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The Great Big Alveolar TI Cell: Evolving Concepts and Paradigms

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Key Words

Type I cell • Alveolar • Lung • Epithelium • Ion transport • Proliferation

Abstract

Pulmonary alveolar type I cells (TI cell) are very large (~5400 μm^2 in surface area) squamous cells that cover more than 98% of the internal surface area of rodent lungs. In the past, TI cells were believed to serve only passive barrier functions, with no active functional properties in the lung. The fairly recent development of methods to isolate TI cells has permitted investigation of functions of this cell type for the first time. Resolvable by electron microscopy, TI cells contain microvilli and organelles typically associated with metabolic functions, such as mitochondria, abundant smooth and rough endoplasmic reticulum and Golgi apparatus. TI cells contain the molecular machinery necessary for ion transport and take up Na^+, K^+, and Cl^-, from which one can infer that it is likely that they play a role in ion and fluid transport *in vivo*. Because the abundance/μm^2 of highly selective Na^+ channels (HSC channels, consisting of all three ENaC subunits) is the same in TI and TII cells and because TI cells cover the majority of the lung internal surface, TI cells may play the major role in bulk transport of Na^+. *In vitro*, TI cells can proliferate and exhibit phenotypic plasticity, raising the question of whether this cell type may play a role in development and lung repair after injury. From gene expression analysis of TI cells, one can infer a variety of other possible functions for TI cells. The development of techniques to administer transgenes specifically to TI cells will permit direct study of this cell type *in vivo.*

Introduction

More than 99% of the internal surface area of the lung resides in the alveolar compartment, which is lined by two types of epithelial cells, called Type I (TI) and Type II (TII) cells. Although TI cells comprise only ~10% of the cells in the lung, they cover ~98% of the internal surface area of the lung, with the remaining ~2% accounted for by TII cells [1]. TI cells are very large squamous cells whose thin (50-100 nm) cytoplasmic extensions form the air-blood barrier essential for normal gas exchange. TII cells are much smaller cuboidal cells that synthesize, secrete, and recycle surfactant components, transport ions, participate in lung immune responses, and function as progenitor cells in response to lung injury. Until fairly recently, the accepted paradigm

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was that TII cells were the "biologically active cells" in the alveolar epithelium, with "terminally differentiated" [2] TI cells playing merely a passive role in forming the air-blood barrier, being incapable of either proliferation or cell plasticity. Because TII cells have been studied *in vitro* since 1974 [3], our knowledge of this cell type is extensive. In contrast, successful isolation of TI cells had to overcome many difficulties, including the need to use EM for cell characterization, the lack of biochemical or molecular tools to isolate or evaluate isolated cells, and the extremely fragile nature of TI cells. Overcoming these difficulties has occurred in a step-wise, incremental fashion, permitting preparation of TI cells in high degrees of purity. From studies of TI cells in vitro in the last few years, it seems likely that the concept of terminally differentiated, biologically inactive TI cells is far too restrictive, and that TI cells may play an extensive role in alveolar functions. Several different lines of evidence support this hypothesis, including studies of gene expression profiling, studies of ion transport, and studies of cell proliferation in TI cells.

Morphologic characteristics of TI cells

Questions have been raised in the literature about whether TI cells display morphologic evidence of microvilli and other subcellular structures necessary for various biosynthetic pathways. These statements may have been based on the fact, as a result of the extremely large size of TI cells and small sampling area of EM, that many published images have included very limited portions of a TI cell. In fact, TI cells have microvilli, as well as all morphologic hallmarks of metabolically active cells, such as mitochondria, abundant Golgi, numerous small vesicles, caveoli, and rough and smooth endoplasmic reticulum (Fig. 1).

Methods of isolating TI cells and brief comparison to the cultured TII cell model system

The method of isolating TI cells is continually evolving, each iteration making incremental improvements. Published methods have all used rat lungs, immuno-selection [4-6], initially with magnetic particles followed by density gradient centrifugation, [5] and more recently by FACS sorting (Fig. 2) [7]. Purities have increased from 60-86% in the initial isolations to > 99% more recently. The development of transgenic mice expressing enhanced green fluorescent protein (EGFP) in TI cells [8] should facilitate isolating TI cells from mouse lungs.

TII cells cultured on flat substrata in the presence of FBS rapidly change their state of differentiation, ceasing to express important markers of the TII cell phenotype, while starting to express some markers associated with the TI cell phenotype. However, freshly isolated and cultured TI cells exhibit many important differences, as well as similarities, in comparison to this cultured TII cell model. These differences can be briefly categorized in relation to ion transport, proliferation, and gene expression profiling.

