The influence of hepatocyte growth factor during phagocytosis by retinal pigment epithelium

By

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Abstract

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Processing of photoreceptor outer segments (OS) by the RPE is critical for maintaining the health of the neural retina. If any portion of OS processing is disrupted, or if the RPE suffer injury, subsequent inhibition of OS processing has deleterious effects. It is therefore crucial to understand OS processing in order to maintain visual health.[1] Sub-retinal clearance of OS by RPE is facilitated by phagocytosis featuring both RPE-specific and FcγR associated signaling cascades.[2] Integration of these two pathways renders RPE capable of internalizing both specific and non-specific targets. Therefore, there must be specific pathways available to present the cell with the protein machinery necessary for binding, internalizing and processing the OS. The discovery that lack of c-Met signaling results in impaired phagocytosis in alveolar and hepatocyte macrophages suggests c-Met's role as modulator of phagocytosis.[3] These data also suggest a role for hepatocyte growth factor (HGF), the natural ligand for c-Met activation, in preparing phagocytes for clearance of cellular debris. We propose that HGF activation of c-Met in RPE prepares these cells for phagocytosis by initiating a signaling cascade that includes activation of

phosphatidylinositol-3 kinase (PI3K). Subsequent activation of Rac1 by PI3K may regulate phagosome formation.[4,5]

Dedication

The author of this work would like to recognize the individuals who have made this research possible- a special group of people whose support has inspired a career.

To parents whose unconditional love has made the toughest storms feel like a passing breeze. Their words, hopes and teachings are my shelter.

To a brother whose leadership and sacrifice stand as constant motivation- his accomplishments are a reminder of what is possible when talent and dedication are inseparably welded.

To a girl whose purity and patience are impossible to capture in words. I begin and end my days with thoughts of her smile. I look forward to our life together.

To friends that have tolerated my temper, paranoia and obsessive nature. Their loyalty, encouragement and company have made the ride worthwhile.

Finally, to an educator, scientist and friend who took a chance on a wayward student- a man whose vision and intellect ignited a fire...the realization of this pupils dream is a reflection of his devotion.

To all, this author is indebted.

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CHAPTER I: Background & Significance

The mammalian retina is supported largely by the retinal pigment epithelium (RPE), a polarized monolayer of pigmented cells separating the neural retina from Bruch's membrane and choriocapillaris. The RPE performs several tasks within the sub-retinal space including, but not limited to, nutrient absorption and distribution, re-isomerization of photo-excitable compounds, sequestering of errant light energy and phagocytosis of spent photoreceptor outer segments (POS).[2] Clearance of this debris generated as a by-product of photoreceptor renewal facilitates proper visual function; interference of this process, whether physical or physiological, often results in pathological sequelae. RPE dysfunction or failure initiates, or at minimum influences, the severity of multiple retinopathies, including the most prominent, age-related macular degeneration (AMD).[6, 7]

While many hypotheses attempt to describe a physiological origin of AMD, it is unlikely that a single event is responsible for the manifestation of this disease. RPE failure is a likely contributor because of the consequences of incomplete or improper phagocytosis, a complex process that can be perturbed by a litany of stressors including oxidative damage, genetic abnormalities and senescence. Though two types of AMD present clinically, geographic atrophy of the macula (dry AMD) is present in nearly all cases;[8, 9] this condition, marked by the accumulation of drusen upon the choroid and choriocapillaris is particularly significant as it has been linked directly to improper clearance of the interphotoreceptor matrix.

Since nearly all AMD patients are seniors, it is believed that aged RPE undergo changes that yield undesirable products and/or altered function. There are likely three key events

responsible for AMD susceptibility. The first event involves the mitochondria and mtDNA of RPE. Studies of aging retina report significant decreases in both size and number of mitochondria in human RPE cells, abundance of mtDNA deletions, increased mtDNA damage and down-regulation of DNA repair enzymes in the neural retina, RPE and choroid of rodents. These observations are consistent with findings of AMD patients whom exhibit high levels of large mtDNA deletions/rearrangements in the retina, unreported and amino acid-changing single nucleotide polymorphisms (SNP) in the coding genome and more SNP's per person in the non-coding MT-D loop region.[10-13,14] In studies of macula-derived RPE, investigators found that these cells have greater mtDNA damage and diminished repair capacity compared to RPE from the periphery, suggesting that a primary defect resulting in AMD occurs within the RPE of the macula.[12] Primary antioxidants, their respective chaperones, antioxidant enzymes and specific repair systems work to protect RPE from ROS induced damage. Reductions of the substrate-specific repair enzyme 8-oxoG DNA glycosylase 1 in RPE contribute to accumulation of oxidative damage.[15, 16]

A second condition promoting RPE failure is the overload of cellular iron, an essential dietary component whose total body concentration accumulates with age since excretion is minimal. While important for many metabolic processes within RPE, excess iron generates highly reactive hydroxyl radicals that damage both lipids and proteins.[15] Coupled with the formation of ROS produced as a byproduct of normal POS degradation, where oxygen consumption is highest in the retina, the RPE suffers from tremendous oxidative load.[15] Multiple groups have illustrated that increased levels of intracellular iron results in decreased rate of POS internalization.[15] Fenton reactions resulting from

iron accumulation yields hydrogen peroxide, which at nontoxic concentration inhibits RPE phagocytosis.[17] Additionally, high intracellular iron prevents maturation of the lysosomal enzyme cathepsin D (CatD), the enzyme responsible for rhodopsin degradation. Compromised CatD results in increased build up of undigested POS debris.[15, 18, 19] Iron overload also interferes with phagocytosis of POS by inhibiting activation of focal adhesion kinase (FAK), a key enzyme partially responsible for phagosome formation and POS internalization following activation of its upstream regulator integrin $\alpha_V\beta5$.[8]

The final component of RPE failure is the release of waste products by exocytosis. During normal sub-retinal clearance (removal of OS by RPE), phagosomes unite with lysosomes to initiate and complete degradation of POS. RPE recycling of visual pigment pre-cursors (all-trans retinol), essential fatty acids (docosahexaenoic acid (DHA)) found abundantly in outer segment phospholipids) and neurotrophins are contingent upon successful clearance of the sub-retinal space. Newly formed endosomes are directed toward the RPE basolateral membrane where exosome contents are released and carried toward the blood. To evaluate the consequences of genetic abnormalities, markers of exosomes were targeted in culture systems where mtDNA of RPE is damaged. The findings of that study suggest that exosomal activity is increased under these conditions. Markers of increased exosome release are found in the plasma of AMD patients. Drusen, the lipid/protein rich deposits of classical dry AMD, appear to contain the same materials excreted by RPE during exocytosis, including DHA. DHA is the neurotrophin precursor of neuroprotectin D1 (NPD1), which inhibits apoptotic behavior mediated by A2E (bispyridinium bisretinoid), a lipofuscin fluorophore component whose toxic effects

manifests as drusen during AMD development. In response to these stressors, RPE secrete chemokine [C-C motif] ligand 2 (CCL2), resulting in leukocyte recruitment and activation of complement cascades. Invasion of RPE by leukocytes is normal and during homeostasis would contribute in clearing the accumulating cellular debris,[8] however, the increased immunological activity cannot accommodate the increased volume of waste generated as a byproduct of compromised mtDNA, increased iron content, slowed phagocytosis, and amplified excretion. Accumulation of A2E during normal aging causes RPE to undergo apoptosis, which precedes photoreceptor dysfunction and death.[20]

Anatomy of the Retina

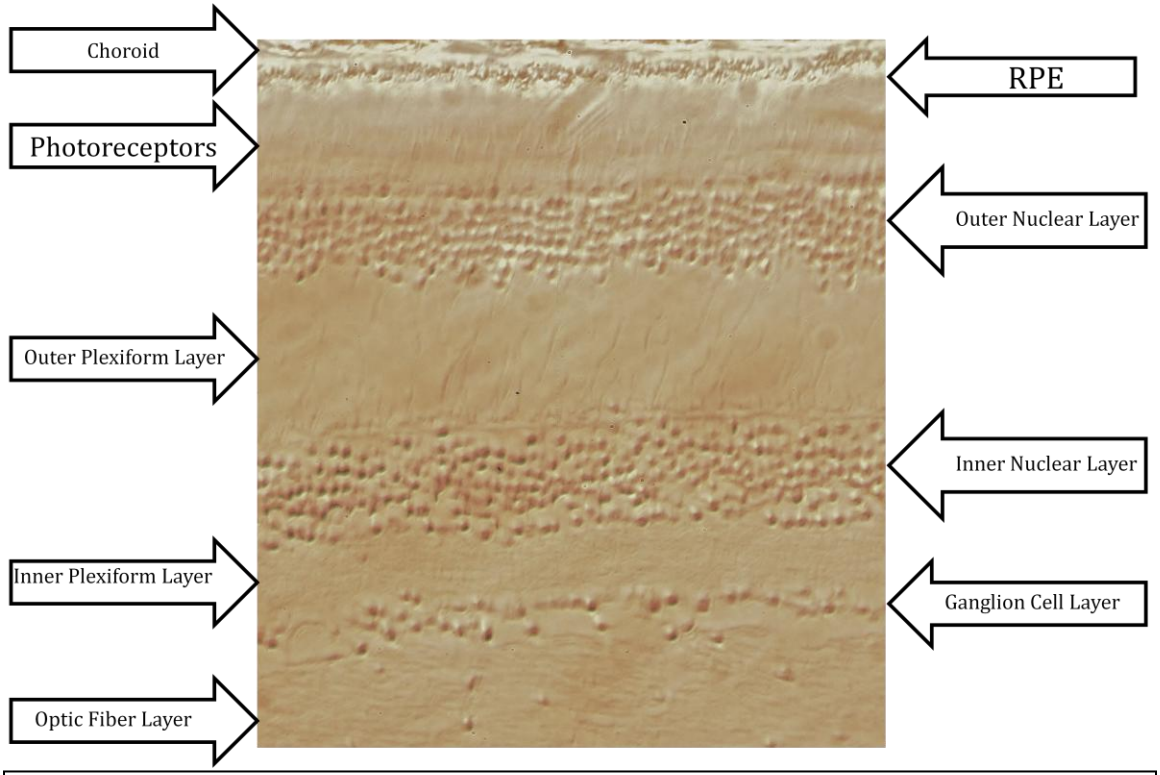

Figure 1. **Histological section of mammalian retina**

The retina is a multilayered light sensing membrane that occupies the innermost surface of the human eye. When viewed sagittally, ten subdivisions of this 500 µm region can be distinguished (Figure 1), each contributing to four basic stages of vision including light detection, neural transmission to bipolar cells, subsequent transmission to retinal ganglion cells and signal propagation along the optic nerve. The anterior most region of the retina, referred to as the optic fiber layer, consists of the inner limiting membrane and the nerve fiber layer. The inner limiting membrane (ILM) contains Müller cells, supportive glial cells for the retina that are capable of reverse differentiation, resulting in the formation of multipotent progenitor cells capable of re-differentiating into a variety of cell types including photoreceptors, upon injury.[21] Immediately posterior to the ILM is the nerve

