PERSPECTIVE

Tunneling nanotubes and actin cytoskeleton dynamics in glaucoma

Glaucoma and the actin cytoskeleton: Glaucoma is an optic neuropathy, with pathophysiological changes affecting anterior and posterior tissues of the eye. The trabecular meshwork (TM) in the anterior segment regulates intraocular pressure (IOP), while photoreceptors in the posterior retina convert light into signals that retinal ganglion cells (RGC) transmit to the brain. The TM is a small, fenestrated tissue located in the anterior chamber angle, between the iris and cornea (**Figure 1A1**). In humans, the majority of aqueous humor fluid drains through the TM into Schlemm's canal. If the outflow channels become blocked, as in glaucoma, IOP starts to increase, pushing the lens and vitreous back onto the optic disk. Pressure-induced damage to the optic nerve head causes a progressive loss of RGCs and their axons, which leads to irreversible blindness. Surgical or pharmacological management of IOP prevents RGC damage in glaucoma patients. Standard pharmacological therapies either reduce production of aqueous humor fluid, or increase aqueous drainage via the TM or the uveoscleral outflow pathways. Recently, a new class of glaucoma therapies targeting the actin cytoskeleton were approved by the Food and Drug Administration. These are known as the Rho kinase inhibitors, and they act on the Rho/Rho-associated protein kinase signaling pathway to disassemble actin stress fibers in TM and Schlemm's canal cells (Rao et al., 2017; Lin et al., 2018). While the molecular details are only partially understood, perturbing the actomyosin system can alter cell shape, volume, contractility, and adhesion of cells to extracellular matrix (Tian et al., 2009), which in turn allows greater aqueous outflow and a reduction in IOP. In addition to forming stress fibers, filamentous actin assembles into actin bundles, which are a major component of filopodia, long finger-like projections that emanate from the cell surface, as well as the related cellular protrusions known as tunneling nanotubes (TNTs) .

TNTs: Classical cell communication forms involve establishment of extracellular signaling molecule gradients, where a signal released from one cell diffuses through the extracellular space to bind its target on the surface of an adjacent cell. However, in the anterior eye, secreted signals are diluted in aqueous humor, which carries them into Schlemm's canal thereby reducing the likelihood of them interacting with their molecular receptors. Coordinated cellular responses to elevated IOP includes additional non-diffusion based communication mechanisms. In 2005, TNTs were described as a new mechanism of cellular communication. These long, actin-rich cellular processes extend between cells, forming a transient tubular connection through which various organelles and signaling cargoes are shared (Marzo et al., 2012). TNTs are thought to be formed by fusion of two filopodia arising from adjacent cells and a thin tube connects their cytoplasms. TNTs have now been described for many other cell types participating in physiological and pathological processes such as neurological diseases. In 2017, we described TNTs as a novel mechanism of cellular communication by TM cells (Keller et al., 2017). Live cell confocal microscopy was used to demonstrate the transfer of fluorescently-labeled vesicles between adjacent TM cells. The actin-rich TNTs showed characteristics typical of these structures such thin diameter (0.5–1 μm) and long length (~240 μm) (**Figure 1A2**). A similar phalloidin-stained, TNT-like structure was found in TM tissue (**Figure 1A3**). Thus, TNTs can provide a direct and specific mode of cellular communication over long distances in TM tissue, effectively overcoming difficulties posed by diffusion-based signaling in a fluid environment.

