The Cytoprotective Role of Ras Signaling in Glomerular Epithelial Cell Injury

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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>ESRD</td>
<td>end stage renal disease</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal regulated kinase</td>
</tr>
<tr>
<td>F-actin</td>
<td>filamentous actin</td>
</tr>
<tr>
<td>Fx1A</td>
<td>fraction 1A</td>
</tr>
<tr>
<td>G-actin</td>
<td>globular actin</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GBM</td>
<td>glomerular basement membrane</td>
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<tr>
<td>GEC</td>
<td>glomerular epithelial cell</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-S-transferase</td>
</tr>
<tr>
<td>GTPase</td>
<td>guanine-triphosphatase</td>
</tr>
<tr>
<td>HIS</td>
<td>heat-inactivated human serum</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor 1</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol (1,4,5)-trisphosphate</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun NH$_2$-terminal kinase</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
</tr>
<tr>
<td>MEK</td>
<td>mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>MN</td>
<td>membranous nephropathy</td>
</tr>
<tr>
<td>MnSOD</td>
<td>manganese superoxide dismutase</td>
</tr>
<tr>
<td>NHERF-2</td>
<td>Na$^+$/H$^+$-exchanger regulatory factor 2</td>
</tr>
<tr>
<td>NS</td>
<td>normal human serum</td>
</tr>
<tr>
<td>PDK1/2</td>
<td>phosphatidylinositol-dependent kinase 1 and 2</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>PHN</td>
<td>passive Heymann nephritis</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositol 3-kinase</td>
</tr>
<tr>
<td>PIP$_2$</td>
<td>phosphatidylinositol-4,5-bisphosphate</td>
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</table>
PIP₃: phosphatidylinositol-3,4,5-triphosphate
Rac: Rac1 GTPase
SGK: serum and glucocorticoid inducible kinase
Abstract
In experimental membranous nephropathy, complement C5b-9-induced glomerular epithelial cell (GEC) injury leads to breakdown of glomerular permselectivity and proteinuria. This study addresses mechanisms that limit complement-mediated injury, focusing on Ras. Complement-mediated injury was attenuated in cultured GEC expressing a constitutively active form of Ras (V_{12}\text{Ras}), compared with Neo (control) GEC. V_{12}\text{Ras} GEC showed constitutive activation of phosphatidylinositol 3-kinase and extracellular signal-regulated kinase pathways, but inhibition of these pathways did not reverse the protective effect of Ras. V_{12}\text{Ras} GEC showed smaller and rounder morphology, decreased F- to G-actin ratio, decreased activity of the Rho GTPase, Rac, and decreased Src activity. In V_{12}\text{Ras} GEC, disruption or stabilization of the F-actin cytoskeleton reversed the protective effect of V_{12}\text{Ras} on complement-mediated injury. Thus, the protective effect of V_{12}\text{Ras} may be dependent on remodeling of the actin cytoskeleton. Furthermore, the reduction of Src activity due to Ras activation may alter the equilibrium in activities of Rho GTPases, a family of proteins known to regulate the actin cytoskeleton. Activation of Ras signaling is a novel pathway to consider in developing strategies for cytoprotection in complement-mediated injury.
Résumé
Dans la néphropathie extra-membraneuse expérimentale, le complément C5b-9 s’attaque aux cellules épithéliales glomérulaires (GEC), causant une perte de la sélectivité glomérulaire ainsi que de la protéinurie. Cette recherche aborde les mécanismes qui restreignent les dommages causés par le complément tout en tirant l’attention sur le rôle de Ras en particulier. Les dommages causés par le complément ont été moins importants chez les GEC exprimant une forme active de Ras (V12Ras) que chez les GEC contrôles. Les GEC V12Ras sont caractérisés par l’activation constitutive du phosphatidylinositol 3-kinase et des voies de kinase, extracellular signal-regulated kinase. Cependant, l’inhibition de ces voies d’activation n’a pas inversé l’effet protecteur de Ras. Les GEC V12Ras sont de morphologie plus ronde et plus petite que les GEC contrôles, en plus de démontrer un ratio actine F sur actine G réduit et une baisse de l’activité de Rho GTPase, Rac et Src. L’effet protecteur de Ras chez les GEC V12Ras peut être inversé soit par la désorganisation ou la stabilisation du cytosquelette d’actine F. Ainsi, l’effet protecteur de V12Ras serait dépendant de la réorganisation du cytosquelette d’actine. De plus, la réduction de l’activité de Src due à l’activation de Ras pourrait altérer l’équilibre d’activation des Rho GTPases, une famille de protéines reconnue pour son activité régulatrice sur le cytosquelette d’actine. L’activation de Ras est une nouvelle voie à considérer pour le développement de stratégies visant à protéger les cellules contre les attaques du complément.
Acknowledgements

I never thought I would have been doing this 4 years ago today, considering I just got a poor grade in an introductory molecular biology class in my sophomore year. It was in my junior year of undergraduate studies that I met my mentor, Dr. Louise Larose, who shared with me her enthusiasm and dedication to her work in molecular biology. I would like express my gratitude to Dr. Larose for introducing to me the fascinating world of molecular biology, as well as helping me throughout my graduate studies as part of my supervisory committee.

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1.0 Introduction

1.1 The Kidney and Glomerulus

The kidney represents the principal organ for maintaining homeostasis in the human body. Its central role is to regulate water concentration, ion composition, the volume of the internal environment, and to excrete metabolic and non-metabolic wastes in the urine. To do this, the kidneys process the plasma portion of blood through its glomeruli and tubules to create urine, which is excreted from the body. The functional subunit of the kidney is the nephron, of which there are 1.2 million in the adult kidney. The nephron processes plasma by filtering, reabsorbing and secreting certain substances, then concentrating to form urine.

This filtration unit of the kidney is the glomerulus, which is located in the kidney cortex. The glomerulus serves to isolate the water and small solutes from the blood and prevent the loss of critical blood proteins such as albumin and clotting factors (1). When the function of the glomerular filter is impaired, proteinuria often occurs and can lead to the development of end stage renal disease (ESRD). In the patient with ESRD, dialysis and transplantation are needed, leading to significant morbidity and mortality. The cost attributed by Medicare to caring for patients with ESRD in the United States alone was estimated to be $18.5 billion in 2004 (2).

1.2 Structure of the Glomerular Filter

The first filtration barrier of the glomerular filter is the monolayer of endothelial cells that line the capillary lumen. The numerous fenestrations of the endothelial cell contribute a size selective barrier 70 to 100nm in diameter. The second major barrier is the glomerular basement membrane (GBM), an acellular matrix 300 to 350nm thick with negative charges that serve as an additional restriction mechanism. The GBM also supports the attachment of the podocytes or visceral glomerular epithelial cells (GEC) on the outer aspect of the glomerular capillary loop. The final filtration barrier is the ~40nm filtration slit formed between adjacent GEC lining the outer aspect of the glomerular capillary. The overall barrier function of the endothelial cell, GBM, and GEC serves to
isolate a cell and protein free filtrate of the blood that can be properly processed by the tubules of the nephron.

1.2.1 Role of the Glomerular Epithelial Cell (GEC) and the Filtration slit

The GEC is terminally differentiated and contains many finger-like extensions called foot processes, which protrude and interdigitate with the foot processes of adjacent GEC to cover the entire GBM (reviewed in (3)). The basal domain of the GEC is connected to the GBM, and the apical domain is covered in sialoglycoproteins that provide an overall negative charge. Between foot processes exists a meshwork of proteins that make up the slit diaphragm, which forms a zipper like structure that connects to the intracellular actin cytoskeleton. These proteins include nephrin, P-cadherin, CD2AP, ZO-1, FAT, podocin, and Nephl (4). Mutation and inactivation of these corresponding proteins leads to foot process effacement and development of proteinuria. Therefore, these proteins are crucial to maintaining a barrier to large molecular weight proteins. Overall, the interdigitating foot processes and the slit diaphragm structure compose the final filtration barrier. This ~40nm filtration slit represents the final layer of the glomerular filter, from which the final filtrate is formed. GEC injury, which is possible through a variety of causes, leads to a breakdown in the final filtration barrier and is often the cause of diseases associated with proteinuria.

1.2.2 The GEC cytoskeleton

The cytoskeleton is what enables eukaryotic cells to adopt a variety of shapes and to carry out coordinated and directed movements. The cytoskeleton is composed of three structural elements: actin microfilaments, intermediate filaments, and microtubules. Microtubules and intermediate filaments make up the majority of GEC body cytoskeleton, including the major processes (5). Actin cytoskeleton is localized in the foot processes of GEC and is crucial to its unique ultrastructure and function.

GEC foot processes are dynamic structures, functioning to support the varying hydrostatic pressures on the glomerular capillary and itself. The actin cytoskeleton is linked to the membrane through different mechanisms. In the apical membrane, podocalyxin associates with the actin cytoskeleton through interactions with ezrin and the
scaffolding Na+/H+-exchanger regulatory factor 2 (NHERF2) (6, 7). In the basal membrane, actin connects to the GBM through α3β1 integrin and α-β dystroglycans, and signals through integrin linked kinases (8). In the slit diaphragm, nephrin protein is linked to the actin cytoskeleton at least in part via Nck adaptor protein (9, 10). Disturbance in these interactions leads to foot process effacement in vivo. This leads to an increase in microfilament density that creates intercrossing stress fibers at the sole of foot processes (11).

Rho family guanine triphosphatases (Rho GTPases) also play an important role in actin cytoskeleton remodeling in GEC, including Rac, Cdc42, and RhoA (12). Mice lacking Rho GDP dissociation inhibitor-alpha (RhoGDI-α), develop massive proteinuria (13). In various cell systems, RhoA can mediate stress fiber formation, while Rac and Cdc42 mediate formation of lamellipodia and filipodia formation, respectively (14).

Src family kinases can also play a significant role in the modulation of the GEC actin cytoskeleton. In GEC, Src family kinases phosphorylate the cytoplasmic domain of the slit diaphragm protein nephrin. This leads to the recruitment of the adaptor protein Nck, which finally mediates the assembly of actin filaments (10, 15). In addition, there is evidence of crosstalk between Src family kinases and Rho GTPases in various cell systems (16).