fiber layer (NFL), containing axons that originate from the third layer, the ganglion cell layer (GCL). As its name implies, this layer houses retinal ganglion cells that project to the axons NFL and receive input from the inner plexiform layer (IPL), a region containing synapses that join the ganglion cell dendrites and the bipolar cell axons. The bipolar cell somas are found in the inner nuclear layer (INL), which represents the final layer of the inner plexiform. The outer plexiform layer (OPL), directly posterior to the INL contains projections of rod and cone photoreceptors, synapsing with bipolar dendrites. The outer nuclear layer (ONL) contains the photoreceptor cell bodies (rods and cones) and precedes the external limiting membrane (ELM) separating the inner segments of photoreceptors (Figure 2) from their morphologically distinct outer segments (OS). The RPE represents the posterior most layer of the retina, forming the blood retina barrier anterior to the choroid. The choroid is a highly vascularized region between the neural retina and the sclera and contains the capillary region the choriocapillaris. The choriocapillaris serves to supply nutrients to the RPE and photoreceptors while removing waste from the posterior cell layers.

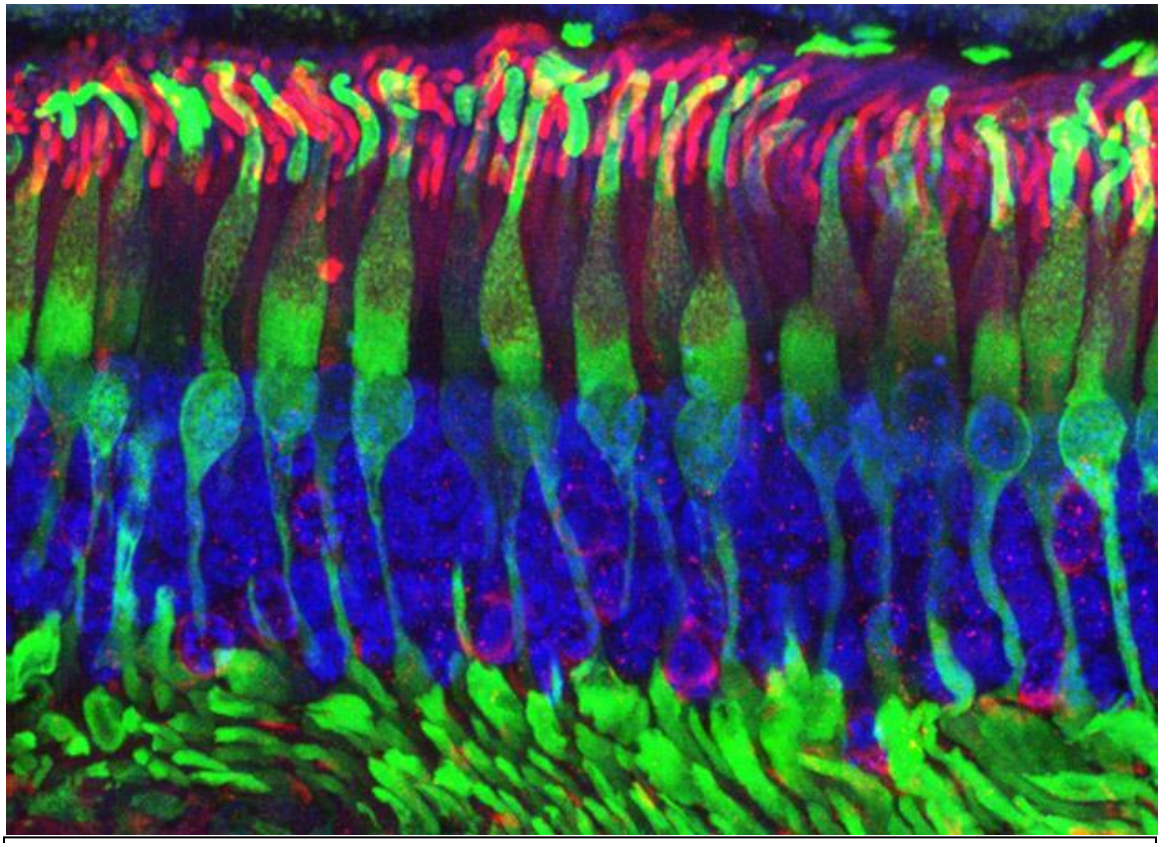

Figure 2. **Fluorescence micrograph of photoreceptors**

Confocal micrograph of retina showing the location and morphology of rods (red), and cones (green). Nuclei are stained blue. Image from Dr. James Sanzo (American Cardiovascular Research Institute) and obtained from Olympus Bioscapes.

While the RPE provides photoreceptor outer segments with nourishment by way of the vascularized choroid, the ophthalmic artery delivers blood to cells of the inner and middle layers of the retina via uveal and retinal circulation. Uveal circulation enters the optic cup through the optic nerve, while retinal circulation passes the optic nerve as a branch of the ophthalmic artery known as the central artery.

Human Phototransduction

Photons travel from the anterior to the posterior retina, where they either activate photoreceptors or are absorbed by RPE melanosomes. The RPE are not only important in this aspect of visual acuity, but also in supplying the photoreceptors with the components

needed for phototransduction. Phototransduction is the light dependent conversion of a photon into an electrical signal by photoreceptors and ganglion cells of the retina.[22] This process is a hallmark of mammalian vision and requires orchestrated signaling events, initiated by the activation of G-protein coupled receptors (GPCR) embedded within the plasma membrane of photoreceptor outer segment discs.

Retinylidene opsins are a class of G-protein coupled receptors (GPCR), seven transmembrane proteins that use retinal as a chromophore for the reception of light. In humans, rhodopsin is the protein that covalently binds to retinal and facilitates the initiation of signaling pathways. Rhodopsin undergoes a conformational change when the chromophore 11-cis-retinal (retinaldehyde/Vitamin A aldehyde) is struck by a single photon. This polyene chromophore is located within the central pocket of the seventh helix at a lysine residue of opsin, forming retinylidene. When initiated, 11-cis retinal undergoes photoisomerization, yielding the opsin agonist all-trans retinal;[23, 24 23 25, 26] the result is a loss of covalent binding to the GPCR, which itself undergoes a conformational change becoming metarhodopsin II. Metarhodopsin II is unstable and splits quickly to free both opsin and all-trans-retinal. All-trans retinal is then transported to the pigment epithelium where it undergoes it is reduced to all-trans retinol (Vitamin A), a precursor needed for the synthesis of 11-cis retinal.[27] The soluble sub-retinal protein IRBP (interphotoreceptor retinoid binding protein) has recently been thought to facilitate the release and delivery of all trans-retinol from photoreceptors to RPE. [28-32]

Metarhodopsin II activates transducin, a heterotrimeric G protein with three polypeptide chains that are distinguished as subunits Tα-GDP, Tβ and Tγ. Upon activation, the Tα-

GDP subunit utilizes a guanine exchange factor to exchange the bound GDP for cytoplasmic GTP once dissociated from the Tβ and Tγ subunits. Unbound T_{α}-GTP then activates cyclic guanosine 3'-5' monophosphate (cGMP) phosphodiesterase which cleaves cGMP bound to the cytoplasmic face of specific ion channels at the outer segment disc membrane. Pre-stimulus, the resting membrane potential of photoreceptors is approximately -40 mV, facilitated in large part by a depolarizing inward current expedited by activated cGMP-gated channels. Upon cleavage by cGMP phosphodiesterase, these channels become deactivated and the depolarizing inward current is reduced. Eventual hyperpolarization of the photoreceptor results in closure of voltage-gated Ca^{2+} channels, decreasing the intracellular concentration of said ion, preventing exocytosis of the neurotransmitter glutamate. The biochemical cascade is halted by a variety of self-regulating mechanisms, including the hydrolysis of activated transducin by GTPase. Additionally, the rhodopsin protein becomes a target for opsin kinase; once phosphorylated the regulatory protein arrestin can interact with rhodopsin resulting in rapid deactivation.

A decrease in the concentration of synaptic glutamate results in the hyperpolarization of the photoreceptor and either de- or hyperpolarization of adjacent bipolar neurons. Bipolar neurons of the retina display center-surround properties such that interaction with a single photoreceptor can evoke varying responses depending of dendritic arborization. Persistent glutamate release by photoreceptors, typical in the absence of light, results in the hyperpolarization of on-center bipolar neurons, and while the mechanism for this phenomenon is unclear and likely different for cells downstream of rods and cones, it is believed that glutamate opens K^{+} selective ion channels that facilitate an outward

hyperpolarizing current. Alternatively, glutamate signaling may result in the closure of cGMP-gated channels that conduct inward Na^+ current in other cells. A light-induced reduction in glutamate signaling depolarizes on-center bipolar neurons. Conversely, off-center neurons become hyperpolarized in the absence of glutamate, or when a light stimulus is present. This occurs as a result of the neurotransmitters concentration dependent activation of Na^+ channels associated with off-center neurons.

Bipolar neuronal signaling initiated by light stimulus is then propagated toward retinal ganglion neurons that share a similar center-surround receptive field orientation. Action potentials generated by the retinal ganglion cells as a result of the communication with bipolar neurons are carried to the first to the lateral geniculate nucleus of the thalamus before reaching the primary visual cortex of the occipital lobe.[27]

Shedding of outer segments: good for vision; bad for RPE?

As the phototransduction occurs in the outer segments, these are certainly the most important structures in generation of impulses that ultimately are interpreted in the visual cortex as an image. But these outer segments have a finite life span.