Actin-binding proteins as TNT regulators: Many of the same molecules participate in filopodia and TNT formation, which directly or indirectly affect assembly or disassembly of these filamentous actin containing structures. Perhaps the best known molecule involved in TNT formation is Myosin-X (Myo10). Overexpression of Myo10 increased the number of TNTs emanating from the cell surface as well as increased vesicle transfer, while RNAi gene silencing had the opposite effects (Gousset et al., 2013). Thus, Myo10 appears to play a role in TNT formation. Interestingly, in neurons, Myo10 has two forms, one of which lacks the actin-binding motor domain and is known as "headless" Myo10. Studies show that 'headless' Myo10 inhibits axon outgrowth in cortical neurons both *in vitro* and *in vivo* (Raines et al., 2012). Other actin-associated proteins are also involved in TNT formation. Cdc42, IRSp53 and VASP inhibit TNT formation and negatively regulate vesicle transfer, whereas Eps8 positively regulates TNTs and vesicle transfer. Our studies implicated the Arp2/3 complex as having a role in TM TNTs (Keller et al., 2017). Arp2/3 governs formation of branched actin networks underlying the cell membrane and are a nucleation point for filopodia formation. CK-666 is an Arp2/3 inhibitor that inactivates the complex and inhibits assembly of branched actin. When TM cells were treated with CK-666, there was a reduction in TNT number and length, as well as significantly reduced vesicle transfer (**Figure 1A4**) (Keller et al., 2017). These functional effects were accompanied by the appearance of thick actin stress fibers. Therefore, the effects of the Rho kinase inhibitor, Y27632, which promotes disassembly of actin stress fibers, were also tested. In contrast to CK-666 treatment, Y27632 treatment increased the number of TNTs and increased vesicle transfer. We speculated that disassembly of actin stress fibers may enrich the cellular pool of G-actin available for

