Apical Lumen Formation in Renal Epithelia

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ABSTRACT

The ability to form epithelial lumina is a central architectural characteristic of virtually all organs and indispensable for their function. Ontogenetically, the kidney is one of the best-characterized organs, but concepts of the regulated formation of its hollow epithelial structures are still emerging. Epithelial cell lines provide the opportunity to study molecular mechanisms in simplified assays modeling cyst and tube formation. In these systems, several groups have identified molecules implicated in lumen formation, and their downregulation results in either multiple-lumen or no-lumen phenotypes. On the basis of these phenotypes, we propose a working model, assigning proteins to groups with similar functions. Defects within these specific protein groups lead to distinct epithelial phenotypes. Studies of mesenchymal-to-epithelial transition underline the importance of these protein groups, but converting these basic models of lumen formation to an understanding of the mesenchymal to tubule formation during kidney development is still challenging.


Epithelial tubes are essential for the structure and function of many organs. The kidneys are among the best-characterized organ systems in developmental biology. During kidney organogenesis, hollow epithelial structures newly form from mesenchymal cells at several stages. They serve as precursors of virtually all kidney structures, including the glomeruli and tubule systems. Thus, generation of hollow epithelial vesicles and tubes is a hallmark of renal development. The mechanisms underlying the spatially and temporally well-regulated formation of lumina de novo are only beginning to emerge. This review examines current concepts of molecular mechanisms underlying apical lumen formation, which are derived mainly from tissue culture models. These concepts will be applied to mesenchymal-to-epithelial transition (MET) and lumen formation in the developing metanephros in vivo.

TISSUE CULTURE MODELS USED FOR THE STUDY OF LUMEN FORMATION

Tissue culture models are the most simplistic approach to studying the molecular mechanisms underlying epithelial lumen formation. Cell lines used for these studies display strong apicobasal polarity, such as Madin-Darby canine kidney (MDCK) cells, which exhibit characteristics of distal kidney tubules. Epithelial cells grown in monolayers are frequently used as a model for epithelial polarity. Although monolayers allow the analysis of polarized trafficking, lumen formation cannot be analyzed in this model. However, the formation of the apical membrane can be studied in monolayers. The primary model used to characterize the initial stages of polarization is the calcium switch where MDCK cells are placed in low calcium media to disrupt cadherin-mediated intercellular adhesion. The loss of adhesion disrupts apicobasal polarity, but adhesion and polarity can be restored when calcium is added back to the media.

The study of apical membrane proteins during calcium switch has led to the identification of the vacuolar apical compartment (VAC). This compartment is created when cells are kept in low calcium and represents endocytosed apical membrane proteins. When calcium is restored, the VACs are exocytosed to reform the apical membrane. This has led to the conclusion that the apical membrane is formed during polarization by exocytosis of internal membranes. However, VACs have been argued to be an artifact of low calcium manipulation. Fullekrug and coworkers have found in nonpolarized MDCK cells that apical proteins do not enter VACs but rather distribute throughout the membrane and resegregate during repolarization. Thus, controversy continues as to the source of the apical membrane during early cell polarization in monolayers.

This is not the only limitation of the monolayer model of polarization. In a monolayer, the polarity of the cells is pre-
defined with the basolateral side facing the support plastic or matrix, whereas tissue culture media covers the apical pole. Thus, completely characterizing mechanisms underlying initial cell polarization or lumen formation is impossible. Accordingly the study of apical membrane and lumen formation is better approached in three-dimensional culture models, like the cyst or tube formation assay.11

**CYST FORMATION ASSAY**

Embedding of epithelial cells into a homogenous extracellular matrix (ECM) provides them with an indifferent environment without predefined polarity, offering ideal conditions for studying endogenous mechanisms underlying polarization and lumen formation.4,12–15 Lubarsky and Krasnow concluded that lumen formation is always related to the delivery of apical membrane.16 In this model, with MDCK cells, the internal membrane, possibly VACs, are exocytosed to form the apical membrane, which is then followed by lumen formation.17 The next steps involve cell proliferation and formation of a large hollow lumen.18

