

# Cilia containing 9 + 2 structures grown from immortalized cells

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Cilia depend on their highly differentiated structure, a 9 + 2 arrangement, to remove particles from the lung and to transport reproductive cells. Immortalized cells could potentially be of great use in cilia research. Immortalization of cells with cilia structure containing the 9 + 2 arrangement might be able to generate cell lines with such cilia structure. However, whether immortalized cells can retain such a highly differentiated structure remains unclear. Here we demonstrate that (1) using *Ela* gene transfection, tracheal cells are immortalized; (2) interestingly, in a gel culture the immortalized cells form spherical aggregations within which a lumen is developed; and (3) surprisingly, inside the aggregation, cilia containing a 9 + 2 arrangement grow from the cell's apical pole and protrude into the lumen. These results may influence future research in many areas such as understanding the mechanisms of cilia differentiation, cilia generation in other existing cell lines, cilia disorders, generation of other highly differentiated structures besides cilia using the gel culture, immortalization of other ciliated cells with the *Ela* gene, development of cilia motile function, and establishment of a research model to provide uniform ciliated cells.

**Keywords:** cilia, 9 + 2 arrangement, immortalization, cell line, differentiation, *Ela* gene

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## Introduction

Immortalized cells or cell lines can persist in culture permanently [1]. In contrast, primary cells that are not immortalized can only live for a limited period of time, which restricts studies of these cells [2]. Immortalized cells can be obtained by transformation of primary cells, typically with gene transfections or chemical treatments [3]. Immortalized cells can save animals by providing researchers with an unlimited number of uniform cells. However, transfected or immortalized cells may not retain all normal cellular structures and may even exhibit tumorigenic signs [4]. In general, the more highly differentiated a cellular structure

is, the less likely the immortalized cell can retain such a structure [5]. For example, a cilium (cell's hair) containing a 9 + 2 arrangement of microtubules is a very highly differentiated cellular structure. A cilium is an organelle extended from a eukaryotic cell, which can be found in plants, unicellular organisms, and animals. Cilia are predominantly associated with epithelial cells, although they are also present on endothelial cells, neurons, and many other cell types [6]. A cilium can be depicted in two ways: by *structure* and by *function*.

The general structure of a cilium consists of a core of microtubules called an axoneme, which is surrounded by the cell membrane. The arrangement of the microtubules in an axoneme varies in different types of cilia. The two most common types are *primary* cilia and *motile* cilia. The axoneme of primary cilia contains a structure of 9 + 0 arrangement whereas the axoneme of motile cilia contains a structure of 9 + 2 arrangement. A cross section of a 9 + 2 axoneme shows nine microtubule doublets plus one central

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pair of microtubules, whereas a 9 + 0 axoneme does not contain the central pair [7]. There are also accessory proteins forming periodic arms extending between neighboring doublets. The primary cilia usually appear as one cilium per cell, whereas the motile cilia appear as multiple cilia per cell. The primary cilia may appear with a necklace (a ring-like widening) near its base.

The functions of these two types of cilia are different. Cilia with a 9 + 0 arrangement, typical of those seen in the kidney, are normally immotile [8]. The immotile cilia (primary cilia) generally are thought to function as sensory organelles involved in chemoreception, photoreception, and mechanoreception [9-10]. The 9 + 2 cilia, such as those found on epithelial cells lining the trachea, oviduct, and brain are motile where they are associated with either cell locomotion or fluid movement. The 9 + 2 arrangement is a critical structure and the premise for motile cilia to function normally with their rhythmic motion. This motile function of cilia depends on many different well-organized proteins [11]. However, exceptions may exist, for example, the 9 + 0 cilia found on the surface of the embryonic node are capable of rotational beating [8].

The two types of cilia are obviously different in their structure (9 + 2 vs 9 + 0) and function (motile vs immotile, respectively). While cell lines for the study of primary cilia (9 + 0) exist, the study of motile cilia (9 + 2) has relied upon a combination of primary tissue explants and flagellate single-celled organisms. This fact indicates that cell lines with motile cilia are difficult to establish, and as a result the study of such a cell line and ciliogenesis is a very difficult task in the field. In one model, ciliogenesis was proposed to be based on an intraflagellar transport of complexes of proteins [12]. However, this model is based on the research of *Caenorhabditis elegans* and *Chlamydomonas*. Therefore, little is known about ciliogenesis of motile cilia in mammalian cells [12]. There have been indications that de-ciliation would occur when cells are separated whereas ciliation (or re-ciliation) would appear when the cells are aggregated [13]. The epithelial cells that are ultimately destined to produce multiple cilia may first extend a single cilium. The function of this phenomenon is unknown and remains as a largely unexplored research area because its elucidation may require the whole animal developmental studies or an ideal ciliated cell line. The lack of such a cell line has hindered the advance of cilia research, such as understanding the mechanism underlying the differentiation of cilia structure with the 9 + 2 arrangement. Therefore, there is a clear need for cell lines that model the motile cilia.

