms
Microtubules (MT)	Defects in peripheral MT number or structure
	Transposition, e.g., one or more peripheral MT doublets in the center of the cilium replace the central pair
	Discordant orientation of the central pair in adjacent cilia
Whole cilium	Swollen cilia
	Compound cilia
	Complete lack of cilia

of cilia may display secondary defects of the ciliary ultrastructure (Afzelius 1981; Wisseman et al. 1981; Smallman and Gregory 1986; Pifferi et al. 2001).

Ultrastructural defects of the internal ciliary anatomy can have various functional consequences, from a reduced ciliary beat frequency (CBF; below the normal range of 11–16 Hz) (Bush et al. 2007), to different changes in the pattern of ciliary movement. Nearly 20 various erratic beat patterns have been described so far (Chilvers et al. 2003); many of these patterns can be ascribed to a specific defect of the ciliary architecture (Afzelius 1979; Schidlow 1994).

Absence of the dynein arms, the most severe defect, causes an eggbeater-like rotation of the cilium or movement of only the distal part of the axoneme, and a reduced CBF. Defects of radial spokes result in the increased axonemal flexibility, leading to a corkscrew rotational beat. Lack of the central MT doublet causes a shift from the whip-like to rotational beating, while transposition defects cause an increased rigidity of the proximal parts of cilia, resulting in a grabbing-like motion of their distal parts. In some cases, cilia with the normal internal architecture only quiver. On the other hand, cilia with the discordant orientation of the central MT pairs have an apparently normal beat pattern, but their beating is not synchronized (Castleman et al. 2009).

Mutations in PCD

Full characterization and understanding of the hereditary defects of the ciliary structure and function require identification of the causative mutations. In some cases, it is possible to link the ultrastructural and/or functional defects with the underlying protein defect(s) (Table 2). For

example, lack of the dynein arms is often the result of mutations in the dynein chains (Morillas et al. 2007), while abnormal localization of MTs may reflect mutations in radial spoke proteins (Castleman et al. 2009) (Table 2). In addition, defects in the ciliary ultrastructure can also be due to the mutations in proteins not directly involved in the structure, but only implicated in the assembly of the ciliary elements (Omran et al. 2008) (Table 2). The cases of immotile cilia with normal ultrastructure illustrate the problems in linking the ciliary defect with the mutation in one of the ~200 polypeptides that constitute the cilium (Meeks and Bush 2000). Full classification, linking the molecular defect with the ultrastructural impairment, and, further, with the deficiency in the ciliary motility, is far from being complete. This would require knowledge of the mutations in the respective genes, but, so far, the genetics of PCD is not fully explained, due to the high genetic heterogeneity of the disease (Geremek and Witt 2004). Numerous linkage studies have indicated several genetic regions potentially involved in PCD pathogenesis (Geremek and Witt 2004; Blouin et al. 2000; Meeks and Bush 2000; Jeganathan et al. 2004).

To date, only a few genes are confirmed to be directly associated with PCD pathogenesis (Escudier et al. 2009). Mutations in two of them, *DNAI1* (9p13.3) and *DNAH5* (5p15.2), are responsible for PCD in 30–40% of the affected families (Morillas et al. 2007). Both genes encode axonemal dyneins, intermediate chain 1 and heavy chain 5, respectively. Mutations in the genes which encode other proteins involved in the ciliary ultrastructure (*DNAH11*, *DNAI2*, *TXNDC3*, *RSPH9*, *RSPH4A*) or in the assembly of axoneme (*KTU*) were reported only in single PCD families (Escudier et al. 2009; Morillas et al. 2007). The genes responsible for the remaining ~60% of PCD cases remain to be identified. In addition, mutations in the known PCD genes are characterized by the very high allelic heterogeneity—to date, approximately 80 mutations were found in *DNH5* and about 20 in *DNAI1* (Pennarun et al. 1999; Guichard et al. 2001; Zariwala et al. 2001, 2006; Olbrich et al. 2006; Hornef et al. 2006; Failly et al. 2008, 2009; Zietkiewicz et al. in press). In summary, the analysis of the genetic background of PCD is difficult, and often inconclusive, due to the extensive genetic and allelic heterogeneity of the disease.