Ion channels

Physiologic studies in whole animals showed that exogenously-added lung liquid was rapidly cleared from the alveolar space, from which it could be inferred that Na^+ channels with a high conductance and/or larger numbers of channels with a lower conductance were involved in clearance. From studies of ion channels and transport in TII cells cultured on flat surfaces in the presence of FBS (the method that was state-of-the-art at that time), it was believed that TII cells contained non-selective Na^+ channels, composed of the alpha subunit of ENaC alone rather than the higher-conductance HSC channels, composed of all three ENaC subunits. More recent studies have shown that freshly isolated TI and TII cells and TII cells cultured on Matrigel express high levels of HSC channels (for detailed review, see [9]).

Proliferation

Comparing recent studies in our laboratory [7] with previously published reports [10-13], the conditions for supporting proliferation of TI cells are dissimilar from those necessary for supporting proliferation of TII cells, demonstrating another difference in these two systems that is important for designing *in vitro* experiments studying proliferation of alveolar epithelium.

Gene expression profiling

Gene expression profiling is a powerful tool to examine global gene expression of various cell phenotypes. There are almost as many differences in gene expression between cultured TII cells and freshly isolated TI cells as there are between cultured TII cells and freshly isolated TII cells [6]. The stoichiometry of TI cell marker genes is quite different in freshly isolated TI cells and cultured TII cells, with cultured TII cells expressing up to 200 fold more or 80% less of various marker genes