Two theories exist regarding the details of OS formation-[33] the first suggests that small vesicles are inserted into the plasma membrane that contains folds at its base,[33, 34] which either remain constant with the membrane as in the cones, or are physically separated to form enclosed discs as in the case of rods.[33, 34] The alternate hypothesis suggests that small protein-containing vesicles fuse and flatten, yielding discs that are inserted into the cell membrane of cones, or kept isolated within rods.[33, 35]

Molecularly, it appears that peripherin, a type III intermediate filament protein prominently found within the peripheral nervous system, and rds (retinal degeneration slow) are key membrane proteins specific to OS and maintained at the apical rim of rod discs and cone folds. Peripherin/rds exists as a homodimer under normal physiological conditions, before coupling with a homologous homodimer to form a homotetramer or a heterotetramer with non-glycosylated homolog rom-1 linked by one or more disulfide bonds. It is speculated that the lumenal loops of peripherin/rds and rom-1 interact to stabilize the complex and to form and maintain highly curved regions of the disc rim.[33, 36] Mutations or expression anomalies associated with this complex have been quickly identified in human autosomal dominant retinitis pigmentosa. Recent models of nascent disc membrane formation require the aforementioned tetrameric complexes to facilitate zippering fusion events.[33, 36-42] Mice selectively bred to exclude rhodopsin transcription are unable to produce OS, suggesting the importance of this chromophore in membranogenesis.[33] In *Drosophila*, rhodopsin is required for proper development of the rhabdomere, the structural equivalent of OS.

There is strong evidence suggesting that photoreceptor OS renewal is influenced by circadian rhythm, featuring a burst of phagocytosis shortly after the onset of light. The Finnemann lab has done remarkable work in identifying phosphatidylserine (PS) externalization by OS as a direct inducer of phagocytosis by RPE in culture. Though the mechanism for externalization remains unclear, diurnal regulation of the anionic phospholipid PS may represent a definitive link between OS clearance and reception of external cues.[43]

Autoradiological studies reported by Young in his landmark paper of 1967 demonstrate that photoreceptor outer segments are comprised of hundreds of closely packaged discs containing light sensitive visual pigments.[44] Young injected tritiated amino acids into vertebrate eyes in an effort to determine both the physiological path and time required during OS renewal. Radiolabeled amino acids appeared within the rod inner segments nearly 10 minutes after injection; appearance of radio labeled molecules appeared at the base of rod OS approximately two hours post injection. Radiolabeled molecules were viewed as they formed within the ciliary plasmalemmal, progressed toward the distal tip of the OS body, and eventually freed from the photoreceptor completely. Radiolabeled bands housed within discs are soon found within phagosomes of the RPE, having traveled toward the posterior region of the optic cup.[45] Our understanding of OS formation is based on the conclusion derived from these studies: the synthesis of opsin proteins are typically housed within the photoreceptor inner segment where they become packaged within continuously forming membranous discs that are eventually displaced by newly synthesized discs at the basal surface of the outer segment. While cones vary slightly with regards to disc formation, (radiolabeled injections are diffused throughout the photoreceptor OS and are devoid of a characteristic band), the synthesis of membrane proteins and displacement of membranous discs occurs in similar fashion.[33] Cone outer segment formation was particularly evident when studying the cone-rich retinas of squirrels.[46] There are differences between the renewal of cone and rod OS, and a central disparity appears to involve longevity with renewal of one complete cone occurring once every 30 or so days in many mammals, and featuring bursts of membrane shedding occurring nocturnally.[33] In addition, cone outer segments are formed via accumulation of

lamellae produced as a product of plasma membrane folding, not the synthesis of discs as in rods. Morphologically, basal folding of lamellae yields a much shorter body, with a conical, narrow distal tip that produces smaller phagosomes in RPE.[33] Furthermore, while rod OS rely on RPE to re-isomerize chromophores necessary for vision, recent evidence suggests that cone OS maintain innate isomerase activity.[33, 47-49]

Calculations suggest that each single RPE cell individuals 80 years of age have engulfed 10^8 shed OS.[44] Given the frequency of OS clearance, perturbations resulting in the disruption of the equilibrium that exists between OS formation and removal can have deleterious consequences for retinal function and the survival of photoreceptors.[33]

Age-related macular degeneration and oxidative stress

Age related macular degeneration (AMD) is a disease that affects the small central cone-rich area of the retina responsible for central vision, the macula. In human populations over 50 years of age, AMD is the most common cause for severe visual loss and legal blindness in all industrialized countries[50] and occurs in 25% of the world's population over 65.[51] Conservative estimates predict that the number of individuals suffering from this disease will increase to 30 million in the next decade.[9] As a result, considerable efforts have been made to target the underlying causes and consequences of this retinopathy.

Studies suggest that morphological and behavioral degeneration within several ocular components (including RPE) may act as potential inducers of this disease. AMD is a multifactorial condition with both genetic and environmental factors influencing

progression.[52, 53] Studies attribute that the physiological failure of RPE contributes to either form of clinically recognized macular degeneration, including geographic atrophy (dry) and exudative (wet) AMD, given either the appearance of drusen bodies or neovascularization of the RPE/choroid, respectively.[9] Many of the molecular errors that precede either event are unclear, thus characterizing RPE activity prior to AMD onset may help in circumventing pathological manifestation. Considerable attention has been devoted to OS phagocytosis, as inability to complete this task is a hallmark of RPE aging, imminent failure and subsequent disease onset.

RPE display a number of significant functional, structural and physiological changes when aged, including the loss of melanin granules, an increase in the number of residual bodies, accumulation of basal deposits on the Bruch's membrane, microvilli atrophy and disorganization of basal enfolding.[54] Oxidative damage has particular significance in aging, and has long been suspected as a contributor to age-related macular degeneration in light of hypothesis supported by the administration of antioxidant vitamins and or zinc and the subsequent slowing of progression from high risk atrophic AMD to neovascular AMD. Concurrent studies support the argument that oxidative stress bears heavy influence during aging, stemming from the identification of many oxidized proteins in the postmortem eyes of patients with AMD by mass spectroscopy.[55] RPE is particularly vulnerable to chronic oxidative injury and inflammation due to the high levels of cumulative irradiation they are exposed to, the accumulation of long chain polyunsaturated fatty acids (PUFA) that are easily oxidized[56] and accumulation of lipofuscin granules, the lattermost being primarily responsible for the intrinsic fluorescence of the human ocular fundus.[57]

Accumulation of lipofuscin in the lysosomal compartments of the RPE is a common characteristic feature of various retinopathies that result in blindness including AMD; as such the formation of these lipid/protein rich bodies has been implicated as an inducer of retinopathy onset. The bulk of lipofuscin granules contain molecules that can neither be degraded nor released from the cell and are toxic at high concentrations. High lipofuscin concentrations have been linked to non-vision related conditions including cardiac hypertrophy, cirrhosis of the liver and neuronal ceroid lipofuscinosis.[50] RPE concentrations of lipofuscin are elevated because of their role of phagocytosing large numbers of membranous discs that are shed by photoreceptors. The highest accumulation of lipofuscin occurs beneath the macula, likely because of the higher photoreceptor to RPE cell ratio.[57]

While consisting of a complex mixture of molecules, the major hydrophobic component of lipofuscin is the fluorophore A2E, which arises from the reaction of two molecules of all-trans-retinal with ethanolamine, both found within photoreceptor OS.[57] Recent studies demonstrate that increased intracellular A2E concentrations inhibit neither binding nor engulfment of OS or degradation of OS proteins, they do however delay digestion of lipid components in FITC labeled OS. The lack of binding and uptake may be influenced by a selective inhibition by A2E of lipid hydrolases phospholipase A_2 or phospholipase C. The presence of a selective block in lipid trafficking caused by A2E that prevents entry into lysosome containing lipid-degrading enzymes may also contribute to this inhibition.[51] Additionally, A2E load can reduce mitochondrial respiration, which may in turn reduce OS uptake and lipid breakdown via ATP-dependent phagocytosis.[58] While minor lysosomal A2E load and moderate mitochondrial

inefficiency may not harm RPE independently, RPE functions are significantly impaired when both occur simultaneously.[58]

Oxidative stress is a cumulative term that refers to direct or indirect injury of a cell as a result of reactive oxygen species (ROS) including hydroxyl radicals, hydrogen peroxide, superoxide anions and singlet oxygen.[59] Since ROS are byproducts of aerobic respiration, mitochondria serve as a major source of ROS. Accumulative ROS can then disrupt the mitochondria, as well as soluble cytoplasmic lipids and proteins.[59] Oxidative stress is believed to play a role in many diseases in addition to those related to aging because of the confounding events that occur downstream of mitochondrial damage by ROS[60] including reduced energy production and compromised cellular function. Since mtDNA are retained during mitosis, oxidative damage to the mtDNA can be passed to daughter cells, potentially amplifying physiological consequences over time. Although the RPE *in vivo* are post-mitotic, they can regain mitotic divisions upon disruption of the monolayer during retinal detachment or other injuries.

