

**ANALYSIS OF THE EFFECT OF VARIOUS
PKA AND CILIARY MODULATORS ON THE
MIGRATION OF RPE CELLS**

By

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Submitted to the University of Michigan-Flint

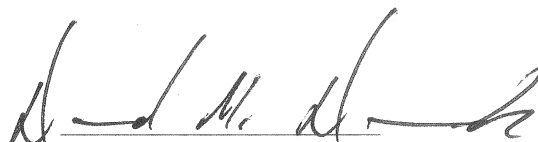
In partial fulfillment of the requirement for the degree of

Master of Science in Biology

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June 30, 2017

ABSTRACT

Cellular migration is a common activity for many different types of cells. If cellular migration is disrupted, cells may be unable to repair damaged tissue (wound repair), migrate appropriately, or correctly signal their cellular state. In several situations, primary cilia have been implicated in helping to determine the direction of cellular migration. cAMP-dependent Protein Kinase A (PKA) regulates many of the proteins involved in the migration process, but its effects vary with cell types. PKA is also known to localize in primary cilia. Using Matrigel invasion chambers as a 3D model of cellular migration, various PKA and ciliary reagents were tested for their ability to alter retinal pigment epithelial (RPE) cell motility. We used ARPE.19 cells, a human RPE cell line capable of forming cilia in culture, as the cell model. Our results showed that 8-Br-cAMP, LiCl and H89 significantly inhibited migration, while bFGF and NaCl promoted migration in ARPE.19 cells. Forskolin was significant as well in reducing migration. While KT5720 reduced migration, it was not significant, although it appears to be trending that way. These results suggest that PKA may be a key link between ciliary signaling and the direction of cellular migration.

ACKNOWLEDGEMENT

I would like to express my deepest gratitude to Dr. Frank Miskevich for accepting me as a thesis student, and giving me the opportunity to gain immense research experience. His expertise and continuous guidance has been a rich learning experience and a constant source of inspiration. I would like to thank Dr. Sucic and Dr. Duriancik for agreeing to be on my thesis committee, and for clarifying little doubts throughout the course of the program. Their timely advice always helped me approach problems better. I also extend my thanks to the Biology Department Staff for their patience and support. I would like to thank the Office of Research, Bryer Foundation, and the Graduate Student Research Assistantship, for the financial support. A special thanks to my parents, who patiently stood by my decisions about my career at all times. And to all the people unmentioned, thank you.

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INTRODUCTION AND REVIEW OF LITERATURE

Cell Migration

Any directed movement of cells that changes their position or location within a body is called migration (Kramer et al. 2013). Cell migration is a fundamental process that establishes the functional organization of all organisms. It is central to embryonic development and morphogenesis, wherein cells migrate during gastrulation and development of the nervous system (Treat et al., 2012). Large numbers of cells migrate together as sheets during gastrulation to form the three layers of an embryo. Cells eventually migrate to various locations, while differentiating into specialized cells leading up to organogenesis (Ridley et al., 2003). In the adult organism, cells continue to migrate for tissue repair and regeneration. In physiological and pathological conditions, cells of the immune system migrate to the site of infection, and fibroblasts and vascular endothelial cells migrate to the wound site for tissue repair (Ridley et al., 2003). A common case for migration in pathology is metastatic cancer, wherein tumor cells migrate from one location to another via the circulatory system (Lauffenburger & Horwitz, 1996). Vascular diseases, osteoporosis, chronic inflammatory diseases like rheumatoid arthritis, and multiple sclerosis are some of the other pathological conditions that involve cell migration (Ridley et al. 2003). Cell migration is a complex process that is dynamically regulated by a network of signaling pathways involving second messengers, kinases and guanosine triphosphatases (GTPases), motor proteins and cytoskeleton-modifying proteins (Welf & Haugh, 2011). The Rho GTPases class of proteins – RhoA, Rac 1, and Cdc42 – play a crucial role in the process (Veland et al., 2014).

Mechanism of cell migration

The migration mechanism can be broken down into four sequential and highly interrelated steps – protrusion, adhesion, translocation, and retraction (Howe, 2004; Lauffenburger & Horwitz, 1996; Chen et al., 2008). Before migration can occur, cells acquire a morphological asymmetry in the form of polarity with distinct anterior and posterior ends (Lauffenburger & Horwitz, 1996). Cell polarization occurs in response to spatial cues, such as chemoattractants or disrupted contact with neighboring cells, and provides a sense of direction during migration. Reorganization of the organelles in the polarized cell and cytoskeleton dynamics are major driving forces in migration. Protrusions formed by the polymerization of filamentous actin (F-actin), like lamellipodia or filopodia, extend from the membrane at the leading end of the cell. The lamellipodium is a specialized subcellular structure that is important during migration (Chen et al., 2008). Facilitated by integrins, the protrusions attach and anchor the cell to the substrate, forming transient cell-matrix adhesion complexes (Veland et al., 2014). Actin polymerization and structural reorganization, along with myosin-associated motor activity, generate the internal force required to contract and translocate the cell body (Lauffenburger & Horwitz, 1996). Depolymerization of F-actin and release of cell-substrate attachments allows the cell to retract the rear-end of the cell. The cytoskeleton dynamics and extra-cellular matrix (ECM) remodeling near the cell are influenced by changes in pH and ion concentrations (Veland et al., 2014).

Cell types differ in the way they migrate in terms of speed and morphology. For example, fibroblasts form lamellipodium-like extensions at the leading edge, whereas neurons form

extensively branched protrusions (Veland et al., 2014; Vicente-Manzanares et al., 2005). As a slow-moving cell type, fibroblasts distinctly display the different stages in migration. In contrast, fast-moving cells like leukocytes and keratocytes smoothly glide over the matrix without the formation of observable cell-substrate attachments (Horwitz & Webb, 2003).

Primary Cilia

The history of cilia dates back to over 300 years when ciliary motility was first described by Antony Leeuwenhoek in 1675, making it one of the oldest known cell structures (Satir, 1995). Cilia project from the cell surface, and their primary function is not just help the cell move, but also sense the environment the cells move in (Wheatley, 2005). While cilia were associated with motility and cell structure, the discovery of primary cilia in 1898 changed the way cilia were studied, and has led to cilia being classified as motile or primary. The primary cilia were considered a vestigial part of the cell, and it has only been in recent years that they have gained importance as a much more functional part of the cell (Norris and Grimes, 2012).

Motile cilia usually occur as tufts or clusters of 100-300, and are found in distinct populations of differentiated epithelial cells like those lining the airways, ventricles, and oviducts (Jain et al., 2010). In contrast, the primary cilia are found in most cell types as a solitary or single cilium. They are found on both epithelial and non-epithelial cells like chondrocytes, fibroblasts, neurons and Schwann cells (Jain et al., 2010; Satir & Christensen, 2007). Primary cilia are also present in specialized cells in the retina, nasal

cavity, and on luminal surfaces of bile ducts, renal tubules and vascular endothelial cells, where they function as sensory organelles. Differentiated cells of myeloid or lymphoid origins and hepatocytes are the only types of cells known to lack primary cilia (Pazour et al., 2000).

Structure of cilia

Cilia are microtubule-based organelles that are membrane-bound, and have been conserved evolutionarily in both prokaryotes and eukaryotes (Liang et al., 2016). Cilia consist of a central structure called the axoneme, a ciliary membrane, and the matrix between the axoneme and the membrane, as shown in Figure 1 (Liang et al., 2016). The axoneme is composed of a ring of nine microtubule (MT) doublets, arranged radially around a central singlet core. The presence or absence of the central core, indicated as 9+2 or 9+0 arrangements respectively, differentiates between the motile and primary cilia. Motile cilia possess the 9+2 axoneme, and associated structures like central pair sheath, dynein arms linking the central and outer microtubules and radial spokes. These structures together coordinate the motility of cilia (Liang et al., 2016). Primary cilia lack both the central core and the associated structures, rendering them immotile (Satir & Christensen, 2007).