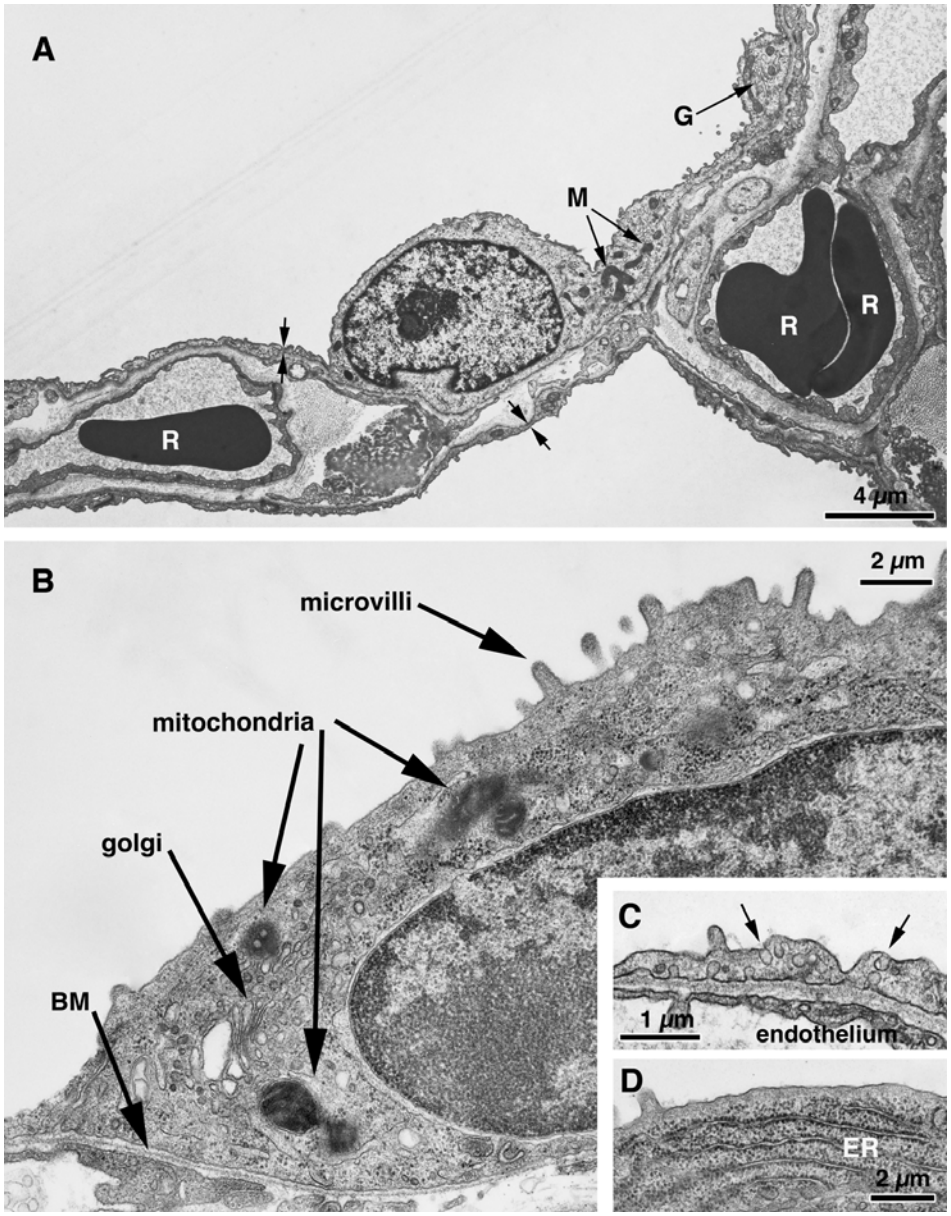

Fig. 1. Electron micrographs of TI cells. Each image contains a labeled micron bar. A) Low magnification transmission EM of alveolar epithelium. Arrows designate the very thin cytoplasmic extensions of the type I cells, which can be seen on both alveolar surfaces. M, mitochondria; G, Golgi; R, red blood cell. B) Higher magnification showing relevant intracellular structures; BM, basement membrane. C) Higher magnification of TI cell extension containing small vesicles and caveoli (arrow). D) This portion of a TI cell shows abundant rough encoplasmic reticulum. Taken from [9, 28].

Table I. The ratios of various parameters between TI and TII cells. Cell surface areas for Sprague-Dawley rats were taken from [1]. The relative abundance of each type of channel was determined by measuring the channel abundance in >500 patch clamp measurements (each ~ 1sq mm) of freshly isolated and briefly cultured TI and TII cells [21]. In that study, the relative densities of both types of Na^+ channels were similar in TI and TII cells, whereas CNG and K^+ channels were present in TI cells (but not detectable under the same experimental conditions in TII cells) and CFTR was more abundant in TII cells than in TI cells. The ratio of channel density was then normalized by relative internal lung surface area covered by each cell type [1, 9].

	TI: TII cell ratio	Refs.
Cell surface area	43	[1]
Osmotic water permeability	7	[5]
Na^+ and K^+ uptake/μg protein	~3	[16]
Apical Na^+ channels/cell	~40	[21]
CNG and K^+ channels	*	[21]
CFTR	6	[21]

*Present in TI cells, not in TII cells

than freshly isolated TI cells.

Taken together, these observations emphasize the need to assess the relative benefits of each in vitro system in order to ascertain the appropriateness of the system to study the biologic topic of interest.

TI cells: roles for ion and water transport

A recent paradigm for fluid transport in the alveolus proposed that Na^+ transport takes place across TII cells, electroneutrality is maintained by Cl^- transport paracellularly or via CFTR in TII cells [14, 15], and water moves across both cell types via aquaporins. For a more detailed description, please see [9]. In the last decade, this paradigm has evolved. TI cells take up Na^+ and Rb^+

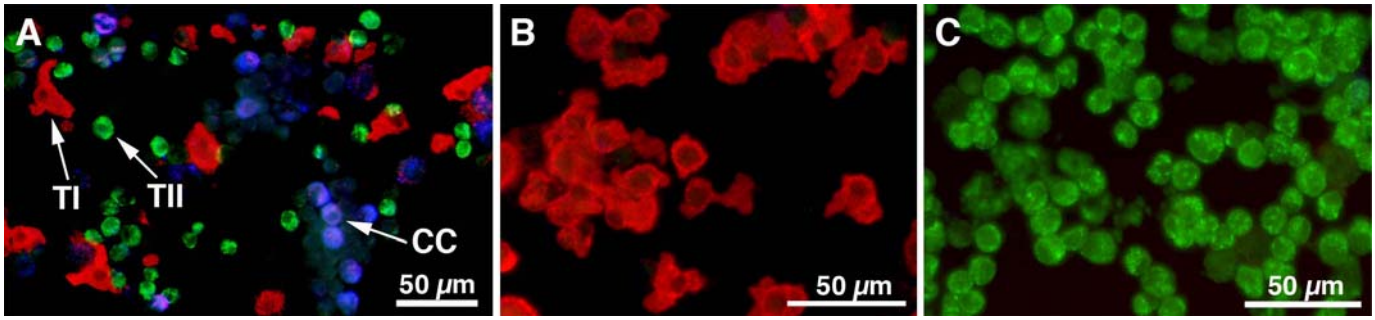

Fig. 2. Isolation of homogenous preparations of TI and TII cells by FACS. All cell preparations were stained for markers respectively for TI cells, TII cells, and Clara cells: RTI40 (red), RTII70 (green), and CC10 (blue). A) The mixed cell preparation resulting from digestion of lung surface cells with elastase prior to FACS sorting showed that all three cell types are present in the initial material. B) FACS sorting for only RTI40, showing purified TI cells (>99% TI cells) with a large surface area and pleomorphic appearance, without TII cells or Clara cells. C) FACS sorting for RTII70, yielding >99% pure population of TII cells, which are smaller and more homogenous in size and shape than are TI cells. (Taken, with permission, from [7]).