Diagnostic problems

In light of the genetic heterogeneity of PCD, the practical diagnostic methods have to rely on the analysis of both ciliary structure and function. As detailed above, the most serious problem is that the primary and secondary defects largely overlap. The ambiguous relation between the ultrastructural defect, ciliary beat pattern, and clinical phenotype is the reason why the TEM assessment, although

Table 2 Ciliary phenotypes associated with mutation in the known PCD genes

Genes	Encoded protein	Ultrastructural defect	Defect of function	References
DNAI1	Dynein intermediate chain 1	Lack of ODA	Immotile cilia	Pennarun et al. 1999; Guichard et al. 2001; Zariwala et al. 2001, 2006; Failly et al. 2008
DNAH5	Dynein heavy chain 5	Lack of ODA	Immotile cilia	Olbrich et al. 2006; Hornef et al. 2006; Failly et al. 2009
DNAH11	Dynein heavy chain 11	No defect	Reduced bending capacity, hyperkinetic beat	Bartoloni et al. 2002; Schwabe et al. 2008
X-linked RPGR	Retinitis pigmentosa guanosine triphosphatase regulator	Complex defect (dynein arms, central MT)	Immotile and motile cilia	Moore et al. 2006
TXNDC3	Thioredoxin–nucleoside diphosphate kinase	Partial lack of ODA	Partially immotile cilia	Duriez et al. 2007
DNAI2	Dynein intermediate chain 2	Lack of ODA	Not known	Loges et al. 2008
RSPH9	Radial spoke protein	Lack of central MT pair	Immotile cilia	Castleman et al. 2009
RSPH4A	Radial spoke protein	Lack of central MT pair	Immotile cilia	Castleman et al. 2009
KTU	Kintoun—involved in the preassembly of dynein arm complexes	Partial or full lack of ODA and IDA	Immotile	Omran et al. 2008

widely accepted as a diagnostic tool, does not always allow for firm PCD diagnosis and discrimination between PCD and SCD. Similarly, a singular observation of the abnormal CBF or beat pattern in the material obtained from the patient also does not provide an ultimate proof that the disease is attributable to the genetic rather than environmental causes.

To differentiate congenital genetic defects from acquired abnormalities that are focal and transient, the presence of the defect should be demonstrated in different areas of the respiratory tract and in specimens sampled at different times (Pifferi et al. 2001); it requires repeated sampling of the mucosa from the patient. Alternatively, a similar effect can be achieved through culturing the respiratory epithelial cells in vitro, to allow the regeneration of the cilia in a controlled environment, free of the agents inducing SCD.

In vitro cell cultures of the ciliated cells from the RE

The culturing of differentiated cells and tissues derived from the human RE seems to be the best solution both for the differential diagnosis of PCD and SCD and for studies of genotype–phenotype relations in genes involved in the ciliary dysfunction. In addition, it may also be used for the research on ciliogenesis, in studies on drug development and administration, and on the influence of pollutants and pathogens on the functioning of the RE (Jorissen et al. 1991; Dimova et al. 2005; Wilson et al. 1992).

Source tissues

The informed choice of the source tissue used to initiate primary cultures of the ciliated cells requires good knowledge of the localization and structure of the RE (Fig. 2a). Basement membrane composed of several types of extracellular matrix (ECM) molecules (Fanucchi et al. 1999), together with the RE, forms the continuous layer of mucous membranes (mucosa), which line the main conducting airways from the nasal cavity, through the trachea down to the bronchial tree.

The RE is composed of four types of cells: ciliated columnar cells with hundreds of cilia on their apical side, non-ciliated columnar cells with microvilli, mucous-producing goblet cells, and, the least numerous, small basal cells (Fig. 2b) (Schmidt et al. 1998; Crystal et al. 2008; Jones 2001). While the arrangement of cell nuclei in the RE suggests its multi-layered organization, all four cell types grow in one layer, contacting the basement membrane; the RE is, therefore, often referred to as pseudostratified epithelium (Schmidt et al. 1998).

Basal cells, which rest in the deeper layers of the RE and do not reach the airway lumen (Crystal et al. 2008), are anchored to the basement membrane by the use of hemi-desmosomes. Goblet and columnar (ciliated and non-ciliated) cells contact the basement membrane only by cell-adhesion molecules (Mygind and Dahl 1998), but they also form tight contacts with the adjacent basal cells (Mygind and Dahl 1998).