The cilium extends from the basal body, which is a modified centriole, located at the base of the cilium. The basal body is composed of nine triplet microtubules, and provides the nine-fold symmetric template from which the ciliary axoneme protrudes. It determines the orientation and position of the cilium to ensure proper functioning. The ultrastructure of the cilium reveals three sub-regions: the transition zone at the base, the middle doublet

zone containing motility-associated proteins, and the singlet zone at the tip of the cilium (Fisch et al., 2011). The triplet microtubules become doublets in the axoneme in the transition zone that links the cilium to the basal body. This short region contains Y-shaped structures extending from the microtubules to the ciliary membrane, and the ciliary necklace. Best studied via freeze fracture electron microscopy, the ciliary necklace is composed of multiple distinct and parallel strands of membranous particles that surround the ciliary shaft (Rohatgi & Snell, 2010; Gilula & Satir, 1972). The necklace is proposed to function as a filter for non-ciliary proteins (Liang et al., 2016). The ciliary tip may play a role in determining the length of cilia by the addition or subtraction of microtubule units at the tip (May-Simera et al., 2017).

The axoneme is enclosed within a ciliary membrane that is continuous with the plasma membrane, and the border between the two membranes is known as the periciliary membrane. Transition fibers that lie proximal to the transition zone provide anchorage between the periciliary membrane and the mother centriole (Verhey & Yang, 2016). The ciliary matrix between the ciliary membrane and the axoneme is akin to the cell cytosol (Brown & Witman, 2014).

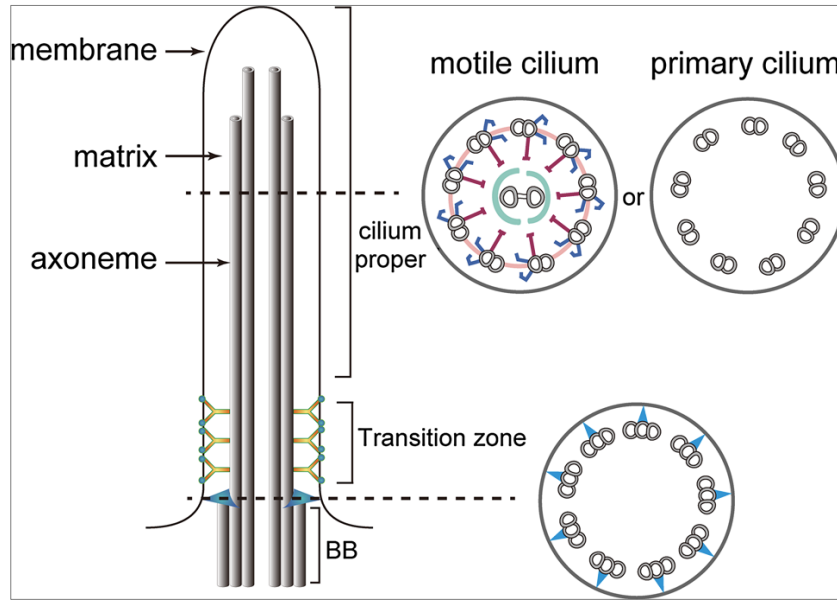


Figure 1. Structure of a cilium (Liang et al., 2016)

Despite the topological continuity, functional barriers separate the ciliary and plasma membranes, making it more of a specific compartment in the cell (Liang et al., 2016; Nachury et al., 2010). The ciliary membrane has a unique composition of proteins and lipids that is different from that found in plasma membrane (Rohatgi & Snell, 2010), and has the capacity to enrich specific receptors (Follit & Pazour, 2013). The protein complement of the membrane includes receptor tyrosine kinases, ion channels, G-protein coupled receptors (GPCRs), extra-cellular matrix proteins, and growth factor receptors (Verhey & Yang, 2016).

The functional separation between the plasma and ciliary membranes allows cilia to enrich specific ions. Most prominent channels appear to be members of the polycystin family, and the transient receptor potential (TRP) channels. A calcium-activated chloride channel, ANO1, has also been detected (Pablo et al., 2016). The resting concentrations of calcium

in the primary cilium appear to be sevenfold greater than in the cell. While changes in Ca^{2+} concentration in the cytoplasm propagate into the cilia, the reverse does not happen due to the larger cytoplasmic volume that buffers and dissipates the calcium ions (Pablo et al., 2016).

Studies on the lipid composition of the ciliary membrane found that several sphingolipids, such as GM1 and GM3, localize to the cilia, including mammalian primary cilia. Cilia are also enriched in the products of inositol 5-phosphate (INPP5E), which localize to the primary cilia (Nachury et al., 2010). Mutations affecting INPP5E localization have been linked to Joubert syndrome (Verhey and Yang, 2016) and other ciliary dysfunctions in humans and mice (Nachury et al., 2010). Sterols may be involved in the localization of proteins at the ciliary membrane, such as in the case of Smo, which is induced by oxysterols (Rohatgi & Snell, 2010).

Cilia lack the machinery required for protein synthesis, and rely on the intraflagellar transport (IFT) system for the bidirectional movement of cargo essential for cilia formation and function (Liang et al., 2016). This specialized mechanism, first described in the flagellated and single-celled green algae, *Chlamydomonas reinhardtii* (Rodriguez & Badano, 2009), moves proteins along the axonemal microtubules inside the ciliary membrane by associating IFT scaffold with non-membrane-bound (ciliary) proteins. IFT in the anterograde direction from the base to the tip of the cilium carries particles for the assembly and maintenance of the axoneme and ciliary membrane, and is mediated by kinesin. Transport in the retrograde direction from the ciliary tip to the base recycles the

IFT and motor proteins, and is facilitated by dynein (Rosenbaum & Witman, 2002; Rodriguez & Badano, 2009).

Primary cilia are formed post-mitotically in differentiated cells or in stem cells during the G₀ phase of the cell cycle. The cilium is resorbed when the cell re-enters the cell cycle. The assembly and disassembly of cilia is a highly regulated and balanced mechanism, making primary cilia dynamic structures (Satir et al., 2010; Liang et al., 2016).

Primary cilia and cell signaling

A study by Pazour et al. in 2000 on polycystic kidney disease (PKD) was key to further understanding the functional importance of primary cilia. It linked development of PKD to defective ciliary assembly resulting from mutations to genes encoding IFT proteins (Pazour et al., 2000). Mouse mutants lacking a homolog of a *Chlamydomonas* IFT protein known as IFT88 were developed as a model for human autosomal recessive PKD (ARPKD). In an IFT88 mutant, kidney cells (and other cells) lacked normal primary cilia, causing defective ciliary signaling, which in turn resulted in PKD. Similar conclusive results were obtained in the case of autosomal dominant PKD (ADPKD). Polycystins 1 and 2 (PC 1, 2) together form a transient receptor potential (TRP) calcium ion protein complex, which was found to be non-functional in ADPKD models. PC1 and PC2 are normally localized in the primary cilia, but were either absent or mislocalized in ADPKD condition (Satir et al., 2010). Defective ciliary assembly due to mutations in IFT88 disrupts the localization and function of PC1 and PC2 (Yoder et al. 2002). Although IFT is not required to move PC2

into the cilium, it is required to recycle the protein into the cell body (Pazour et al. 2002; Brown & Witman, 2014).

Research on primary cilia increased after the discovery that they play crucial roles in development and diseases. Primary cilia play a key role in cell signaling because of the presence of multiple receptors, ion channels, and the localization of their downstream effector molecules to the basal body and/or cilium. They coordinate processes that are central during organismal growth and development like tissue homeostasis, cell migration, cell proliferation, differentiation, and apoptosis (Satir et al., 2010). One of the well characterized pathways that takes place in primary cilia is the Hedgehog (Hh) signaling pathway. It is important in neural tube and limb patterning (Nachury et al., 2010). The receptor, Patched (Ptch), is concentrated in the cilium in the absence of a ligand. Upon activation, Ptch exits the cilium and is replaced by a GPCR-like receptor, Smoothed (Smo). This activates Gli transcription factors, Gli2 and Gli3, that become enriched at the tip of the cilia (Nachury et al., 2010; Pablo et al., 2016). Transmembrane proteins involved in Platelet-derived growth factor (PDGF α) signaling are also concentrated in the ciliary membrane (Nachury et al., 2010).

As a sensory organelle, primary cilia respond to environmental signals such as mechanical stimulation via bending of the cilium, chemosensation via recognition of ligands, growth factors and hormones, osmosensation, and phototransduction (Waters & Beales, 2011). Mechanical stimulation triggers calcium signaling. When fluid passes over the cilium, it bends, triggering calcium channels to open, and inducing a rapid increase in intracellular

calcium. This increase in intracellular calcium spreads to neighboring cells in a wave-like manner, and occurs in neurons, glial cells, osteoblasts, hepatocytes, epithelial cells, and endothelial cells (Khamidakh et al., 2013).