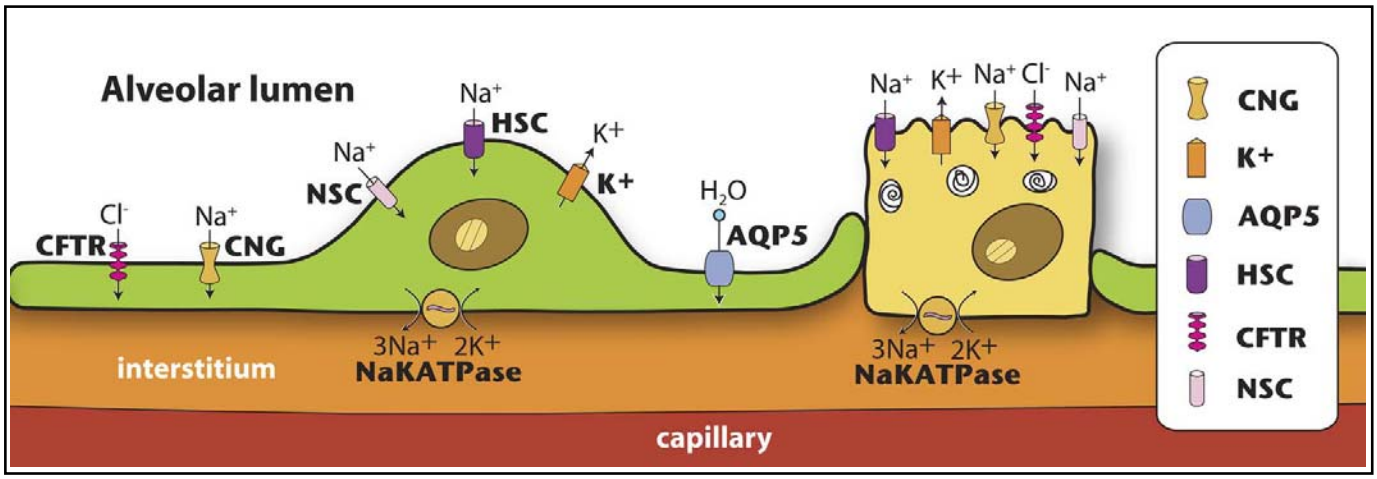

Fig. 3. Cartoon showing the evolving schematic depiction of alveolar ion and fluid transport. Under conditions of net absorption of Na^+, Na^+ is absorbed from the apical surfaces of both TI (indicated by green color) and TII (indicated by yellow color) cells via ENaC (HSC and NSC channels) and via CNG channels in TI cells. Electroneutrality is conserved with Cl^- movement through CFTR in TI and TII cells (and possibly other anion channels) and/or paracellularly through tight junctions. Na^+ is transported from the basal surface of both cell types into the interstitial space by Na^+, K^+, -ATPase. K^+ may be transported from alveolar epithelial cells via K^+ channels located on the apical surface of TI or TII cells. If the directionality of net ion transport is from the apical surface to the interstitium, an osmotic gradient would be created, which would in turn direct water transport in the same direction, either through aquaporins or by diffusion. Conversely, in the formation of alveolar liquid, there may be net Cl^- secretion mediated by CFTR (and possibly other Cl^- channels). Under these conditions, there would be net secretion of Na^+ by unidentified pathways. For details, see text. HSC: highly selective Na^+ channel; NSC: non-selective Na^+ channel; CNG: cyclic nucleotide-gated channel; K^+: potassium channel; CFTR: cystic fibrosis transmembrane regulator; AQP5: aquaporin 5. (Adapted from [21] and [9]).

(a surrogate for K^+) [16], express all three subunits of ENaC, the rat sodium epithelial channel [16, 17], and express α-1-, α-2-, and β-1-Na^+-, K^+-ATPase [16-18]. Electrophysiologic studies of isolated TI cells and lung slices demonstrated the presence of channels characteristic of ENaC (HSC and NSC channels), less specific sodium channels, cyclic nucleotide gated (CNG) channels, K^+ channels, CFTR and other anion channels ([19-21] and Johnson, unpublished data). The ratios between TI and TII cells for various parameters are shown in Table I, from which one can conclude that TI

Initial # cells/well	# wells	# wells containing colonies at 7 days	Total # colonies at 7 days
1	68	34	34
2	96	69	89

Table II. Proliferation of TI cells at very low cell densities. TI cells were plated at very low density and individual wells were inspected for the number of cells adherent in each well every 24 hours. After a 3-day lag period, in which cells adhere to the surface, extend filopodia, and become motile, they begin to proliferate. Each well was then tracked for 7 days and the final number of colonies in each well was recorded [7].

