Investigating the Effects of ZBP1 KD and Cdc42 Inhibition on Epithelial Cyst Morphogenesis

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Abstract

Cell division control protein (Cdc42) is a Rho-protein that regulates signaling for pathways involved in maintaining epithelial cell polarity and morphogenesis. The exact molecular mechanisms involving Cdc42 are not clear. In this project, we used Madin-Darby Canine Kidney (MDCK) cells with and without knockdown of ZBP1, or zipcode-binding protein-1, in an attempt to more clearly define the role Cdc42 plays in 2D and 3D cell cultures, particularly in regulating the Cdc42-aPKC-Par complex and cytoskeletal proteins such as actin and alpha-tubulin.

Both *in vivo* and 3D cultures of epithelial cells show cyst formation. Cysts characteristically have an apical, basal, and lateral surface while serving as barriers for anatomical organs. Understanding how this organization is regulated is vital for elucidating the mechanisms responsible for proper organogenesis. ZBP1 is known to play a role in localizing mRNA transcripts so proteins are synthesized in the proper location. Our lab has seen perturbation in cyst morphology following the knockdown of the ZBP1 protein. By using a Cdc42 inhibitor, we further categorize the effect that the loss of ZBP1 has in maintaining the 3D morphology in MDCK cells. Using immunofluorescence imaging, we show that active Cdc42 and ZBP1 expression is required for 1) the correct morphological organization in MDCK cysts, 2) proper localization of the lateral and apical domains, and 3) correct mitotic spindle orientation. The overall conclusion of this work provides evidence that Cdc42 expression is required for epithelial morphogenesis, as its inhibition leads to a similar phenotype such as ZBP1 KD. We had planned to develop a bioreporter for investigating active Cdc42 using live-cell imaging model, but this research was halted prematurely due to the COVID-19 pandemic.

Introduction

Proper polarity in epithelial cells is important in all organisms possessing multiple organs; epithelial cells form lumen in internal organs and are present in the integumentary system as well. Errors in forming and maintaining proper junctional contacts may result in improper permeability of membranes, as well as disturbance in organogenesis.

We are interested in investigating the morphogenesis mechanism in MDCK cells via observing mRNA translation and localization. In mice, ZBP1 KD has resulted in severe deformity in organ size, shape, and function. Interestingly, our lab has described how ZBP1 dependent localization of the beta actin mRNA to adherens junctions complex is required to stabilize the cell-cell adhesion. Furthermore, disrupting this localization event lead to delayed junction formation and loss of epithelial barrier function. However, investigating the role of ZBP1 in establishing epithelial morphogenesis still remains largely unknown.

Many proteins are involved in both forming and mediating the junctions between epithelial cells. In the MDCK model, we hope to probe the mechanisms that are involved in polarity and cyst structural integrity by using molecular biological techniques such as immunofluorescence in a ZBP1 KD cell line. A small Rho GTPase, Cdc42 is a member of the Cdc42-aPKC-Par complex, which localizes to the apical domain as polarity is established during cyst formation. Observing the molecular interactions between these molecules in relation to the effects of mislocalization of beta actin will provide a better understanding of how microtubule stability, and thus, polarized cell behavior, is regulated. Previous studies have shown that communication between Rho GTPases and microtubule dynamics extends both ways. Understanding the consequences of these associations through mitotic spindle orientation is crucial for investigating epithelial tissue formation.

Methods

Spindle Orientation Components

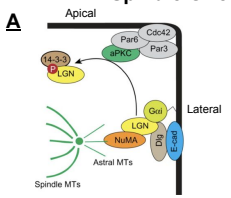

Figure 1. (A) As cells undergo mitosis, spindle microtubules are regulated by various complexes. One of these complexes is the Cdc42-aPKC-Par complex, which localizes near the apical domain in a cyst.

Epithelial Cell Polarity and Junction

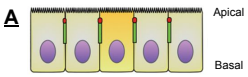

Figure 2. (A) Epithelial cells, such as MDCK cells, have a polarity, as shown. MDCK cells grow in both 2D and 3D cultures, which allows investigation of polarity mechanisms. Many types of normal epithelial cells will die without those cell-cell contacts and growth factors created by surrounding cells.

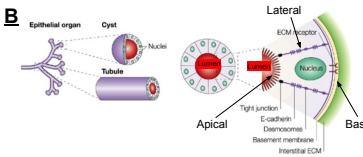

Figure 2. (B) An epithelial organ consists of cells in a cyst or tubule structure. A closer look at the proteins and junctions between cells shows the polarity and characteristic localization that results in the 3D shape. Lateral junctions are made of β-catenin and E-cadherin, whereas tight junctions consist of ZO-1, with the localized apical marker GM130.

Results

Lateral and Apical domains are disturbed in ZBP1 KD cells

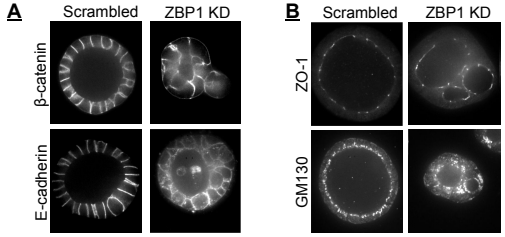

Figure 3. (A, B) Different proteins are shown using IF in both the Scrambled and ZBP1 KD; these proteins are involved in maintaining the integrity of the cytoskeleton and cyst structure. Unlike the ZBP1 KD cysts, the Scrambled control has the single-cell, single-lumen phenotype without any cells seen in the central lumen.The "messy" appearance of the ZBP1 KD cysts seem to correspond with the mitotic spindles being misoriented, which occurs by the process in Fig. 4.