Formation of solitary lumina depends on the pace of polarization relative to proliferation. Thus, the ECM influences the mechanism of lumen formation. In the presence of laminin, providing a strong basolateral cue, MDCK cells undergo rapid polarization, leading to prompt formation of a solitary lumen.18–21 MDCK cells growing in a collagen monoprotein matrix polarize more slowly but proliferate at the same speed, resulting in the trapping of cells within the central lumen. Subsequently, a single central lumen is cleared through apoptosis as a corrective mechanism.18

Numerous apically localized proteins play an important role in the proper establishment and maintenance of the apical lumen. Simons and coworkers performed extensive knockdown studies of apical membrane proteins in MDCK cells and divided the resulting phenotypes into multiple-lumen versus a no-lumen phenotypes.22 Other authors made similar observations in RNA interference (RNAi) knockdown or protein inhibition studies, although the distinct phenotypes often have not been quantified.3–5,10,23–25 Although the molecular functions of many of these proteins have been well characterized, the mechanisms underlying these phenotypes remain unclear.

**CELL POLARITY AND APICAL POLARITY PROTEINS**

Apical membrane and lumen formation are tightly coupled to apicobasal polarity. Upon polarization, vertebrate epithelial cells form tight junctions (TJs), separating an apical from a basolateral membrane domain, forming a tight seal between neighboring cells and thus regulating paracellular permeability.26,27 Two highly conserved apical protein complexes and one basolateral complex seem to complement each other to establish apicobasal polarity.28 The apically located Crumbs (Crb) complex consists of the transmembrane protein Crb, interacting through its C-terminal cytosolic tail with Protein Associated with Lin Seven One (PALS1), which itself interacts with the PALS1-associated TJ protein (PATJ).29 The second apical Par complex consists of Partitioning defective protein 3 (Par3), Par6, and atypical protein kinase C (aPKC).27 These apical polarity complexes are able to physically interact.30,31

Crb3 is the major epithelial form of the three mammalian Crumbs isoforms and is expressed in mammalian kidney tubules.27,32 It is a small transmembrane protein that localizes to the apical membrane domain, whereas the other members of the Crb complex localize predominantly to the TJ. The basolateral Scribble complex consists of Scribble, Discs large, and Lethal giant larvae.33 Cell adhesion is mediated by adherens junctions located basally of the TJ. The function of the polarity complexes and the initial establishment of cell junctions and polarity are reviewed elsewhere in detail.26,27 We and others have conducted experiments to characterize the role of these proteins in lumen formation. The results are summarized in the following section.

**MOLECULAR MECHANISMS OF KIDNEY EPITHELIAL LUMEN FORMATION AND MAINTENANCE**

Lumen formation is a complex process relying on cellular machineries that have to function in parallel. On the basis of the finding that lumen formation is closely connected with apicobasal polarity and junction formation, we postulate the following working model: To form a solitary lumen, cells must polarize and define the apical membrane where the lumen is to be formed, maintain the correct positioning of the apical membrane, and open up the lumen. To fuel these processes, cells must possess a polarized trafficking machinery to deliver the constituents involved in these processes. Interference with any of these processes leads to a specific phenotype in the cyst or tube formation assay (Figure 1).

**DEFINING THE APICAL MEMBRANE: CRUMBS PROTEINS**

Crb is a highly conserved apolarity protein that is expressed in a large variety of tissues.27,34 Overexpression of Crb proteins leads to an increase in size of apical structures in Drosophila.35 In MDCK cells, excessive Crb3 levels delay the TJ formation after the calcium switch,3 but moderate overexpression results in expansion of the apical domain during cyst formation. (Margolis and Schlüter, unpublished observations). MCF10A cells, nontumorigenic mammary epithelial cells, express low Crb levels and do not form TJs. Exogenous Crb3 expression induces these cells to form distinct apical and basolateral domains and TJs.36 Thus, Crb3 possesses a defining role for apical membrane identity. Accordingly, its RNAi-mediated knockdown causes a predominant no-lumen phenotype in MDCK cells, relocating the actin meshwork to the basal side of the
cells. Residual small lumina with proper polarization probably result from cysts with an incomplete Crb3 knockdown (Margolis and S. Fan, unpublished observations). Because Crumbs defines the apical membrane where the lumen will be formed, complete downregulation of Crumbs genes should result in total absence of apical membrane and complete loss of lumen formation (Figure 1B).