Owing to the need and difficult nature of obtaining a cell line with motile cilia, a strategy of multiple steps and a separation of the final goal into several sub-goals may

be beneficial. The steps or sub-goals are to obtain a cell line, to generate the *cilia structure*, and then to acquire *cilia function*. The final goal is the development of a cell line with motile cilia. The first tracheal cell line had been obtained before 1993 [14, 15], which was regarded as a first milestone toward the final goal. However, no cilia structure was generated from that cell line. Despite the attempts by many scientists with decades of tremendous efforts, whether immortalized cells could generate the 9 + 2 cilia structure remains unclear [16-20]. So far, no cilia structure has been generated from the initial tracheal cell line as well as from other tracheal cell lines that have been established since then. This indicates that generation of a 9 + 2 cilia structure from cell lines is a very difficult task. In this report we demonstrate that the second step (generation of the 9 + 2 cilia structure from immortalized cells) can be achieved. Therefore only the third step, acquiring cilia function (i.e. the motility), has not yet been accomplished, although we have observed a trace of motile phenomenon in the cell line, which will be discussed later. It should be noted that more than 12 years have passed from obtaining a tracheal cell line without a cilia structure to establishing a cell line with a 9 + 2 cilia structure. Moreover, *cilia motile function* is a complicated feature dependent on as many as 250 different types of proteins, which must be well organized to function properly [11]. Thus, it seems likely that it will take some substantial time before the final step (acquiring motile cilia from cell lines) can be realized. In this article, we mainly focus on *in vitro* generation of the 9 + 2 cilia structure but not on the cilia motile function.

We report here that immortalized cells generate the cilia structure containing a 9 + 2 arrangement. Cell lines with a 9 + 2 cilia structure provide an opportunity for next-step studies in areas such as development of cilia motile function, cilia differentiation mechanisms, and cilia disorders. Our results also provide clues for appropriate immortalization methods and culture conditions, which might be useful to other investigators when differentiating other important cell structures of interest.

## Materials and Methods

### *Isolation and immortalization of rat tracheal epithelial cells*

Adult Sprague-Dawley rats (Harlan Inc., Indianapolis, IN, USA), used to obtain tracheal epithelial cells, were raised in a virus-free environment. Tracheal epithelial cells were isolated according to the methods previously described [21], with some modifications as follows. Tracheal cells were isolated following an overnight incubation at 4 °C in the LHC-9 medium containing 0.1% protease (Sigma, MO, USA). Cells were then harvested the next day and plated in the LHC-9 medium. After several hours of incubation, cells settled down on the bottom of the culture plates. The medium was carefully replaced with a DMEM medium containing vectors that carried the 12S *E1a* gene produced by  $\psi$ 2 cells [22, 23]. The  $\psi$ 2 cells

were obtained from ATCC (Manassas, MD, USA) and cultured in a DMEM medium with 10% fetal bovine serum (Sigma, MO, USA). The *E1a* vectors were released from  $\psi$ 2 cells into the medium. The tracheal cells were exposed to the *E1a* medium in the presence of 8  $\mu$ g/ml of polybrene [24] for 3 h daily for 3 d in a culture incubator. In 10–14 d, 0.25 mg/ml of geneticin G418 (Sigma, MO, USA) was added [25] to destroy cells that were not transfected with the *E1a* vector. The transfected cells survived and were maintained in the LHC-9 medium.

#### *Detection of cDNA using PCR, electrophoresis, and hybridization [26]*

On the basis of the *E1a* mRNA sequence available in the GenBank, a pair of 18-mer primers was synthesized as follows: primer 1 corresponding to position 592–610 (5'-ACC GAA GAA ATG GCC GCC-3') and primer 2 corresponding to position 1 551–1 569 (5'-CAC ACA CGC AAA TCA CAG G-3'). For the target 12S cDNA, the size of the amplified fragment is expected to be ~725 bp in length. The PCR reaction mixture was prepared as follows: 0.2  $\mu$ M each primer, 200  $\mu$ M each dNTP, 40 ng target DNA, sterile water, and 10 $\times$ reaction buffer. The reaction volume (100  $\mu$ l) was overlaid by an equal volume of mineral oil. The main cycling was performed at 94  $^{\circ}$ C for 1.5 min, at 55  $^{\circ}$ C for 1.5 min, and at 72  $^{\circ}$ C for 3 min, and repeated for 30 cycles. Products were electrophoresed on 1.4 % agarose gels containing ethidium bromide (0.5  $\mu$ g/ml) and each lane was loaded with an aliquot of amplified DNA fragments. For hybridization, a probe of an *Eco*RI-*Sac*I digest of a plasmid containing the 12S *E1a* cDNA was labeled with  $^{32}$ P and used in Southern blot analysis.