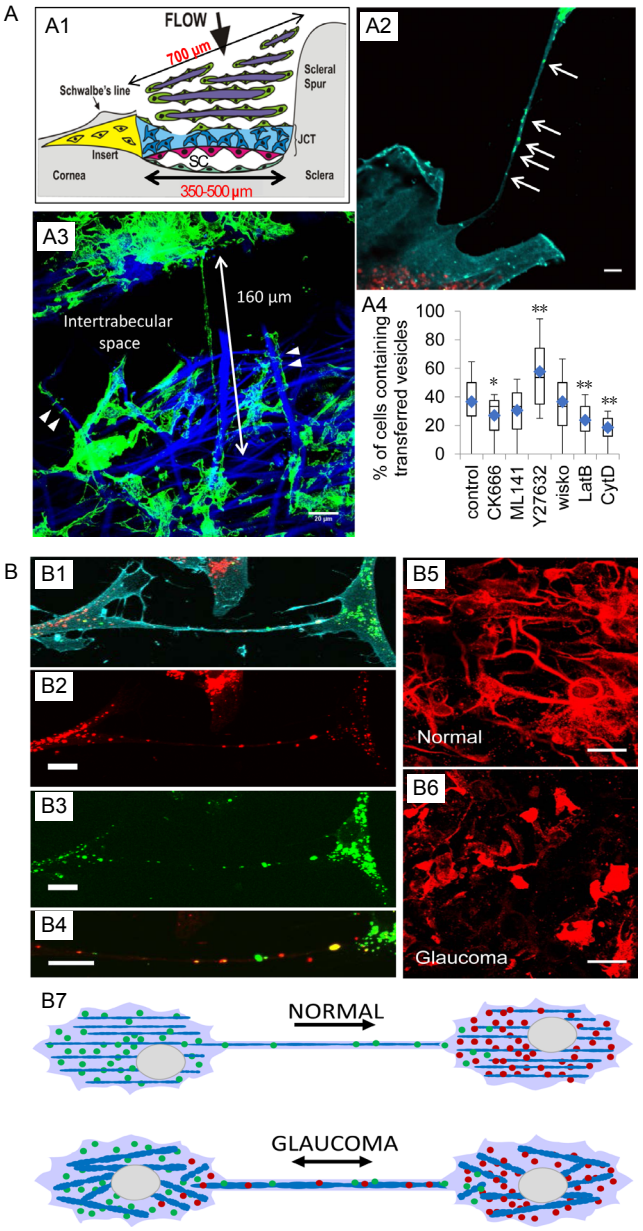

Figure 1 Tunneling nanotubes and actin cytoskeleton dynamics in glaucoma. (A1) Schematic of a cross-section of the trabecular meshwork (TM) with TM cells (green), corneoscleral beams (purple), the juxtacanalicular (JCT) region (blue), the inner wall cells of Schlemm's canal (pink), the putative stem cell insert region (yellow) and Schlemm's canal (SC). (A2) A long TNT connects a DiO-labeled cell (green) with a DiD-labeled (red) cell and DiO-labeled vesicles are clearly visible (arrows) within a long cell process connecting the two cells. Anti-CD44: cyan. Scale bar: 5 μm. (A3) Filamentous actin-rich cell process (160 μm long) bridges an intertrabecular space between corneoscleral beams (double-headed arrow). Green: Phalloidin; Blue: DAPI staining and autofluorescence of collagenous TM beams. Scale bar: 20 μm. (A4) The effect of actin inhibitors on vesicle transfer in TM cells. The Arp2/3 inhibitor, CK666, significantly decreases vesicle transfer, whereas the Rho kinase inhibitor, Y27632, increases transfer. $*P = 0.02$ and $**P < 0.002$ by analysis of variance. Other actin manipulators ML141 (a Cdc42 inhibitor), wisko (Wiskostatin, an N-WASP inhibitor), LatB (Latrunculin B) and CytD (Cytochalasin D) were also tested. Adapted from Keller et al. (2017). (B1-4) Red and green vesicles are clearly seen within a TNT arising from a glaucoma TM cell. Antibodies to CD44, a cell surface receptor, were used to visualize the plasma membrane. Cyan: CD44. Scale bar: 20 μm. (B5, 6) Immunofluorescence of Myo10 protein in normal (B5) and glaucomatous (B6) age-matched TM tissue. Scale bars: 20 μm. Adapted from Sun et al. (2019a). (B7) Normal TM cells have thin filamentous actin stress fibers, which support unidirectional vesicle transfer, whereas glaucomatous TM cells have thicker, less dynamic actin stress fibers, which support the bidirectional transport of cellular cargoes.

TNT formation and vesicular transfer. In addition to the effects on TNTs, CK-666 treatment and Myo10 silencing reduced outflow in an *ex vivo* model of IOP regulation, suggesting that TNTs and filopodia have an important role in IOP regulation (Sun et al., 2019b). As the study of TNTs becomes more widespread, it is likely that additional actin-associated proteins that affect TNT formation and growth will be identified.

TNTs and glaucoma: Recently, we reported TNT formation by glaucomatous TM (GTM) cells (Sun et al., 2019a). GTM cells had 40% fewer filopodia on their cell surface, but the cellular protrusions were 19% longer than normal TM cells. Although there were fewer TNTs, there was increased vesicle transfer in GTM cells. This is consistent with another study that suggests a regulatory balance between the two structures such that fewer filopodia may result in more TNTs (Gousset et al., 2013). Live-cell imaging of the actin cytoskeleton showed that vesicle transfer in GTM was slower than in normal TM cells (Sun et al., 2019a). Furthermore, GTM cells had thicker, less dynamic actin stress fibers. Our results suggest that stable actin may prolong TNT connections between cells, allowing a greater number of vesicles to transfer between cells. Additionally, both green and red fluorescently-labeled vesicles were observed in the same TNT (**Figure 1B1–4**) suggesting that glaucomatous TM cells may allow bidirectional transfer of vesicles. Unidirectional transport of cargoes from one cell to another is commonly described in the literature. However, some studies report distinct subsets of TNTs, which provide bidirectional transport. Thick TNTs (> 0.7 μm diameter) showed bidirectional transport of vesicles (late endosomes, lysosomes) whereas thin (< 0.7 μm) TNTs did not. The thicker TNTs contained tubulin in addition to actin, whereas the thinner TNTs did not. Since the molecular and phenotypic characteristics appear to influence the identity of cargoes transported, further studies are needed to measure TNT diameters in GTM cells. Moreover, a closer examination of the molecular components of TNTs in GTM cells and tissue is warranted given that Myo10 protein had a more punctate distribution in glaucomatous TM tissue compared to age-matched control tissue (**Figure B5** and **6**).