**Figure 1.** Phenotypes in the MDCK cyst formation assay derived from RNAi studies of proteins’ participation in epithelial lumen formation. (A) Regular cyst formation. When embedded into ECM, single cells divide and form small clusters of cells. Apical membrane (green), initially facing ECM at the one-cell stage, is taken up and transported in a polarized fashion to the cell side facing away from the ECM. Here, it defines the site where the lumen is to be formed. (B) No-lumen phenotype. In the absence of molecules mediating apical membrane identity, the missing apical membrane leads to impaired targeting of molecules involved in opening up the lumen, as seen in a Crb3 RNAi knockdown. Similarly, the absence of molecules mediating membrane detachment may lead to a similar phenotype. (C) Multiple-lumen phenotype. Dysregulation of molecules involved in the maintenance of apicobasal polarity and tight junctions (red) lead to an improper delineation of apical membrane, resulting in the formation of multiple lumina. Disruption of single apical trafficking pathways is likely to lead to either a no- or multiple-lumen phenotype, depending on the transported cargo.

**Figure 2.** MET and lumen formation in the metanephric mesenchyme in vivo. In a first step, Wnt9b from the ureteric bud and autocrine stimulation with Wnt4 induce the metanephric mesenchyme to initiate MET, downregulating EMT transcription factors. Expression of cadherin molecules promotes adhesion and cell aggregation. In a second step that requires silencing of Wnt signaling and possibly involves downregulation of additional EMT transcription factors, induced cells likely start to express Crb and other polarity proteins. This initiates the establishment of a solitary lumen, leading to the formation of the renal vesicle.

**Figure 3.** MET and lumen formation in the MDCK cyst formation assay derived from RNAi studies of proteins’ participation in epithelial lumen formation. (A) Regular cyst formation. When embedded into ECM, single cells divide and form small clusters of cells. Apical membrane (green), initially facing ECM at the one-cell stage, is taken up and transported in a polarized fashion to the cell side facing away from the ECM. Here, it defines the site where the lumen is to be formed. (B) No-lumen phenotype. In the absence of molecules mediating apical membrane identity, the missing apical membrane leads to impaired targeting of molecules involved in opening up the lumen, as seen in a Crb3 RNAi knockdown. Similarly, the absence of molecules mediating membrane detachment may lead to a similar phenotype. (C) Multiple-lumen phenotype. Dysregulation of molecules involved in the maintenance of apicobasal polarity and tight junctions (red) lead to an improper delineation of apical membrane, resulting in the formation of multiple lumina. Disruption of single apical trafficking pathways is likely to lead to either a no- or multiple-lumen phenotype, depending on the transported cargo.

The boundaries of the apical and basolateral membrane domains are maintained by two mechanisms: spatial exclusion and physical separation. Phosphorylation-dependent signaling mechanisms mediate the apical exclusion of lateral proteins and vice versa, resulting in distinct apical and basolateral membrane domains. For example, aPKC at the apical membrane phosphorylates Lethal giant larvae and Par1, excluding both from the apical membrane. At the basolateral side, Par3 is phosphorylated by Par1, facilitating Par3 binding to 14-3-3 and sequestering it from Par6 and aPKC. Thus, the assembly of the Par complex is spatially restricted to the apical membrane. On the boundary between both domains, the TJ forms to re-
strict the flux of membrane components between the compartments.26

On the molecular level, several transmembrane proteins, Claudin, Occludin, and junctional adhesion molecules (JAMs) and cytosolic proteins such as PATJ, the Par complexes, and the Zonula Occludens (ZO) proteins constitute a dense protein meshwork. This network provides a connection to the cytoskeleton and initiates signaling pathways involved in the maintenance of cell polarity, proliferation, and differentiation.26,27 The role of the TJ proteins in lumen formation has been studied by several groups, but phenotypes have rarely been quantified as no-lumen or multiple-lumen phenotypes.