1.3 Injury to the glomerulus

A breakdown in glomerular filtration often leads to nephrotic syndrome, which is characterized by massive proteinuria, generalized edema, hyperlipidemia and hypercholesterolemia. Membranous nephropathy (MN) is the second most common cause of nephrotic syndrome after diabetic nephropathy in adults. MN is an immune complex disease of primarily unknown etiology, and represents a major primary cause of nephrotic syndrome. It is diagnosed by kidney biopsy, and is characterized by greatly thickened capillary walls as a result of the basement membrane being 5 to 10 times thicker than normal (17). Upon visualizing the glomerulus by electron microscopy, there exist numerous electron dense immune complexes in intramembranous and sub-epithelial locations. The deposits demonstrate IgG and C3 by immunofluorescence. There is also diffuse effacement of the GEC foot processes.
A model of membranous nephropathy is passive Heymann nephritis of the rat (PHN) (18). In this model, heterologous anti-Fx1A (anti-Fraction 1A) antibodies is first generated by immunization of animals with an antigenic preparation rat proximal tubule brush border proteins (Fraction 1A). After injection of the generated anti-Fx1A antibodies into the rat, the plasma complement system is activated and C5b-9 membrane attack complex is deposited on the GEC. The resulting kidney immunohistochemical and functional features of this rat model closely resemble that of human membranous nephropathy. From this model, it was found that damage to GEC is mediated by complement activation, and that this triggers a cascade of intracellular signaling events. This model is also applicable to in vitro studies of GEC, where incubation with anti-Fx1A antibodies would first “prime” the GEC culture for activation of the classical complement pathway. Subsequent incubation with normal serum would cause deposition of C5b-9 complexes to cause cell injury (19).

The complement system is composed of soluble plasma proteins which functions include binding and destroying invading pathogens. The end effect of complement activation is recruitment of inflammatory cells, opsonization of pathogens, or killing of pathogens through the C5b-9 membrane attack complex. Formation of the C5b-9 complex can happen either through the classical pathway, which involves antigen-antibody binding, or the alternative pathway, which involves spontaneous formation of C5b-9. In contrast to non-nucleated cells, nucleated cells such as GEC have mechanisms to counteract injury by C5b-9. These include removal of C5b-9 by exocytosis and expression of complement regulatory proteins. In GEC, the C5b-9 complex can be transported intracellularly and extruded into the urinary space, where it is excreted in the urine.

Cytolysis of nucleated cells display multi-hit kinetics, as numerous C5b-9 lesions is required for cell death. GEC loss has been demonstrated in PHN and in vitro, and a substantial body of literature has implicated this as contributing to the progression of glomerulosclerosis (20-22).

Ischemia reperfusion injury is also commonly encountered in mature kidneys and in allografts. Ischemia, or loss of blood flow, is a major cause renal tubular injury, and may also injure glomerular cells. Depending on the severity of ischemia, this can lead to
acute renal failure. Reperfusion, or restoration of blood flow, is associated with the production of reactive oxygen species. In vitro, it is possible to mimic ischemia-reperfusion injury in GEC using chemical anoxia followed by re-exposure to glucose. During these processes, GEC signaling pathways are activated which can either exacerbate or attenuate cell injury.

Ultimately, GEC injury and cell death, along with the inability for differentiated GEC to proliferate and repair the damaged glomerulus, plays a pivotal role in the progression of glomerular scarring (23). It is thought that GEC loss leads to an area of bare GBM, first leading to the bulging of the capillary loop and adherence to the Bowman’s capsule. This represents the first step in the development of focal segmental glomerulosclerosis and leads to scarring in the capillary loop(24).

1.4 Signaling pathways activated by C5b-9 in GEC

In PHN, C5b-9 disrupts the GEC plasma membrane integrity through the formation of a transmembrane channel. As a result, an assortment of subcellular signaling cascades is activated. These include activation of phospholipases, protein kinases, transcription factors, growth factors, proteinases, and others (19). Some of these signaling pathways significantly exacerbate injury, whereas others appear to limit the extent of injury, or promote recovery. However, some signaling pathways may lead to both protective and detrimental responses.

C5b-9 leads to a general increase in cytosolic calcium (25). Extracellular calcium flows through the transmembrane channel into the cytoplasm and there is also release of calcium from intracellular stores.

C5b-9 mediates the transactivation of a variety of growth factor receptor tyrosine kinases, which can serve as scaffolds for protein interactions and cell signaling (26). In cell culture and PHN, activation of phospholipases by C5b-9 leads to the liberation of arachidonic acid from the plasma membrane, endoplasmic reticulum (ER), and nuclear membrane (27, 28). Arachidonic acid functions as a key substrate for subsequent metabolism into eicosanoids. It has been shown that the pathway of arachidonic acid metabolism could alter kidney function in PHN (19).
Mitogen activated protein kinases (MAPK) are modules of serine/threonine kinases that respond to a variety of extracellular stimuli to regulate cellular activity. The MAPKs modules c-Jun N-terminal kinase (JNK), extracellular signal regulated kinase (ERK), and the p38 MAPK have all been shown to become activated in response to C5b-9 deposition in GEC (29-31).

The ER stress response has been documented to become activated upon C5b-9 deposition in GEC, including upregulation of ER stress chaperones bip and grp94 (32) and induction of the unfolded protein response (33). These changes happen in a cPLA\textsubscript{2} dependent manner. In these studies, inducing the ER stress response with tunicamycin or adriamycin prior to complement injury has been shown to have a beneficial effect in kidney function during PHN.

C5b-9 also results in the production of reactive oxygen species (29), which can either directly contribute to injury through lipid peroxidation or activate other signaling pathways in low doses (19). Activation of the NF-κB transcription factor has also been documented upon C5b-9 deposition.

Finally, C5b-9 may also lead to the disruption of the actin cytoskeleton. Upon complement injury, actin filaments condense at the base of effaced podocyte foot processes, which is accompanied by disruption of the filtration slits (34, 35). At a subcellular level, Rho family of GTPases, including RhoA, Cdc42, and Rac are modulated (12). In GEC, actin cytoskeleton has been shown to facilitate the activation of cPLA\textsubscript{2} during complement mediated injury (36).

**1.5 Signaling pathways that may protect or facilitate recovery from C5b-9 injury in GEC**

The signaling pathways that are activated upon exposure to the C5b-9 complex are numerous. The following sections will describe signaling pathways that may potentially mediate cytoprotection from C5b-9 induced injury in GEC.

**1.5.1 Ras signaling**

Ras is a small 21kDa GTPase that is highly conserved from yeast to humans. It is activated upon activation of various membrane receptors, and is responsible for a wide
variety of signaling pathways that control cytoskeletal integrity, proliferation, cell adhesion, cell migration, and apoptosis. It cycles between two conformations: a GTP bound “on” state, and a GDP bound “off” state. Therefore it acts as a molecular switch. Guanine nucleotide exchange factors (GEFs) promotes the exchange of GDP for GTP on Ras, thereby allowing Ras to form complexes and activate downstream effector target molecules. GTPase-activating proteins (GAPs) stimulate the intrinsic GTPase activity of Ras to promote formation of the GDP bound, and hence inactive, form of Ras (37). The GTPase activity of Ras requires the action of p120 RasGAP, as Ras is a poor catalyst on its own (38, 39).

Ras signaling is dependent on localization. In order for Ras to signal, it associates with the cytoplasmic leaflet of cellular membranes (40). In Ras C-terminus lies a hypervariable region that terminates with a CAAX motif. Depending on the modification of this motif, Ras becomes associated with different membrane domains. In particular, palmitoylation and farnesylation targets Ras to lipid rafts and caveolae, respectively (41).

To date, Ras signaling is dependent on many pathways. The first and most well characterized pathway is the Raf to mitogen-activated protein kinase kinase (MEK) to extracellular-signal-regulated-kinase1/2 (ERK) pathway (42). In GEC, transfection with a constitutively activated form of Ras (V12Ras), has been shown to supplant the requirement of extracellular matrix signaling to enable proliferation (43). Transfection of GEC with a constitutively active form of MEK (R4F-MEK) or focal adhesion kinase (FAK), showed ERK activation and decreased apoptosis in a manner that is dependent, at least in part, on the actin cytoskeleton (44). Therefore, Ras signaling through ERK appears to be important in maintaining GEC properties of normal adhesion and apoptosis. As well, ERK is activated during complement mediated injury in a manner that is dependent, at least in part, on Ras activation and on the actin cytoskeleton (31).

In addition to ERK, there are numerous reports that Ras can activate other MAPK pathways. Expression of a dominant negative form of Ras has been shown to decrease the effect of platelet derived growth factor on activating p38 phosphorylation (45). Ras has been shown to activate MEKK (46), and granulocyte colony stimulating factor activation of JNK has been shown to be Ras dependent (47). Therefore, Ras can potentially activate p38 and JNK in the context of GEC.
Perhaps the second most characterized effector of Ras is the PI3K pathway. GTP-bound Ras has been shown to interact with, and hence activate, the p110 subunit of PI3K (48). In GEC, transfection with a form of Ras that constitutively activates PI3K, but not the ERK pathway, does not supplant the requirement of extracellular matrix for proliferation (49). This suggests that Ras mediated activation of PI3K by itself is not sufficient to supplant the ECM for growth and survival.

Ras has also been characterized to associate with other GEFs, which activate other GTPases. This includes Ral GEFs (Ral-GDS, Rgl, Rgl2/Rlf, Rgl3), and the Rac GEF, Tiam1 (50). RalB has been shown to be required for survival of tumour cells, whereas RalA is required for anchorage independent proliferation.

Finally, Ras has been shown to activate phospholipase C epsilon, which catalyzes the conversion of phosphatidylinositol-4,5-bisphosphate (PIP$_2$) to the second messengers inositol (1,4,5)-triphosphate (IP$_3$), and diacylglycerol (DAG) (51). IP$_3$ interacts with IP$_3$-sensitive Ca$^{2+}$ channels to elevate Ca$^{2+}$ ions, whereas DAG activates protein kinase C (PKC) (37).

Ras is known for its pro-survival effects in variety of cell systems (52), which is primarily thought to involve PI3K signaling and Akt activation (53). However, cell survival dependent on Ras mediated activation of the Raf to (MEK) to ERK1/2 pathway has also been demonstrated (54).

Ras-mediated signaling also affects the actin cytoskeleton. Microinjection of constitutively activated Ras protein in cultured fibroblasts induces membrane ruffling (lamellipodia) due to cortical actin rearrangement (55). This membrane ruffling phenotype, was also observed after incubation with growth factors and microinjection of cells with constitutively active Rac, a Ras-like GTPase known to become activated by Ras (56).

Ras is activated upon assembly of C5b-9, and this activation was shown to be dependent on G$\beta$γ subunit of G-protein coupled receptors (57). In the context of GEC, Ras mediates the C5b-9 dependent activation of ERK, as the expression of a dominant negative Ras inhibited complement-dependent ERK activation (31).
Overall, the effect of Ras is dependent on the cellular context, such as the cell type, developmental stage, and intensity of Ras activation. In the context of GEC, Ras signaling may serve as an important mediator of complement injury.