RPE & Phagocytosis

Although multiple signaling pathways are involved in the various mechanisms for internalization of extracellular material, phagocytosis initiation and optimization is facilitated via expression of cell surface receptors and stabilizing proteins to initiate binding. Apical surface receptors and microvilli are employed by RPE to promote stabilization and adhesion to OS; activation of these components by circadian rhythm regulates daily phagocytic behavior.[61] Several receptors are implicated in sub-retinal clearance by RPE, including the integrins $\alpha v\beta 5$ and $\alpha v\beta 3$, CD36, MERTK (also called c-

Mer) and $F_c\gamma$ receptors (FcγR). The integrin αvβ5 is widely expressed in epithelial tissue and binds extracellular ligands. CD36 binds many ligands including collagen, oxidized low density lipoprotein, native lipoproteins, oxidized phospholipids, and long-chain fatty acids. The MERTK/c-Mer and FcγR are transmembrane proteins that participate in phagocytosis. Additional ligands including Gas6 and MFG-E8 may potentially positively influence phagocyte activity by RPE.[61, 62]

The RPE occupy the sub-retinal space where they form a portion of the blood retina barrier.[63-68] In normal eyes the apical region of RPE line photoreceptors and contribute to the renewal of the visual cycle by way of debris clearance and metabolite regeneration (Figure 3). The basal region of RPE faces the Bruch's membrane, preventing leakage from the fenestrated endothelium of the choriocapillaris.[2] RPE of the macula region are small, at approximately 10-14 microns, but become flattened toward the periphery where their diameter can reach 60 μM.[69]

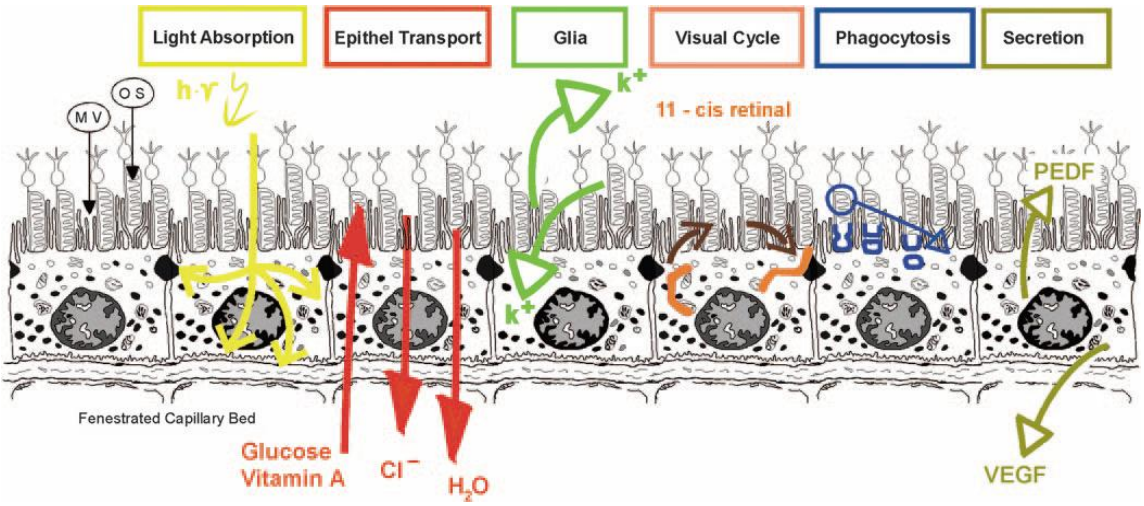

Figure 3. Illustration of RPE responsibilities (Strauss, 2005)

To maintain excitability, photoreceptors rely on RPE to re-isomerize 11-cis retinal from all-trans retinal and deliver it to photoreceptor outer segment discs where it will be reabsorbed and covalently bound to opsin.[2] Approximately 45 photoreceptors are supported by a single RPE,[69] suggesting that increased surface area of the epithelial cells has a convergent evolutionary advantage. In order to accomplish this task, RPE recycle other metabolic necessities of specialized neurons, and reduce physical stress or deformation within the sub-retinal space. As mentioned, RPE phagocytose spent OS in accordance with diurnal cues. First reported in Young in 1967, formation of discs at the proximal ends of outer segments is coordinated with the shedding of distal, aged OS tips.[43] The predictive nature of OS shedding is synchronized to external lighting cues and qualifies for distinction as being under circadian control. Unlike many mammalian systems that follow circadian patterns, OS shedding appears to be mostly independent of suprachiasmatic nuclei (SCN) input; however, retinal innervation by the SCN is required to reestablish light initiated shedding when synchronicity has been lost.[43]

The cue that exists between the RPE and photoreceptors within the interphotoreceptor matrix is a reflection of the rate at which photoreceptors shed their outer segments. As a result, RPE are responsible for the daily phagocytosis of hundreds to thousands of shed membranous discs.[70, 71] *In vitro*, RPE respond to OS challenge slowly and in distinct phases: binding, internalization and trafficking. Binding occurs during the first two hours following OS presentation; internalization may take between an additional two to six hrs.[72, 73] *In vivo*, phosphatidylserine (PS) expressed by the distal tips of photoreceptors initiate binding by RPE indirectly (similar to phagocytosis of apoptotic cells by professional macrophages), when milk-fat globule protein E-8 (MFG-E8) binds to both PS and membrane bound integrin αvβ5.[1, 74] While disputed for nearly a decade, recent findings by Finnemann et al. (2008) demonstrate that RPE rely on a number of membrane surface proteins to complete the daunting task of OS engulfment, but only the integrin αvβ5 localized to the apical plane *in vivo* and *in vitro* is required for recognition of MFG-E8 and PS. Simple IgG blocking studies have shown that without participation from this integrin, OS binding by RPE is abolished, yet internalization of already bound OS is not affected.[72, 75] CD36 and tyrosine kinase Mer (MerTK) mediate OS engulfment, as evidenced through similar blocking techniques whereby internalization was abolished.[76,77] These findings suggest that αvβ5 initiates a signaling response that includes subsequent activation of MerTK and many other downstream analogs including focal adhesion kinase (FAK) (Finnemann, 2003). Upon activation of αvβ5, downstream effector focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase, co-localizes with integrins and heavily influences cellular responses that involve cytoskeletal reorganization.[78-80] While FAK can become phosphorylated of any of its six tyrosine

residues, only those at positions Tyr576 and Tyr577 directly reflect increased enzymatic activity.[81, 82] The only autophosphorylation site on FAK is Tyr397[83], as such, increased levels of phosphorylated Tyr397 correlate well with FAK activation.[71] Finally, the addition of phosphate at residue Tyr861 promotes binding to αvβ5 and FAK autophosphorylation.[71, 84, 85] Data supports the hypothesis that FAK forms a complex with αvβ5 at the apical surface of RPE in addition to MerTK, and that FAK signaling is essential for internalization of bound particles by way of MerTK activation. A transmembrane protein with two fibronectin type-III domains, two Ig-like C2-type domains, and one tyrosine kinase domain, MerTK is a key factor during OS engulfment. Over-expression of MerTK is preceded by redistribution to internalized OS sites *in vitro.*[70, 71, 86, 87] One could argue that this intimate association with sites of endosome formation demonstrates its role as a component of the phagocytic machinery. Additionally, RPE cells respond to OS challenges with increased phosphorylation of MerTK tyrosine residues.[70, 71, 86] However, while MerTK is a downstream target of phosphorylated FAK, αvβ5 binding and subsequent activation of FAK can occur independently of MerTK. Furthermore, experiments using RPE from MerTK defective Royal College of Surgeon rat (RCS) rats suggest that expression of FAK without MerTK induces internalization of OS by non-professional phagocytes.[70] The latter suggests that FAK or a comparable signaling molecule such as PYk2 (a non-receptor tyrosine kinase of the FAK family) can act in some compensatory fashion during integrin dependent phagocytosis in a various cell types.[70] A necessity for engulfment, MerTK phosphorylation is also achieved through binding with linker molecules Gas6 and Protein S to the same PS patches expressed by photoreceptors to initiate binding.[1] Additional

studies evaluating the role of αvβ5 show that knockout mice RPE cells challenged with isolated OS fail to phagocytose this debris and that β_5 deficient retina lack the synchronized burst of RPE phagocytosis usually preceded by rod shedding in rodent retina.[88] Furthermore, primary cultures of mouse RPE devoid of β5 do not increase tyrosine phosphorylation of FAK or MerTK when challenged with POS. These data suggest that αvβ5 also influence rhythmic activation of FAK and MerTK during RPE phagocytosis. [62, 88]

Morphological assessment of RPE *in vivo* has revealed the presence of extensive sheet-like microvilli emanating from the apical surface and projecting into the interphotoreceptor matrix.[89, 90] Typically, between 30 and 40 microvilli of varying length will bind intimately to photoreceptors; although it is not uncommon for a single projection to encircle a one rod or cone multiple times.

The expression of apical microvilli by RPE may contribute to photoreceptor binding via establishment of frictional or electrostatic interactions with outer segment discs; consequently, interference between RPE and OS can result pathological sequelae. This hypothesis is supported by clinical evidence from patients who underwent retinal re-attachment and were unable to regain normal vision because restoration of a proper RPE–microvilli-POS relationship could not be attained.[89, 90] Furthermore, studies on the effect of age and visual health show a decrease in number and length of apical projections. Additional research focusing on the constituents of microvilli suggest that ceruloplasmin, a protein abundant in the apical microvilli of rat RPE is greatly reduced in patients with aceruloplasminemia, an autosomal recessive disorder that manifests in

amongst other traits, retinal degeneration.[89, 90] Apical microvilli contain an internal core bundle of actin filaments and ezrin. While it is known that ezrin is a linker of actin filaments and plasma membrane proteins, less well understood is the extent at which ezrin facilitates the trafficking of membrane proteins to specific regions affiliated with apical microvilli in a potential effort to maximize phagocytic efficiency.

Receptor tyrosine kinase activation pathway / HGF (Hepatocyte Growth Factor)

It is well established that cultured RPE can secrete growth promoters for themselves or neighboring cells[91] in an effort to establish proper morphological development (which occurs in concert with the neural retina,[66, 67, 92-101] respond to cellular insult, and regulate phagocytosis.[102-105] Several growth factors are involved or implicated in the pathogenesis of age-related macular degeneration, especially for their participation in choroidal neovascularization associated with exudative AMD.[106] Of particular significance are the growth factors platelet-derived growth factor (PDGF), vascular endothelial growth factor, and transforming growth factor beta. PDGF was initially identified as a potent mitogen for muscles, but is now known for stimulation and chemotaxis for a variety of cell types, including epithelial.[107-109] In the eye, PDGF is associated with the differentiation of oligodendrocytes and type 2 astrocytes of the optic nerve, and is linked to proliferative vitreoretinopathy (PVR), as this potent chemoattractant induces RPE migration when paired with fibronectin.[110-113]

Vascular endothelial growth factor (VEGF) is yet another key protein whose activity has several consequences in the mature eye. Initially characterized as a tumor-secreted

protein that stimulates neovascularization and angiogenesis, increased VEGF concentration during hypoxic conditions can induce a litany of pathologic sequelae including age-related macular degeneration and diabetic retinopathy.[114-117]

Transforming growth factor beta (TGF-β) has a dual role in retinal health, and may be related to which isoform is produced. Early evidence suggested that during PVR, there is a significant increase in the TGF-β concentration in the vitreous of PVR patients, and that this increase is likely due to a proliferative role in the pathology. Lamoreaux et al. demonstrated that increased TGF-β concentrations may be related to stabilizing the PVR membrane by decreasing release of matrix metalloproteinases.[118] During choroidal neovascularization, TGF-β positively regulates leukocyte infiltration, matrix deposition, scarring, and negatively regulates reepithelialization.[106]