Our laboratory has extensively studied the role of the Crb/PALS1/PATJ complex in apical membrane and lumen formation: In the MDCK cyst assay, knockdown of either PALS1 or PATJ, which both associate with Crb3 at the TJ, does not completely abolish apical membrane and lumen formation but results in a predominantly multiple-lumen phenotype with many small lumina.4,5 Crb3 expression is retained in both cases, although reduced in the PATJ knockdown, underlining the presence of membranes with apical characteristics. Similar results are obtained for members of the Par complex: Overexpression of a Par3 mutant incapable of interacting with 14-3-3 results in a multiple-lumen cyst phenotype with many small lumina.4,5 Crb3 expression is retained in both cases, although reduced in the PATJ knockdown, underlining the presence of membranes with apical characteristics. Similar results are obtained for members of the Par complex: Overexpression of a Par3 mutant incapable of interacting with 14-3-3 results in a multiple-lumen cyst phenotype. Inhibition of aPKC yields a similar phenotype, pointing out a regulatory role of the Par complex.25,42

Data on the TJ transmembrane proteins are more sparse: Knockdown or overexpression of dominant interfering mutants of JAM-A resulted in defective membrane formation or undergo apoptosis.23 but experiments testing the major structural components Occludin and Claudin have not yet been carried out in this context and would be interesting, because interference with Occludin and Claudin function leads to changes in cell adhesion and paracellular permeability, respectively.26 ZO-1 to -3 are all involved in TJ assembly,26 and knockdown of ZO-1 interferes with MDCK cyst formation.24 The transcription factor ZONAB and the heat-shock protein Apg-2, which have roles in cell cycle progression and proliferation, compete for the same binding site on ZO-1. RNA interference with both proteins impairs regulated cyst formation.24,43

We speculate that interference with the cytoplasmic TJ protein network is a primary cause of the multiple-lumen phenotype often seen in cyst assays. We hypothesize that defects in TJ signaling lead to an inability to aggregate and segregate apical membrane, and this failure results in the formation of multiple ectopic lumina (Figure 1C). Another model was suggested by Mostov and coworkers: They propose that epithelial cells possess the ability to sense the presence of three different surfaces: a basal surface through interaction with ECM proteins, a lateral surface adhering to neighboring cells, and an apical or luminal surface lacking interactions with ECM proteins. Cells lacking one of the surfaces would either try to create the missing surface through apical membrane formation or undergo apoptosis. This process is repeated until a cyst possesses a solitary lumen and a single-layered wall.11 Because the TJ proteins mediate adhesion to neighboring cells, regulate apicobasal polarity, and play a role in cell cycle control and proliferation,24 it is conceivable that they are part of a suggested sensing system. Thus, knockdown of these proteins leads to impaired surface sensing, resulting in reduced surface formation or apoptotic removal of obstructing cells.

OPENING UP THE LUMEN

Mostov and coworkers suggest that the opening of the lumen might require membrane detachment caused by steric repulsion through large transmembrane glycoproteins.11 A candidate is Podocalyxin (also known as gp135), initially identified as the major podocyte sialoglycoprotein. It is expressed in various cells, including MDCK cells and kidney tubules at lower levels, where it localizes to the apical poles.44–50 Due to heavy glycosylation, the molecule carries a highly negative surface charge and confers antiahesive properties to MDCK cells.44,50–52 Podocalyxin interacts with NHERF-2, linking it to the actin cytoskeleton.53–56 Podocalyxin knockdown in MDCK cells impairs lumen formation.10 Others failed to reproduce this, although demonstrating a role during tube formation after human growth factor stimulation, where lumina are formed de novo from chains of cells.50 It is also interesting to note that Podocalyxin knockout mice do not display a tubular phenotype, but this may be due to possible functional redundancy of other glycoproteins.57 It should also be noted that in addition to detaching opposing apical membranes epithelial cells also secrete fluid to open the lumen. This can occur through transport and exocytosis of fluid-filled vesicular structures, including VACs (see above).17,58 “Transcellular NaCl and water transport also enhances lumen formation.”59,60