1.5.2 PI3K signaling

PI3K is responsible for a diverse group of functions that are crucial for mediating cell growth and survival. Structurally, it is a heterodimer that is composed of an inhibitory regulatory (p85) subunit and a catalytic (p110) subunit. Upon activation by one of a variety of mechanisms, including receptor tyrosine kinase activation and activation of Ras, PI3K is recruited to the plasma membrane. PI3K phosphorylates the lipid second messenger PIP2 into phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 recruits proteins containing the pleckstrin homology (PH) domain to the membrane, including phosphatidylinositol-dependent kinase 1 and 2 (PDK1, PDK2), and Akt. The function of PI3K is antagonized by the PIP3 phosphatase PTEN, which converts PIP3 to PIP2.

The primary downstream mediator of PI3K is the serine/threonine kinase Akt. Akt activation leads to phosphorylation of a wide variety of substrates that regulate cell survival, cell cycle progression, cell growth, and cell metabolism (58). Full Akt activity is achieved via phosphorylation on serine 473 by PDK1. Akt itself promotes an anti-apoptotic phenotype directly through phosphorylation of Bad, a pro-apoptotic protein and a member of the Bcl-2 family. This phosphorylation leads to the binding of Bad to 14-3-3, therefore forming an inactive complex to prevent Bad from inactivating anti-apoptotic proteins. Therefore, PI3K activation serves to mediate a transient assembly of proteins in order to mediate a sustained cellular signal for survival. Akt has also been shown to phosphorylate Caspase-9 to attenuate its activity.

Akt exerts control over transcriptional activity of other apoptotic machinery. Akt can phosphorylate members of the FoxO transcription factor family. FoxO transcription factors regulate expression of the cyclin-dependent kinase inhibitor p27, which inhibits cell cycle progression, pro-apoptotic genes such as FasL, and manganese superoxide dismutase (MnSOD), which may protect the cell against oxidative stress. Upon phosphorylation, FoxO transcription factors unbind from DNA, become excluded from
the nucleus, and are subsequently degraded. Three members of the FoxO family of transcription factors are phosphorylated by Akt, which leads to its inactivation via exclusion from the nucleus. This leads to a general shutdown of the transcription of pro-apoptotic machinery.

In addition, Akt could be involved in phosphorylating Mdm2, an E3 ubiquitin ligase for p53. p53 promotes transcription of pro-apoptotic genes such as Bax, PUMA, Noxa, Fas, and DR5. The phosphorylation of p53 could result in the inactivation and degradation for an overall anti-apoptotic effect.

Finally Akt may lead to cell survival by modulating cell metabolism. Akt activation has been shown to lead to the translocation of the glucose transporter 4 (GLUT4) to the plasma membrane. Akt can also lead to the phosphorylation and inactivation of glycogen synthase kinase GSK-3 to promote the storage of glucose as glycogen. The end result is an increased storage of energy that may be utilized to counter cell injury.

Serum and glucocorticoid inducible kinase (SGK), is an early response gene that is activated by PI3K following serum or glucocorticoid treatment. It is activated by PI3K and shares a 45-60% homology to the catalytic kinase domain of Akt (59). It is regulated at both transcriptional and post-translational levels. Hyperosmotic stress by sorbitol induces accumulation of SGK mRNA transcripts and protein in mammary epithelial cells, which is dependent on the upstream activation of p38 MAPK (60). In GEC, complement has been shown to activate p38, which exerts a protective effect (30). Therefore, a possible downstream mechanism for this cytoprotection may be due to the upregulation of SGK. Full catalytic activity of SGK requires phosphorylation of amino acid residues Thr256 and Ser422, by the PI3K dependent kinases PDK1 and PDK2 (61, 62).

Functionally, SGK has been shown to induce cell survival to various forms of stress. In a study by Leong et al, SGK became upregulated in mammary epithelial cells in response to osmotic stress, heat shock, ultraviolet irradiation, and oxidative stress in a p38 MAPK dependent manner. Expression of a constitutively active SGK protected these cells against apoptosis induced by these stimuli (63). In addition, SGK is upregulated in animal models of neurodegeneration, which is thought to exert a protective role against
oxidative stress induced death (64). Overall, SGK may play an important role in mediating cytoprotection against injury in GEC.

The localization of nephrin to the slit diaphragm suggested that it served a critical function in regulating filtration (65). It was later discovered that the cytoplasmic tail of nephrin, together in a complex with CD2AP, is able to activate PI3K, leading to the phosphorylation and activation of the serine/threonine kinase Akt and its downstream substrates (66). Thus, this suggests that the structure and functional integrity of the slit diaphragm, including proper nephrin signaling, may be required to regulate cell survival. A recent study showed that insulin-like growth factor I (IGF-1), prevented apoptosis from etoposide in human glomerular epithelial cell cultures (67). Therefore, there is evidence that PI3K signaling is important in regulating GEC survival and morphology.

PI3K has also been shown to become activated during C5b-9 deposition in a variety of cell types (68, 69). In oligodendrocytes, C5b-9 was shown to activate PI3K and protect against apoptosis through the intrinsic pathway (68) and extrinsic pathway (70). In human aortic smooth muscle cells, C5b-9 induced activation of PI3K and Akt (69, 71). Therefore, there is substantial evidence that C5b-9 may activate PI3K in GEC.

Overall, PI3K may serve as an important mediator of GEC injury.
2.0 Hypothesis and approach

The signaling cascades that are known to become activated in response to C5b-9 in GEC are numerous. However, the understanding of the functions of these signaling pathways in complement-mediated injury is less complete. Therefore, our approach was to identify, from a functional standpoint, novel signaling pathways that play a cytoprotective role during C5b-9 mediated injury in GEC, focusing on Ras and PI3K signaling.

Although Ras was originally identified as a proto-oncogene, studies have shown that Ras plays crucial roles in cell proliferation, differentiation, and survival without necessarily causing cell transformation. Our hypothesis was that Ras mediated signaling in GEC is protective against injury. PI3K has also been well characterized in its role in cell survival in many cell systems. Our hypothesis is that signaling via the PI3K pathway also plays a protective role against injury. The rationale for our hypothesis is based on findings from slit diaphragm signaling and C5b-9 mediated PI3K activation in other cell systems. Our overall goal is to address the role of Ras and PI3K during complement injury in GEC. To do this, we utilized a complement injury model in GEC cultures. We then assessed the role of Ras and PI3K pathways using a variety of molecular biology techniques including immunoblotting, immunoprecipitation, pharmacological modulation of signaling pathways, and fluorescence microscopy.
3.0 Materials and methods

3.1 Materials
Tissue culture reagents Dulbecco’s modified Eagle’s medium (DMEM), Ham’s F-12, Opti-MEM, NuSerum were purchased from Gibco (Burlington, ON). Electrophoresis and immunoblotting reagents were purchased from Bio-Rad laboratories (Mississauga, ON). MG-132 and PD98059 were purchased from Calbiochem (Mississauga, ON). Insulin like growth factor-1, wortmannin, LY294002, epidermal growth factor, dexamethasone, SP600125, SB203680, latrunculin B, cytochalasin D, phalloidin-rhodamine, were all purchased from Sigma-Aldrich (Mississauga, ON). H₂O₂ was purchased from Fisher Scientific (Pittsburgh, PA). Lipofectamine 2000 was purchased from Invitrogen (Burlington, ON). Anti-phospho-Akt and anti-phospho FKHR antibodies were purchased from Cell Signaling (Danvers, MA). Anti-SGK, anti-Rac, and anti-190 RhoGAP antibody was purchased from Upstate Biotechnologies (Charlottesville, VA). Anti-phospho-Src antibody was purchased from Biosource International (Burlington, ON). Sepharose 4B beads were purchased from Pharmacia Biotech (Baie d’Urfé, QC). Anti-phospho-tyrosine antibody was purchased from BD Biosciences Pharmingen (San Diego, CA). Enhanced chemiluminescence detection reagents were purchased from Amersham Biosciences (Piscataway, NJ). HA-SGK plasmid was a kind gift from Dr. Michael Greenberg (Harvard University). Plasmid HO6T1 containing the V¹² Ras gene was kindly provided by Dr. Morag Park (McGill University). The G-LISA RhoA activation assay biochemistry kit was purchased from Cytoskeleton Inc. (Denver, CO).

3.2 Cell culture and transfection
Primary cultures of rat GEC were established as described previously (22). Briefly, rat glomeruli were explanted onto culture dishes coated with collagen gels. After 7-9 days, colonies of GEC were marked and excised from the collagen and replated onto collagen-coated multiwell dishes. The wells growing pure colonies of GEC were selected for. The selection criteria includes polygonal shape, cobblestone appearance at confluency, cytotoxic susceptibility to low doses of puromycin aminonucleoside, positive immunofluorescence for staining for cytokeratin, and presence of junctional complexes.
by electron microscopy. A subclone of GEC growing on plastic substratum was created by plating GEC growing on collagen gels onto plastic. Many of these cells eventually became non-viable and detached, but colonies emerged after 3-4 weeks. These cells were used for further studies and transfection. The standard medium used to maintain GEC culture, K1, is a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 medium, supplemented with 5% NuSerum and hormones. According to manufacturer, NuSerum contains 25% fetal calf serum, 5ng/ml epidermal growth factor, and hormone supplements.

All cultures were maintained at 37°C in an atmosphere of 95% air and 5% CO₂. Upon confluence, GEC were passaged. Briefly, GEC were suspended by incubating with 0.05% trypsin-0.02% EDTA mixture in Ca²⁺ and Mg²⁺ free Hank’s balanced salt solution. Cells were resuspended in K1 medium and replated onto tissue culture dishes. Studies were done with cells between passages 10 and 90. Unless otherwise noted, experiments were performed on a subclone of GEC that contains the neomycin-resistance gene (Neo GEC).

Transfection of HA-SGK plasmid was performed on human embryonic kidney 293T cells. Briefly, 10⁶ cells were plated into 100mm plates in DMEM plus 10% FBS. After growing for 24 hours, cells were transfected with 1μg HA-SGK plasmid according to manufacturer’s instructions. After overnight incubation, the cell medium was supplemented with 7ml DMEM + 10% FBS. After 24 hours, cells were harvested and proteins were analyzed by Western blot.