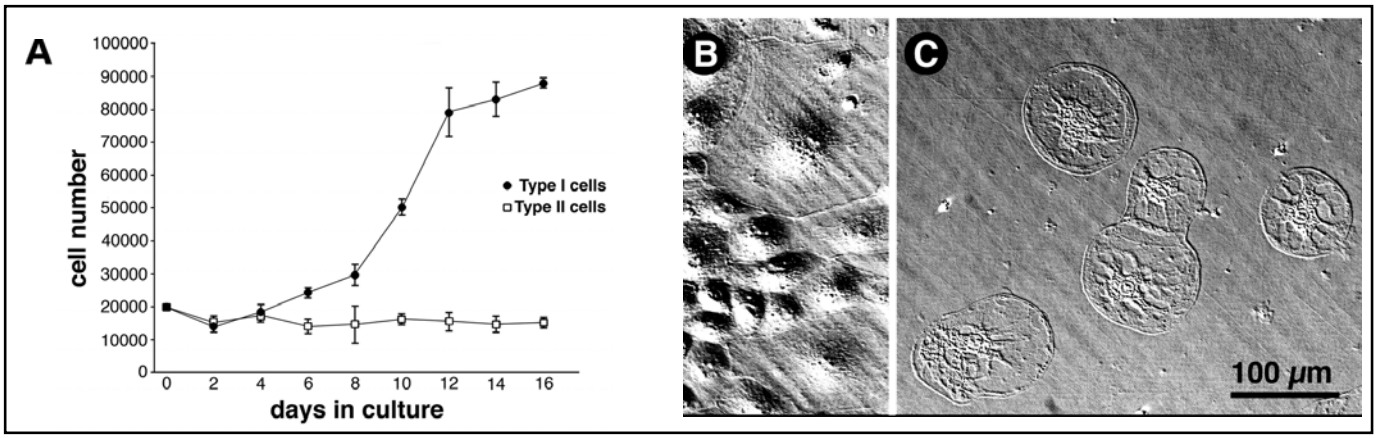

Fig. 4. TI cells proliferate in culture under conditions in which TII cells do not proliferate. Cells were plated at densities of $2x10^4$ cells/transwell with media containing 20% FBS. At various time points, duplicate samples were taken and cells were counted with a hemacytometer. Growth curves of TI and TII cells are shown in (A); TI cells proliferate until they are contact inhibited. Under the conditions of the experiment shown here, the number of TI cells increases ~6-fold. Hoffman modulation contrast optical images of TI cells (B) and TII cells (C) cultured for 7 days. TI cells are very large, pleomorphic cells, that become contact inhibited. TII cells are smaller cells that do not proliferate and assume a flattened, „fried-egg" appearance. Marker bar of 100 µm applies to both B) and C) (from, with permission, [7]).