The shear stress-induced release of Ca^{2+} occurred in both ciliated and un-ciliated cells, and was found to be ATP-dependent. A study analyzed stress detection in kidney cells, MDCK, at various stages of primary cilium development (Rodat-Despoix et al., 2013). It was found that ATP release, and the ensuing mobilization of different purinergic receptors (P2 receptors), was key to shear stress response. ATP binds to two types of P2 receptors, P2X and P2Y. P2X receptors are ligand-gated, and the P2Y are GPCRs (Rodat-Despoix et al., 2013). While in un-ciliated cells, the calcium response was primarily through P2X receptors; the response in ciliated cells was via P2Y receptors (Rodat-Despoix et al., 2013).

A study in 2015 presented a method that selectively captured the ciliary proteome, wherein cilia-targeted proximity labeling enzyme (cilia-APEX) was used to selectively biotinylate ciliary proteins. Along with identifying known ciliary proteins like IFT-A and B, Kinesin 1, and dynein 1b, some unexpected proteins were also identified. Cilia-APEX classified cAMP-dependent PKA as a ciliary signaling molecule, able to regulate Hedgehog signaling via phosphorylation of Gli3 in primary cilia (Mick et al. 2015). Calcium-dependent protein kinase (PKC), mitogen-activated protein kinase (MAPK), intracellular cAMP and cAMP-dependent protein kinase A (PKA) are some intracellular molecules that regulate ciliary function and/or length in endothelial cilia. Studies also showed that only intracellular cAMP and PKA both affect cilia length and function. According to their working model,

pharmacological drugs affect PKA, and thereby ciliary function, through intracellular cAMP. But PKA can also regulate cilia length through MAPK and phosphatase-1 (PP-1), causing cofilin dephosphorylation, actin rearrangement and change in cilia length (Abdul-Majeed et al. 2012).

Ciliopathies

The spectrum of developmental diseases or disorders associated with genetic mutations that result in defective ciliary assembly or signaling is termed ciliopathies (Liang et al., 2016). Some of the physical manifestations of dysfunctional cilia include retinal degeneration and blindness, fluid accumulation in the brain, renal diseases and diabetes (Brown & Witman, 2014). Ciliopathies are also linked to brain disorders such as hydrocephalus, mental retardation, defective neural tube formation, and cerebellar hypoplasia (Narita & Takeda, 2015).

Hydrocephalus is a condition where the movement of fluids inside the brain is interrupted, leading to an increase in pressure of the cerebro-spinal fluid (CSF), ultimately causing the ventricles to expand (Kausi and Katsanis, 2016). It is a relatively common condition caused by infections, genetic factors, dysfunctional ciliary fluid flow sensing, and severe brain injuries (Kausi and Katsanis, 2016). The incidence of congenital hydrocephalus is 0.5-1 out of 1000 live births, and that of acquired hydrocephalus in developed countries is 3-5 out of 1000 births (Muir et al., 2016). Studies show that hydrocephalus occurs in a number of ciliopathies, implicating defective primary cilia and their role in fluid flow sensing as a potential causative agent (Valente et al., 2014).

There is so far no definitive cure for hydrocephalus. For most cases, a ventriculo-peritoneal (VP) shunt is used to relieve pressure in the ventricles by diverting CSF to other sites in the body. But this mode of treatment has been associated with complications like shunt extrusion, infections, breakage, overdrainage and obstructions in infants, causing mortality (Bigio and Curzio, 2015; Muir et al. 2016). Nearly half of the shunts fail within the first 2 years, resulting in the need for multiple revisions in a lifetime. In developed countries, the incidence of VP shunt infections range between 2-9%, whereas in developing countries, this range falls between 8.6-50% (Muir et al., 2016). Although surgical alternatives exist, the risk of complications outweighs their benefits. Current research is underway to develop alternative non-surgical methods to manage hydrocephalus.

Cell migration and primary cilia

Multiple studies link structurally or functionally defective primary cilia to cell migration-associated conditions and disorders. A seminal work published by Albrecht-Buehler in 1977 discusses the orientation of primary cilia in 2D cultures of 3T3 fibroblasts during their growth-arrested phase (Christensen et al., 2008). According to that study, ciliary orientation appeared random in confluent cell cultures with no migration, while in non-confluent cultures, the cilia on cells that migrated oriented in the direction of migration, and parallel to the substrate. The functional relationship between cell polarity and primary cilia that regulates cell migration seems to occur during the reorganization of the cytoskeleton (Christensen et al., 2008; Veland et al., 2014; Albrecht-Buehler, 1977). Other wound-healing studies have produced similar results, confirming that the cilium of

migrating cells reorients itself at the wound site, directly interacts with the ECM, and regulates the orientation of the the centrosome to the front of the nucleus (Veland et al., 2014). Besides just orienting itself in front of the nucleus, in the direction of movement, studies have shown that the primary cilium also coordinates a network of signaling pathways to aid migration. This link was realized in the last decade or so, when platelet-derived growth factor receptor alpha (PDGFR α), which is a cell growth arrest-specific protein, was found to play a role in directional cell migration (Christensen et al., 2013). There is increasing evidence linking migration-related disorders, including abnormal neurodevelopment, to defective formation or functioning of primary cilia. However, mechanisms by which ciliary-mediated signaling induces a migratory response remain largely unknown (Veland et al. 2014).

Adenosine 3',5'-cyclic monophosphate

Some extracellular signals (also called first, or more commonly primary messengers) like hormones or neurotransmitters cannot directly enter the cell to relay a signaling pathway. The cell surface receptors convert such signals into mediators, known as “second messengers”, to relay and trigger intracellular signaling that result in physiological effects (Krebs, 1989). Some common second messenger systems include cyclic AMP, cyclic GMP, diacylglycerol, inositol triphosphate (IP3) and calcium ions (Yan et al., 2016).

cAMP is a key second messenger in multiple signaling pathways that affect important physiological functions (Yan et al., 2016). cAMP is a nucleotide that was first discovered as a second messenger by Earl Sutherland in 1958 while studying the effects of epinephrine

and glucagon on glycogen in liver. It was found that for the epinephrine to take effect, an increased intracellular concentration of cAMP was required. Similar signaling was observed during muscular activity, wherein epinephrine signals the conversion of glycogen to glucose (Cooper, 2000; Yan et al., 2016; Krebs, 1989).

Cyclic AMP is derived from adenosine triphosphate by the enzymatic action of adenylate cyclase and hydrolyzed to AMP by cAMP phosphodiesterase. Adenylate cyclases are activated by the specialized G-proteins linked to the GPCRs. In the absence of a signal, the α -subunit of the heterotrimeric G-protein binds GDP (guanosine diphosphate). Binding of a ligand to GPCR triggers the dissociation of the α subunit from the $\beta\gamma$ dimer of G-protein and the exchange of GDP to GTP on $G\alpha$. The $G\alpha$ subunit activates adenylate cyclase leading to an increase in the concentration of cAMP (Murray, 2008; Gerits et al., 2008). Since the phosphodiesterase (PDE) group of enzymes breaks down cAMP, inactivation of PDE also leads to an increased level of cAMP in the cells.

Protein Kinase A

One of the primary targets of cAMP is PKA, which in turn phosphorylates specific proteins to trigger cellular activities. Cyclic AMP response-element binding-protein (CREB) is a transcription factor that, upon being phosphorylated, regulates the transcription of various genes mediating important physiological processes (Yan et al., 2016; Cooper, 2000).

PKA is a member of the protein kinase family of enzymes that is responsible for phosphorylating target proteins (Cheng et al., 1998). First discovered by Walsh and Krebbs

in 1968, PKA is a serine/threonine kinase with targets in the membrane, cytoplasm, nucleus, mitochondria and the cytoskeleton (Howe, 2004; Cooper, 2000; Cheng et al., 1998). Structurally, it is a heterotetrameric enzyme made up of two regulatory (R) and two catalytic (C) subunits. While the R subunits are encoded by 4 genes – RI α , RI β , RII α , RII β , the C subunits are encoded by 3 genes - α , β , and γ . The R subunits play a major role in the enzyme's functionality as a cAMP target. Depending on the type of R subunit, PKA holoenzyme may either be type I or type II. In its inactive state, the R subunits are bound to the catalytic cleft of the C subunits. When 2 molecules of cAMP bind to each R subunit, the C subunits are released as active catalytic subunits, allowing the now active enzyme to affect signaling pathways (Howe, 2004). A schematic illustrating the activation of PKA is shown in Figure 2. For the catalytic subunits to phosphorylate targets, they have to bind to ATP. A-kinase anchoring proteins (AKAPs) compartmentalize PKA to subcellular regions, allowing it to act in a small and defined domain with specificity (Murray, 2008; Howe, 2004).