Creation of a stable GEC line expressing V¹²Ras was described previously (43). Briefly, HO6T1 plasmid containing a constitutively active HA-Ras gene (Gly¹²→Val¹² mutation) and G418 (neomycin) resistance was transfected into GEC adherent to plastic using the CaPO₄ technique according to the manufacturer. After transfection, GEC were cultured in K1 medium containing 0.5mg/ml G418. GEC clones resistant to G418 were isolated, expanded, and assessed for expression of Ras protein by Western blot.
3.3 Immunoblotting

Cells were scraped into homogenization buffer containing 50mM HEPES, 0.25M sucrose, 1mM EDTA, 1mM EGTA, 20μM leupeptin, 20μM pepstatin, 0.1mM PMSF (4°C). Proteins were solubilised in buffer containing 0.5% Triton X-100, 50 mM β-glycerophosphate, 2mM MgCl₂, 1mM dithiothreitol, 20μM leupeptin, 20μM pepstatin, 0.2mM PMSF, 1mM Na₃VO₄, 1mM EGTA, pH 7.2 (4°C). The mixture was centrifuged at 14,000g for 1 minute and the supernatant was used for immunoblotting. Samples were boiled in Laemmli sample buffer and 50 to 100μg protein were loaded onto a 7.5% polyacrylamide gel and subjected the SDS-PAGE under reducing conditions. Proteins were electrophoretically transferred onto nitrocellulose paper and blocked with 5% bovine serum albumin, incubated with primary antibody, and then horseradish-peroxidase conjugated secondary antibody. The blots were processed using enhanced chemiluminescence technique (ECL; Amersham Pharmacia Biotech). Protein signal via ECL was analyzed using scanning densitometry, using National Institutes of Health ImageJ software.

3.4 Rac and RhoA activation assay

For Rac activity assessment, GST fused to the Rac-GTP binding domain of PAK, CRIB, was first purified as described previously (12). Briefly, *Escherichia coli* BL21 cells transformed with GST-CRIB were grown at 37°C to an absorbance of 0.7. Expression of the recombinant protein was induced by addition of 0.1mM isopropylthiogalactoside for 2hrs. Cells were resuspended in sonication buffer (50mM Tris-HCl, pH 8, 2mM MgCl₂, 0.2mM Na₂S₂O, 10% glycerol, 20% sucrose, 2mM dithiothreitol, 1μg/ml leupeptin, 1μg/ml pepstatin, and 1μg/ml aprotinin) and sonicated. Cell lysates were centrifuged at 4°C for 20 min at 45,000g and the supernatant was incubated with glutathione Sepharose 4B beads for 30min at 4°C. GST-CRIB bound to the beads were washed three times in immunoprecipitation lysis buffer, and then protein quantity was estimated using Coomassie blue stained 10% SDS-PAGE gels. GEC culture lysates were prepared by scraping into homogenization buffer and solubilizing using Mg²⁺ lysis buffer containing 25 mM HEPES (pH 7.5), 1% NP-40, 10mM MgCl₂, 100mM NaCl, 5% glycerol, 5mM NaF, 1mM Na₃VO₄, 1mM PMSF, 10μg/ml aprotinin, 10μg/ml leupeptin. Lysates were
then incubated with GST-CRIB bound on beads for 1 hr at 4°C. The beads and protein were then washed three times with Mg\textsuperscript{2+} lysis buffer and subjected to SDS-PAGE and immunoblotting with anti-Rac antibody.

Assessment of RhoA activity was performed using the G-LISA RhoA activation assay biochemistry kit from Cytoskeleton Inc. according to manufacturer’s instructions. Briefly, cell lysates were prepared quickly on ice using provided lysis buffer. Cos-1 cells (33) transiently transfected with L\textsuperscript{63}-RhoA were used as a positive control. Protein concentration was equalized to 1mg/ml and incubated in 96-well plates containing a Rho-GTP binding protein linked to the wells. Wells were washed and RhoA was then detected using a RhoA antibody. The colorimetric signal for RhoA was developed and assessed using an ELx808 ultra microplate reader (Bio-Tek Instruments Inc).

3.5 p190 RhoGAP activation assay

GEC lysates were first diluted with immunoprecipitation buffer to a concentration of 1μg/μl total protein. Immunoprecipitation was achieved by incubating 1mg of total protein with 4μg of anti-p190 RhoGAP antibody at 4°C overnight. Then, the mixture was immunocomplexed by mixing with additional 100μl of protein G agarose bead slurry at 4°C for 2 hours. The mixture was then centrifuged at 14,000g and the resulting beads were washed with immunoprecipitation buffer three times. The isolated immunocomplexes bound to beads were then resuspended in 2x Laemmli buffer and boiled for 5 minutes. The resulting solubilised immunocomplex was then resolved using SDS PAGE, and immunoblotted using anti-pY69 antibody to assess tyrosine phosphorylation. The blot was later stripped and reprobed using anti-p190 RhoGAP antibody to verify identity.

3.6 In vitro GEC injury

For complement mediated injury, GEC growing in monolayer culture (48hr after replating) were incubated in 5% v/v rabbit anti-GEC antiserum diluted in modified Krebs-Henseleit buffer (measurement buffer) containing 145mM NaCl, 5mM KCl, 0.5mM MgSO\textsubscript{4}, 1mM Na\textsubscript{2}HPO\textsubscript{4}, 0.5mM CaCl\textsubscript{2}, 5mM glucose, and 20mM Hepes, pH 7.4, for 40
minutes at 22°C. GEC were then incubated with complement-containing normal human serum (NS), or heat-inactivated (decomplemented) human serum (HIS), diluted to various concentrations in measurement buffer. For studies of cytolysis, higher concentrations (5% to 15%) of NS were used. Complement has been shown to not become activated in the absence of anti-GEC antibody (28).

For anoxia-recovery injury, GEC were washed with glucose free measurement buffer, and then incubated with 10µM antimycin A plus 10mM 2-deoxyglucose (anoxia) for 90min. Cells were subsequently washed with glucose free measurement buffer and reincubated with glucose-replete medium for overnight (recovery). For assessments of cytolysis, LDH release was measured in the medium.

3.7 Measurement of complement-dependent cytotoxicity (LDH)

Complement-mediated cytotoxicity was assessed by measuring the release of lactate dehydrogenase (LDH) into the cell treatment medium. Samples of the medium were mixed into lactate/glycine buffer at pH 8.9 containing 58mM lithium lactate, 0.2M glycine, 4.8mg/ml NAD⁺. Spectrophotometric readings were performed at 340nm at 30 second intervals for 2 minutes to assess LDH activity. % LDH release was calculated LDH release for sample versus LDH release for triton-X soluble fraction. Specific LDH release for complement mediated injury was calculated as:

\[
\text{% Specific LDH} = \frac{[\text{NS} - \text{HIS}]}{[100 - \text{HIS}]}
\]

Where NS and HIS is the percentage of total LDH released into the medium upon their respective treatments (5%, 10%, 15% v/v for NS, 15% v/v for HIS).

The specific LDH release for anoxia-recovery mediated injury was calculated as:

\[
\text{% Specific LDH} = \frac{[\text{A/R} - \text{K1}]}{[100 - \text{K1}]}
\]
Where A/R is the percentage of total LDH release into the medium during anoxia-recovery treatment and K1 is the percentage of total LDH release released into the medium without treatment (medium alone).

3.8 Fluorescence microscopy
GEC adherent to glass cover slips were fixed with 3% paraformaldehyde in PBS, permeabilized with 0.5% Triton X and blocked with 0.1% glycine. They were then incubated with phalloidin-rhodamine (0.043μg/ml) and DNase I conjugated to Alexa-Fluor 488 (9μg/ml) diluted in 3% BSA for 30min. Cells were then washed and mounted onto glass slides and photographed using a Nikon Diaphot fluorescence microscope and a Nikon Coolpix 995 digital camera. Photographs were taken under rhodamine and fluorescein filters of the same visual field.

Filamentous/globular (F/G) actin ratio was determined by the mean red/green intensities of 3 microscopic visual fields per slide and corrected for their respective background intensities. Photographs were analyzed using Adobe Photoshop software for red/green intensities.

3.9 Statistics
Data are presented as means ±SEM. One way analysis of variance (ANOVA) between the groups was used to determine significant differences. Where significant differences were found, individual comparisons were made using the t-statistic and correcting the critical value using the Bonferroni method. Two-way ANOVA was used to determine significant differences in multiple measurements amongst groups. Paired t-tests were performed to determine significant differences between groups.
4.0 Results

4.1 Ras signaling is cytoprotective against complement mediated injury in GEC

To determine the role of Ras in GEC injury, we utilized a GEC line that expresses a constitutively active form of Ras (V12Ras), as described previously (43). V12Ras contains a mutation on the 12th amino acid from a glycine to a valine, thus rendering it insensitive to GTPase activating activity by p120RasGAP. Thus, V12Ras is locked in a GTP-bound active state. We previously isolated several GEC clones that express V12Ras by stable transfection. Among these, Ras514 expresses high levels of V12Ras, whereas Ras25 expresses lower levels of V12Ras protein (Figure 1A) (43).

In vitro complement stimulation was accomplished by first sensitizing GEC with anti-Fx1A antibody, then incubating with indicated concentrations of normal human serum or decomplemented heat inactivated human serum. To assess cytotoxicity on V12Ras expressing GEC following complement treatment, lactate dehydrogenase (LDH) release was measured by assessing LDH activity in the complement treated medium. Ras514 GEC showed a marked decrease in specific LDH release compared to Neo control at all concentrations of complement (Figure 1B, top graph, Neo control: 5%, 26.5%±2.9%; 10%, 54.7%±3.8%; 15%, 66.5%±3.4%; Ras514: 5%, 6.5%±0.7%; 10%, 14.6%±1.6%; 15%, 23.1%±1.8%, n=12). Ras25 GEC showed a significant decrease in LDH release compared to Neo control, particularly at the higher concentrations of complement (Figure 1C, bottom graph, Neo control: 5%, 26.4%±4.4%; 10%, 54.7%±5.9%; 15%, 61.9%±5.2%; Ras25: 5%, 24.3%±2.9%; 10%, 37.6%±4.5%; 15%, 42.4%±3.6%, n=8). Therefore, these results indicate that activation of the Ras signaling pathway is cytoprotective against complement mediated cytolysis in GEC. In addition, the decreased LDH release in Ras514 versus Ras25 in response to complement suggests that the cytoprotective effect of Ras is dose dependent.

To determine whether the cytoprotective effect of Ras is specific to complement mediated injury, we subjected Ras and Neo GEC to anoxia-recovery injury, a model of ischemia-reperfusion injury. LDH release after 24hr post-anoxia recovery was also significantly decreased, although less drastically, in Ras514 GEC compared to Neo GEC (Figure 1C, Neo control: 44.6%±11.1%; Ras514: 30.83%±7.1%). Therefore, this result
suggests that the protective effect of Ras signaling provides a generalized protective effect in GEC.

4.2 Ras mediated activation of PI3K by itself does not afford protection against complement

A well characterized effector of Ras mediated survival signaling is PI3K, which in turn activates the Akt pathway. Activated Ras binds to the p110 subunit of PI3K and recruits it to the cell membrane where it can exert its action. This study assesses whether Ras mediated PI3K activation by itself is necessary for conferring cytoprotection to GEC against complement injury.