Basal cells are the stem cells of the pseudostratified zone (Crystal et al. 2008), and they are responsible for the growth of the RE and its regeneration after injury (Rock et al. 2009). Although basal cells have the highest division potential, columnar cells can also divide (Randell 2006). In addition, columnar cells can 'transdifferentiate' into all of the remaining cell types without dividing themselves (Randell 2006). The relative contribution of goblet and ciliary cells in the composition of healthy pseudostratified epithelium is not

Fig. 2 The pseudostratified respiratory epithelium (RE). a Localization in human airways; *inset shows* localization in the nasal cavity and the *dashed lines* encircle areas where the pseudostratified RE is localized. b Structure of the pseudostratified RE
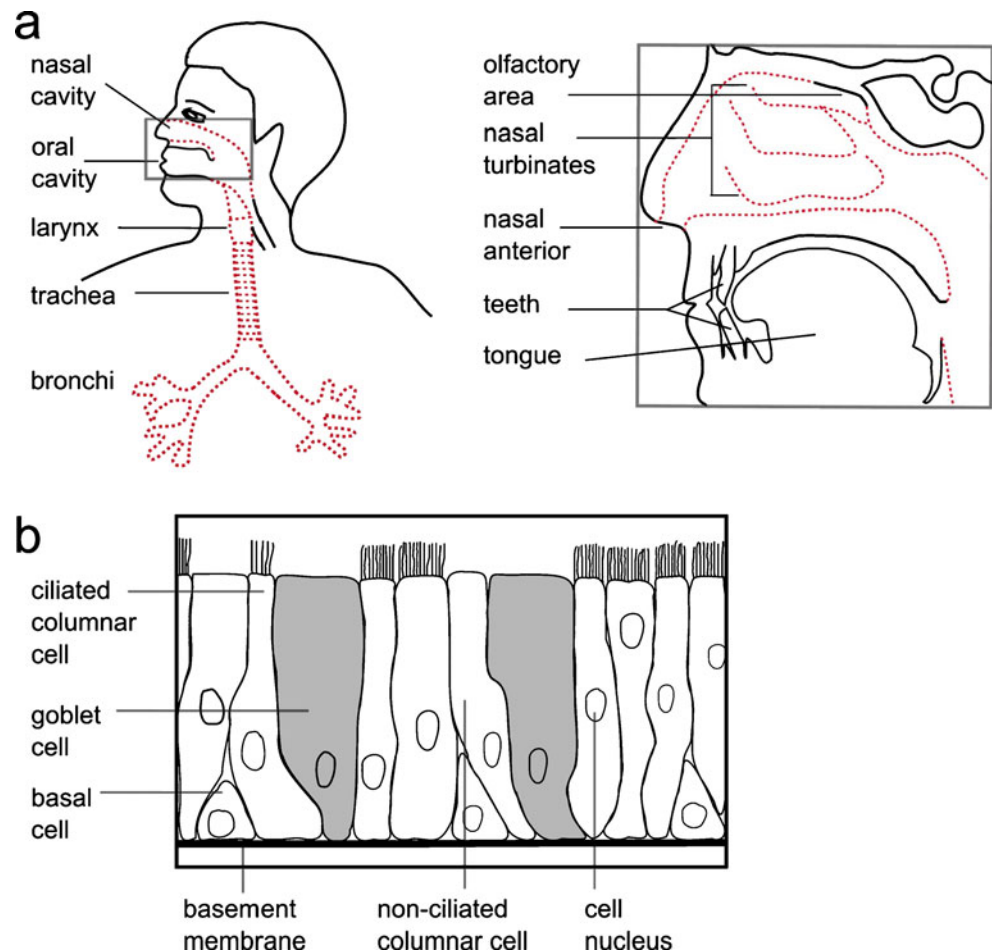
uniform; for example, goblet cells are especially frequent in the regions of high air flow (Schmidt et al. 1998).

Pseudostratified RE specimens for initiating primary cultures can be theoretically collected from any segment of the conducting airways, but not all of the segments are equally accessible and useful. Bronchial biopsies are relatively hard to obtain (surgical intervention is necessary) and often contaminated with infectious agents (Wu et al. 1985). Nasal polyps, the most frequently exploited, easily accessible abundant source of the respiratory tissue, show defects in ion transport (increased Na^+ absorption and Cl^- permeability) and cannot be used for studies of drug permeation and metabolism in nasal epithelium (Schmidt et al. 1998). Normal epithelial tissue collected from the nasal cavity allows overcoming of the above-mentioned restrictions.

In the nose, the pseudostratified RE occupies the central part of the nasal cavity, bordered by the squamous and transitional epithelium in the nasal anterior, and by the olfactory epithelium in the upper part of the cavity (Mygind and Dahl 1998) (Fig. 1a inset). The tissue in the central part of the nasal cavity expresses important features of the lower airway RE, and is relatively easily accessible, allowing sampling to be performed in the ambulatory settings.