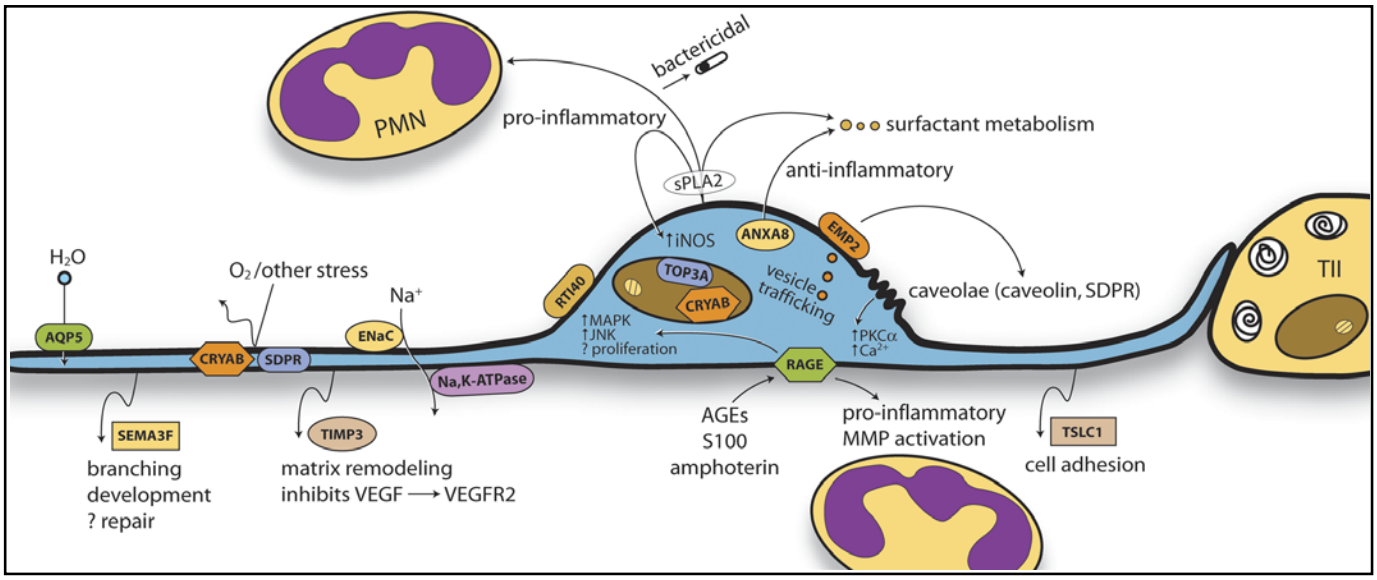

Fig. 5. Cartoon representing known and hypothetical functions of type I cells. Water transport across the apical surface occurs via AQP5 (aquaporin 5); Na^+ and K^+ transport via ENaC and Na^+ ,K^+-ATPase. TIMP3 is critical for maintenance of normal extracellular matrix and in preventing emphysema. CRYAB and SDRP may be important in the defense against oxidative and other stress. TSLC1, a member of the immunoglobulin superfamily, mediates cell adhesion and cell–cell interactions. The function of RTI40 is unknown, but the gene has been shown to be important for normal lung development. RAGE is localized mainly to the basal membrane; the receptor binds various proteins such as AGEs, S100, and amphoterin, resulting in diverse downstream biologic events, such as stimulating MAPK and JNK pathways, proliferation, activation of MMPs, and proinflammatory responses. EMP2 has complex properties, including facilitating protein trafficking, altering adhesion of cells to matrix proteins, and inhibiting caveolin-1 expression. sPLA2 also has many potential functions, including bactericidal and proinflammatory functions, stimulation of intracellular iNOS, and surfactant metabolism. Of the genes shown in this diagram, all with the exception of ENaC and Na^+, K^+, -ATPase are expressed much more in type I cells than in type II cells; ENaC and Na^+, K^+, -ATPase are expressed in both cell types. The diagram is not to scale (from [27]).

cells are likely to play a major role in ion transport.

Ridge and coworkers calculated that α-2-Na^+-, K^+-ATPase, which is expressed in TI cells but not TII cells, is responsible for ~60% of the unstimulated fluid transport in the lung [18]. Taken together, these and other observations have contributed to a revised paradigm, in which both TI and TII cells contribute to ion transport, with TI cells contributing the major portion of bulk Na^+