Less well known, however, is the role of hepatocyte growth factor (HGF) in the eye. Mature HGF is an 80 kDa heterodimeric protein, consisting of a 69 kDa α-subunit linked by a disulfide bond to a 34 kDa β-subunit[119 120] and is formed from a 728 amino acid residue precursor.[121] Human HGF is encoded by a gene on chromosome seven, approximately 70 kb in length containing 18 exons and 17 introns.[122-131] The alpha chain of mature HGF contains a hairpin loop at its NH2 terminus and four Kringle domains formed by three internal disulfide bridges where the beta chain features a serine protease like structure.[132, 133] The mature protein induces a multitude of morphological, mitogenic and motogenic responses including tubulogenesis, branching morphogenesis, cytoskeletal rearrangement, loss of intercellular junctions and active migration following cell dissociation (scattering).[134, 135]

HGF is secreted as a biologically inert precursor under conditions of extracellular stress including tissue damage. It is hypothesized that proteolytic digestion of pro-HGF is facilitated by urokinase plasminogen activator (uPA) or by a protease homologous to factor XII.[136, 137]

Initially discovered as a progenitor of liver regeneration, mRNA of HGF is induced in stromal epithelium by inflammatory cytokines IL-1, -6 and TNFα, suggesting that increased expression of HGF and its receptor is a response to extracellular insult.[138] Mediation of pro- or anti- inflammatory responses are associated with professional phagocytes including macrophages and neutrophils and can stimulate phagocytic associated behavior including release of toxic microbiocides.[139]

Separate signaling pathways in traditional professional phagocytes such as monocytes control phagocytosis and production of cytokines, though both can be activated via stimulation of immunoglobulin receptors. HGF is considered an important trigger for wound healing or protection against the risk of apoptosis or necrosis in wounded RPE or the ischemic retina. RPE secrete HGF to maintain retinal homeostasis and visual function or to repair itself or adjacent cells after injury.[139, 140] Binding of HGF to its cognate receptor c-Met initiates signaling cascades that result in morphological changes, including cytoskeletal rearrangement. The changes in the cytoskeletal architecture is observed in fibroblasts stimulated with HGF, inducing cellular proliferation and migration and earning the growth factor the monogram of scatter factor (SF).[141]

Cytoplasmic signaling molecules activated as a result of HGF/c-Met binding and cytokine exposure include PI3K, ERK, Ip3 phospholipase D, DAG, MAPK and NFKB;

several of which, specifically PI3K, PLC, MAPK, and ERK, participate in phagocytosis. These phagocytic influencers are only four of several messenger molecules that are activated by HGF activation of c-Met, suggesting that overlapping signaling cascades can be used to manipulate the phagocytic ability of cells expressing generic machinery. An interesting phenomenon is the strengthening of phagocytic response when PKC activation is greater in monocyte-differentiated macrophages. For these reasons we believe that through the recruitment of PI3K, ERK and PKC, professional phagocytes optimize phagocytic activity.

c-Met (Mesenchymal Epithelial Transition Factor)

The mature HGF receptor c-Met is a proto-oncogenic receptor tyrosine kinase belonging to a sub-family that includes Ron and Sea.[142] The 195 kDa heterodimer is comprised of a 50 kDa alpha chain and a 145 kDa beta chain. The completely extracellular alpha chain is linked by disulfide bonds a beta chain that transverses the plasma membrane ad features a tyrosine kinase domain.[138] These large polypeptide chains are derived post-translationally from a single chain precursor by proteolytic cleavage much like its ligand.[138] This β-subunit has two tyrosine residues whose responsibilities include autophosphorylation and two additional tyrosine residues that mediate high-affinity interactions with SH2 proteins.

HGF up-regulates c-Met receptors by way of transcriptional regulation. Consistently, the catalytic activity of c-Met is soundly up-regulated by autophosphorylation. Tyrosine residues positioned at 1234 and 1235 are responsible for the increased rate of autophosphorylation; negative regulation of c-Met occurs through two pathways, each

terminating with the phosphorylation of serine residues in the cytoplasmic domain by protein kinase C or by a Ca^{2+} dependent serine kinase.[143] Critical for phosphorylation and subsequent activities are the tyrosine residue docking sites 1356 and 1349.[144-146]

Kinase activation of the Met receptor results in induction of cellular responses, variance of those Met substrates that are activated will dictate mito-, morpho-, or motogenic behavior. Scattering and growth response pathways have been described; the former is initiated by the activation of phosphatidylinositol 3-kinase (PI3K) or by the p85 adaptor subunit.[144, 147] Since scattering and pseudopod extension requires cytoskeletal rearrangement that can begin with PI3K activation, an early hypothesis has suggested that HGF induces cytoskeletal arrangement via PI3K.[148] These substrates contain the Src homology 2 domain (SH2) which have high affinity for the Y1349/Y1356 docking site.[144, 149] In renal cells, PI3K is critical for chemotaxis response to HGF in addition to tubulogenesis. Wortmannin has been used in renal epithelial cells to inhibit PI3K, which blocks motogenesis and morphogenesis.[150] Mitogenesis is not significantly inhibited in these renal cells, but greatly inhibited in murine mammary carcinoma cells (SP1), suggesting that genotypically normal cells may involve PI3K within the scattering pathway while cancer cells activate PI3K in the growth pathway.[151]

PI3K along with GrB2, GAP, PLCγ, Src, and MAP kinase are implicated as regulators the motogenic/mitogenic pathway as well.[148, 152] Cell growth influenced by HGF is associated with significant increases in cytoplasmic Ca^{2+} via a protein kinase C mechanism.[153, 154] Activation of the GrB2 adaptor protein, which couples receptor tyrosine kinases to the Ras signaling pathway, relies upon Jun kinase (JNK) activation.[155]

There appears to be no connection between inactivation of JNK and inactivation of MAP kinase, suggesting that JNK and MAPK require separate enzymes for activation. Grb2 binding induces Ras activation by recruiting SOS.[152, 156, 157] This leads to activation of Rac1 which then activates Cdc42.[155, 156, 158, 159] Subsequent activation of SEK and then JNK concludes with cJun activation, which increases DNA synthesis.[156]

The potential roles of PI3K & Rac1 during OS phagocytosis by RPE

FcγR-mediated phagocytosis requires the activation of structural proteins belonging to the Dos/Gab subfamily of scaffolding molecules.[160] Once FcγR has bound to the Fc region of an external IgG coated particle, rapid actin polymerization facilitates the formation of a phagosome that engulfs the external target. FcγRI and RIII are high and low (respectively) affinity receptors expressed by murine macrophages, each contains ligand binding a chains and subunits that feature immune receptor-based tyrosine motifs (ITAMs).[160] Activation of membrane associated Src tyrosine kinases occurs downstream of FcγR binding; subsequent activation of ITAMs by Src proteins via tyrosine phosphorylation[161] is thought to be critical, as deletion of select Src tyrosine kinases significantly reduce the rates of phagocytosis. Genetic removal of Syk, a cytoplasmic non-receptor tyrosine kinase, inhibits the formation of the phagocytic cup in mice. [162, 163]

Phosphoinositide 3-kinases (PI3K) are a family of signal transducing enzymes capable of phosphorylating the third position hydroxyl group of the inositol ring within phosphatidylinositol molecules thought to be involved in pseudopod extension during phagocytic cup formation.[164,165] Studies demonstrate that cells lacking the p85 α and β subunits of PI3K essentially blocks phagocytosis.[166] Upon activation, one

target of PI3K phosphorylation is phosphatidylinositol (3,4,5)-triphosphate (PIP3). Typically associated with cell survival, studies show that PIP3 lipid generation is highly restricted to the phagocytic cup during FcγR phagocytosis.[167]

Scaffolding proteins including the Grb2 associated binding protein 2 (Gab2) are key regulators of PI3K activation in FcγR-mediated mast cell signaling.[168] A member of the Dos family of scaffolding proteins, Gab2 contains an amino-terminal pleckstrin homology (PH) domain, many tyrosine phosphorylation sites and several proline-rich motifs. While phosphoinositides binding specificity has not been fully characterized, the Gab1 PH domain binds with high selectivity to PIP3.[169] FcγR activation leads to the tyrosyl phosphorylation of Gab2, which recruits SH-2 containing signal relay molecules Shp-2 and p85. As mentioned, p85 is a regulatory subunit of PI3K and is critical for cytokine and FcγRI-evoked PI3K activation[168,170] while Shp-2 influences cytokine induced early gene expression and growth factor induced MAPK activation.[171] Similarities in FceR1 and FcγR signaling[172] have led groups to study the possible molecular overlaps during macrophage response. In bone marrow derived macrophages (BMM), Gab2 becomes tyrosine phosphorylated and associates with Shp-2 and p85 upon FcγR activation as it does during FceR1 signaling.[173] Furthermore, BMM devoid of Gab2 display reduced activation of Akt, a serine/threonine-specific protein kinase downstream target of PI3K, suggesting that Gab2 regulates this pathway during FcγR mediated phagocytosis.[173]

Rac1 (Ras-related C3 botulin toxin substrate), is a 21 kDa protein encoded by the RAC1 gene and member of the Rho family of GTPases. Similar to other members of this super

family, Rac appears to regulate a diverse set of cellular events including growth, cytoskeletal reorganization, and activation of protein kinases.[174] Since Rac1is a binding protein that can control lamellipodia formation and membrane ruffling in fibroblasts, it is likely that it plays a role in at least one stage of phagocytosis. Indeed, Rac1 and Cdc42 regulate Fc receptor-mediated phagocytosis in macrophages by controlling various steps of membrane and actin dynamics resulting in particle engulfment.[175] As mentioned, RPE share many phagocytic mechanisms from their professional macrophage counterparts, therefore it is likely that Rac1 plays a role in OS phagocytosis. Further analysis revealed that there is a critical role for Rac1 in clearance phagocytosis by cultured RPE cells and those *in vivo*.[4] Mao and Finnemann were the first to identify Rac1 activation changes in any form of phagocytosis in vivo in intact tissue, and demonstrate that Rac1 phosphorylation is essential for of OS by RPE cells.[4] Furthermore, the inhibition of Rac1 stimulation with peak OS phagocytosis in $B5^{-/-}$ and MFG-$E8^{-/-}$ retina suggests the dependence of Rac1 and engulfment signaling initiated downstream of $\alpha v\beta 5$ ligation.[4] Results of the aforementioned experiments clearly indicate that inhibition of only Rac1 is sufficient in preventing F-actin recruitment to the local membrane where extracellular particles are bound to RPE.[4]

Cell Lines: ARPE-19

ARPE-19 (American Type Culture Collection, Manassas VA) is a commonly used cell line used to study RPE function *in vitro*.[176] ARPE-19 are a rapidly growing spontaneously formed human cell line derived by Amy Aotaki-Keen from the normal eyes of a 19-year-old male donor, enucleated two hours post mortem. This cell line is

particularly valuable as it retains characteristic features of RPE *in vivo*, including morphologic hallmarks, normal karyology, apical microvilli, junctional complexes basal enfoldings, expression of RPE marker proteins RPE-65 and CRALBP and polarized distribution of organelles when plated on laminin-coated Transwell-COL filters in medium with a low serum concentration (polarization of pigment granules are not achieved by ARPE-19).[177] Prior to depositing at ATCC, these cells were subjected to selective trypsinization for the first four passages to remove superficial cells before passaging the cuboidal basal layer (as described by ATCC).