There are three different groups of techniques used for the sampling of the nasal epithelium: traumatic methods, atraumatic methods, and postmortem biopsies (Schmidt et al. 1998). Traumatic methods include surgical biopsy, surgical removal of nasal polyps (polypectomy), or nasal turbinates (turbinectomy), as well as plastic surgery (face reconstruction). The biggest advantage of these methods is the usually high amount of harvested cells (Schmidt et al. 1998). This is reflected in the literature, where most of the primary epithelial cell cultures are started by the use of traumatic techniques. The main disadvantage is that these techniques require at least local anesthesia, and that collecting samples from the same area of the nose can rarely be repeated (Schmidt et al. 1998). Postmortem biopsies are similar to the traumatic methods in their advantages and shortcomings. They provide a high harvest of epithelial cells, but repeated sampling is not possible. In addition, artifacts caused by medication, stress, and variable ischemia time can exist (Schmidt et al. 1998).

In contrast, atraumatic sampling techniques do not require anesthesia and they offer the possibility to repeat the tissue sampling (Bridges et al. 1991a, b). In addition, material can be sampled from a precise area of the nasal cavity (Schmidt et al. 1998). Among many methods of

atraumatic sampling described in the literature (Schmidt et al. 1998), only nasal scraping (using a small curette) and brushing (using a small cytobrush) give the yield of epithelial cells high enough to start a primary cell culture (Bridges et al. 1991b; Lopez-Souza et al. 2003). Specimens collected by these methods usually include only the epithelium, without the deeper layers of the mucosa. Importantly, atraumatic methods provide nasal epithelium, without the ion transport abnormalities typical for nasal polyps (Bernstein and Yankaskas 1994; Schmidt et al. 1998).

Mucociliary phenotype in the in vitro cultures of RE cells

The primary goal of in vitro cell culture systems is to achieve differentiated morphology and biochemical features, resembling original tissue as closely as possible (Dimova et al. 2005). This can mean maintaining the differentiated state of the source cells or reconstituting the differentiated state in the cells following their in vitro proliferation; the latter is especially important if the culture is to be used in the differential diagnosis of PCD and SCD.

The history of development of culturing methods that would allow achieving the expression of typical functions of the RE, such as barrier formation, metabolic capacity, vectorial transport of solutes, as well as mucus production and ciliary activity, shows that it was not an easy task (Dimova et al. 2005). One of the important issues to start with was that lifespan of differentiated cells in cultures of nasal RE cells which in the beginning was only a few days. Initial culture conditions were optimized for cell attachment, proliferation, and further subculturing. As a consequence, freshly seeded mucociliary-differentiated cells would lose their cilia and secretory granules within a few days (Bernacki et al. 1999). At the same time, newly proliferated cells followed the pathway of squamous differentiation, which is induced in vivo under conditions of chemical or mechanical injury or vitamin A deprivation (Rearick and Jetten 1989). In effect, a stratified epithelium was formed, with the top layer of squamous, flattened cells and without any ciliated or secretory cells (Rearick and Jetten 1989). The retention of the mucociliary phenotype lasted slightly longer in explant outgrowth cultures (see below), presumably due to the supportive influence of non-epithelial cell types (Agu et al. 1999; Dimova et al. 2005; Neugebauer et al. 2003).

With time, conditions of long-term cultures were established, supporting both cell proliferation and mucociliary differentiation of newly formed cells, together with the retention of the pseudostratified phenotype. A success in mucociliary differentiation was achieved using a number of different methods (Chevillard et al. 1991, 1993; de Jong et al. 1994; Neugebauer et al. 2003; Jorissen et al. 1989). The direction and extent of the differentiation depended on the culturing system and many other factors described below.

Culture systems

There are two basic approaches to establishing the culture of differentiated RE cells: explant growth cultures and cultures of dissociated tissues (Table 3).

Explant outgrowth cultures were one of the first systems developed. Small specimens of tissue (biopsies) were cultured on uncoated (Steele and Arnold 1985) or coated plastic supports (collagen, ECM molecules, etc.) (Wiesel et al. 1983). Sometimes, also a fibroblast feeder layer was used (de Jong et al. 1993). The most promising aspect of this system was its high reproducibility. Explants could be serially replated up to seven times and, after the removal of the explant, cultures retained ciliary activity (Wiesel et al. 1983). Although high levels of cell differentiation were achieved (Chevillard et al. 1991), the results of the experiments were not easy to predict and to interpret, due to the presence of non-epithelial cell types in explants. Another disadvantage was the longer time needed to establish the cell culture, compared with the cultures starting from dissociated cells (Dimova et al. 2005). The use of explant outgrowth cultures became, therefore, less popular; presently, this approach is most often used in studies on the immune response to pathogens and allergens (Ooi et al. 2007; Liu et al. 2007), and on the epithelial barrier function in allergic rhinitis.