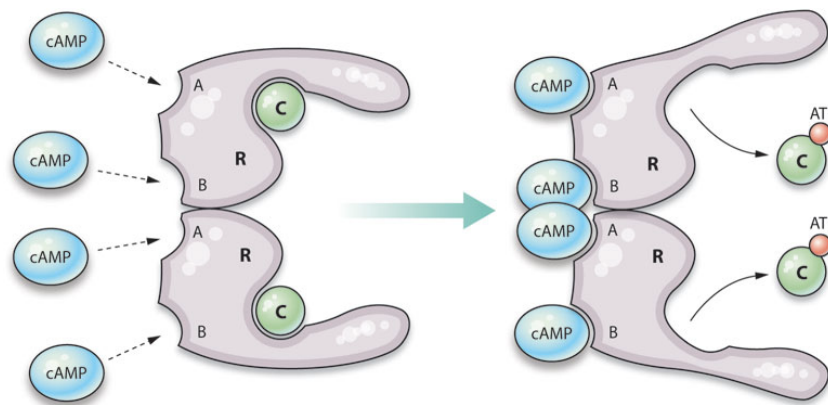


Figure 2. PKA activation by cAMP (Murray, 2008)

Cyclic AMP/PKA in cell migration

The process of cell migration is delicately regulated by cAMP and PKA. Critical signaling events like cell polarization and cytoskeletal reorganization are regulated by PKA. PKA controls activation of RhoA, Rac and cdc42, wherein it activates Rac1, but inhibits the activation of RhoA (Holthauzen et al., 2008). Studies have shown a conflict in the role PKA plays in cell migration and cytoskeletal remodeling, in that it exerts both inhibitory and activating effects. An example is the migration of $\alpha v\beta 3$ -dependent epithelial cells in vitronectin, wherein PKA exhibited both positive and negative effects (Chen et al., 2008). On the other hand, studies showed that elevated cAMP levels and PKA activation were required during migration of mammary epithelial cells on laminin (Howe, 2004).

In a study by Chen et al. in 2008, cAMP inhibited migration of mouse embryonic fibroblasts (MEF) and mouse 4T1 breast tumor cells by interfering with their ability to form lamellipodia at the leading edge (Chen et al., 2008). An important target of PKA is myosin light chain (MLC) kinase, which phosphorylates and activates myosin II. Flow of F-actin in the lamella is regulated by myosin II, thereby regulating actin-driven migration. PKA lowers the activity of MLC kinase by phosphorylating it (Chen et al., 2008).

PKA and ciliary modulators

Protein kinase inhibitors have been central to understanding the mode of action of particular kinases. However, developing specific and cell permeable kinase inhibitors has been a challenge, specially given the large size of the kinase family, and the structural similarity within (Lochner & Moolman, 2006). Two widely used potent and specific PKA

inhibitors are H89 and KT5720 . H89 is an isoquinoline derivative developed from the nonspecific PKA inhibitor, H8 (Murray, 2008). It has been used in studying the role of PKA in heart muscles, osteoblasts, hepatocytes, epithelial cells, smooth muscles and neuronal tissue (Lochner & Moolman, 2006). KT5720 belongs to a family of compounds synthesized from the fungus, *Nocardioopsis* sp (Murray, 2008). The mechanism of action of both the inhibitors is similar, as shown in Figure 3, i.e. as competitive antagonists of ATP. They bind to the catalytic subunits, blocking the sites for ATP binding, thereby preventing phosphorylation of PKA substrates (Murray, 2008; Lochner & Moolman, 2006).

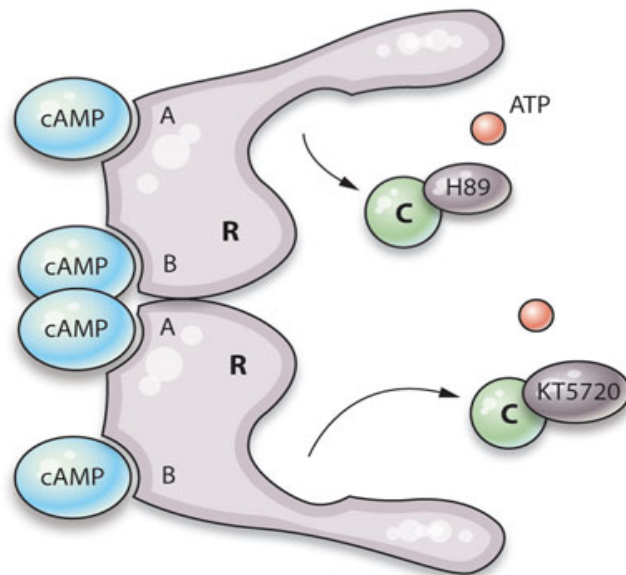


Figure 3. Mechanism of action of H89 and KT5720: they bind catalytic subunits of PKA and prevent phosphorylation of targets (Murray, 2008)

Although developing inhibitors that act as ATP antagonists is common, it comes with distinct challenges. The IC_{50} value (the concentration at which 50% of activity is inhibited) of an inhibitor depends on the concentration of ATP in the cell. And since ATP levels vary,

It is difficult to determine the concentration of inhibitor required for effective blockade of kinase activity. Also, it is easy for PKA inhibitors targeting ATP-binding sites to act nonspecifically due to the abundant availability of ATP and ATP receptors in the cell (Murray, 2008). Multiple studies have reported non-PKA based activity of H89 and KT5720, where other protein kinases and other enzymes were inhibited by them (Murray, 2008). One of the studies found that H89, at a concentration of 10 μ M, inhibited at least 8 other kinases by 80-100%, of which 3 were inhibited with similar or greater potency than that found for PKA (Lochner & Moolman, 2006). Alternative PKA inhibitors act as competitive antagonists of cAMP binding sites on PKA. An example of this is R_p -adenosine-3',5'-cyclic monophosphorothioate (R_p -cAMPS), which binds to cAMP-binding sites, preventing the release of active catalytic subunits (Murray, 2008). Forskolin is an adenylate cyclase activator that is used to induce or increase the intracellular concentration of cAMP formation (Yan et al., 2016). LiCl is a psychotropic drug used in the treatment of bipolar disorder (Miyoshi et al., 2009) and arthritis (Thompson et al., 2016). Multiple studies have shown the effect LiCl has on primary cilia and Hh signaling. LiCl is also known to increase the length of primary cilia (Thompson et al., 2016). An electron microscope image of LiCl-induced lengthening of primary cilia is shown in Figure 6.

Basic fibroblast growth factor (bFGF) belongs to the FGF family of structurally related proteins, and acts via cell surface FGF receptors. bFGF is known for its involvement in the development of the nervous system including its cell growth, differentiation, movement and maintenance. Literature indicates that bFGF acts as a trophic factor for retinal cells

(Driscoll et al., 2007). In this study, bFGF was used as another control to study RPE migration.

The Retinal Pigment Epithelium

The eyes of all organisms, from insects to higher vertebrates, are composed of the photoreceptor cell and the pigmented cell, forming the light-sensitive photoreceptor (PR) layer and the retinal pigment epithelium (RPE), respectively. The two layers are dependent on each other for functional differentiation, even during embryonic development, and their continued interaction is essential for physiological functioning (Strauss, 2011). In a vertebrate eye the RPE is located between the photoreceptor layer and the choroid blood supply, and is composed of polarized cells that are hexagonally arranged into a tight junction monolayer. The apical surface of the RPE is in contact with the matrix in the subretinal space through which it interacts with the outer segments of the photoreceptor layer. The basolateral membrane of the RPE interacts with the choroid blood vessels through the Bruch's membrane (Strauss, 2011).

The RPE cells possess two types of microvilli on the apical surface, thin microvilli of 5-7 μm and specialized microvilli called photoreceptor sheaths that extend into the outer segment of the rods and cones, the length covered being greater in the case of rods. This expands the surface area available for trans-epithelial transport and facilitates the functional interaction with the photoreceptor layer (Sparrow et al., 2010). Figure 4 is an illustration of the retina that shows the RPE and its interaction with the photoreceptor cells via the microvilli.