GETTING THE ACTORS ON STAGE: THE APICAL TRANSPORT SYSTEM

Apical trafficking of proteins is equally important for lumen formation. Delacour and Jacob comprehensively reviewed current concepts of apical transport.7 Briefly, heterogeneous sorting signals mediate apical protein delivery. These include glycosylphosphatidylinositol (GPI) anchorage, N- and O-glycosyl chains,61 and structural features in the transmembrane and cytoplasmic domains. Several sorting mechanisms function in parallel. Lipid rafts are cholesterol- and sphingolipid-rich membrane microdomains formed in the trans-Golgi network, and they transport their cargo on routes distinct from lipid-raft-independent mechanisms.62 A subpopulation of apical proteins, among these GPI-anchored proteins, is recruited to sites in the trans-Golgi network that exclude basolateral proteins. Apical receptors interact with apical proteins through these sorting signals, linking them to the apical sorting machinery. Among these, VIP17/MAL is found in lipid rafts and is involved in apical protein delivery of GPI-anchored proteins. VIP17 knockdown in

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**BRIEF REVIEW**

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**Apical Lumen Formation**

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MDCK cysts results in a multiple-lumen phenotype. The lipid-raft-associated Annexin-13 has two functionally distinct isoforms both involved in apical transport. In contrast to other studied trafficking proteins, combined knockdown of both isoforms resulted in a no-lumen phenotype. Galectin-3 mediates raft-independent apical trafficking and interacts with several apical glycoproteins. Its inhibition results in basolateral mis-targeting of its apical binding partners. In vivo, Galectin-3 has been associated with kidney differentiation and cystic disease. Depletion of Galectin-3 in MDCK cells causes a multiple-lumen phenotype in the cyst formation assay, implicating Galectin-3 in the transport of proteins with functional relevance for lumen formation.

A MODEL FOR EPITHELIAL LUMEN FORMATION

Decreasing expression of certain apically trafficked proteins leads to either a no-lumen or a multiple-lumen phenotype during cyst formation, but the mechanisms underlying these phenotypes remained unclear. After reviewing the current data on lumen formation, we postulate the following hypothesis (Figure 1): Proteins involved in the definition of apical membrane lead to a no-lumen phenotype, as shown for Crb3, because the lumen will only form after establishment of an apical cell pole. Proteins involved in opening of the lumen may interfere with lumen formation as well, although the importance of this is still under dispute. Knockdown of proteins regulating TJ formation does not generally interfere with the ability to form apical membrane, but cells fail to appropriately limit the dimensions of the lumen due to incorrectly located TJs, ultimately yielding multiple ectopic lumina. To fulfill their tasks, the protagonist proteins in lumen formation have to be transported to their proper locations. The trafficking machinery comprises various mechanisms targeting each protein to its destination, but exact mechanisms involved in apical trafficking are only emerging.

The RNAi-mediated inhibition of trafficking proteins results in either no-lumen or multiple-lumen phenotypes, and we speculate that the phenotype ultimately corresponds to the loss-of-function phenotype of the transported cargo. Thus, interference with a trafficking route involved in Crb3 trafficking should lead to a no-lumen phenotype, whereas inhibition of a pathway transporting TJ regulatory proteins would lead to a multiple-lumen phenotype.

Still a mystery is the order of these events during lumen initiation. What proteins initially demarcate the apical membrane to allow transport mechanisms to define and reinforce the apical domain? In a uniform ECM, nonpolarized epithelial cells initially aggregate via cadherins. Our own unpublished studies show an overlapping aggregation of Crb3 in the initial area of cell adhesion, which may demarcate a nascent but immature apical surface. Increasing amounts of apical proteins in this area suggest polarized transport of apical membrane already at this stage. We theorize that exclusion of basolateral proteins from the nascent apical domain leads to accumulation of basolateral proteins at the opposite pole of the cell, where thereafter apical proteins might be excluded, generating two distinct membrane domains. They are finally separated and fortified by a newly established TJ. We hypothesize that failure to segregate apical and basolateral membranes at this early point, as might be seen due to defects in polarity proteins such as Par3 or PALS1, sets the stage for a multiple-lumen phenotype.