Cell Lines: RPE-J

RPE cell lines from a handful of species are commercially available, though many have been intentionally modified by transformation with oncogenes or viral proteins. An example of the latter is the RPE-J line of rat RPE cells (American Type Culture Collection, Manassas VA). To establish this cell line, retinas were removed from a seven day old Long Evans rat, transfected with a temperature sensitive SV40 virus, and clones displaying epithelioid morphology and expressing tight junctional protein ZO-1 were isolated.[177, 178] While less difficult to maintain when compared to primary cultures, the polarity of Na^+/K^+-ATPase and N-CaM differs from *in vivo* localization of these proteins; still, RPE-J retain many differentiated features of RPE including the ability to synthesize melanosomes and apical/basolateral polarity[177] (as described by ATCC).

Specific Aims

Dysfunction of the retinal pigment epithelium (RPE) has been shown to induce photoreceptor injury. As a result, complete understanding of the cellular miscues that precede failure is crucial for maintenance of visual health and disease prevention.[1] The most prominent retinopathy, age-related macular degeneration (AMD), is the leading cause of central vision loss both domestically and abroad.[9] AMD manifests as either accumulation of drusen upon the macula (geographic atrophy) and/or neo-vascularization of sub-retinal basement membranes (exudative). Consistent with either scenario is the death of RPE caused by a myriad of complications including oxidative stress. While it has been shown that deposition of lipid dense granules and aggressive/invasive blood vessel expansion cause undesirable effects that occlude visual ability. The decline of sub-retinal maintenance by RPE, particularly phagocytosis of spent outer segments (OS), exacerbates photoreceptor degeneration.

OS phagocytosis by RPE is a key component of visual regularity, as retinal, the excitable polyene chromophore that initiates signaling within photoreceptors, is converted to all-trans retinal upon photon exposure. Since all-trans retinal is unresponsive to light, RPE are required to reisomerize inert all-trans retinal into an 11-cis configuration that is returned to photoreceptors, binds to opsin and effectively renew this cycle. Perturbations within this process inhibit recycling of excitable compounds and result in accumulation of OS within the sub-retinal space, blocking activation of opsin signaling and physical distension of the retina respectively. Furthermore, the membranous discs contain the essential fatty acid docosahexaenoic acid (DHA) a precursor of neuroprotectin D1

(NPD1) which inhibits apoptotic behavior mediated by A2E (bispyridinium bisretinoid), a lipofuscin fluorophore component whose toxic effects manifests as AMD.[51, 179]

As a non-classical macrophage, RPE rely on the collective machinery that drive signaling events observed during FcγR mediated phagocytosis and OS phagocytosis to remove non specific debris *in vitro*. OS clearance features a unique cascade that appears to be specific to photoreceptor renewal. It is believed that this process begins with the OS externalization of phosphatidylserine patches, a behavior exhibited by apoptotic cells conveying an 'eat me' signal' to circulating phagocytes.[180] These terminal primary amino acid groups facilitate recognition as they are bound by milk-fat globule EGF factor-8 (MFG-E8) which in turn binds to integrins αvβ5 and αvβ3 (and scavenger receptor B CD36) at the extracellular membrane.[70] Active αvβ5 complexes with tetraspanin CD81 resulting in mobilization of focal adhesion kinase (FAK) to the cell membrane where it becomes autophosphorylated and initiates phosphorylation of MerTK and the Src signaling pathway. Finnemann et al. have shown that while CD81 and αvβ3 contribute to OS binding, αvβ5 is the key mediator of OS recognition; however, simultaneous activation of MerTK by OS is required for internalization. Linker proteins Gas6 and protein S mediate the interaction between the tyrosine kinase and OS; MerTK's continued phosphorylation directly by FAK and indirectly by PS causes an increase in cellular production of inositol 1,4,5-triphosphate (InsP3) potentially to partially regulate internalization through increase of intracellular Ca2, cAMP and PKC which act as downstream modulators of phagocytic activity.[1] Formation of the phagocytic cup relies on cytoskeletal reorganization mediated almost entirely by motor protein myosin II. Royal College of Surgeon (RCS) rats possessing a defective MerTK, fail to redistribute

myosin II post OS binding, suggesting that this receptor is of crucial during OS engulfment .[1]

Substrate recognition by traditional macrophages results in the recruitment of FcR to the membrane contact site. Multiple kinases are activated thereafter, resulting in actin polymerization mediated by GTPase, Rac1, Rac2, and cell division control protein 42 (cdc42),[181] Rac1 effectors Wiskott—Aldrich syndrome protein and actin related protein 2/3 (ARP2/3). Furthermore, actin regulator annexin a2 has been implicated as a modulator of OS internalization. Recently annexin a2 was shown to nucleate actin polymerization of latex beads coated with phosphatidylinositol-4, 5-bisphosphate (42,43), a product of phosphatidylinositol 3-kinase activation. Castellano et al., have demonstrated that the membrane recruitment of Rac1 is a necessity for FcγR mediated phagocytosis; additionally it has been shown that Rac1 activation by avb5 is also required for phagocytosis of OS by RPE.[182]

The discovery that lack of c-Met signaling results in impairment of phagocytosis in alveolar macrophages by Hu et al. suggests c-Met's role as modulator of this activity in post-mitotic cells secreting HGF. As activated PI3K has also been implicated as a necessity for FcR mediated phagocytosis, particularly during phagosome closure,[183] it appears likely that activation of c-Met by HGF and their subsequent effectors will influence OS clearance by RPE. Preliminary data supports this hypothesis when RPE are challenged with non-specific targets *in vitro* (Blaize, not yet submitted). Although it is well established that loss of the Mer tyrosine kinase receptor will result in RPE failure, the activity of c-Met during OS phagocytosis has yet to be characterized. It appears that

signaling pathway cross talk evoked by tyrosine kinase receptor activation may elicit a variety of yet to be observed behaviors. Taken together, we propose a) that c-Met activation influences each phase of RPE response during non-specific target challenge, b) this modulation occurs through the activation/up-regulation of PI3K c) application of PI3K inhibitors will prevent HGF induced augmentation of phagocytosis.

1a) Does RPE respond to application of exogenous HGF *in vitro*? 1b) Does activation of c-Met result in subsequent activation of messenger molecules?

1a) In order to understand the effect of HGF influence on OS or non-specific debris processing by RPE we will first identify the time required to induce maximal c-Met phosphorylation. To accomplish this, RPE will be exposed to 0, 25 or 50 ng/ml HGF (concentrations shown to induce up-regulation post 24 hr exposure) with cell lysates harvested in 20 minute intervals up to but not exceeding one hour. Phosphorylated c-Met will be measured by western blotting and immunocytochemical techniques. 1b) In a concurrent study we will investigate the expression of Grb2, STAT3, PI3K and $\alpha v\beta 5$ to determine if expression or phosphorylation of these key signaling molecules/integrin are dependent upon activation c-Met.

2a) Will an increase of extracellular HGF result in accelerated/increased phagocytic behavior among RPE *in vitro*? 2b) Does chemical inhibition of PI3K interfere with phagocytosis?

2a) To determine the extent of pathway activity during exposure to increased growth factors with and without the stress of an external target, RPE will be cultured and

exposed to either 0, 25 or 50 ng/ml HGF (concentrations shown to exhibit maximal response *in vitro).* 2b) Once second messenger expression profiles have been determined, RPE will be challenged with a PI3K antagonists prior to OS exposure as before (Aim 1). Cells will then be prepared for time-lapse microscopy, confocal and scanning electron microscopy in an effort to characterize phagocytosis. *The experiments of Aim 2 are designed to better understand the role of c-Met effectors (specifically PI3K) as mediators of RPE phagocytosis. While it is likely that numerous signals can influence the efficiency of this process, PI3K activation by HGF has not yet been evaluated during specialized clearance facilitated by non-traditional macrophages.*

CHAPTER II: The influence of HGF on c-Met and facilitators of OS binding: HGF induces up-regulation of c-Met and αvβ5 in RPE

Introduction

The realization that HGF is expressed by epithelial cells did not occur until the late 1990's when it was described in both human and rabbit epithelium.[184, 185,186] While RPE were known to secrete a variety of substances prior to this discovery (including metalloproteinases in response to cytokine stimuli),[187] little was known about the influence of HGF in RPE behavior. In 1998, He et al. discovered that RPE respond chemotactically to HGF through constitutive tyrosine phosphorylation of c-Met *in vitro*, suggesting its role as an autocrine growth factor and its involvement during retinal development.[186] The same year, HGF levels in patients with proliferative retinopathies were described, suggesting that the presence of HGF was related to a disease state.[188, 189] Since RPE have long been associated with support and recruitment of astrocytes and monocytes via secretion of chemoattractants[184] during retinal injury, it is unsurprising that they themselves are capable of responding to chemoattractant stimuli, witnessed during retinal pathology manifestations (proliferative vitreoretinopathy, AMD and retinal detachment).[184]