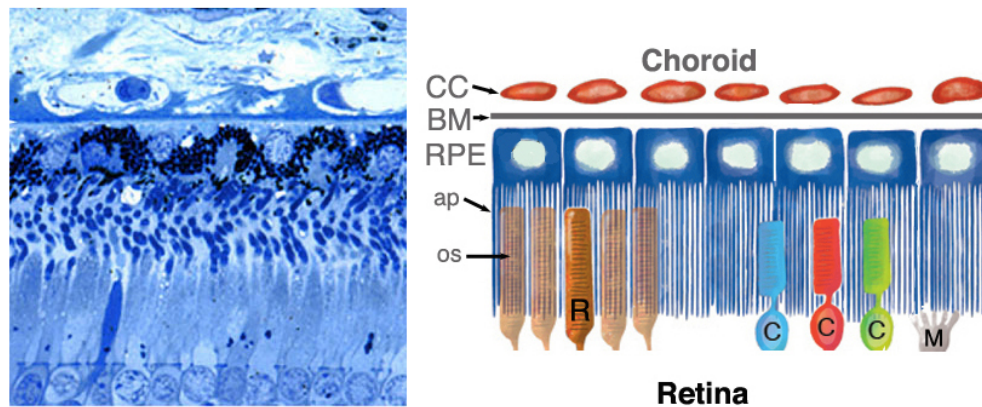


Figure 4. Light micrograph (left) and cartoon (right) of the RPE (Strauss, 2011)

The RPE cells carry out multiple functions that are critical to the normal functioning of the retina, such as retinoid conversion and storage, phagocytosis of used up photoreceptor outer segment, maintenance of fluid and electrolyte balance, trans-epithelial transport, scar tissue formation, and light absorption and dissipation of heat energy (Strauss, 2011; Choudhary et al., 2016.; Sparrow et al., 2010; Mazzitello et al., 2009; Sharma et al., 2005). Ca^{2+} is found to be highly involved in RPE functioning (Khamidakh et al., 2013).

The RPE is exposed to strong photo-oxidative energy on the retinal side and excess oxygen on the side of the choroid blood supply. The outer segments of the photoreceptor layer require constant renewal due to the damage they receive from photo-oxidation. The RPE degrades the damaged and shed photoreceptor disks via phagocytosis, releasing free radicals in the process. RPE cells contain pigment granules or melanosomes that act as their first line of defense against photo-oxidative damage. Further lines of defense include

the cell's DNA repair mechanism and anti-oxidants that are both enzymatic and non-enzymatic (Strauss, 2011).

Although the degradation happens quickly in young, healthy RPE cells, older cells accumulate partially digested materials in the form of lipofuscin, a yellow pigment composed of protein and fat damaged by free radicals (Mazzitello et al., 2009; Strauss, 2011). The tight-junction feature of the RPE allows it to provide protection to the eye by forming a barrier between the inner eye and the blood stream. The RPE interacts with the immune system via modulators, such as interleukin-8 and complement factor H, to suppress or activate an immune response in a healthy or infected eye, respectively (Strauss, 2011). Malfunction of the RPE is associated with diseases such as proliferative vitreoretinopathy (PVR), diabetic retinopathies, and age-related macular degeneration (AMD), often resulting in loss of sight (Choudhary et al., 2016).

RPE cell lines have been widely used in ciliary studies, the most popular being ARPE.19 cell line (May-Simera et al., 2017). Derived from the eye globes of a 19-year-old male donor, the ARPE.19 cells are “diploid, non-transformed human RPE cells that display properties typical of differentiated RPE *in vivo*” (Dunn et al., 1996; Sharma et al., 2005). Figures 5 and 6 are electron microscope images of ARPE.19 cells containing primary cilia and microvilli. Figure 6 shows LiCl-induced elongation of a primary cilium in an ARPE.19 cell.

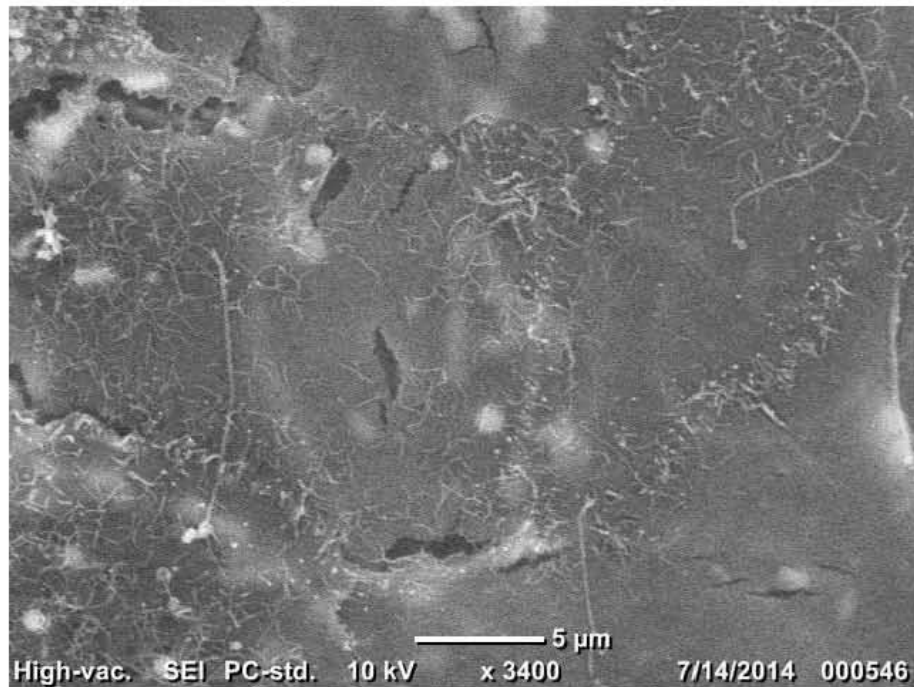
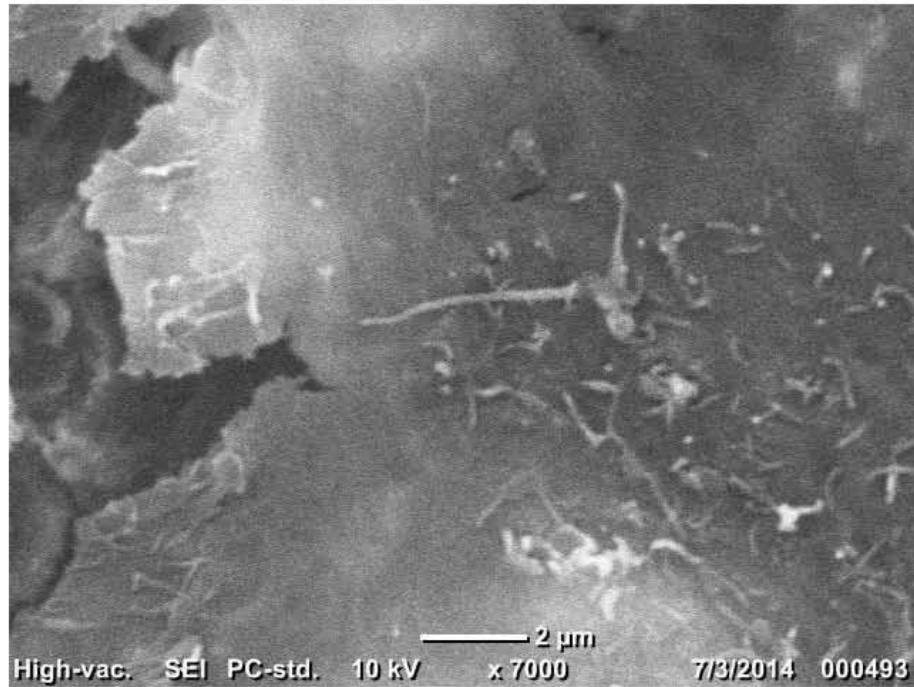


Figure 5. Electron Microscope images of ARPE.19 cells with microvilli and primary cilia (Paul Fox, unpublished data)