Potential for TNTs in the posterior segment: While our studies have described TNTs in the anterior segment, TNTs have not yet been reported for glaucoma-related cell types located in the posterior optic disc. Because TNTs are sensitive to common fixatives, it is possible that TNTs have inadvertently been destroyed during experimental procedures. Lack of a TNT biomarker also hinders their identification. However, TNTs in retinal pigment epithelial cells have been described and there are several reports of TNTs in non-ocular neuronal cells. For example, in the central nervous system, heterotypic TNTs can form between neurons and astrocytes, and between neurons and glia (Mittal et al., 2018). By extension, there is a strong possibility that neuronal cells in the optic disc may communicate via TNTs. Evidence for TNTs in glaucoma-related cell types may also be informed by studying TNTs in other neurodegenerative diseases, such as Creutzfeldt-Jakob, Alzheimer's, Parkinson's and Huntington's diseases (Mittal et al., 2018). In these diseases, TNTs are involved in the intercellular transport of misfolded protein aggregates. For example, TNTs are involved in the intercellular transport of Tau, a protein involved in Alzheimer's disease. Extracellular Tau fibrils appear to nucleate TNT formation, which subsequently facilitates the TNT-mediated transport of Tau aggregates between neurons. A similar mechanism has been described for TNT-mediated transfer of α-synuclein aggregates in astrocytes of Parkinson's patients, as well as for prions in Creutzfeldt-Jakob disease. In all cases, TNT transfer of misfolded protein aggregates into healthy cells appears to cause correctly folded molecules to transform and aggregate. This sets off a cascade of degeneration that contributes to the demise of a once healthy cell.

TNTs transport mitochondria between cells. Several studies reported the TNT-mediated transfer of mitochondria from healthy cells to cells experiencing stress, thereby preventing their apoptosis. While mitochondrial transfer may prevent further deterioration of stressed cells in the short-term, a reduction in the number of mitochondria in the healthy cells may have the unintended effect of rendering the donor cells susceptible to degeneration over the long-term. A link between mitochondria dysfunction and glaucoma is also emerging. Age-related changes in mitochondria function may impact the availability of ATP to maintain normal RGC function and thus, over time, render the RGCs more susceptible to injury and apoptosis. Interestingly, there is some evidence for the heterotypic transport of mitochondria in ocular neuronal cells. Mitochondria derived from RGCs can be degraded by optic nerve astrocytes, although TNTs were not reported to be involved in this transcellular process (Davis et al., 2014). Nevertheless, TNT-mediated transfer of mitochondria in the optic disk seems likely.

Finally, knockout of molecules regulating TNT formation are beginning to emerge and several of these models have ocular phenotypes. Myo10 knockout mice show defects in their retinal vasculature with 50% fewer filopodia formed *in vivo* (Heimsath et al., 2017). There was also a loss of filopodia branch points, which may reduce the number of cell-cell contacts thereby reducing TNT formation in these *Myo10*$^{-/-}$ mice. Another group showed similar findings when full-length, but not 'headless', *Myo10* was ablated. Genetic manipulation of additional TNT actin regulators should provide new information on the *in vivo* function of TNTs in anterior and posterior ocular tissues.

Conclusions and perspectives: Identification of TNTs in neuronal cells of the posterior segment, or in other retinal cells such as those in the microvasculature or lamina cribrosa, still await detection. However, our studies have demonstrated the existence of TNTs in normal and glaucomatous TM cells and tissue. TNTs directly transfer signals between cells to allow efficient communication in the aqueous environment of the anterior chamber. This overcomes barriers of diffusion-based signaling. Our studies also suggest that TNTs have a role in IOP regulation. Pharmacological treatments targeting the actin cytoskeleton were found to increase or decrease cellular communication via TNTs (Keller et al., 2017), providing additional insight into how the Rho kinase inhibitors may act to reduce ocular hypertension (Rao et al., 2017; Lin et al., 2018). However, further studies are required to investigate the molecular components involved in the formation, maintenance, and dissolution of normal and glaucomatous TNTs, as well as to determine whether different molecular cargoes are transported.

The author dedicates this manuscript to the late Mr. John M. Bradley.

This work was supported by National Institute of Health RO1 grant EY019643 and EY010572 (P30 Casey Eye Institute Core facility grant) and an unrestricted grant to the Casey Eye Institute from Research to Prevent Blindness, New York, NY, USA.

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***Received:** *January 9, 2020*
***Peer review started:** *January 16, 2020*
***Accepted:** *March 7, 2020*
***Published online:** *May 11, 2020*

***doi:** *10.4103/1673-5374.282254*

***Plagiarism check:** *Checked twice by iThenticate.*
***Peer review:** *Externally peer reviewed.*

***Open peer reviewer:** *Jiaxing Wang, Emory University, USA.*

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P-Reviewer: Wang J; C-Editors: Zhao M, Li JY; T-Editor: Jia Y