Another effective approach to culturing differentiated RE cells is based on use of dissociated tissue samples (Table 3). Digestion of a sample with a protease (pronase) at a low temperature has proven to be one of the most efficient ways to isolate a pure population of epithelial cells, and dispose of non-epithelial cells (Dimova et al. 2005; Schmidt et al. 1998; Wu et al. 1985). Dissociated cells also allow a faster establishment of the culture (Dimova et al. 2005). The system underwent a long evolution since the first reports in the 1980s. The conditions of pronase digestion were optimized, together with other techniques reducing the fibroblast contamination (serum-free media, preplating of the dissociated cells mixture on plastic supports), and cell cluster formation (filtering the cell suspension through filters or sieves) (reviewed in Dimova et al. 2005). Of note, in contrast to solid-tissue samples, samples collected by nasal

Table 3 Culture systems

Explant growth
Dissociated tissue
Adherent:
• Submersion
• Air–liquid interface (ALI)
Suspension
Sequential:
• Submersion monolayer followed by suspension

scraping or brushing techniques do not require additional digestion. The collected cells are already present in the form of mucosal sheets, single cells, and cell clusters, similarly to tissue samples which were digested with protease but not sieve-filtered (Bridges et al. 1991b; Neugebauer et al. 2003).

There are many types of cultures in which the dissociated RE cells are used (Table 3). They include adherent culture (which can be further divided into submersion and air–liquid interface [ALI]), suspension culture, and sequential submersion monolayer-suspension culture (Gray et al. 1996; de Jong et al. 1994; Jorissen et al. 1989; Bridges et al. 1991b).

In the adherent cultures, dissociated epithelial cells were originally seeded in plastic uncoated vessels (Gray et al. 1996) or on a layer of fibroblast feeder cells (Claass et al. 1991; de Jong et al. 1993; Wu et al. 1985). Later, it was found that the adhesion and proliferation of epithelial cells were better promoted by the use of different growth supports/matrix (plastic dishes, floating gel, semipermeable membranes), and vessel/membrane coating with ECM molecules (collagen type I, laminin, etc.), better mimicking the composition of basement membrane, which supports the RE in the in vivo setting (Neugebauer et al. 2003) (Table 4).

Finally, the submersion method used in most of the adherent cultures was found to have inhibitory effects on the process of mucociliary differentiation; differentiated cells were losing cilia and proliferated cells remained undifferentiated (Bernacki et al. 1999), probably due to the lack of signals such as air interface or contact with ECM molecules, which induce cell polarization.

A better degree of differentiation was obtained in suspension cultures (Table 3). When epithelial cells collected by nasal brushing were cultured in the form of polarized multicellular spheroids, with only minimal adherence of epithelial cells to the support, cells retained the ciliated phenotype for over 14 days (Bridges et al. 1991b). After a longer culture time (21 days) in a similar setting (nasal brushing without cell dissociation), the de novo formation of cilia could be observed (Pifferi et al. 2009).

An important modification of the adherent cultures is the use of the ALI (Table 3). In this method, adherent cells growing on inserts with porous bottoms are fed only from their basolateral side, and their apical surface has contact with the air, reflecting the in vivo situation in the RE (de Jong et al. 1993; Wu et al. 1986). Such polarization of the cell layer allows much higher levels of differentiation than a typical submersion feeding system (Whitcutt et al. 1988; Kondo et al. 1991, 1993). The ALI is now a widely recognized "gold standard" for culturing RE cells. Due to the presence of a permeable insert supporting adherent cells, ALI systems are especially useful for measuring the transport and metabolism of drugs across the cell layer and within the cells (Schmidt et al. 1998; Dimova et al. 2005). The drawback of this method is a rather short time for which the layer of differentiated cells is available: in some cases, cells started to detach from the culture vessels already ~14 days after reaching confluence (Werner and Kissel 1995).

The long life of a differentiated culture is the main advantage of the sequential monolayer-suspension culture method (Jorissen and Bessems 1995) (Table 3). During the first phase, the source cells are cultured as an adherent layer in culture vessels coated with collagen type I gel. When cells reach confluency (the time depends on the seeding density), the monolayer is detached from the vessel by use of a collagenase, mechanically fragmented, and transferred into the suspension culture. During the next week, suspended cells are mechanically rotated, in order to promote the formation of closed spheroids from monolayer fragments. Cells in the spheroids are well differentiated and cilia are developed at this stage. Then, ciliated spheroids can be cultured in a stationary incubator, for up to 7 months (Jorissen et al. 1989). The main disadvantage of the sequential method is that it is time-consuming and rather costly.