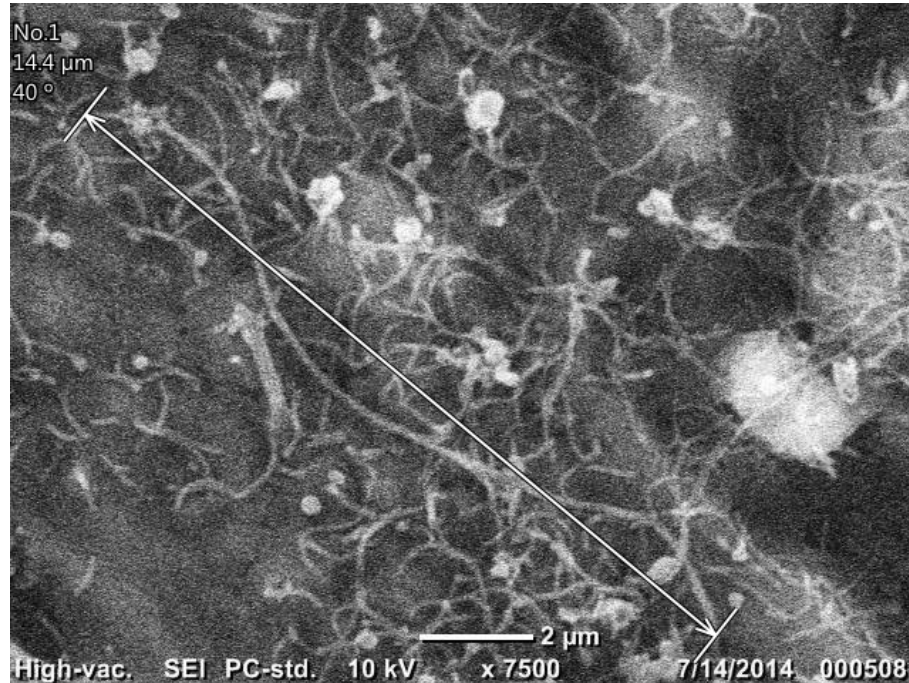


Figure 6. Electron Microscope image of ARPE.19 cell with elongated primary cilium induced by treatment with LiCl (Paul Fox, unpublished data)

Cell migration and invasion assays

There are numerous ways to study cell migration and invasion, with two of the most common methods being the cell wound closure assay and the transwell invasion assay. These assays help study migratory behavior in detail and facilitate molecular studies of the mechanism involved (Justus et al., 2014). The wound closure assay is one of the simplest techniques useful in determining migration in cell masses. It analyzes a cell line's ability to move and gradually close a scratch (wound) made on a confluent cell dish. Along with being able to determine the velocity of cell movement, this assay allows for studying cell migration dynamics by live cell imaging in real-time using time-lapse fluorescence microscopy. Despite the versatility of the scratch assay, it is not a suitable method for

studying non-adherent cells, and those cell lines that tend to dislodge from the plate once a scratch is made (Justus et al., 2014).

The transwell invasion assays are used to study the way single cells sense and respond to chemo-attractants like growth factors, chemokines, and nucleotides, through physical barriers. By adding a layer of ECM to the transwell membrane, the assay can be used to study directional cell invasion that mimics the process of physiological ECM invasion. The limitation of this technique is that obtaining time-lapse data and live-cell imaging is complicated (Justus et al., 2014).

AIM AND OBJECTIVES

We analyzed the effects of cyclic AMP-dependent protein kinase A and ciliary modulators on the motility of human retinal pigment epithelial cells. Using ARPE.19 cells as the cell culture model, we carried out cell invasion assays to test various reagents. Forskolin, H89, 8-Bromo-cyclic AMP, KT5729, basic fibroblast growth factor, ATP, lithium chloride, sodium chloride were the reagents tested.

Some of the signaling pathways involved in cell migration seem to overlap with those that occur within primary cilia or are required for proper ciliary functioning. Our hypothesis is that primary cilia play a role in cell migration, and that cAMP-dependent PKA signaling is important in mediating this process.

MATERIALS AND METHODS

Materials

The human retinal pigment epithelial cell line ARPE.19 was used as a model for the cell invasion assays. It was purchased from American Type Culture Collection (ATCC). H89, KT5720, 8-Bromo cyclic AMP, Forskolin (Tocris), bFGF, ATP, NaCl, and LiCl (Sigma) were the reagents tested for their effect on ARPE.19 cell migration.

Methods

Maintenance and sub-culturing of cells

Cells were maintained in cell culture dishes in at 37°C and 5% CO₂ in 1:1 DMEM/F12 media (Sigma) supplemented with 10% fetal bovine serum (Sigma) and penicillin/streptomycin (Sigma) used as directed. To passage the cultures, the old media was aspirated, and 2 mL of 0.05% trypsin was added and incubated for 5 minutes at 37°C to facilitate dissociation of cells. The dissociated cells were collected and centrifuged (at approximately 5000 RPM) for 5 minutes. The trypsin was aspirated carefully without disturbing the pellet, and 2 mL media was added to the tube. The cells were re-plated at 1:25 dilution, i.e., 400 µL of cells to 10 mL media in a 10 cm culture dish. The dish was labelled and incubated as described above.

Cell invasion assay

Corning BioCoat Matrigel invasion chambers were used to perform cell invasion assays. As illustrated in Figure 7, the cell culture inserts contain a polyethylene terephthalate (PET)

membrane of 8 micron-sized pores coated with a thin layer of Matrigel matrix. The latter obstructs the pores of the membrane, serving as a “reconstituted basement membrane *in vitro*”, so as to block non-invasive cells from moving through the membrane. Invasive cells, on the other hand, can invade through both the Matrigel and the PET membrane.

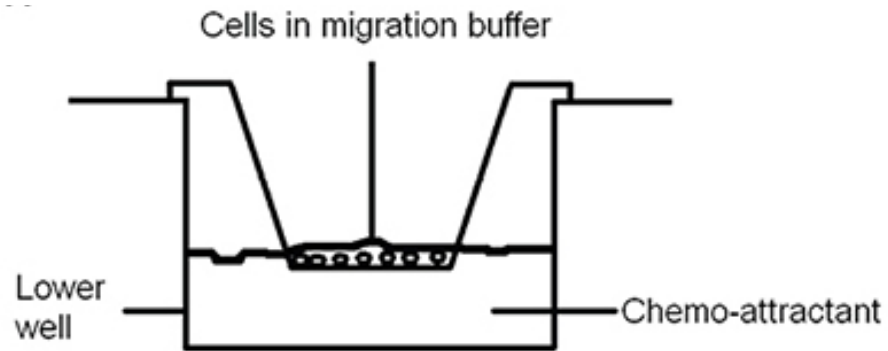


Figure 7. Schematic of an invasion chamber (Justus et al., 2014)

The assay protocol, as described by BD Biosciences Matrigel invasion chamber guidelines, includes 3 steps – rehydration of the inserts and wells, addition of cells and reagents, and measurement of invasion.

1. Rehydration

The package containing the cell culture inserts was allowed to come to room temperature, and the required number of inserts were transferred into the wells of a 24-well plate.

500 μ L of warm serum-free DMEM/F12 was added to the interior of the inserts and bottom of wells, and allow to incubate for 3 hours at 37°C, 5% CO₂ atmosphere. After rehydration, the medium was removed without disturbing the layer of Matrigel matrix.

2. Invasion

ARPE.19 cell suspension containing 75,000 cells/mL, counted using hemocytometer, was prepared in serum-free media. 750 μ L of chemo-attractant – 5% fetal bovine serum - was added to the wells of the 24-well plate. The inserts were then transferred into the wells containing the chemo-attractant, while making sure no air bubbles were trapped beneath the membrane. 500 μ L of cell suspension, containing 37,500 cells, was immediately added to the inserts. H89, KT5720, ATP, 8-Bromo cAMP, Forskolin, bFGF, NaCl, and LiCl were each added to the inserts respectively. Concentrations of the reagents used were as follows: H89 and KT5720 at 20 μ M, Forskolin at 50 μ M, ATP at 5 μ M, LiCl and NaCl at 100 mM, and 50 ng/mL of bFGF. The inserts were incubated for 72 hours.

3. Measurement of cell invasion

Non-invading cells were removed by gently scrubbing the inside of the matrigel inserts using cotton swabs. The swabbing was done quickly to avoid the cells adhering to the bottom side of the membrane from drying up. The cells were then fixed using 100% methanol for 2 minutes, stained using crystal violet for 2 minutes, thoroughly rinsed with double distilled water to remove excess stain, and air dried. The membranes were carefully removed from the insert housing using a scalpel and mounted on microscope slides. The slides were observed under the inverted microscope and the cells were counted in nine fields across the membrane to calculate the mean number of cells invaded.

The data were analyzed by one-way analysis of variance, followed by Tukey posthoc test for pairwise comparisons using R with significance defined as $p < 0.05$.

RESULTS AND DISCUSSION

We used invasion assays to analyze the effect of various ciliary and PKA modulators on cell migration of ARPE.19 cells. Images were taken of nine different areas of each membrane and cells within them were counted using ImageJ software.