The phosphorylated form of Akt on serine 473 is required for full catalytic activity of Akt. Using a specific antibody against phospho-serine 473 on Akt, we performed an immunoblot to compare the relative PI3K activation levels of Ras514 GEC versus Neo GEC. Ras514 GEC showed an increased basal phospho-Akt signal compared to Neo GEC (Figure 2A; lane 2 vs. lane 3). Ras514 GEC treated with the PI3K inhibitors wortmannin and LY294002 showed a lowered phospho-Akt signal compared to untreated Ras514 (Figure 2A; lane 3 vs. lanes 4 and 5). Therefore, the increased phospho-Akt signal of Ras signaling is PI3K dependent. This is consistent with the commonly accepted model of Ras signaling (51).

To assess whether Ras mediated activation of PI3K was responsible for cytoprotection against complement, cells were pretreated with wortmannin and LY294002 and were then stimulated with complement. Cytotoxicity was determined by LDH release. Pretreatment with wortmannin did not significantly reverse the cytoprotective effect of Ras signaling (Figure 2B). Similarly, pretreatment of GEC with LY294002 did not reverse the cytoprotective effect of Ras signaling (Figure 2C). Therefore, inhibition of the PI3K pathway does not reverse Ras mediated cytoprotection against complement.

4.3 Assessment of the PI3K pathway during complement mediated injury in GEC

Although Ras mediated PI3K signaling by itself does not afford protection against complement injury, the possibility exists that activating PI3K through a Ras independent
pathway may mediate cytoprotection against complement injury. It has been reported that C5b-9 protects oligodendrocytes from death by activating the PI3K pathway (68). To assess PI3K following complement injury in GEC, we stimulated GEC with complement, and performed immunoblots on the GEC lysate for phospho-Akt Ser473. Concentrations of normal human serum at 0.625%, 1.25%, 2.5%, and 5% for 30 min were used. Heat-inactivated decomplemented human serum (HIS) at the highest concentration (5%) was used as a negative control. 2.5% was noted as the highest concentration of NS that did not cause lytic injury. There was little to no basal Akt phosphorylation in untreated GEC and no Akt phosphorylation with cells treated at different concentrations of NS for 30 min and no difference from that of HIS treated control GEC (Figure 3A). To assess longer-term effect of complement, cells were stimulated with 2.5% NS, for 30 min, 90 min, and 4 hours (Figure 3B). However, there was no difference in Akt phosphorylation between NS and HIS. Densitometry for phospho-Akt signaling for 2.5% NS stimulation on GEC for 60 min did not show a significant difference from HIS treated control GEC (Figure 3C). Therefore, these results strongly suggest that Akt is not activated upon complement stimulation.

SGK is a kinase related to Akt that is activated through a PI3K dependent mechanism. To assess the potential function of SGK in complement mediated GEC injury, we stimulated cell cultures with complement and assessed SGK upregulation via immunoblot using an anti-SGK antibody. 293T cells transiently transfected with HA-SGK plasmid were used as a positive control. Basal levels of SGK in GEC were undetectable by immunoblot. Incubation of GEC with 5 μM MG-132, a 26S proteasome inhibitor, for 2 hours induced a detectable expression of SGK (Figure 3D). This is consistent with the report that in many cell types, the basal expression of full length SGK is low because of extensive polyubiquitination and degradation by the 26S proteasome (72). To assess whether complement had any effect on SGK upregulation, GEC were treated with 2.5% NS for 1, 2, 5 hour periods. Compared to HIS treated control GEC, SGK expression levels remained undetectable (Figure 3E). To bring out expression levels of SGK that can be distinguished with immunoblot, NS and HIS were also co-incubated with MG-132. However, complement still did not seem to have any effect on
the expression level of SGK. Therefore, these results suggest that SGK is not upregulated by complement.

Our next goal was to further assess the functional possibility of PI3K in mediating cytoprotection from complement injury in GEC. To do this, we attempted, but failed to create a GEC clone that stably expresses myristoylated p110α. It has been reported by others that the viability of other cell lines is impaired by expression of this protein. IGF-1 has been shown to inhibit apoptosis in podocytes through the PI3K pathway (67). Specifically, IGF-1 binds to the IGF-1 receptor, which subsequently dimerizes and recruits PI3K to the cell membrane. In order to test whether exogenously activating PI3K is protective against complement mediated injury, we pretreated Neo GEC with IGF-1. Incubation of GEC with 100ng/ml IGF-1 for 30min stimulated Akt phosphorylation (Figure 3F, top immunoblot). Pretreatment of Neo GEC with IGF-1 did not affect LDH release upon complement stimulation (Figure 3F, bottom graph). Therefore, exogenously activating PI3K did not confer a protective effect against complement in GEC.

In the absence of Akt activation, FoxO transcription factors may serve a protective effect (73). JNK is activated during complement stimulation in GEC (29). JNK has been shown to activate FoxO transcription factors in *drosophila melanogaster*, which in turn upregulates MnSOD to prevent cell injury via oxidative stress. Because of the lack of Akt and SGK activation in GEC during complement injury, we hypothesized that FoxO transcription factor activation may serve as a mechanism to protect against cell injury. We assessed the phosphorylation of FKHR, one of the FoxO transcription factors known to become phosphorylated in a PI3K dependent manner. However, we were not able to assess the differences between complement treated and heat inactivated serum treated GEC lysate, since both treatments led to increased FKHR signal (data not shown).

Overall, these results suggest that exogenous, Ras-independent PI3K activation does not confer a protective effect against injury in GEC.

4.4 Ras mediated activation of MAP Kinases by itself is not responsible for Ras mediated protection against complement

Ras dependent activation of the MAPKs ERK, p38, and JNK have been documented in many cell systems. Here, we assess whether these potential effectors of
Ras are important in mediating protection against complement in GEC. To do this, we utilized specific inhibitors for each of these pathways. Ras514 GEC exhibited an increased phospho-ERK signal, compared to Neo GEC as determined by immunoblot (Figure 4A: top immunoblot; lane 3 vs. lane 2). This is consistent with Ras being an upstream activator of Raf-1, which activates MEK, which subsequently phosphorylates and hence activates ERK. PD98059 is a specific inhibitor of MEK, a MAP kinase kinase directly upstream of ERK. Pretreatment with 75\(\mu\)M PD98059 for 30min in Ras514 GEC completely ablated the phospho-ERK signal (Figure 4A: top immunoblot; lane 4 vs. lane 3). Pretreatment of Ras514 GEC with 75\(\mu\)M PD98059 for 30min prior to complement stimulation did not reverse the cytoprotective effect of Ras (Figure 4A: bottom graph). Therefore, these results suggest Ras mediated activation of ERK by itself is not responsible for mediating the cytoprotective effect of Ras.

Ras has also been documented to activate the p38 MAP kinase pathway (45), and activation of this pathway has previously been shown to be cytoprotective against complement mediated injury in GEC (30). Ras514 GEC showed a greater basal phospho-p38 signal compared to Neo GEC (Figure 4B: top immunoblot; lane 3 vs. lane 2). To assess whether Ras mediated activation of p38 confers the cytoprotective effect of Ras, we utilized SB203680, a p38 specific inhibitor. Incubation of Ras514 GEC with 10\(\mu\)M SB203680 for 30min decreased the phospho-p38 signal to levels comparable to untreated Neo GEC (Figure 4B: top immunoblot; lane 4 vs. lane 3). Pretreatment with 10\(\mu\)M SB203680 for 30min prior to complement stimulation did not significantly affect complement-mediated cytotoxicity for Ras514 GEC (Figure 4B: bottom graph). Therefore, inhibition of p38 does not reverse the cytoprotective effect of Ras signaling.

Ras has also been documented to activate JNK, and activation of this MAPK pathway has also been shown to limit injury against complement in GEC (29). To examine the effect of JNK on Ras mediated cytoprotection, SP600125, a specific inhibitor of JNK, was used. Pretreatment with 10\(\mu\)M SP600125 for 30min failed to reverse the cytoprotective effect of Ras signaling in GEC, but tended toward increasing complement injury in Neo GEC (Figure 4C). This is in keeping with a previous study which shows that the functional role of JNK is to limit or protect GEC from complement attack (29).
However, this result suggests that Ras mediated activation of JNK does not play a role in mediating cytoprotection from complement.

Overall, these results suggest that Ras activation of each of the three main arms of the MAP kinase pathways does not, individually, play a significant role in mediating cytoprotection from complement injury.

4.5 Interference with actin cytoskeleton remodeling abrogates the protective effect of Ras against GEC injury

Activation of Ras has been documented to have profound effects on the actin cytoskeleton, leading to the transformed phenotype seen in many oncogenic cell lines. This study addresses the potential effect of Ras-mediated actin cytoskeleton remodeling in cytoprotection of GEC against complement. To assess the effect of the actin cytoskeleton on Ras mediated cytoprotection, we utilized drugs that affect actin turnover.

Latrunculin B sequesters monomeric globular actin (G-actin) in the cytosol to inhibit its polymerization into filamentous actin (F-actin). Incubation of GEC with 1μM latrunculin B for 30min significantly disrupted the F-actin staining pattern for both Neo and RasS14 GEC (Figure 5A, second row panels). Pretreatment at this dose of latrunculin B significantly reversed the cytoprotective effect of RasS14 to complement injury at 10% and 15% serum incubation (Figure 5C: RasS14 untreated; 10% serum, 7.1%±0.8%; 15% serum, 11.9%±1.2%; RasS14+LB: 10% serum, 22.5%±3.2%; 15%, 40.8%±8.1%, n=6). Furthermore, pretreatment of Neo GEC with latrunculin B showed significant protective effect against complement at 10% and 15% serum incubation (Figure 5C: Neo untreated; 10% serum, 28.1%±5.3%; 15% serum, 41.6%±7.5%; Neo+LB: 10% serum, 18.5%±4.3%; 15%, 27.5%±4.4%, n=6).

Swinholide A disrupts actin turnover by sequestering actin as dimers, and also exhibits F-actin severing ability (74). Incubation of GEC with 1μM swinholide A for 30min also significantly disrupted the F-actin staining pattern for both Neo and RasS14 GEC (Figure 5A, third row). Pretreatment at this dose of swinholide A significantly reversed the cytoprotective effect of RasS14 to complement injury at all concentrations of serum incubation (Figure 5D: RasS14 untreated; 5% serum, 13.4%±4.5% 10% serum, 27.3%±7.0%; 15% serum, 37.3%±7.2%; RasS14+Sw: 5% serum, 23.8%±4.1% 10%
serum, 41.1%±5.2%; 15% serum, 52.1%±5.3%; n=6). Pretreating Neo GEC with swinholide A also showed a significant protective effect against complement (Figure 5D: Neo untreated; 5% serum, 13.4%±4.5% 10% serum, 27.3%±7.0%; 15% serum, 37.3%±7.2%; Neo+Sw: 5% serum, 23.8%±4.1% 10% serum, 41.1%±5.2%; 15% serum, 52.1%±5.3%; n=6).