DOWNREGULATION OF EMT TRANSCRIPTION FACTORS INDUCES MET

Recent research led to the identification of factors that regulate the expression of epithelial proteins during epithelial-to-mesenchymal transition (EMT) and MET. The Snail transcription factors directly suppress transcription of epithelial proteins such as E-cadherin by binding to E-box motifs in their promoters, allowing the cells to acquire a mesenchymal phenotype. The Snail transcription factors play a highly conserved role during mesoderm formation.

LUMEN FORMATION DURING KIDNEY ORGANOGENESIS

Tissue culture models provide insights into molecular mechanisms underlying lumen formation. However, considering how these mechanisms relate to the development of epithelia within the kidney is important. Critical to this process is the development of epithelia from mesenchyme.
Snail1 and Snail2 (Slug) are upregulated upon differentiation of the intermediate and lateral mesenchyme. Their expression is lost in the newly forming renal epithelia, allowing expression of E-cadherin and indirectly Cadherin-16, resulting in the formation of pretubular aggregates. Thus, Snail genes play a role during kidney development, acting as switches between either an epithelial or mesenchymal phenotype. Additionally, several other EMT transcription factors are likely to take part in this process.

Upon adhesion, the pretubular aggregate cells establish apicobasal polarity. In mature kidney tubules, the lumen is bordered by the apical membrane, which is separated from the basolateral membrane by continuous TJ strands. EMT factors negatively regulate the expression of TJ components, as shown for Occludin, several Claudins, and ZO-1; thus, EMT and most likely MET events seem to be mediated through regulated expression of adherens and TJ components. Recently, our group demonstrated that Snail also represses Crb3 expression and that the polarity proteins PALS1 and PATJ are partially downregulated. Thus, expression of Crumbs, polarity, and TJ proteins are almost certainly upregulated as the cells advance from the mesenchymal aggregate stage to fully polarized epithelia.

The exact factors that mediate the progression from loose mesenchyme to aggregate mesenchyme to epithelia are just being defined. A connection between Wnt signaling and the expression of EMT transcription factors has been shown, because Wnt signaling regulates Snail1 and Snail2 transcription and Snail1 stability and function in different tissues. However, because cadherins, Crumbs, and TJ proteins are all regulated by Snail proteins, it is not clear if they are induced all at once or in sequential fashion as the mesenchyme progresses to epithelia. There are many other transcription factors such as Twist and zinc finger E-box binding proteins that can also repress E-cadherin and Crumbs that may have a role in spatially defining expression of cadherins versus polarity proteins.

Several groups have presented microarray data on gene expression of different tissues during kidney organogenesis in vivo and organ culture. Some of these studies compare gene expression at different stages of metanephric mesenchyme development; however, no significant differences in proteins associated with cell polarity or lumen formation were found. Gene arrays of the whole metanephric mesenchyme only provide pooled gene expression data of all included cell types and lack information about spatial expression. None of these studies compares protein expression of isolated renal vesicles to the surrounding metanephric mesenchyme, an approach that might be more informative in identifying MET and cell-polarity-associated proteins in vivo. Furthermore, these studies neither examined protein levels, which can differ from mRNA levels, nor post-translational modifications. Accordingly more work needs to be done to understand the molecular mechanisms underlying the progression from mesenchyme to luminal epithelia.

CONCLUSIONS

In this review, we have surveyed the factors that regulate apical lumen formation and lead to apicobasal polarity of epithelia. We understand that proteins such as Crumbs and cadherins initiate these processes and that in the developing kidney these regulatory proteins are de-repressed in the mesenchyme, leading to the transition to epithelia. The exact molecular mechanisms whereby the induction of these proteins can lead to the generation of a distinct apical surface and lumen formation are still being elucidated. However, good tissue culture models of apical membrane and lumen formation exist, and they will provide the tools to further understand this process.

NOTE ADDED IN PROOF

Recent work has demonstrated a role for Cdc42, a small GTPase involved in cell polarity, in the regulation of the axis of epithelial cell division and the generation of a multiple lumen phenotype.

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DISCLOSURES

None.

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