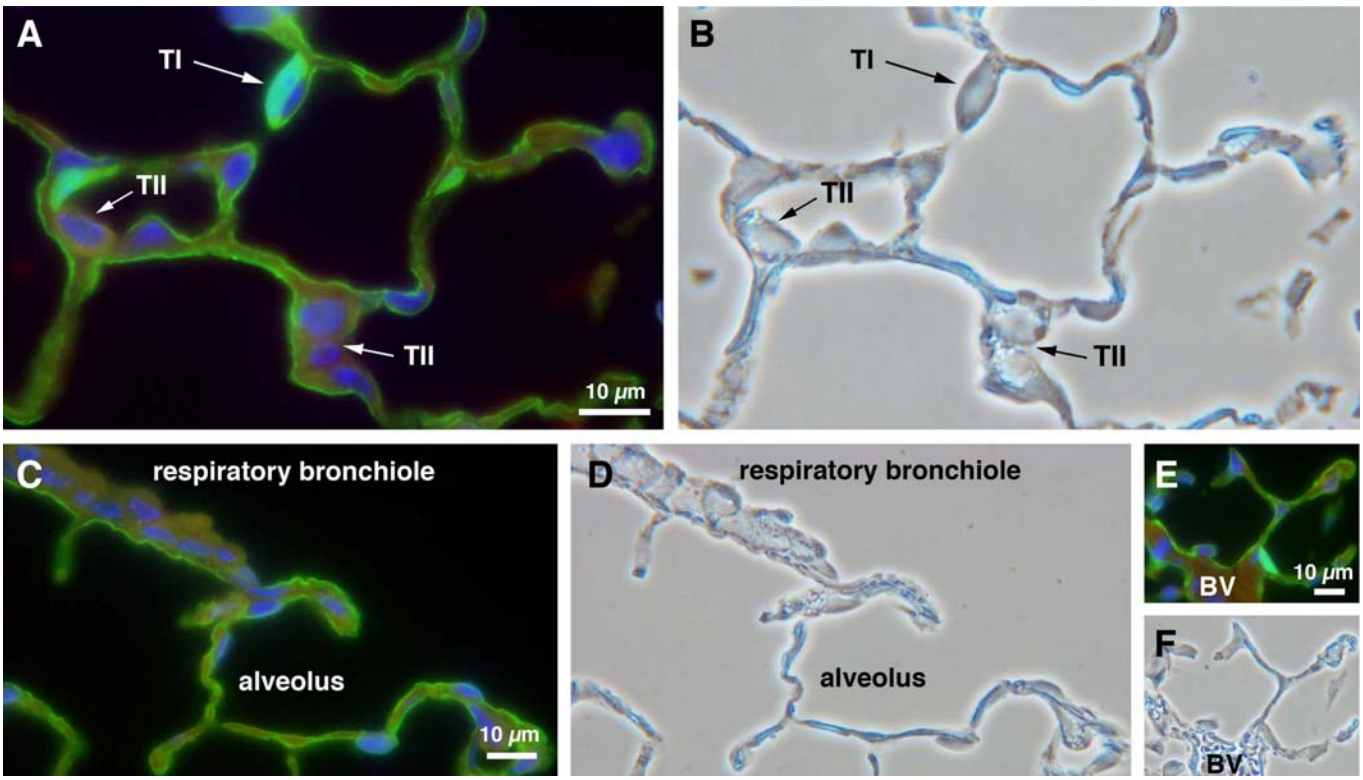

Fig. 6. Expression of EGFP transgene specifically in TI cells. Paired immunofluorescence (A, C, E) and phase contrast (B, D, F) views of 2-3 μm thick cryosections of fixed and cryoprotected lung tissue. RTI40-BAC-EGFP transgenic mice express EGFP (green) throughout the TI cell, including the perinuclear area (A, B; TI arrow) and the very thin cytoplasmic extensions. Nuclei (blue, DAPI stain) are visible in the TI perinuclear areas, where they display a slight shift in color from green to blue-green. A and B) TII cells, indicated by arrows, do not express EGFP. C and D) Respiratory bronchiolar cells do not express EGFP, but at the junction with the alveoli, TI cells express EGFP. E and F) Neither endothelial cells nor red blood cells express EGFP (adapted from [8]).

flux. Cl^- transport may occur both by paracellular transport and by transcellular transport across TI cells. Under conditions of net absorption of Na^+ in which an osmotic gradient is created, there would be passive flow of water via aquaporins (Fig. 3). A more detailed review of the data, analysis, and discussion can be found in [9].

Proliferation of TI cells i*n vitro*

Based on the classic studies of Evans [22] and Adamson [23], who used techniques of autoradiography in oxidant-injured rodent lungs and the analysis of TI cell morphology of TI cells by Weibel, the currently accepted paradigm regarding alveolar epithelial cell lineage and differentiation is that TII cells have the capacity to proliferate and transdifferentiate to TI cells, whereas TI cells are terminally differentiated and do not have the capacity to proliferate. Although the concept that TI cells may proliferate has been suggested [24], it has not gained widespread acceptance.

We have recently found that TI cells proliferate in vitro until they become contact inhibited. In the first few days after they have been placed in culture on fibronectin-coated surfaces in the presence of 20% FBS, they adhere to the surface and start to spread (24-48 h), extend filopodia and become motile, and start to divide (72 h), going through ~5 rounds of division before they become confluent (Fig. 4) [7]. Approximately 50% of cultured TI cells are positive for the proliferation marker Ki67. When the cells are plated to achieve very low densities (1-2/well), ~50% of the cells have the capacity to form colonies (Table II).

Freshly isolated and cultured TI cells express OCT-4A [7], a protein that has been implicated in establishing and maintaining the undifferentiated pluripotent state of stem cells. The significance of OCT-4A expression is ultimately unknown, but OCT-4A is common to two other lung cell populations that have been proposed to act as progenitor cells within the lung [25, 26]. Finally, when proliferating TI cells *in vitro* are removed from fibronectin and recultured on Matrigel in the presence of KGF, the