Factors influencing differentiation in vitro

Mucociliary differentiation has been demonstrated in many different culture systems, but it remains a difficult task, with only a few laboratories having achieved success in this field (Bridges et al. 1991b; Chevillard et al. 1993). The broad range of conditions reported for the successful in

Table 4 Cell supports, membranes, and coatings used in cultures of differentiated respiratory epithelium (RE) cells

Cell support material	Coating
Uncoated plastic	Collagen type I
Membranes	Rat tail collagen
Polyester	Calf-skin collagen
Polyethylene terephthalate	Bovine dermal collagen (Vitrogen 100)
Hydrophilic polytetrafluoroethylene	Bovine placental collagen
Polycarbonate	Collagen type IV
Mixed cellulose esters	Human placental collagen
Collagen membranes CD-24	Mixtures:
Hyaluronate derivatives	Collagen type IV:laminin:heparan sulfate proteoglycan

vitro culturing of the mucociliary tissue reflects the complexity of the mucociliary differentiation in vivo (see Dimova et al. 2005; Wu et al. 1985). Factors to be considered cover different levels of the culturing procedure, and include: processing of the source tissue or cells, seeding density, confluence and cell differentiation status, type of cellular support used (membrane versus cell culture vessel, ECM molecules used for coating), medium composition, feeding regimen, culture time, and number of passages. None of these factors alone appear indispensable or sufficient to direct the differentiation of respiratory cells in vitro.

Among a variety of aspects involved in mucociliary differentiation, **the cell-to-cell interactions** seem to be one of the most important. In vitro, these interactions depend on several conditions, like the processing of the source tissue, seeding density, the presence of cell support, coating vessels with ECM molecules, and the number of passages. The processing of the source tissue (i.e., inoculating culture with non-dissociated versus dissociated cells) appears to have the most immediate consequences. Undisrupted cell contacts are present in suspension cultures inoculated with undigested mucosal sheets (collected by nasal brushing). Mucociliary phenotype in these cultures directly reflects the differentiation status of the source tissue. In contrast, in cultures inoculated with dissociated cells, high seeding density is required for the promotion of cell proliferation and adhesion, which accelerates cellular confluence (Neugebauer et al. 2003; Kowalski et al. 1998).

Once the culture reaches confluence, epithelial cells are forced to change shape, increasing contact with their neighbors. This results in a better transduction of the differentiation signals, which prompt polarization of the cell layer, formation of tight junctions on the lateral sides of the cells, and, finally, the growth of microvilli and cilia on the apical cell surfaces (Neugebauer et al. 2003). However, the successful cell differentiation can proceed in vitro only in the presence of medium containing a balanced composition of factors that facilitate both cellular proliferation and differentiation.

The intact network of cell-to-cell interactions together with the proper medium composition are sometimes sufficient to maintain or induce cell differentiation, as evidenced by the success of suspension cultures initiated from mucosal sheets (Bridges et al. 1991b; Neugebauer et al. 2003). In cultures initiated from dissociated cells, the development of cell-to-cell interactions is required for proper **polarization** of the cell layer and for the further differentiation process. Following the initial orientation of the apical and basal surfaces of the cells, further steps of the differentiation occur, ending in the formation of typical secretory or columnar (ciliated or non-ciliated) RE cells (Crystal et al. 2008). The polarization and differentiation

processes can be promoted by additional external signals, such as a specific type of culture (suspension or ALI culture described above), special cell support (culture matrix or vessel coating), or the composition of medium supplements (reviewed in Schmidt et al. 1998; Dimova et al. 2005).

ECM molecules, constituents of the basement membrane in RE, are naturally involved in the RE cell proliferation, migration, and differentiation. They are also essential for the development of a successful culture of human RE cells. Different ECM molecules have been used for that purpose (Table 4), but collagen seems to be the most promising. Coating of the culture vessels with collagen (especially type I), as opposed to laminin, fibronectin, and polylysine (Table 4), has been reported to positively influence attachment, growth (Wu et al. 1985), and differentiation (Neugebauer et al. 2003) of the RE cells in culture.

In submerged cultures, the effect of collagen coating depends on its physical structure. RE cells grown on a derivatized collagen showed better differentiated phenotype (monolayer with columnar/cuboidal morphology), compared to cells grown on a fibrillar or polymerized collagen, which promoted squamous and multilayered phenotype (Agu et al. 2001). In cultures grown on porous membranes (submerged and ALI), the effects of collagen coating are not so obvious/straightforward. Some investigators reported the improvement of cell attachment efficiency, cell proliferation, and differentiation (Yankaskas et al. 1985; Clark et al. 1995), while others reported no influence (Werner and Kissel 1995) or even negative effects on cell growth (Wu et al. 1985). These contradictory results suggest that other factors, such as media composition and possibly the source of the cells, are important for the full expression of the ECM influence.