Jasplakinolide is a cell-permeant compound that binds to actin filaments and inhibits depolymerization at the pointed ends of the filaments. Therefore, it serves to stabilize F-actin. Jasplakinolide competes with phalloidin for the F-actin binding site; therefore visualization of F-actin using phalloidin was not possible. Pretreatment with 10µM jasplakinolide for 30min significantly increased LDH release at all serum concentrations (Figure 5E: Ras514 untreated; 5% serum, 15.0%±2.0% 10% serum, 33.0%±3.1%; 15% serum, 41.9%±2.9%; Ras514+Jp: 5% serum, 29.3%±4.7% 10% serum, 48.6%±3.4%; 15% serum, 54.4%±5.8%; n=7). Pretreating Neo GEC with jasplakinolide also significantly protected Neo GEC against all concentrations of complement (Figure 5E: Neo untreated; 5% serum, 34.0%±6.0% 10% serum, 74.8%±4.4%; 15% serum, 84.8%±3.3%; Neo+Jp: 5% serum, 21.0%±4.8% 10% serum, 55.3%±5.6%; 15% serum, 72.0%±4.5%; n=6).

Cytochalasin D is a molecule that caps the barbed end of actin filaments and is known to also sever actin filaments. Treatment of GEC with 20µM cytochalasin D for 30min significantly disrupted the F-actin staining (Figure 5A: bottom row panels). However, pretreatment with this dose of cytochalasin D did not significantly affect LDH release in both Ras514 and Neo GEC clones (Figure 5F).

Overall, these results suggest that mechanisms of actin cytoskeleton turnover may underlie the protective role of Ras.

4.6 Ras signaling alters the GEC actin cytoskeleton

We sought to further characterize the actin cytoskeleton in V12Ras GEC. GEC were stained with rhodamine-phalloidin to visualize F-actin using confocal microscopy (Figure 5A). Untreated Neo GEC had broad cortical F-actin staining and exhibited a tight cobblestone appearance, whereas Ras514 GEC appeared smaller, and exhibited a more
rounded appearance (Figure 5A, top panels). Therefore, at a qualitative level, the actin
morphology of Ras514 GEC is altered.

We simultaneously stained GEC for both F-actin with phalloidin-rhodamine (appears red under fluorescence microscope) and G-actin with DNase I conjugated to Alexa Fluor 488 (appears green under fluorescence microscope). We then determined the F to G actin ratio. To do this, we assessed the intensity of the red signal to green signal for Ras514 and Neo control using Adobe Photoshop software. Ras514 exhibited a significant decrease in F-actin to G-actin ratio versus Neo control (Figure 5B, Ras514 0.533±0.019; Neo 0.889±0.018, n=8, p<0.001).

These results suggest that there exists a fundamental difference in the actin cytoskeleton in Ras514 GEC.

4.7 Ras activation is associated with decreased Src and Rac activity

In this study, we aimed to assess signaling pathways in V12-Ras GEC that may potentially affect the actin cytoskeleton. Rho family GTPases, including the Rho, Rac, and Cdc42 proteins, orchestrate cytoskeletal dynamics. We set out to address effect of Ras signaling on the activation of each of these Rho GTPases. To do this, we performed pull-down assays of the GTP-bound, and therefore catalytically active form of each of these Rho GTPases and performed immunoblots. Using GST-CRIB, the binding domain of PAK for GTP-bound Rac and Cdc42, we were able to perform pull-downs on the activated forms of these Rho GTPases. Ras514 GEC showed a significantly decreased level of active Rac, compared to Neo GEC (Figure 6A, B: Ras514 0.336±0.07, Neo 1.000±0.00, p<0.0001, n=5). Ras514 did not differ significantly from Neo GEC in the activation state of Cdc42 (data not shown). We then analyzed RhoA activity in Neo and Ras514 GEC, and we used Cos-1 cells transiently transfected with constitutively active L63-RhoA as a positive control. In two independent experiments, there were no apparent differences in RhoA activity between Neo and Ras514 cells (data not shown).

Src family of non-receptor tyrosine kinases are involved in cytoskeletal reorganization in a variety of cell systems. To address the activation of Src, we performed immunoblots against pY418-Src, the positive regulatory region of Src. The region surrounding this phosphotyrosine residue is highly conserved. Therefore it is
likely that this antibody recognizes many members of the Src family, including Fyn and c-Src. Ras514 GEC exhibited a decreased phospho-Src signal compared to Neo GEC (Figure 7A: Ras514 0.613±0.072, Neo 1.000±0.00, p<0.05, n=3). This decreased level of activated Src may also play a role in the mediating Ras dependent actin cytoskeleton remodeling. To address whether Src activation is cytoprotective, we incubated GEC with PP2, an inhibitor for Src family kinases. Incubation with 5μM PP2 for 30min prior to complement stimulation significantly increased LDH release for Ras514 and Neo GEC (Figure 7B). Therefore, Src activation seems to mediate cytoprotection for both Ras514 and Neo clones.

Src is known to regulate RhoA activity through its modulation of p190 RhoGAP phosphorylation. In order to assess the effect of Ras signaling on p190 RhoGAP phosphorylation, we performed a pulldown of p190 RhoGAP, and performed an immunoblot using an anti-phospho-tyrosine antibody (pY-69). However, there was no significant difference between Neo and Ras514 GEC (Figure 7C).
5.0 Discussion

In MN in vivo, assembly of C5b-9 in GEC plasma membranes is associated with sublethal GEC injury and proteinuria. In this study, we addressed signaling mechanisms that protect GEC from complement-mediated injury, focusing on Ras and its effectors. It is not practical to address such mechanisms in vivo, and we therefore adopted the model of GEC in culture. However, assaying proteinuria in cell culture models is not feasible. Instead, in cultured GEC, we have assayed C5b-9-mediated injury as cytolysis (LDH release), and it has been shown that cytolysis in culture correlates with proteinuria in vivo, in the context of injury associated with generation of prostanoids (75), p38 MAPK activation (30), and ER stress (32). Our results show that activation of Ras is protective against complement mediated injury in GEC in a manner that is dose dependent. This suggests that in vivo, Ras signaling in GEC may function to attenuate proteinuria.

Ras is known to be involved in numerous pathways affecting different apoptotic processes. There have been many reports on the protective effect of Ras signaling. In hematopoietic cells, both the Ras-mediated activation of the Raf/ERK and PI3K pathway prevents apoptosis induced by IL-3 withdrawal (76). Oncogenic Ras has also been shown to suppress p53 mediated apoptosis through the Raf/ERK pathway (77). Apoptosis by the DNA damaging agent cytosine arabinoside is suppressed by enhanced Ras/Raf/ERK signaling (78). The following sections will discuss our findings on how Ras signaling may mediate cytoprotection against injury in GEC.

5.1 PI3K does not play a significant role in complement cytoprotection

In many cell types, PI3K and Akt activation provides the predominant survival signal (reviewed in (79)). Akt directly inhibits pro-apoptotic machinery, modulates transcription of apoptosis machinery, and promotes cell survival possibly through modulation of cell metabolism. In this study we show that PI3K is not upregulated during complement attack in GEC (Section 4.3). IGF-1, which transiently activates PI3K upon binding to IGF-R, does not confer a significant protective effect against complement in GEC (Section 4.3). Constitutive Ras mediated elevation of PI3K signaling by itself does not confer protection against complement attack in GEC (Section 4.2). Therefore, our
results suggest that PI3K activation does not protect against complement-mediated injury in GEC.

The lack of effect of PI3K illustrates a GEC death mechanism that may bypass the pro-survival effects of PI3K. Although PI3K activation has been reported to confer a survival phenotype in the face of noxious stimuli, there are numerous other mechanisms of injury that are not suppressed by the activation of PI3K. In bone marrow derived Baf-3 cells, it has been shown that activation of PI3K and Akt by interleukin-3 does not protect against DNA damage induced apoptosis by UV irradiation, which functions via a p53 dependent pathway to induce cell death (80). In another study, sublytic C5b-9 induces DNA damage in rat GEC, which was associated with increased protein levels of p53, p21, GADD45, and checkpoint kinases-1 and -2 (20). Another possibility is that PI3K may be part of a redundant pro-survival signaling pathway. Therefore, another Ras-initiated signaling pathway may impinge on the same downstream PI3K effector(s) in order to compensate for PI3K inhibition.

It has previously been shown that JNK is activated during complement stimulation in GEC (29). JNK has been shown to activate FoxO transcription factors in *drosophila melanogaster*, which in turn upregulates MnSOD to prevent cell injury mediated by oxidative stress (73). In our study, we hypothesized that the lack of Akt and SGK activation may increase activated FoxO transcription factors. We therefore attempted to assess the phosphorylation level of FKHR, a FoxO transcription known to become phosphorylated and inactivated by Akt and SGK. However, our results were inconclusive, as there seemed to be an increased phospho-FKHR signal for both NS and control HIS treated GEC. This illustrates a potential limitation of our in vitro complement stimulation technique; there may be a serum factor that is present in both NS and HIS that elevates phospho-FKHR. Therefore, activation of FoxO transcription factors still remains a possible mechanism for GEC to protect against oxidative stress during complement injury.

5.2 The protective effect of Ras is not dependent on MAP kinase signaling

The best characterized effector of Ras is the Raf/ERK pathway. Depending on the stimulus and the cell type, activation of this pathway leads to either prevention or
induction of apoptosis. In many cell systems, Ras/Raf/ERK activation has been shown to promote cell survival. Apoptosis induced by cytosine arabinoside, a DNA-damaging agent, is suppressed by enhanced Raf signaling in human leukemia cells (81). Raf overexpression in rat fibroblast cells increased ERK activation and caused an overall inhibition of cytosolic caspase activation, without affecting cytochrome c release (82). Here, we showed that the constitutively elevated ERK signal in V12Ras GEC is not by itself responsible for complement mediated cell death (Section 4.4). This suggests two possibilities. The first is that the mechanisms downstream of constitutively activated ERK may not be responsible for Ras-mediated cytoprotection. The second possibility is that ERK signaling may in fact be protective, but may be redundant in GEC. That is, there may be crosstalk between ERK and another as of yet unidentified signaling pathway that impinge on the same pro-survival effector. For example, in prostate cancer cells, epidermal growth factor activates both the Ras/MEK pathway and Rac/PAK1 pathway, which both leads to Bad phosphorylation and an overall anti-apoptotic phenotype (83). Whether ERK is part of a redundant pro-survival signaling network will require further study.