#### *Immunochemical staining for the E1a protein and cytokeratins, and detection of F-actins*

Cells in culture were fixed with 4–10% formaldehyde, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS), washed with PBS, and exposed to the mouse monoclonal antibodies (10–20 $\times$  dilution in PBS) against the *E1a* protein [27] or cytokeratins 8, 18, and 19 [28]. Cells were then washed with 0.05% Triton X-100, treated with a 1:50 dilution of a fluorescein-conjugated goat-anti-mouse IgG, rinsed with 0.05% Triton again, and mounted on a slide with glycerol-PBS (9:1). For detection of F-actins, the cells were treated with rhodamine phalloidin [29].

#### *Transmission electron, scanning electron, fluorescence, and phase contrast microscopy*

Cells were prepared and examined by transmission and scanning electron microscopy as previously described [30, 31]. The cells in culture were washed three times with Hanks balanced salt solution, fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffers (pH 7.4), and dehydrated in an alcohol series. The cells for transmission electron microscopy were embedded in plastic, thin-sectioned, stained with uranyl acetate and lead citrate, and examined on a transmission electron microscope at 60–75 kV. The specimens for scanning electron microscopy were critical-point dried, coated with gold-palladium, and examined in a scanning electron microscope at 15 kV.

The fluorescence microscope was used to examine cells either processed with immunostaining or stained with rhodamine phalloidin as previously described [27–29]. After preparation as described above, the specimen was placed on the microscope stage. The appropriate dichroic filter was selected so that the filter could reflect the shorter-wavelength excitation light toward the specimens but allow the

longer-wavelength light emitted from the specimens to pass through. The intensity of the excitation light was adjusted in order to generate clear images of the cells. The fluorescent image was observed and captured by a camera. The phase contrast microscope was selected to observe live cells, such as the harvested tracheal cells and cell line cells. The morphology of live cells could be clearly observed using the phase contrast mode, which otherwise would look transparent. The images observed under the phase contrast microscope were captured by a camera at a magnification of 200–400 $\times$ , shown in the Results section.

#### *Measurement of transepithelial cell electrical resistance*

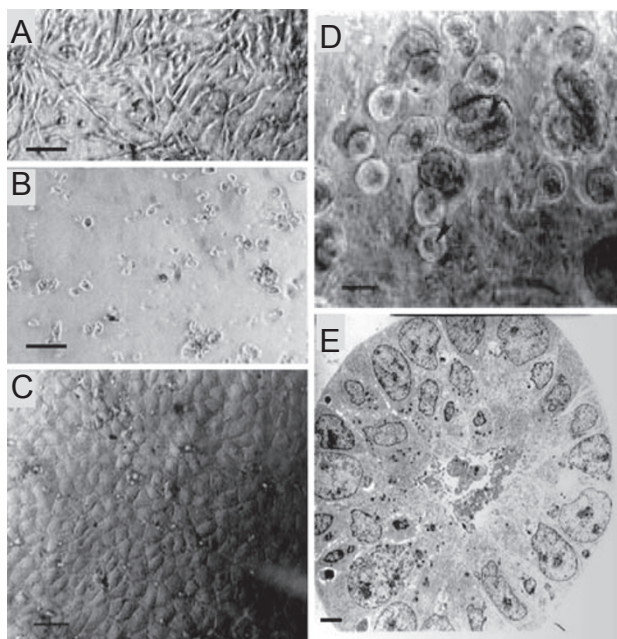
The cells were seeded at a density of  $5 \times 10^5$  on Millicell culture inserts in LHC-9 medium and grown to confluence as a monolayer. Electrical resistance of the transepithelial cell monolayer was measured on days 3–7 after the cells were seeded as previously described [30, 31]. Two electrodes were used in the measurement: one inside the culture insert and the other outside the insert so that the current would flow across the monolayer. The resistance was measured over a period of 10–15 min to ensure that the values remained stable. The results of resistance are reported as the mean of the measurements in triplicate cultures each day.