During fetal development, many organ types including liver, lung, mammary glands, skeletal muscle and sensory neurons express HGF and its receptor.[190-193] In the liver of mice lacking HGF/c-Met signaling, fetuses fail to complete development and die *in utero.* The embryonic liver has reduced size and displays an extensive loss of parenchymal cells.[194] In the lung, kidney and mammary glands, HGF is implicated as a key initiator of

epithelial branching and growth.[190, 193, 195] Sensory neurons rely on the chemoattractant traits of HGF, as HGF enhances axonal growth and survival of neurons that innervate the skin and limbs of the thorax.[192] Within the eye, HGF improves the survival of ganglion cells and promotes axonal regeneration *in vitro* and *in vivo*[196] while inducing retinal angiogenesis through increased urokinase expression.[197] RPE utilize HGF during wound healing, as downstream effectors of c-Met and epidermal growth factor receptor communicate in response to pathologic conditions.[176] Interestingly, there is a lack of HGF immunoreactivity of human RPE explants, suggesting that HGF may be expressed only when RPE are stimulated. This may occur by simply culturing RPE, detaching the sensory retina in explants cultures or growth in the presence of serum or cytokines.[186]

We sought to reveal the effect of exogenous HGF application on RPE cell cultures after 24 hours of exposure; the reasoning behind this methodology was two-fold: 1) an overnight approach would allow for detection of morphological differentiation and cellular proliferation upon exposure to HGF and 2) evaluate the cellular response to chronic exposure to HGF by way of mature c-Met expression. Treatment with various concentrations of exogenous HGF allowed us to establish a dosage threshold, whereby a maximal response could be elicited by exposure to the minimum concentration of the growth factor.

Materials & Methods

Cell growth and treatment

ARPE-19 or RPE-J were cultured in Dulbecco's modified Eagle's media (D-MEM), supplemented with antibiotic/antimycotic, 2.5% sodium bicarbonate and 10% fetal bovine serum. Medium and supplements were purchased from Life Technologies (Carlsbad CA). Cells were seeded at 5,000 cells/cm^2 and maintained at 37°C and 5% CO_2 until they reached ~80% confluence. Individual cultures were then serum starved for 24 hr prior to HGF treatment at concentrations of 0, 15, 25, 50, 75, 100 ng/ml for either 24 hr (chronic) or 1, 20 or 60 min (acute).

Confocal Microscopy

Cells were washed with PBS and fixed in 2.5% paraformaldehyde (PFA) in PBS for 1 hr at room temperature. Non-specific binding of antibodies was eliminated by blocking in 5% nonfat dry milk, 0.05% Tween-20 and 4% normal goat serum in PBS. Following blocking, antibodies were diluted in an antibody dilution buffer (ADB) consisting of 2.5% nonfat dry milk and 2% NGS in PBS. Primary antibody was rabbit anti c-Met (Abcam, Cambridge MA) and was diluted 1:1,000 (0.2 μg/ml) in ADB. Cultures were incubated for 2 hr at 4^oC with constant agitation, followed by three rinses in ADB. Following these rinses, goat anti-rabbit IgG conjugated with Alexa Fluor 488 (Molecular Probes/Life Technologies, Carlsbad CA) was diluted 1:1,000 (2 μg/ml)) in ADB with incubation for 1 hour at room temperature with constant agitation. Cultures were rinsed in PBS, counterstained with ProLong Gold antifade with DAPI (Molecular Probes/Life Technologies, Carlsbad CA), covered with a 1.5 cover glass and imaged with a Leica SP2 AOBS confocal microscope.

Immunoblot

Cells were grown as described above, and harvested through scraping of Corning T25 flasks, or Lab-Tek multi-well plates and employment of a rubber policeman. Cultures were post-treated with ice cold RIPA lysis buffer (0.5M Tris-HCl, [pH 7.4], 1.5M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA and HALT protease inhibitor cocktail (AEBSF-HCl, aprotinin, bestatin, E-64, EDTA, leupeptin, pepstatin A dissolved in dimethylsulfoxide (DMSO) purchased from Thermo Fisher Scientific, Waltham MA) for 15 min (total). Extracts were centrifuged at 12,000 RPM for 12 min at 4° C, and the resulting supernatant was analyzed for total protein concentration and subsequently diluted with sample buffer.

Extracts were diluted with sample buffer containing 60 mM Tris-Cl (pH6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue, and frozen at -80°C until needed. For electrophoretic separation of total proteins 30 µl were separated using a NOVEX 4-20% Tris-Glycine pre-cast poly acrylamide gel (Life Technologies, Carlsbad CA). A fixed volume was used when small sample volume prevented protein determination by way of Bradford, Micro BCA, or Lowry assay. Proteins were boiled for 1 min at 100°C prior to electrophoresis at 80-120V for 3 hours in tank buffer containing 60 mM Tris-Cl and 2% SDS.

To determine changes in protein expression, total proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (Thermo Fisher Scientific, Waltham MA). Proteins were transferred to membrane using a NOVEX Xcell II transfer chamber (Life Technologies, Carlsbad CA) in a tank buffer consisting of 25 mM Tris, 192 mM glycine and 10% methanol for 3.5 hours at 100 mA. Efficiency of transfer was determined using

Ponceau S staining (0.1% (w/v) Ponceau S in 5% acetic acid) followed by destaining in 1M NaOH and distilled H_2O.

Membranes were subjected to several rounds of blocking (Tris buffered saline with 4% normal goat serum, 5% bovine serum albumin and .01% Tween-20) prior to immunological detection employing diluted primary antibodies directed against c-MET and a housekeeping protein (actin or tubulin). For c-Met evaluation, the primary antibody (rabbit anti phosphorylated-c-Met or rabbit anti-c-Met) was diluted 1:250 (0.8 μg/ml). Housekeeping proteins were also diluted in ADB, using a 1:1,000 (50 ng/ml) dilution of rabbit anti-tubulin or 1:10,000 (25 ng/ml) dilution of mouse anti-F-actin. Membranes were incubated in primary antibodies overnight at 4°C with constant agitation. Secondary staining using 1:1,000 to 1:2,000 (100 – 200 ng/ml) goat anti-mouse or rabbit IgG bound to horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Santa Cruz CA) for 2 hours at room temperature with constant agitation. Afterwards, membranes were washed with Tris buffered saline with 0.05% Tween-20 (TBST) before treatment with chemiluminescent substrate. Chemiluminescent bands were captured using the Alpha Innotech FC2 Imager II.

Results

When challenged with an extracellular ligand, cells often increase expression of the cognate receptor to accommodate an increase in second messenger signaling. RPE exposed to increasing concentrations of HGF responded by increasing expression of its cognate receptor c-Met. Figure 4 A-C illustrate punctuate staining reflective of c-Met expression using immunocytochemical analyses. We demonstrated here that exposure to

increased HGF concentrations above 25 ng/ml (2 – 8 μM) resulted in a significant increase ($p<0.001$) in c-Met expression in cultured RPE cells (Figure 4 A-C). The dose 15 ng/ml (1.2 μM) did not show a significant decrease ($p>0.05$) in protein expression (Figure 4D). Fluorescence intensity values from the channel corresponding to c-Met were recorded from no less than 100 individual cells. A one-way analysis of variance (ANOVA) test was used compare fluorescent intensity values of cells treated with various concentrations of the growth factor. Post-hoc tests (Bonferroni and Tukey) were used thereafter to identify statistical relevance between independent variable groups. Our findings suggest that RPE respond to increased exposure to HGF by up-regulating its receptor, and maintain that expression beyond a minimal effective dosage.

While it is likely a percentage of c-Met expression stems from endosomal containment as a means to attenuate signal transduction, we suspect that the overall increase of c-Met in a dose-dependent manner is only one characteristic of a much larger cellular event- that is, there are other consequences of c-Met activation and up-regulation. The discovery that increased ligand availability results in differential expression of receptors was not made recently, and this observation has been used to emphasize the importance of RTK activation among mitotic including CDK compromised cells undergoing tumorigenesis.[198] We conclude that 25 ng/ml (2 μM) HGF is the minimal effective dosage required to evoke a significant cellular response by RPE.

Quantitation of total c-Met by microscopy indicated that there was altered detectable amount of the receptor present in response to increasing HGF concentration, yet this may have been masked by epitope sequestration in the plasma membrane. To determine if c-

Met levels were indeed up-regulated, we turned to an immunoblot analysis of membrane-bound proteins.

Immunohistochemistry has advantages in determining relative amounts of protein expressed at given loci. The disadvantages are that it may not fully describe subtle changes in protein expression. Using the parameters of our immunohistochemical analyses, we were able to confirm that 25 ng/ml (2 μM) HGF as well as the higher concentrations elicited a significant increase in c-Met expression (Figure 5 A, B).

Because of the sensitivity of the immunoblot technique, we also examined lower doses (400 nM and 800 nM). Here we confirmed that RPE showed a response to HGF, where 5 – 10 ng/ml (400 - 800 nM) did not up-regulate c-Met expression but there was a significant increase in expression at 1.2 μM (15 ng/ml) that was consistently elevated through 100 ng/ml (8 μM). The data from the more sensitive immunoblot assay suggested that at the 1.2 μM concentration, c-Met expression was increased, although not demonstrated through immunocytochemistry.

We have demonstrated that HGF up-regulated expression of membrane-bound receptors. It is not unusual for ligands to up-regulate expression of their cognate receptors; however, our original hypothesis was that HGF prepare the RPE for phagocytosis. If true, we predicted that increased extracellular HGF would signal the RPE to prepare for increased binding of OS by up-regulating expression of the αvβ5 integrins. Using the 2 μM concentration as the minimal effective dose to elicit changes in receptor expression, we tested the efficacy of HGF to increase integrin expression by the RPE. RPE up-regulated integrin αvβ5 in response to chronic HGF exposure in a dose-dependent fashion (Figure

6). Similar to the response shown by c-Met, fluorescent intensities of $\alpha_V\beta_5$ increased significantly when RPE were exposed to 25 and 50 ng/ml of HGF ($p < 0.05$).