Representative images of each reagent is shown in figures 8, 9, 10 and 11. According to our results, bFGF and NaCl ($p = 2 \times 10^{-6}$) were significant in promoting migration of the cells. As shown in Figures 8 and 9, bFGF and NaCl have more cells than control. On the other hand, 8-Br-cAMP, ATP (p value of .0001) and LiCl significantly inhibited migration. With a p value of 3.8×10^{-6} , H89 was also significant in reducing migration. Forskolin was slightly significant with $p = 0.05$. KT5720 ($p = 0.127$) was found to not be significant, although it appears to be trending that way.

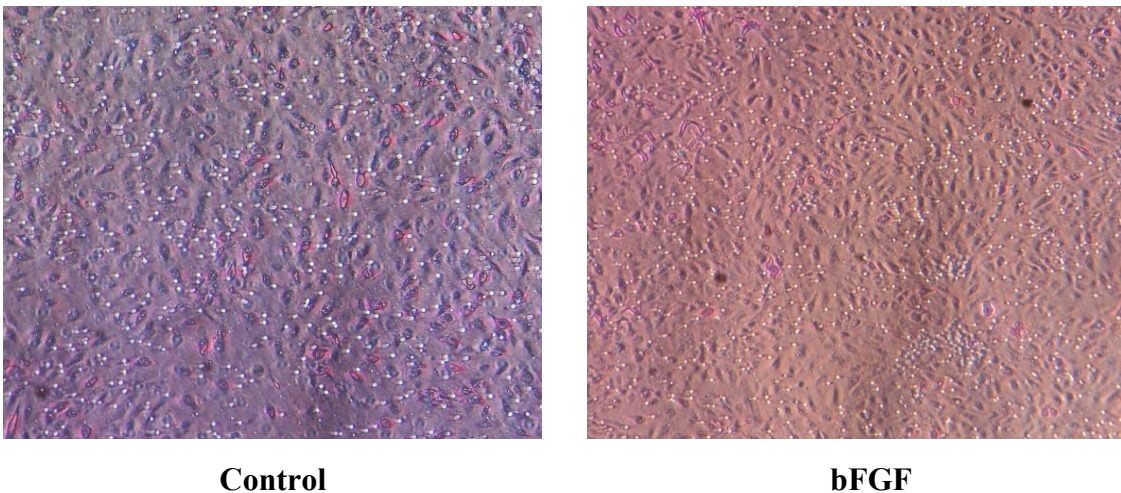
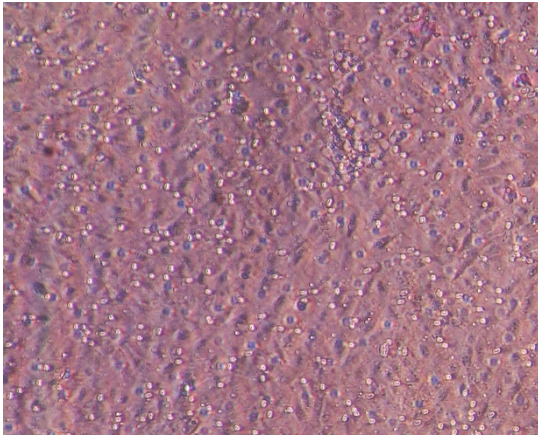
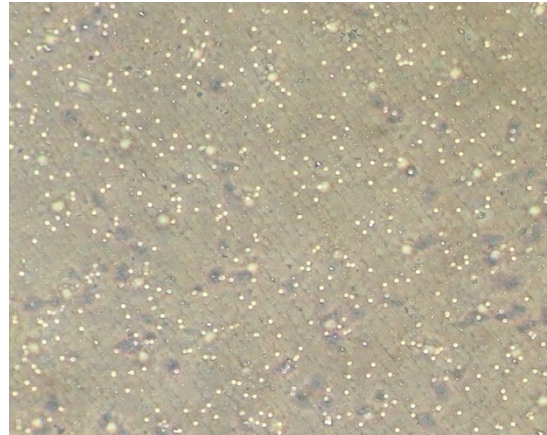


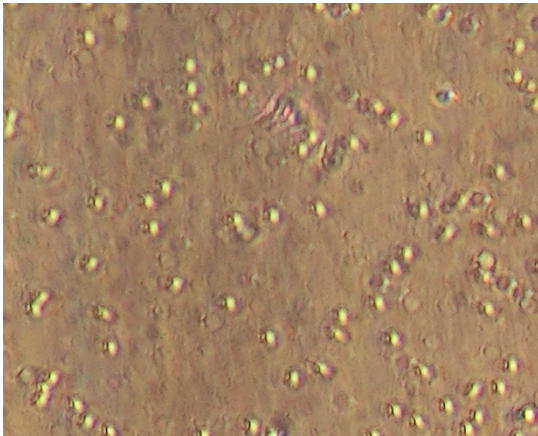
Figure 8. ARPE.19 cells stained after invasion assay. 37,500 cells were incubated without any treatment for control, and with bFGF for 72 hours. More cells are observed in the bFGF image than in the control image.



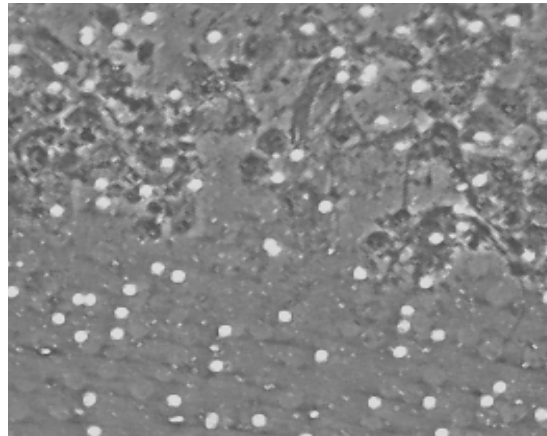
NaCl



8 Bromo cAMP

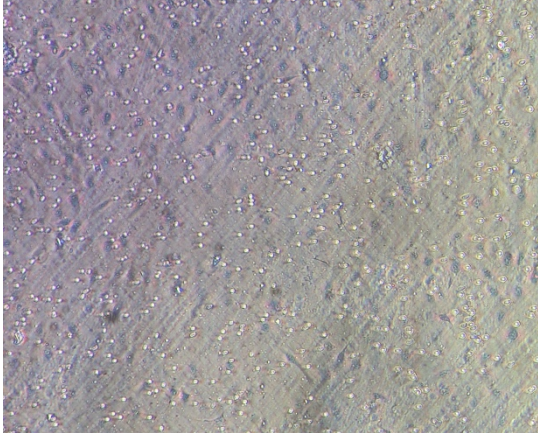


LiCl

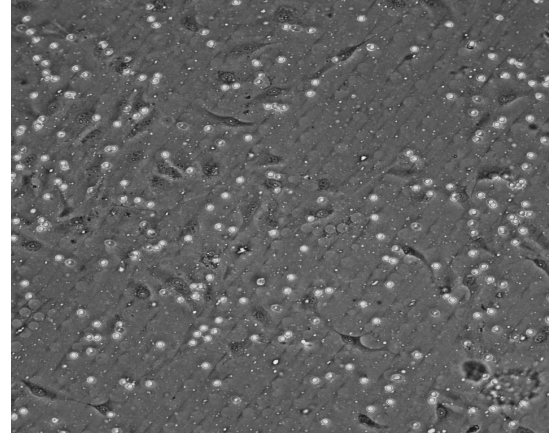


Forskolin

Figure 9. ARPE.19 cells stained after invasion assay. NaCl was used as a control for LiCl. More cells can be seen in the case of NaCl than in LiCl. Although the 8 Br-cAMP image shows more cells than LiCl's, there are fewer cells in comparison to forskolin. Forskolin, LiCl and 8 Br-cAMP modulate PKA via modulation of cAMP.

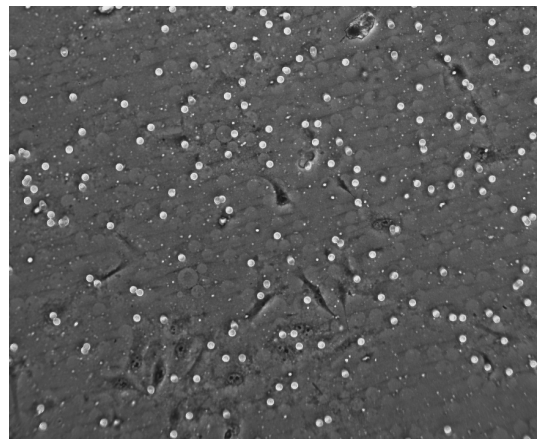


H89



KT5720

Figure 10. ARPE.19 cells stained after invasion assay. The above shows images of representative areas of cells treated with PKA inhibitors – H89 and KT5720. The image of H89 shows fewer cells as compared to KT5720.