## Results and Discussion

#### *Immortalization of tracheal cells with the E1a gene*

Rat tracheal epithelia containing ciliated cells were utilized for immortalization. The rat trachea is small and the epithelial cells are lined inside the tiny lumen of the tracheal tube. To isolate the rat tracheal cells, we modified the enzyme digestion method [21] (see Materials and Methods). The modified process allowed the enzyme to interact with the tissue fully for 24 h to digest more epithelial cells off the tracheal wall. The incubation remained at 4  $^{\circ}$ C so that the cells were in an inactive status and were still quite robust upon harvesting. The harvested ciliated cells were observed under the microscope with cilia still stroking in rhythm. This result indicates that robust tracheal epithelial cells can be harvested with this modified method (Figure 1A). These high-quality cells were good candidates to survive the following immortalization process.

There are many approaches described in the literature to immortalize cells. *E1a* gene transfection was the approach we finally tried, with which we have eventually achieved success. The *E1a* (early region 1a) and its products modulate cell-cycle regulatory proteins and regulate the transcription of S-phase genes [22]. Although the *E1a* gene approach has been used to immortalize other cell types [23], whether or not *E1a* would transform rat tracheal epithelial cells was unknown. To carry out the transfection, we exposed the harvested primary cells to a DMEM medium containing the *E1a* gene vectors, which was prepared from  $\psi$ 2 cells (see Materials and Methods). Our results indicate that *E1a* can immortalize rat tracheal epithelial cells (Figure 1C).



**Figure 1** Morphology of the cells in culture during immortalization. Phase contrast images are shown (A-D, bar = 25  $\mu$ m). (A) Monolayer of mixed types of primary cells from the inner layer of a rat trachea. The cells would be transfected with the *E1a* gene. (B) A few *E1a*-transfected cells survived after majority of non-transfected cells were destroyed by Geneticin. (C) Monolayer with a “cobble stone” appearance was formed by the cell line cloned from transfected trachea epithelial cells. (D) Spherical aggregations with a solid core at initial developing stages formed by the cell line after the cells were seeded in a 3-D gel. (E) Electron micrograph (bar = 3  $\mu$ m) displaying a spherical aggregation at the mid-developing stage, enclosing a small hollow core.

#### Establishment of tracheal cell lines

Geneticin (G418) was used to select transfected cells [25]. After exposure to the *E1a* vector medium, both non-transfected and transfected tracheal cells continued to grow together into a typical heterogeneous monolayer (Figure 1A). The non-transfected epithelial cells were sensitive to geneticin and were destroyed by the geneticin selection, while cells transfected by the *E1a* vector were resistant to geneticin. They survived (Figure 1B) and continued to grow.

We used a dilution method [32] to clone cells and establish cell lines. The surviving geneticin-resistant cells were harvested, diluted with LHC-9 medium, and seeded in multi-well dishes at 0 to 1 cell count per well. A cluster of cells, which was observed growing from one cell in one well, was collected as a cloned cell line. The cloned cell line grew into a homogeneous monolayer composed

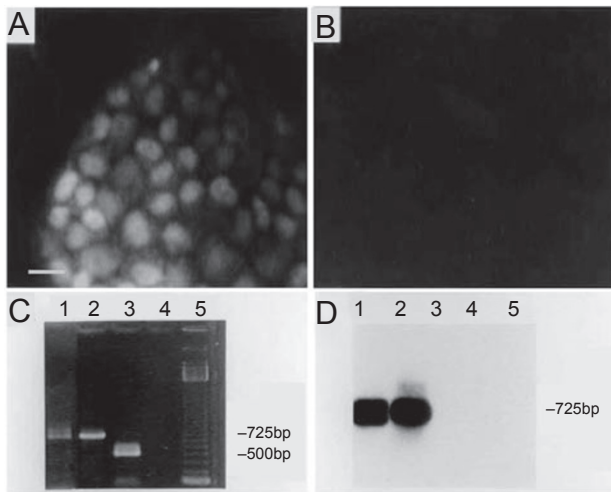
of uniform cells. This monolayer appeared like a surface of “cobble stones” (Figure 1C), which is consistent with the appearance of a normal epithelial monolayer. The cell line has been sub-cultured over 40 times during 18 months, and proved to be stable without undergoing crisis. Three sets of experiments were planned: (1) to verify whether or not the cell line was indeed transfected with the *E1a* gene; (2) to induce the cell line to generate cilia structure; and (3) to examine whether or not the cell line retained other major phenotypic characteristics associated with normal epithelial cells.