Less well characterized effectors of Ras include the p38 and JNK MAPK pathways (45-47). JNK and p38 has been characterized to play a protective role in numerous contexts. In cultured cardiomyocytes, activation of MEKK1 (upstream of JNK) prevents against pressure-overload-induced apoptosis and inflammation (84). During ischemia-reperfusion injury, blockade of JNK activation increased rates of apoptosis in cardiomyocytes, as assessed by caspase 9 activation (85). In GEC, JNK is upregulated in response to complement injury, and this functions to protect GEC from complement attack (29). Similarly, inhibition of p38 by either MKK6 mutants or SB203580 treatment in murine fibroblasts increased TNF-induced apoptosis (86). Activation of p38 has been shown to function in ischemic preconditioning in rabbit hearts prior to ischemia/reperfusion injury (87). In GEC, p38 is transiently activated during complement attack, and the overall functional effect of this activation is protection against injury. In our study, we showed that blocking JNK activation with SP600125, or blocking p38 activation with SB203580 did not significantly affect complement mediated injury in GEC (Figure 4C). Therefore, this suggests that Ras signaling in GEC may not
activate JNK or p38 to a level that causes cytoprotection. Alternatively, there may exist other pathways that impinge on the downstream effectors of p38 or JNK to promote cytoprotection against complement injury. In this case, this renders the p38 and JNK pathway redundant in Ras signaling.

5.3 Role of Ras signaling on GEC actin cytoskeleton and protection from injury

Although increased Ras signaling is characterized for its role in tumorigenesis, increased Ras signaling also has a number of non-oncogenic effects. Ras signaling is well characterized for its ability to remodel the actin cytoskeleton (53). In a variety of cell types, it has been shown that Ras induces membrane ruffling through actin remodeling in a manner that is dependent on PI3K (53). The effect Ras on actin may also be due to the activation of Rac (88). As well, it has been shown that RhoA strongly cooperates with Ras (89). Therefore, Ras is known to have effects on the actin cytoskeleton, and in some cells this may be due to modulation of PI3K or Rho GTPases. However, we have shown that inhibition of PI3K in GEC does not reverse the cytoprotective effect of V12Ras signaling.

In this study, we show that exogenous modulation of the actin cytoskeleton by drugs reversed the protective effect of Ras against GEC injury. Pretreatment of latrunculin B, swinholide A, and jasplakinolide reversed the protective effect of Ras against complement injury (Figure 5C, D, E). This result suggests that there is a fundamental difference in the characteristics of the actin cytoskeleton in V12Ras GEC, which protect these GEC from complement injury. Pretreatment with cytochalasin D, however, did not reverse the protective effect of Ras signaling, despite its ability to disrupt F-actin (Figure 5A, F). Therefore, the ability of actin-modulating drugs to reverse the protective effect of Ras may not be dependent on F-actin disruption alone. It is important to note the mechanistic differences between these drugs. Latrunculin B sequesters actin monomers to inhibit actin polymerization. Swinholide A sequesters actin dimers and severs F-actin. Jasplakinolide binds to F-actin and inhibits depolymerization at the pointed ends of actin filaments. Cytochalasin D caps the barbed end of actin filaments and depending on the concentration, also has F-actin severing ability. It has been reported that cytochalasin D has differing effects depending on the concentration. In
MDCK epithelial cells, cytochalasin D is able to disrupt both apical and basal actin bundles, with the apical actin bundle population being less disrupted with increasing concentrations of cytochalasin D (90). As well, the precise mechanism of cytochalasin D is unclear. There have been reports of cytochalasin D binding to both actin monomers and dimers (91). Although cytochalasin D can induce depolymerization of actin filaments, it can also promote the formation of actin dimers, enhance the initial rate of actin polymerization, and it can sever actin filaments, depending on the cell type and concentration (92, 93). In rat hepatocytes, the concentration of cytochalasin D employed affected the F-actin distribution differently to alter the kinetics of Ca\(^{2+}\) release from store operated channels. Therefore we can conclude that disruption of actin remodeling by latrunculin B, swinholide A, and jasplakinolide reverses the protective effect of Ras signaling. Cytochalasin D, on the other hand, may disrupt F-actin patterning, but may not interfere with actin dynamics in a way that reverses the cytoprotective effect of Ras.

In order to further characterize the actin cytoskeleton between Neo and V\(^{12}\)Ras GEC, we determined the F to G actin ratio. There was a striking decrease in the Ras514 GEC F- to G-actin ratio compared to that of Neo (Figure 5B). In V\(^{12}\)Ras GEC, this may be due to either a decrease in F-actin or an increase in G-actin. This may be directly responsible for the protective effect of Ras. The actin treadmilling cycle itself is known to regulate the expression and activity of genes. In muscle cells, the depletion of the G-actin pool induces the activation of serum response factor, a transcription factor that regulates muscle-specific genes (94). Therefore, the decreased F- to G-actin ratio of V\(^{12}\)Ras GEC may increase the transcription of as of yet unidentified genes that are protective against complement.

In GEC, it has been shown cPLA\(_2\) is dependent, at least in part, on the actin cytoskeleton, as its disruption leads to a significant decrease in cPLA\(_2\) activity and arachidonic acid release (36). Depending on the pathway of arachidonic acid metabolism, this may exacerbate or attenuate GEC injury. Activation of cPLA\(_2\) may serve as a convergence point for much of complement-dependent signaling in GEC, such as ER stress protein upregulation (32), the unfolded protein response (33), and JNK activation (29). Therefore, the actin cytoskeleton in V\(^{12}\)Ras GEC may modulate levels of
arachidonic acid and the kinetics of its subsequent metabolism to cause an overall pro-survival effect.

In order to assess the signaling pathways of Ras that potentially affect the actin cytoskeleton, we addressed the activation level of Rho family GTPases. Activated Ras was shown to activate Rac through its association with Tiam1, a Rac GEF, in a manner that is independent of PI3K (95). However, there is a conflicting report that Ras signaling downregulates Rac activity through downregulation of Tiam1 expression (96). Here, we show that V^{12}Ras GEC have a decreased level of active Rac (Figure 6A). Our finding is consistent with the latter report. The ability of Ras to activate or inactivate Rac may depend on the cell type, developmental stage, and intensity of Ras activation. Rac has also been implicated in apoptotic signaling in several cell systems. In NIH 3T3 cells, Rac induces apoptosis by inducing Fas ligand and ceramide production (97). Chinese hamster ovary (CHO) cells transfected with constitutively Rac strongly induces cell death in response to cytotoxic T lymphocytes and Fas ligand (98). Therefore, in the context of GEC, decreased Rac signaling may be a survival mechanism that decreases extrinsic apoptotic signals in response to injury.

Crosstalk amongst Rho GTPases as a mechanism for cell survival is another interesting possibility to consider. Ras has been shown to activate RhoA signaling (99). Rac activation has also been shown to lead to a downregulation of RhoA activity (100). In neuronal cells, expression of an active form of Rac renders the cell refractory to RhoA-induced signaling (101). Therefore, there is evidence in support of reciprocal activity between RhoA and Rac. Constitutive activation of RhoA signaling has been shown to be protective against complement injury in GEC (12). In this study, we did not see a significant difference in RhoA activation levels between Neo and V^{12}Ras GEC. However, with our finding that V^{12}Ras GEC have significantly decreased Rac activity, the possibility exists that RhoA activity in these cells is unopposed. Morphologically, V^{12}Ras GEC appeared smaller and rounder and with a strong cortical F-actin patterning (Figure 5A). This is consistent with a recent report that GEC expressing constitutively active RhoA are also smaller and rounder with broad cortical F-actin patterning (12). Therefore, although we did not measure an increase in RhoA activity in V^{12}Ras GEC directly, Ras signaling may actually increase the effect of RhoA activity in these GEC.
We propose that the functional outcome of unopposed RhoA activity in GEC is cytoprotection against complement-mediated injury.

Activated Ras has been shown to bind to Src and negatively regulate its function in mouse neuronal cells (102). It has been shown that Src can modulate the actin cytoskeleton upstream of Rho GTPases (16). In GEC, Src kinases phosphorylate nephrin, which leads to recruitment of Nck adaptor protein and assembly of actin filaments (103). In our study we showed that Ras negatively regulated Src signaling (Figure 7A). As well, inhibiting Src activity with PP2 significantly reversed the protective effect of Ras in GEC (Figure 7B). Therefore, this strongly suggests that Src family kinases also play a role in mediating actin cytoskeleton remodeling and protection from complement mediated injury. As a possible mechanism, Src may mediate the phosphorylation of p190RhoGAP, which serves to activate the GTPase activity of, and hence inactivate, RhoA (104). However, our assessment of the p190RhoGAP phosphorylation showed that there was no significant difference in the phosphorylated p190RhoGAP signal between Ras and Neo GEC (Figure 7C). There are other mechanisms by which Src could interact with RhoA. Translocation of Src to the cell periphery has been shown to depend on the control of the actin cytoskeleton by Rho GTPases (105). Whether this is a possibility in GEC will require further study.

Nephrin is a slit diaphragm protein that is phosphorylated by Src family kinases (15). Nephrin has been shown to be transiently phosphorylated by Fyn, a member of the Src family kinases, during GEC injury (103). Therefore, another possibility is that the lowered Src activity in the case of V12Ras GEC clones may serve to lower nephrin phosphorylation and association with actin cytoskeleton. This may explain the characteristic morphology of V12Ras GEC and may also explain its cytoprotective effect against injury. This view will require confirmation by further studies.
6.0 Conclusions

Membranous nephropathy is a major disease, which features injury to the GEC, a major component of the glomerular filtration barrier. GEC injury leads to a breakdown in the slit diaphragm ultrastructure and manifests as proteinuria, which can lead to ESRD. Therefore, it is important to further understand the mechanism of injury in GEC and develop strategies for resisting GEC injury. Here, we show that constitutively elevated Ras signaling within GEC is protective against complement mediated injury in a manner that is dependent on the actin cytoskeleton. Ras-mediated modulation of the actin cytoskeleton may depend on decreased Rac and/or decreased Src signaling.