Spindle misorientation disrupts epithelial tissue formation

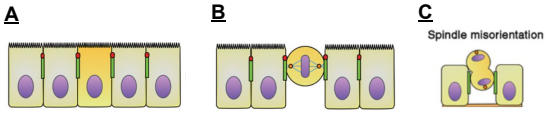

Figure 4. (A) As a cell prepares to divide, (B) the metaphase plate should point toward the center of the cyst so as cells divide, a single-layer forms. (C) Spindle misorientation results in cells sitting on top of each other in a multi-layer.

ZBP1 KD leads to the misorientation of mitotic spindles

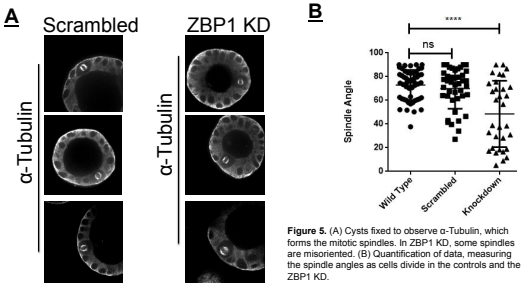

Figure 5. (A) Cysts fixed to observe α-Tubulin, which forms the mitotic spindles. In ZBP1 KD, some spindles are misoriented. (B) Quantification of data, measuring the spindle angles as cells divide in the controls and the ZBP1 KD.

Cdc42 inhibition leads to a ZBP1 KD-like phenotype

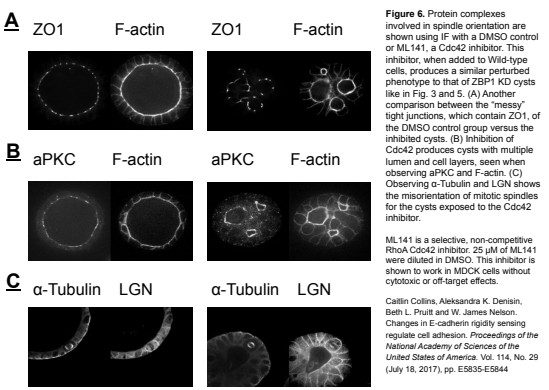

Figure 6. Protein complexes involved in spindle orientation are shown using IF with a DMSO control or ML141, a Cdc42 inhibitor. This inhibitor, when added to Wild-type cells, produces a similar perturbed phenotype to that of ZBP1 KD cysts like in Fig. 3 and 5. (A) Another comparison between the "messy" tight junctions, which contain ZO1, of the DMSO control group versus the inhibited cysts. (B) Inhibition of Cdc42 produces cysts with multiple lumen and cell layers, seen when observing aPKC and F-actin. (C) Observing α-Tubulin and LGN shows the misorientation of mitotic spindles for the cysts exposed to the Cdc42 inhibitor.

ML141 is a selective, non-competitive RhoA Cdc42 inhibitor. 25 μM of ML141 were diluted in DMSO. This inhibitor is shown to work in MDCK cells without cytotoxic or off-target effects.

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Results

Development of bioreporters for Cdc42 and α-Tubulin for use in live-cell imaging

From "PTEN-mediated apical segregation of phosphoinositides controls epithelial morphogenesis through Cdc42": "Fig. 5 (B) GFP-Cdc42 distribution in mature cysts. GFP-Cdc42 cells were grown for 5d and stained for nuclei (blue) and actin (red). Bottom-right panel shows the magnification of the indicated region of the merged panel (bottom left). Arrowheads indicate colocalization of GFP-Cdc42 and actin at the AP PM. Scale bar, 10 μm in upper-right panel and 2 μm in the bottom-right panel."

Martin-Belmonte F, Gassama A, Datta A, et al. PTEN-mediated apical segregation of phosphoinositides controls epithelial morphogenesis through Cdc42. *Cell*. 2007;128(2):383-397. doi:10.1016/j.cell.2006.11.051

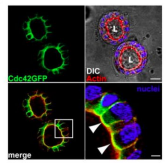

Figure 7. We intended to observe the morphological changes as cyst formation occurs by tracking Cdc42 and α-Tubulin activity. Spindle orientation and any "wobble" would be tracked with these fluorescent bioreporters. This aspect of the project could not be completed due to the COVID-19 pandemic suspending lab work. The image is from an article published in *Cell* (cited above with its description). For Wild-type cysts, our Cdc42 bioreporter would likely produce images such as in the top left panel. ZBP1 KD cysts would have perturbation in morphology, possibly due to mislocalization of Cdc42.

Conclusions

- Immunofluorescence imaging allows observation of mRNA localization, which lets adherens and tight junctions form according to the established cell polarity.
- Post-transcriptional mRNA localization is important in development and morphology.
- ZBP1 KD perturbs MDCK cyst morphogenesis, as seen with multiple lumen and multi-layers forming due to increased mitotic spindle misorientation.
- ZBP1 is required for localization of certain mRNA transcripts, as well as lateral and apical domain organization, which determines polarity.
- Cdc42 is a member of the Cdc42-aPKC-Par complex and defines the apical domain in cyst morphology.
- Inhibition of Cdc42 activity using ML141 has a negative effect on junctional assembly and exhibits a similar phenotype to that of ZBP1 KD cysts.
- Active Cdc42 is critical for establishing proper spindle orientation and cell polarity.

Future Plans

- We were in the process of developing a Cdc42 and α-Tubulin fluorescent bioreporters to track activity during live-cell microscopy of ZBP1 KD and Wild-type cyst development.
- Perturbing other aspects of cyst formation via signaling pathways may yield more knowledge regarding how microtubule stability and organization is controlled by other protein complexes.
- To determine the mechanism by which polarity is perturbed during cyst formation, we plan to further investigate the protein complexes associated with mitotic spindle orientation.

Acknowledgments and References

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