#### Verification of expression of *E1a* gene in immortalized cells

To verify that the cell line was indeed transfected with the *E1a* gene, detection of both the *E1a* protein and cDNA was performed [26, 27]. For detection of the *E1a* protein, the cell line was positively stained with an anti-*E1a* antibody (Figure 2A), while non-*E1a* cells remained unstained (Figure 2B). After PCR amplification (see Materials and Methods), a product with the size of ~725 bp corresponding to the *E1a* cDNA was detected from the cell line by gel electrophoresis stained with ethidium bromide (Figure 2C, lane 2). The correct size of the PCR product was further confirmed against the positive (Figure 2C, lane 1) and various other controls (Figure 2C, lanes 3-5). The *E1a* cDNA sequence in cell line cells was also detected by hybridization to a specific probe labeled with  $^{32}$ P (Figure 2D, lane 2) in a blot with a lane setting corresponding to that in Figure 2C.

#### Generation of cilia structure containing 9 + 2 arrangement with 3-D gel culture

To induce the cell line to generate cilia structure, an environment of 3-D gel culture was developed. A matrigel derived from mouse sarcoma basement membrane extract [33] was used to coat the bottom of the culture plate (~1.0 mm thick). When the cells were seeded on the matrigel bed and covered with shallow LHC-9 medium allowing them to be in close proximity to the air, these cells did not grow into a large flat monolayer as was usually seen on a regular culture surface; instead, they penetrated into the gel and formed spherical aggregations enclosing a hollow core or lumen (Figures 1D, 1E and 3A). This phenomenon of aggregation was rapid, occurring within 48-72 h after the cells were seeded. Spherical aggregations at three developing stages were noticed: (1) spherical aggregations with an almost-solid core during the initial stage (Figure 1D); (2) with a tiny hollow core during the mid-development stage (Figure 1E); and (3) with a well-shaped central lumen at a later stage (Figure 3A). The spherical aggregations persisted for up to 2 weeks. The innermost cells grew as



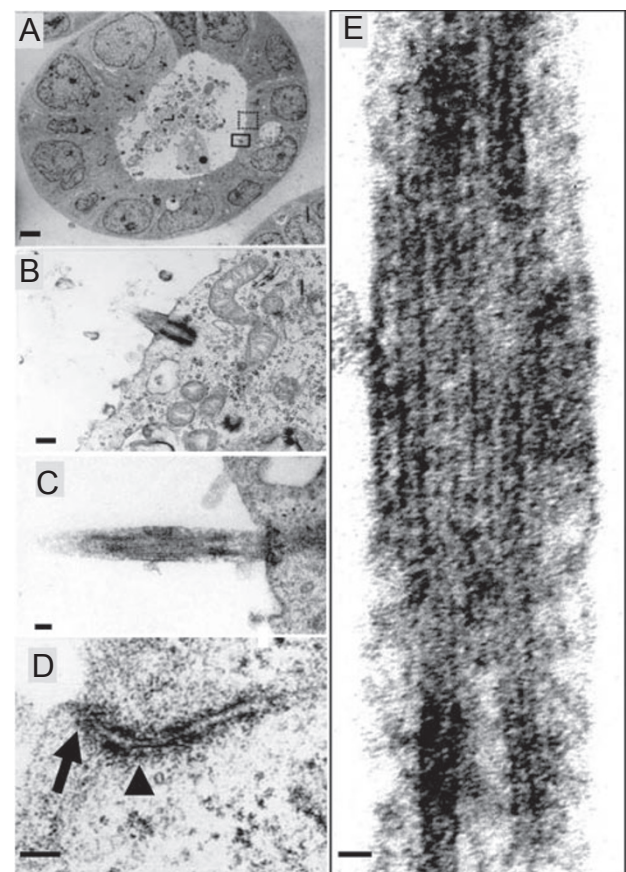
**Figure 2** Expression of *E1a* in the cell line. **(A)** Fluorescent image of immortalized cells showing positive immunostaining against *E1a* proteins, while the staining was negative for non-*E1a* bronchial cells **(B)**. Bars = 25  $\mu\text{m}$  **(A, B)**. **(C)** The  $\sim 725$  bp *E1a* cDNA amplified from the cell line (lane 2) was analyzed in ethidium bromide-stained gel. Lane 1: *E1a* cDNA as a positive control; lane 3: lambda DNA reaction control; lane 4: no-target negative control; lane 5: molecular standard 123 ladder. **(D)** Blot corresponding to **(C)** but using a specific  $^{32}\text{P}$ -labelled *E1a* probe (hybridization). The cDNA sequence from the cell line matched the *E1a* sequence (lane 2), as supported by the positive control in lane 1 and negative controls in lanes 3-5.

a monolayer lining the inside surface of the lumen. The cells of the monolayer were polarized, with their apical pole facing toward the lumen (Figure 3A).