Activation of Ras signaling is a novel pathway to consider in developing methods to protect GEC against various forms of injury. It should be noted that constitutive activation of Ras does not necessarily lead to oncogenic transformation of cells, as certain syndromes have been described where activating Ras mutations are well-tolerated (106). Compounds such as ascorbyl stearate have been shown to inhibit p120 RasGAP activity by binding to its PH domain (107). Such compounds would be able to increase Ras activity, which in GEC could lead to protection against complement mediated injury. Therefore, Ras activation in GEC may eventually serve as a therapeutic target in patients with membranous nephropathy.
7.0 Literature Cited


8.0 Figure Legends

Figure 1. V12Ras expression in GEC protects against injury. (A) Expression of Ras in GEC. GEC were transfected with constitutively active HA-Ras gene (V12Ras). GEC clones (Ras514 and Ras25) were selected and assessed for expression of Ras protein by immunoblotting. Ras514 expresses high levels of V12Ras, whereas Ras25 expresses intermediate levels of V12Ras as previously described by Cybulsky et al (43). (B) GEC expressing V12Ras are resistant to complement mediated injury. Ras514, Ras25, and Neo-transfected GEC were incubated with NS (5, 10, or 15% v/v), or 15% HIS as a control. Cell injury was determined by measuring LDH activity in the supernatant (see methods). (*P < 0.05, ***P < 0.001 vs. Neo control; n=12 for Ras514, n=8 for Ras25). (C) V12Ras protects against anoxia-recovery mediated injury. Ras514 and Neo-transfected GEC were treated for 1hr in medium containing 10μM antimycin A and 10mM 2-deoxyglucose. Then, cells were reexposed to glucose for 16hr. Cell injury was determined by measuring LDH activity in the supernatant. (*P < 0.05 vs. Neo control; n=5)

Figure 2. Ras mediated protection against complement injury is not reversed by PI3K inhibitors. (A) Ras activates Akt in a PI3K dependent manner. After plating for 48hr, cell lysates from untreated Neo, untreated Ras514, and Ras514 treated 30min with 0.1 μM wortmannin, or Ras514 treated 30min with 10 μM LY294002 were separated on a 7.5% polyacrylamide gel and immunoblotted with anti-phospho-Akt antibody. Neo treated 30min with 1mM H2O2 was used as a positive control. (B) Pretreatment of V12Ras GEC with wortmannin does not reverse protection against complement injury. Ras514 and Neo-transfected GEC were pretreated with 0.1μM wortmannin for 30min. Cells were then incubated with complement and cell injury was determined by measuring specific LDH activity in the supernatant. There was no significant difference between treated and untreated groups. (C) Pretreatment of V12Ras GEC with LY294002 does not reverse protection against complement injury. Ras514 and Neo-transfected GEC were pretreated with 10μM LY294002 for 30min. Cells were then incubated with complement
and cell injury was determined by measuring specific LDH activity in the supernatant. There was no significant difference between treated and untreated groups.

**Figure 3.** Assessment of PI3K pathway during GEC injury. (A) Akt is not activated by complement. Neo GEC were stimulated with increasing concentrations of NS and HIS for 30min, or 2.5% NS and HIS for varying timepoints (B). Resulting proteins were separated by SDS-PAGE and immunoblotted with anti-phospho-Akt antibody. (C) Densitometry of 3 blots showed no significant difference in phospho-Akt signals. (D) SGK is degraded via the proteasome. Neo GEC was incubated with or without 10μM MG-132, a 26S proteasome inhibitor, for 2 hours. Proteins were immunoblotted using α-SGK antibody. 293T cell proteins transfected with HA-SGK were used as a positive control (middle lane). (E) Complement does not induce SGK expression. Neo GEC was stimulated with normal human serum (NS) or heat-inactivated human serum (HIS) with or without 10μM MG-132 for 2 hours. Resulting proteins were separated with SDS-PAGE and immunoblotted using anti-SGK antibody. (F) Pretreatment of Neo GEC with IGF-1 activates Akt, but does not confer protection against complement injury. Neo GEC were pretreated or not with 100ng/ml IGF-1 for 30min (top blot). Cells were then incubated with complement and cell injury was determined by measuring specific LDH activity in the supernatant. There was no significant difference between treated and untreated groups (bottom graph).

**Figure 4.** Inhibition of ERK, p38, or JNK does not reverse Ras mediated protection against complement injury. (A) V12Ras activates ERK signaling, but inhibiting ERK does not reverse Ras cytoprotection against complement injury. Cell lysates from untreated Neo, untreated Ras514, Ras514 treated 30min with 75μM PD98059 (MEK inhibitor), were separated on a 7.5% polyacrylamide gel and immunoblotted with anti-phospho-ERK antibody. Neo treated 30min with 1mM H2O2 was used as a positive control (first lane). Cells were then incubated with complement and cell injury was determined by measuring specific LDH activity in the supernatant. There was no significant difference between treated and untreated groups (bottom graph). (B) V12Ras activates p38 signaling, but inhibiting p38 does not reverse the cytoprotective effect of Ras. Cell lysates from
untreated Neo, untreated Ras514, Ras514 treated 30min with 75μM SB203680 (p38 inhibitor), were separated on a 7.5% polyacrylamide gel and immunoblotted with α-phospho-p38 antibody. Neo treated 30min with 1mM H₂O₂ was used as a positive control (first lane). Cells were then incubated with complement and cell injury was determined by measuring specific LDH activity in the supernatant. There was no significant difference between treated and untreated groups (bottom graph). (C) Pretreatment of V¹²Ras GEC with the SP600125 JNK inhibitor does not reverse protection against complement injury. Ras514 and Neo-transfected GEC were pretreated with 10μM SP600125 for 30min. Cells were then incubated with complement and cell injury was determined by measuring specific LDH activity in the supernatant. There was no significant difference between treated and untreated groups.

**Figure 5:** Characterization of the actin cytoskeleton and its relevance to complement-mediated injury in V¹²Ras GEC. (A) Visualization of F-actin in Neo and V¹²Ras GEC. Neo GEC and Ras514 GEC were untreated (top row), pretreated with either 1μM latrunculin B (LB: second row), or 1μM swinholide A (Sw: third row), or 10μM cytochalasin D (CD: bottom row) for 30min. F-actin was then stained with rhodamine-phalloidin and visualized using confocal microscopy. Untreated control Ras514 GEC generally appeared smaller and rounder compared to untreated control Neo GEC. Treatment with latrunculin B, swinholide A, or cytochalasin D significantly disrupted F-actin patterning. (B) F to G actin ratio is lower in V¹²Ras GEC compared to Neo GEC. F-actin and G-actin in Ras514 and Neo GEC were visualized using rhodamine-phalloidin and DNase I conjugated to Alexa Fluor 488, respectively. Relative intensities of F-actin signal (red) and G-actin signal (green) were quantified using Adobe Photoshop software. Ras514 GEC have a significantly lower F to G actin ratio (***P < 0.001 vs. Neo, n=8). (C, D, E, F) Effect of pretreatment of Ras514 GEC with LB (C), Sw (D), jasplakinolide (Jp) (E), or CD (F) on complement mediated injury. Neo-transfected GEC and Ras514 GEC were pretreated with either 1μM LB, 1μM Sw, 10μM Jp, or 10μM CD for 30min. Cells were then incubated with complement, and cell injury was determined by measuring specific LDH activity in the supernatant. There was significant reversal of cytoprotection with LB, Sw, and Jp treatment of V¹²Ras GEC (*P < 0.05, **P<0.01, ***P < 0.001 vs.
Ras untreated; n=8 for LB group, n=6 for Sw, n=7 for Jp). There was no significant difference between cytochalasin D treated and untreated groups.

**Figure 6:** Ras decreases Rac activity in GEC. Cell lysates were subject to pulldown with GST-CRIB to assess for active (GTP-bound) Rac. Resulting protein was resolved using SDS-PAGE and immunoblotted with anti-Rac antibody. (A) Representative immunoblot of GTP-bound Rac (upper panel) and total Rac from 100µg protein (bottom panel). (B) Densitometric analysis. (**P < 0.001 vs. Neo untreated, n=4)

**Figure 7:** Ras decreases Src activity in GEC and inhibition of Src reverses Ras mediated cytoprotection against complement. (A) Src phosphorylation is decreased in Ras514 GEC. Cell lysates were subjected to SDS-PAGE and immunoblotted with anti-phospho-Src antibody. Top: representative immunoblot. Bottom: densitometric analysis. (*P < 0.05 vs. Neo untreated, n=3). (B) Pretreatment of GEC with Src inhibitor abrogates the protective effect of Ras514 GEC during complement injury. Ras514 and Neo-transfected GEC were pretreated with 5µM PP2. Cells were then incubated with complement and cell injury was determined by measuring specific LDH activity in the supernatant. (P < 0.05, PP2 treated vs. untreated Ras514 group; two-way ANOVA, n=4). (C) Ras does not have an effect on p190 RhoGAP phosphorylation. Cell lysates from Neo and Ras514 GEC were subject to immunoprecipitation with anti-p190 RhoGAP antibody, and immune complexes were resolved by SDS-PAGE. Immunoblot was performed using anti-phospho-tyrosine antibody (pY69; top blot). The blot was reprobed for p190 RhoGAP to verify its identity (p190; bottom blot).
Figure 1

A

V\textsuperscript{12}Ras (21 kDa)

B

\textbf{Specific LDH release (%)}

\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Serum (%)} & \textbf{Neo} & \textbf{Ras514} & \textbf{Ras25} \\
\hline
5 & 25 & *** & 25 \\
10 & *** & 75 & *** \\
15 & *** & 75 & *** \\
\hline
\end{tabular}

C

\textbf{Specific LDH release following anoxia-recovery (%)}

\begin{tabular}{|c|c|}
\hline
\textbf{Neo} & 25 \\
\textbf{Ras514} & 50 \\
\hline
\end{tabular}
Figure 2

A

p-Akt (60kDa)

B

C

Specific LDH release (%) vs Serum (%)

Neo
Neo + Ly
Ras514
Ras514 + Ly

Serum (%)

5 10 15
Figure 3
E

HA-SGK (293T) Neo lysate

<table>
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<th>Time (hr)</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>1</th>
<th>2</th>
<th>5</th>
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SGK (60kDa)

MG-132
NS (2.5%)  
HIS (2.5%)

F

p-Akt (60kDa)

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Figure 4

A

Neo + H₂O₂
Neo
Ras514
Ras514 + PD

p-ERK1 (44kDa)
p-ERK2 (42kDa)

B

Neo + H₂O₂
Neo control
Ras514
Ras514 + SB

p-p38 (37kDa)

C

Neo
Neo + SP
Ras514
Ras514 + SP

Specific LDH release (%)

Serum (%)
Figure 5

A

<table>
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<tr>
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+LB

+Sw

+CD
Figure 6

A

GTP-bound Rac (21kDa)

Total Rac (21kDa)

B

Rac activation (Densitometry)

Neo  |  Ras

0.00  |  **0.25**

Neo  |  Ras

0.50  |  **0.75**

0.75  |  **1.00**

0.00  |  Neo  |  Ras

***
Figure 7

A

p-Src (60 kDa)

Neo
Ras514

p-Src (Densitometry)

Neo
Ras514

B

Specific LDH release (%)

Serum (%)

C

pY (190kDa)

Reprobe: p190 Rho GAP

Neo
Ras514
10.0 Appendix