ATP

Figure 11. ARPE.19 cells stained after invasion assay. ATP activates purinergic signaling, thereby increasing calcium ion influx into the cell. The image above of ATP-treated cells show fewer cells than control.

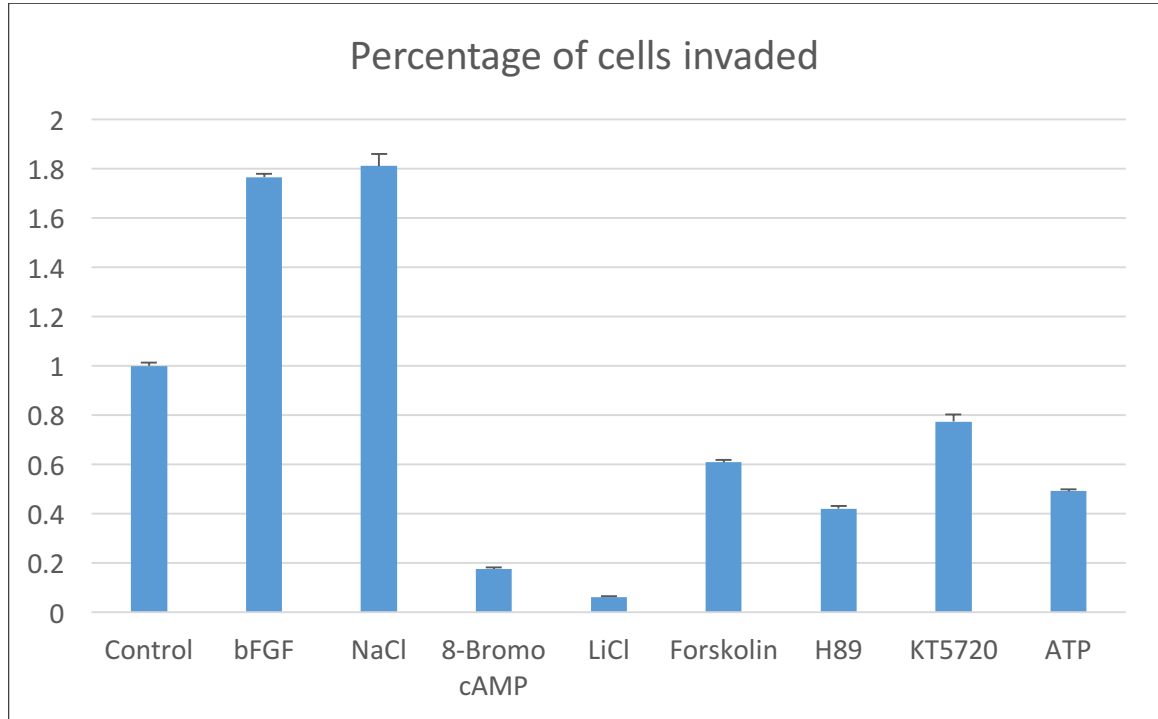


Figure 12. The graph summarizes the effect of the various modulators tested on migration of ARPE.19 cells. The error bars represent standard error. While bFGF and NaCl significantly increased migration, 8 Br-cAMP, LiCl, forskolin, H89 and ATP significantly inhibited migration. Although KT5720 decreased migration, it was not significant.

Discussion

We found that 8-Bromo cyclic AMP significantly reduced migration of ARPE.19 cells. An analog of cAMP, it acts as an activator of PKA. Negative effects of cAMP analog in RPE cells was demonstrated in 1986 by Hackett et al. Increased levels of cAMP, induced by dibutyryl cAMP, was shown to decrease migration of RPE cells. They found that it took a high concentration of dibutyryl cAMP, despite being more permeable than cAMP, to produce even a low intracellular level of cAMP (Hackett et al., 1986). 6-benzoyl-cyclic AMP, another cAMP analog, was found to reduce serum-induced migration of mouse

embryonic fibroblast cells (Chen et al., 2008), and pancreatic cancer cell lines, human Panc1 and MiaPaCa2 (Zimmerman et al., 2016). 8-Br-cAMP was found to suppress migration of human vascular smooth muscle cells (Newman et al., 2003), but stimulate migration of mouse embryonic stem cells (Kim et al., 2015).

Inhibitory effects of forskolin have been observed in other cell types as well. A study carried out in 2008 by Chen et al. found that the addition of 50 μ M forskolin inhibited migration and serum-induced invasion in MEF and 4T1 breast tumor cells. They also found that addition of H89 to MEF cells stimulated serum-induced migration and reduced the inhibitory effects of forskolin. According to their data, PKA played a negative role in MEF cell migration (Chen et al., 2008). Similar results were obtained in another recent study on pancreatic cancer cell lines, human Panc1 and MiaPaCa2, in which forskolin arrested migration in Panc1 (Zimmerman et al., 2016). Basic FGF promoted migration of bovine retinal pigment epithelial cells (Spraul et al., 20014). KT5720 was used to analyze the effect of PKA on migration of bronchial epithelial cells, and the study found that KT5720 reduced migration of those cells (Spurzem et al., 2002).

Our results show that LiCl significantly inhibited migration of ARPE.19 cells. Inhibitory effects of LiCl on serum-induced cell migration has been observed in vascular smooth muscle cells (Wang et al., 2013), and HEK293 cells (Kuang et al., 2009). LiCl is a known inhibitor of adenylate cyclase 3 (AC3) and glycogen synthase kinase-3 (GSK3) (Miyoshi et al., 2009). A recent study by Thompson et al. found that LiCl inhibited Hedgehog (Hh) signaling and altered primary cilia in bovine articular chondrocyte, and human

chondrocytes *in vitro*. LiCl-inhibition of Hh pathway was found to increase the length of cilia (as shown in Figure 6) (Thompson et al. 2016). Similar results were observed in fibroblast-like synoviocytes (Ou et al. 2009). cAMP and PKA are known to affect the length of primary cilia (Wann & Knight, 2012; Abdul-Majeed et al., 2011), and adenylate cyclase (AC) maybe involved (Thompson et al., 2016). While AC inhibition was shown to elongate cilia in synovial fibroblasts, forskolin-induced activation of AC elongated cilia in kidney and bone cells. 8-Bromo cyclic AMP was found to increase cilia length as well (Wann & Knight, 2010). Increased cAMP and resulting PKA activation increased primary cilia length in mammalian epithelial and mesenchymal cells (Besschetnova, 2010). Hedgehog is an important pathway that regulates cell migration, and PKA is a known negative regulator of Hh signaling (Tuson et al., 2011). Based on the above mentioned studies, we can hypothesize that cilia length influences cell migration, but there is no evidence to justify that speculation.

Sodium chloride was used as a control for lithium chloride, and the results we obtained were surprising. NaCl significantly increased migration of ARPE.19 cells. A recent study found that rinsing with NaCl promoted migration of human gingival fibroblast cells (Huynh et al., 2016). Osmotic stress may be a factor driving cells to migrate away from stressful environment created by NaCl, and this could be associated with the vanilloid subfamily of TRP channels (TRPV4). Hypertonicity is known to inhibit TRPV4 channels (Venkatachalam & Montell, 2014). A study carried out on neuroendocrine cells, GN11, linked activation of TRPV4 channels found on lamellipodia to retraction and reduced migration (Zaninetti et al. 2011). The inhibitory effects produced by ATP could be linked to purinergic signaling in the cell. A recent study found that ATP mediated the purinergic

inhibition of migration of breast carcinoma BTEC cells. cAMP was involved in the cytoskeletal remodeling and the resulting anti-migratory effect of those cells (Avanzato et al., 2016).

Conclusions

The results suggest that cAMP signaling at modest levels must be involved in the migration of RPE cells. Multiple studies have shown that cell migrations and invasion either require PKA activity or be inhibited by it. This dichotomy in the activity of cAMP and PKA in cell migration that a balance or an optimum in cAMP-dependent PKA activity is required for successful migration (Howe et al., 2005). Our results support this notion. Also, calcium appears to inhibit migration, though there are no studies linking calcium signaling to migration yet. Further studies are required to link migration and PKA activity to ciliary function. Some of our next experiments could be to study directional migration via live cell imaging. It would also be interesting to see if the length of cilia has an effect on migration of RPE cells.

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