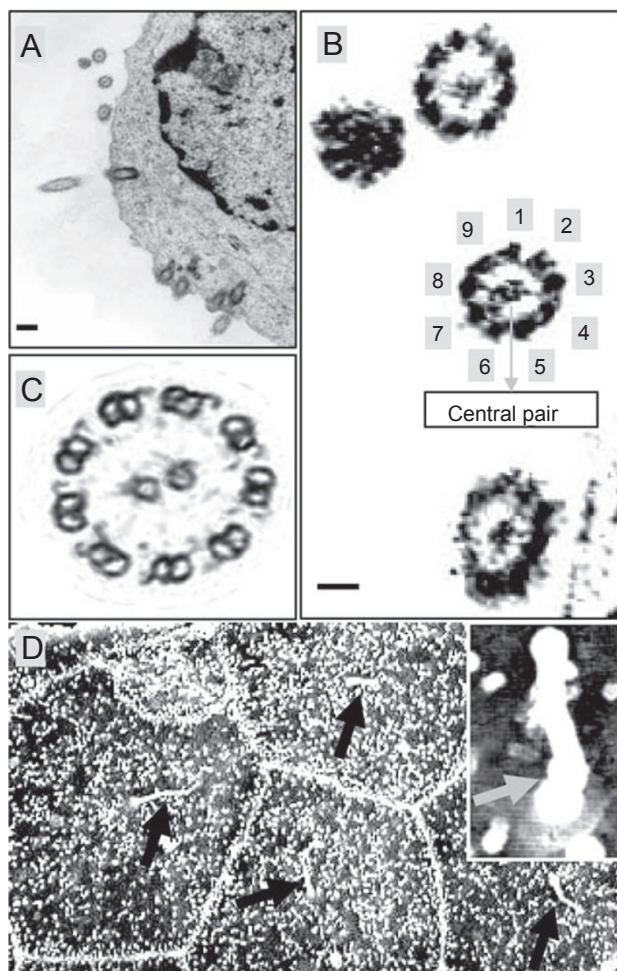
Inside the lumen of the aggregation, short cilia at their early differentiation stage were clearly observed protruding into the lumen from the apical pole of the cells as shown in Figure 3B. Some cilia grew to up to 1.2  $\mu\text{m}$  in length (Figure 3C). Their microtubules were vertically well-organized, cross-linked within the ciliary shaft as shown in Figure 3E, known as the axoneme [34], and rose from the basal body within the cytoplasm at the root of the cilium (Figure 3C). Multiple cilia were also generated from some cells (Figure 4A), and the cilia displayed a highly differentiated structure containing a clear 9 + 2 arrangement (Figure 4B) that is consistent with the descriptions previously reported [20]. To clearly display the cilia structure with the 9 + 2 arrangement, the axonemes were imaged at a higher resolution (Figure 4C). The sampling and imaging processes used to emphasize the 9 + 2 structure have compromised the display of other cellular components. The cytoplasm surrounding the axoneme is lightly visible up to the boundary of the cell membrane (Figure 4C). The ultrastructure of the nine microtubule doublets with an additional central pair of microtubules (9 + 2) is clearly

displayed (Figure 4C).

As for the time course of cilia generation, no cilia structure was observed before the cells became confluent and the aggregations were formed. A single cilium appeared in one or two cells in culture around the fourth day and a significant increase in the number of cells with cilia was observed around the eighth day. We observed that 80% (40/50) of cells generated the cilia in a given culture, of which 88% (35/40) had a single cilium (Figure 4D) and 12% (5/40) had multiple cilia. The 9 + 0 arrangement was predominantly observed in the single cilium. The 9 + 2 arrangement was observed in the multiple cilia. In the case of multiple cilia, at



**Figure 3** Highly differentiated structures of the cell line (electron micrograph). **(A)** Spherical aggregation enclosing a lumen at a development stage later than that in Figure 1E. **(B)** Magnification of the cilium in 3A (low-positioned solid-line box) protruding into the lumen from the apex of the cell. **(C)** A long cilium growing from a cell line cell. **(D)** Magnification of the junctional complexes in **(A)** (upper dotted-line box). The tight junction appeared at the cells' apical surfaces (arrow), and desmosomes appeared at the cells' lateral membranes beneath the tight junction (arrowhead). **(E)** Digital magnification of the cilium in **(C)** displaying vertically well-organized and cross-linked microtubules (axoneme). Bars = 2  $\mu\text{m}$  **(A)**, 200 nm **(B)**, 100 nm **(C, D)**, 20 nm **(E)**.