Media composition strongly influences the viability, proliferation, and differentiation of any cultured cell type, including RE cells. In the absence of a proper medium, efficient mucociliary differentiation is not achieved even in, otherwise most favorable, ALI cultures. Conversely, a proper medium composition can be sufficient to induce ciliogenesis even in cultures grown in suspension or in the absence of coating with ECM molecules (Neugebauer et al. 2003) (Table 5). Successful in vitro ciliogenesis/differentiation can occur in both simple (only 2–3 supplements) (Werner and Kissel 1995; Agu et al. 2001) and more complicated media (Wu et al. 1985) (Table 5). For a detailed review of different media components and their influence on cell growth and differentiation, see Dimova et al. (2005).

Already the early studies had shown that serum-supplemented media, most suitable for the culture of bronchial cells, would limit the cell proliferation and lifespan of tracheal and nasal epithelial cells (Masui et al. 1986). Serum was also shown to induce squamous cell

Table 5 Examples of methods successfully used for culturing differentiated epithelial respiratory cells

Author	Wiesel et al. 1983	Neugebauer et al. 2003		Agu et al. 2001	Jorissen et al. 1991	Bridges et al. 1991a, b
Culture type	Explant outgrowth	Submerged	Suspension	ALI	Sequential	Suspension
Tissue source	Nasal polyps	Inferior turbinates	Inferior turbinates	Nasal polyps or turbinates	Nasal polyps	Bilateral nasal brushing
Dissociation	Mechanical fragmentation	0.01% pronase, 18–22 h at 4°C	0.01% pronase, 18–22 h at 4°C	0.2% pronase, 16–20 h at 4°C	0.1% pronase, 16–24 h at 4°C	No dissociation
Support	Endothelial cell-produced ECM matrix	Plastic dishes coated with mixture of collagen IV: laminin:heparan sulfate proteoglycan (5:2:2)	Uncoated plastic flasks	Cell vessels uncoated or coated with different forms of collagen I	0.2% rat tail collagen gel	
Seeding density	5–7 explants per 35-mm dish	N/A	N/A	10^6 cells/cm^2	Less than 10^3 cells/cm^2	N/A
Medium and supplements	RPMI 1640, 25% FCS	DMEM/F12 (3:1), INS (1 μg/ml), TR (1 μg/ml), HC (0.5 μg/ml), EGF (10 ng/ml), RA (10 ng/ml), L-glutamine (3.2 mM), and 1.25% (v/v) AA	DMEM/F12 (3:1), INS (1 μg/ml), TR (1 μg/ml), HC (0.5 μg/ml), EGF (10 ng/ml), RA (10 ng/ml), L-glutamine (3.2 mM), and 1.25% (v/v) AA	First day: DMEM/F12 (1:1), 5% FCS Later: DMEM/F12 (1:1), 2% UltroSer G, CT (10 ng/ml), strep (50 μg/ml), pen (50 ng/ml)	Monolayer: DMEM/F12 (1:1), 2% UltroSer G, pen (50 μg/ml), strep (50 mg/ml) Suspension: DMEM/F12 (1:1), 10% NuSerum, pen (50 μg/ml), strep (50 mg/ml)	F12 with INS (2 μg/ml), HC (100 nM), CT (10 ng/ml), T3 (2 nM), EGCS (4 ug/ml), EGF (12.5 ng/ml)
Culture time	14 days	5–6 weeks, up to 14 weeks	At least 7 weeks	12 days	Up to 28 weeks	Up to 14 days
Differentiation status	Ciliary activity, cells cuboidal, flat, closely packed	Densely packed, cuboidal cells with small apical surface, microvilli and/or cilia	Cells columnar/cuboidal, densely packed, bearing cilia and/or microvilli	Derivatized collagen I: monolayer with columnar/cuboidal ciliated and non-ciliated cells	In suspension, cells cuboidal to columnar, microvilli and cilia, no goblet cells visible	Polarized cells: microvilli/cilia visible