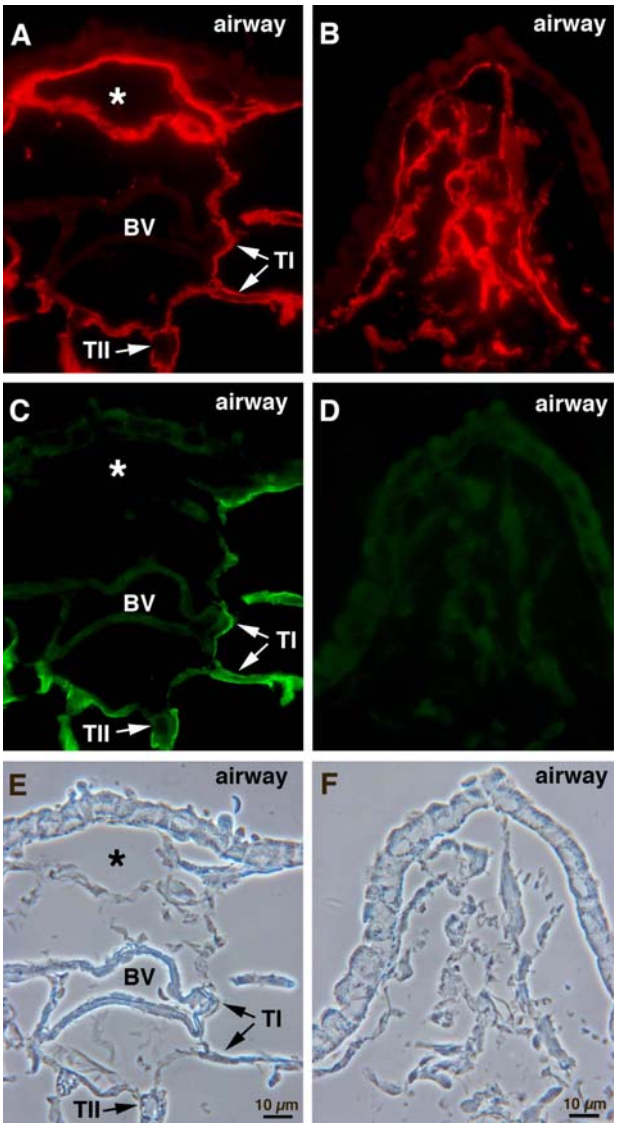

Fig. 7. Expression of the EGFP transgene driven by rat podoplanin BAC promoter is limited within the lung to TI cells, in contrast to expression of endogenous mouse podoplanin, which is expressed in lymphatics as well. Pulmonary expression of the EGFP transgene is more restricted than is the endogenous mouse podoplanin gene. Lung co-stained for mouse podoplanin (red in A and B) and GFP (green in C and D). The corresponding phase contrast images are in E and F. Note extensive mouse podoplanin staining (red) in lymphatic-like structure (asterisk in A) and lymphoid-associated stroma in B. Both are negative for GFP expression. GFP and mouse podoplanin are co-expressed in type I cells within alveoli (A and C); TI, type I cells; TII, type II cells; BV, blood vessel. Scale bars indicate 10 μm. (Taken from [8]).

cells can be induced to express SP-C and CC-10, single markers expressed in the lung by TII cells and Clara cells, respectively [7]. Taken together, these findings in vitro support the concept that, in the lung, TI cells may have the capacity to proliferate and to express at least some markers of other differentiated cell phenotypes and could potentially play a role in lung development and repair after injury. Testing this hypothesis in animal models in which cells can be irreversibly marked will help to answer the question of whether TI cells display these capacities *in vivo*.

Other putative functions of TI cells

Based on results from suppression subtractive hybridization to identify genes differentially expressed in TI cells, we verified differential expression of various candidate genes by immunohistochemistry. Based solely on the expression of these mRNAs, one can speculate about various possible functions of TI cells [27] (Fig. 5).

Transgenic models and future directions

A key biologic question in alveolar research is how do we bridge the gap between studies of animal physiology with those using isolated, cultured cells? One method is to introduce or silence specific genes and proteins selectively at various times in TI or TII cells. Directed expression of transgenes in TI cells has been difficult to achieve. Although transfection studies defined a 1.1-kbp basal promoter for the RTI40 (rat podoplanin) gene, up to 10 kb promoter fragments of either mouse podoplanin or aquaporin-5 have not proven effective in conferring high-level TI cell-specific expression in adult transgenic mice. Based on two lines of reasoning, we elected to use a rat bacterial artificial chromosome (BAC), ~168 kb in size, to direct transgene expression to TI cells. The lines of reasoning were at least twofold: 1) the expression of rat podoplanin was more restricted to TI cells in rat lungs than was the mouse ortholog mouse podoplanin in mouse lungs, which prompted us to use the rat gene; and 2) because we lacked detailed knowledge of the fully functional podoplanin promoter, we used a very large BAC vector containing extensive upstream and downstream sequence. The results of this strategy proved successful [8]. Fig. 6 and 7 show the widespread transgenic expression in TI cells of rat-podoplanin-BAC-EGFP expression, as well as the more restricted expression of the transgene in comparison to endogenous mouse podoplanin. The ability to deliver transgenes to TI cells in a time and spatially-dependent manner will permit the study of either overexpression or ablation of selected transgenes of interest at various points in development or in lung injury.

Summary

From recent experiments *in vitro*, it seems reasonable to reevaluate our concepts about the roles that TI cells may play in the lung. From an initial hypothesis that TI cells served only as a passive barrier in the lungs, without any specific functions, the paradigm has evolved to one in which TI cells may play a major role in lung homeostasis, regulation of ion transport, and possibly contribute both to development and the response to injury. It is likely, based on studies of gene discovery by gene expression profiling, that many more functions of TI cells will be discovered over the next 10 years.

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