**Figure 4** Structure of multiple cilia (A-C, Transmission EM) and single cilium (D, scanning EM) from cells of the cell line. (A) Multiple cilia generated by a single cell of the cell line (Bar = 200 nm). (B) Digital magnification of several multiple cilia exhibiting the 9 + 2 arrangement (labeled with 1-9 for “9” and center pair for “2”) rather than other cellular structures (Bar = 100 nm). (C) High-resolution image showing clear ultrastructure of the cilia 9 + 2 axoneme. (D) Single-cilium cells. Each cell generated one single cilium (four black arrows) and abundant small microvilli appeared on the apical surface of the cells. Inset: digital magnification of a single cilium (gray arrow).

least 12 cilia had been observed to grow from one immortalized cell. Obviously, our culture condition was appropriate to induce the cilia but might not be optimized. Should the condition be optimized, perhaps more cilia could have been generated from one cell, and such optimization will be one of our goals in the future. In addition, a necklace-like structure was observed near the base of the single cilium (Figure 4D, inset). For a single cilium, its 9 + 0 structure and the appearance of the necklace-like structure are con-

sistent with the description of a primary cilium. The result that majority of cells had a single cilium at a given time course may suggest that the single cilium appears before the appearance of 9 + 2 multiple cilia. The mechanism of this time course is unknown, and will be investigated in future studies. On the other hand, how many cells can be ciliated and how many cilia can be generated per cell may be dependent on several factors including the formation of the spherical aggregation, potential nutrition from the surrounding gel, development of the lumen in the aggregation, and the microenvironment inside the lumen. Manipulation of these factors may affect the number of ciliated cells and the number of cilia per cell. All these issues await further studies over the next several years. The potential effect of spherical aggregation is discussed below.

#### *Spherical aggregation, tube formation, and culture microenvironment*

Spherical aggregation is a phenomenon observed in this study, which may be related to two issues: tube-forming tendency and cell-culture microenvironment. (1) With respect to the tube-forming tendency, several points should be noted. First, the cell line cells form a lumen or cyst in the gel culture. Second, the epithelium lining the trachea is relatively flat. Third, one of the potential processes of tube morphogenesis in embryo development is termed “cavitation” [35]. The cavitation process progresses through several stages [35]: aggregation of a group of cells destined to the same purpose, development of a loose core, appearance of a small lumen inside, elongation of the small lumen, and finally cavitation into a large and long tube. Considering these factors, the aggregation observed with the cell line suggests a tendency of tube formation that could be assumed as the behavior of tracheal cells, though this needs to be confirmed in a future study. When a cavity is small and short, it looks like a cyst; when a cyst is large and long, it looks like a tube. A small animal has a small trachea whose epithelium circles a small tube and therefore is less flat than that in large animals. Being flat or less flat is just an issue of relativity, while both tube and cyst are covered by one layer of epithelium. Nevertheless, the aggregation and lumen formation in the cell line may suggest the tendency of tube formation though further confirmation is needed. (2) With respect to the microenvironment, the lumen may create a special culture condition. The microenvironment inside the lumen may be different from that of a regular culture medium. A facilitating condition may be developed inside the lumen during the first 10 days. The fluid inside the lumen may contain the secretions from the epithelium lining the lumen and nutrition potentially from surrounding gel. However, these interpretations are just hypotheses. Because cilia appeared inside the lumen, we can reasonably assume that such fluid

or microenvironment may facilitate the generation of the cilia structure in a period within around ten days. However, further confirmation is needed and the fluid inside the lumen needs to be investigated.

#### *Phenomenon of cilia motile function*

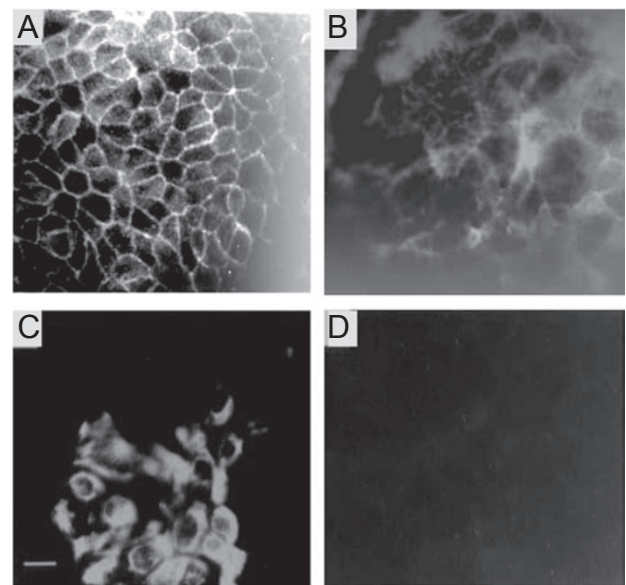
Since the 9 + 2 cilia observed in this cell line were formed inside the cell aggregation, we have not yet been able to observe the cilia motile function; although once we observed indistinctly some cilia on the cells beating in the culture, the beating disappeared during the follow-up. It is possible that this may have resulted from cells derived from an occasionally broken aggregation. Having a cell line with both the proper cilia structure and cilia motile function would of course constitute an ideal model system. Without cilia motile function, the usefulness or utility of the cell line may be limited. Nevertheless, establishing a cell line with the proper cilia structure could be regarded as a significant advancement towards the goal of developing an ideal cell line(s) with the cilia motile function.