DMEM, Dulbecco's Modified Eagle's Medium; F12, Ham's F12 Medium; INS, insulin; TR, transferrin; HC, hydrocortisone, EGF, epithelial growth factor; RA, retinoic acid; CT, cholera toxin; pen, penicillin; strep, streptomycin; AA, antibiotic–antimycotic solution

differentiation and to impair ion transport in respiratory epithelial cells (Van Scott et al. 1988). This inhibition was later shown to be due to the presence of a transforming growth factor β in the blood-derived serum (Masui et al. 1986). With time, the composition of serum-free hormone-supplemented media was optimized to enhance growth and to prolong the lifespan of cultured respiratory epithelial cells (Lechner and LaVeck 1985; Wu et al. 1985). Serum replacements such as UltroSer G, formulated to support the growth of airway epithelial cells, are now commercially available. The most frequently used serum-free media are: DMEM, F12, and BEGM (Agu et al. 2001; Dimova et al. 2005; Jorissen et al. 1989; Schmidt et al. 1998) (Table 5), and, although rather seldom, sometimes also, RPMI 1640 was used (Wiesel et al. 1983) (Table 5). The media are often complemented with antibiotics and fungicides (e.g., penicillin, streptomycin, gentamicin, amphotericin B), in order to reduce microbial and yeast contamination, which is typical for the tissues exposed to the external environment (Jorissen and Willems 2000; Werner and Kissel 1995; Yankaskas et al. 1985; Yoon et al. 2000) (Table 5).

A broad range of supplements can be added to the culture media to promote better attachment, growth, and differentiation of RE cells: insulin, hydrocortisone, epithelial growth factor (EGF), epinephrine, triiodothyronine, bovine pituitary extract, endothelial cell growth supplement, transferring ethanolamine, phosphoethanolamine, retinoic acid and its derivatives, and cholera toxin (Bridges et al. 1991b; Jorissen et al. 1989; Lechner and LaVeck 1985; Sachs et al. 2003; Werner and Kissel 1995; Yankaskas et al. 1985) (Table 5). Insulin is considered to be the most important supplement promoting cell growth (Gray et al. 1996; Lechner and LaVeck 1985; Wu et al. 1986), but for the majority of media components, the physiological importance is either not yet precisely defined or depends on the culture method used. Sometimes, the components are added just in order to follow previously described methods which have been optimized experimentally in cells from other species. As a consequence, the composition of culture media and supplements is very variable and, in many cases, gives contradictory results.

The action of some supplements may depend on the presence and concentration of other media components.

Retinol is considered to be an important factor in maintaining the proliferation and differentiation of RE cultures. Its action is highly dependent on the calcium level (Sachs et al. 2003; Wu et al. 1985) and on specific cellular support (Rearick and Jetten 1989). On the other hand, high calcium level combined with high EGF level is known to suppress mucociliary differentiation and promote the transition of cultured human respiratory cells towards squamous epithelium (Sachs et al. 2003; Van Scott et al. 1988). Therefore, for in vitro mucociliary differentiation of the human respiratory epithelial cells, it is suggested to use media containing a high concentration of Ca ions (~1 mM), high retinoic acid, and low EGF (Sachs et al. 2003). In addition, it is recommended to use collagen matrices (coating, gel, membranes), as they help to express the secretory phenotype in retinoic acid containing cultures (Rearick and Jetten 1989).

The effects of many supplements are not consistent or well-pronounced; their use can depend on the cell origin (species), culture method used, and the scientific goal of the study. For example, insulin, cholera toxin, and bovine pituitary extract were considered to be essential for cell growth in some cases (Wu et al. 1985), while in other settings, sustained cell proliferation was observed only if insulin, EGF, hydrocortisone, ethanolamine, and phosphoethanolamine were present (Lechner and LaVeck 1985). In addition, the optimal concentrations of supplements can differ depending on a specific type of culture; for example, it has been observed that EGF concentrations required for growth and mucociliary differentiation in ALI cultures are much lower compared to those in submerged cell culture type (Schmidt et al. 1998; Dimova et al. 2005).

Conclusions

Differentiated cultures of the respiratory epithelium (RE) cells offer an important augmentation of the experimental toolbox in the diagnosis and analysis of the molecular basis of primary ciliary dyskinesia (PCD). Due to the process of de novo mucociliary differentiation in the absence of harmful environmental factors, the cultures permit the exclusion of any acquired changes in the ciliary architecture and, in consequence, the reliable differential diagnosis of PCD versus secondary ciliary dyskinesia (SCD). By providing the tissue required for the simultaneous analysis of the ciliary ultrastructure and function, the cultures allow deeper characterization of the genotype–phenotype relationships in the ciliary protein mutants.

Several types of successful culture systems of differentiated RE cells have been reported to date, and each of them has its advantages and disadvantages. The existing systems are generally no superior to each other, and still require

better definition and standardization. Still, a specific cell culture system has to be selected depending on the specific application or the scientific goal of the study. Although differentiated cultures of respiratory epithelial cells permit the diagnosis of PCD and/or SCD, the same cultures are not always suitable for the simultaneous research on transport, metabolism, toxicity, and mucociliary differentiation. Therefore, the evolution of a perfect in vitro cell system that would allow separate studying of all of the important processes and cell types of the airway epithelium still remains a goal for the future.

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