#### *Other major normal epithelial characteristics in immortalized cells*

Although our study has focused on cilia, confirmation of epithelial nature of this cell line is also important. We only show several major epithelial characteristics of this cell line here. Details of many other cellular characteristics will not be covered in this report because they have been previously addressed in immortalized cells.

We found that our cell line expressed other normal epithelial phenotypic characteristics as well. The cells did not become aneuploid, as evidenced by cytogenetic and karyotypic analysis with Giemsa banding techniques [36]. The analysis was performed by stopping the cell cycle progression when chromosome pairs were formed, releasing the chromosome pairs from the cell nucleus, organizing the pairs, and observing them for their appearance. The results revealed that the cells retained the diploid karyotype. The behavior of cell growth was also studied. The monolayer in the culture was well formed (Figure 1C), and the cell growth curve (rate) in culture (count of cell number as a function of post-seeding days) appeared as a sigmoid shape with a saturation plateau appearing around 10-14 days. The monolayer in culture (Figure 1C) and saturation in growth suggest that the behavior of the cell line is consistent with that of normal epithelial cells and also suggest that lateral contact inhibition of cells [37] still existed in this cell line. Immunochemical staining was performed to detect components that are expected to be present in normal epithelial cells [28, 29]. The F-actins lined up against the inner side of the plasma membrane in epithelial cells when cell-to-cell contacts were established [29]. The results of our immuno-

chemical studies indicate that the F-actins were organized in an orderly fashion, and displayed as inner rims of cells (Figure 5A). However, no ring structures were observed in the negative control fibroblasts although the F-actins were present (Figure 5B). The intermediate filaments (cytokeratins) expressed in normal epithelial cells [28] were also clearly observed in the cell line (Figure 5C), but were absent in the negative control fibroblasts stained under the same conditions (Figure 5D). In the microenvironment displayed in Figure 3A, cells were polarized. Microvilli and tight junctions were developed at the cell apical surface as reported in the literature for epithelial cells [38]. Formation of tight junctions was observed in electron microscopic images (Figure 3A) and clearly shown in a high-resolution view (Figure 3D). Desmosomes encountered in the junctional complexes [39] were also observed (Figure 3D). In addition to the results demonstrating (1) the monolayer formation in Figure 1C, (2) the F-actin rims in Figure 5A, and (3) the tight junctions in Figure 3A and 3D, the overall function of these three structures was tested by measuring the electrical resistance across the monolayer [31]. A transepithelial electrical resistance across the monolayer formed by the cell line was recorded to be as high as 4 000



**Figure 5** Fluorescent images showing epithelial phenotypic characteristics of the cell line (bars = 25  $\mu$ m). (A) Ring structures of F-actins between the cell line cells labeled with rhodamine phalloidin. (B) No ring structures were observed in the negative control fibroblasts cultured under the same test conditions as in (A), although the F-actins were still present. (C) Cytokeratins were labeled with immunochemical staining in the cell line. (D) No staining of cytokeratins was observed in the negative control fibroblast culture using the same staining procedures.

ohms per cm<sup>2</sup> on the sixth day after the cells were seeded in a culture insert, suggesting the presence of a normal intact seal of the monolayer.

## Summary

This report focuses on the generation of a complex cilia structure in immortalized cells. Although indistinct cilia movement was once observed with this cell line, the report does not cover the cilia motile function, which will be a topic of future studies. No cilia structure has been generated or reported for immortalized cells until this study, since the first tracheal cell line was obtained 12 years ago. We show here that *E1a* immortalizes the tracheal cells, and gel culture induces these cells to generate the cilia structure containing a 9 + 2 arrangement. Although its utility remains to be fully demonstrated, the cell line with a 9 + 2 cilia structure may provide a basis for future efforts to develop an ideal ciliated cell line(s) with cilia motile function, as well as provide a model system to study both cilia structure differentiation and cilia structural disorders. The fact that we were able to obtain properly structured cilia from our cell line indicates that the immortalization approach and the culture condition used are in-principle appropriate, which may impact related studies such as immortalization of other ciliated cells, generation of cilia in other cell lines, and differentiation of other important cellular structures from immortalized cells, which have so far not proven feasible.

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