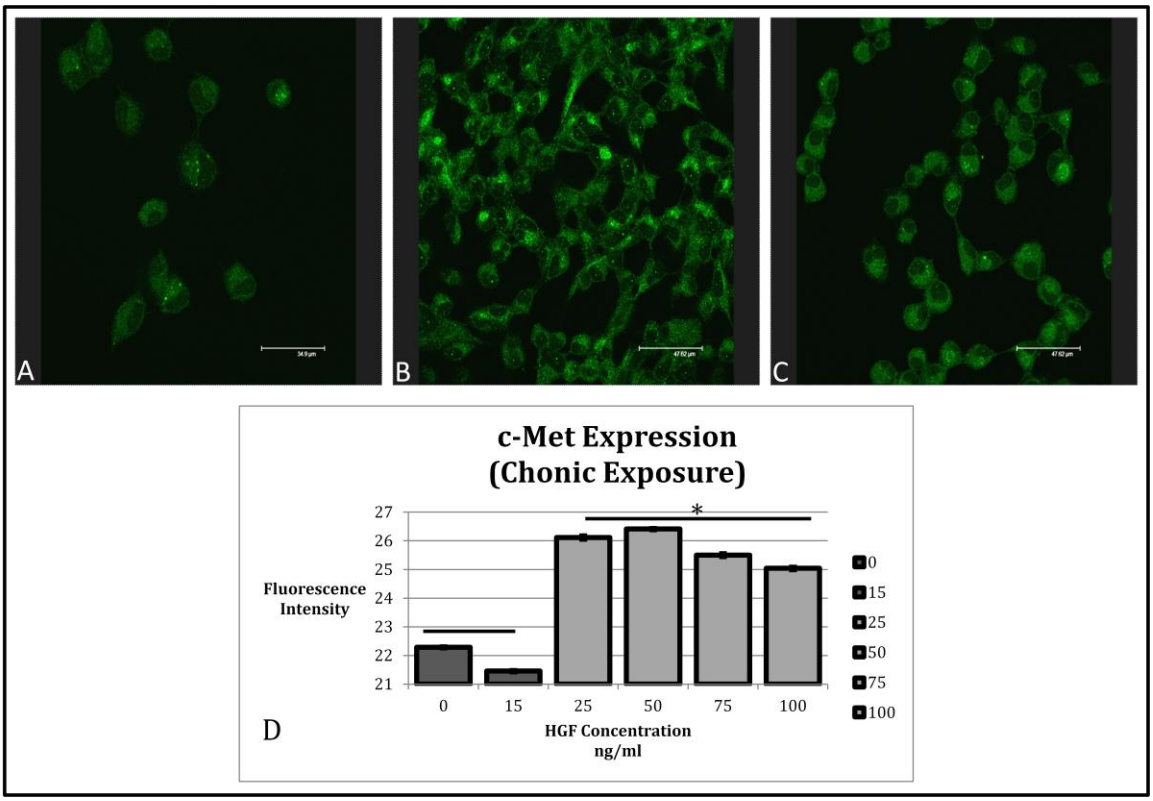

Figure 4. Response of RPE to increasing concentrations of HGF.

In panels A-C, c-Met was up-regulated in response to increasing concentrations of HGF (0, 25 and 50 ng/ml shown). (D) Quantitation of fluorescence intensity revealed a significant increase ($p < 0.001$) in c-Met expression when HGF exceeded 25 ng/ml *in vitro.* There was a non-significant decrease at 15 ng/ml.

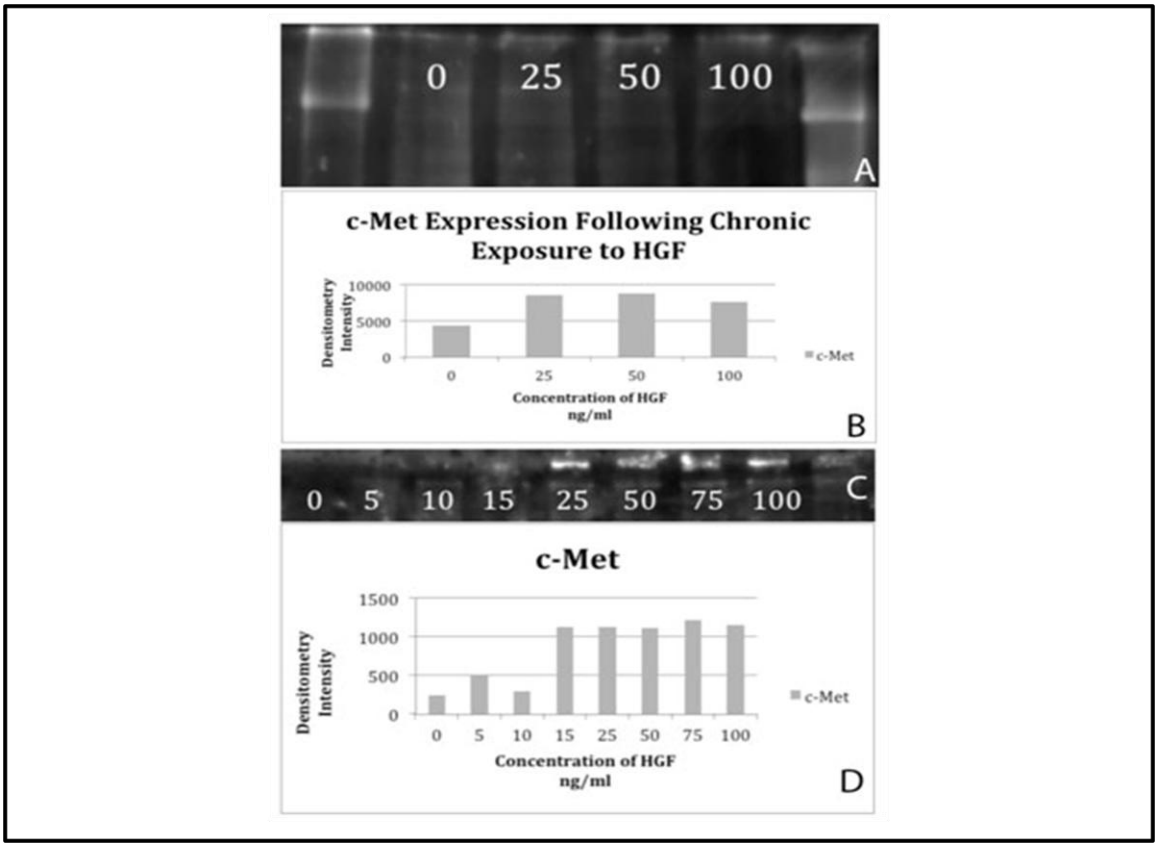

Figure 5. Western blot analyses of c-Met expression by RPE following HGF treatment

A, B. Using the dosage paradigm for the immunohistochemical analyses, c-Met expression was increased with HGF at concentrations of 25 – 100 ng/ml. C, D. To determine if these changes occurred at lower concentrations, we tested HGF at 5 – 15 ng/ml.

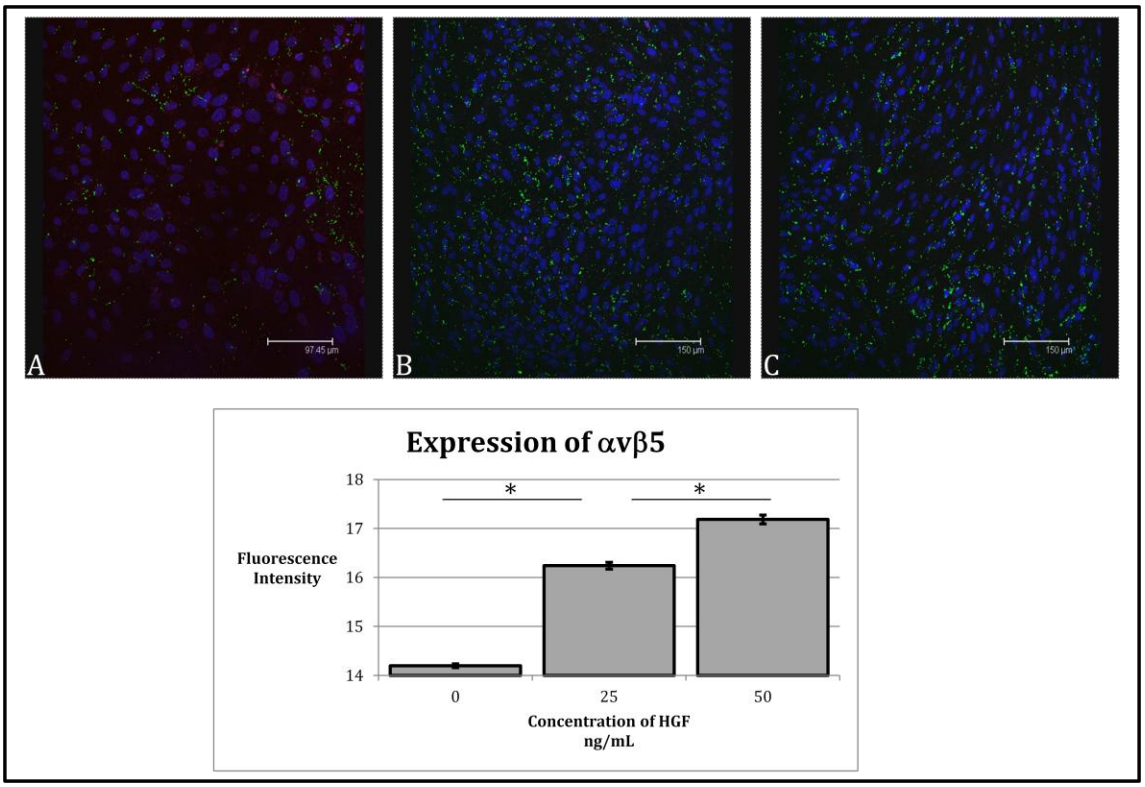

Figure 6. Expression of αvβ5

A-C illustrates the up-regulation of αvβ5 in RPE when exposed to increasing concentrations of HGF (0, 25 and 50 ng/ml shown). αvβ5 were labeled with Alexa Fluor 594 and imaged via confocal microscopy post fixation. (D) Quantitation of fluorescence intensity showed a dose-dependent response to HGF- potentially preparing RPE for recognition of POS and initiation of phagocytosis.

Discussion

Several studies have shown that RPE secrete and respond to various concentrations HGF both *in vivo* an *in vitro*,[176, 186, 199] and while c-Met activation is typically associated with wound healing, proliferation and or migration, our data suggest an alternative role for this tandem as a mediator of phagocytosis by RPE. While elevated levels of HGF could be expected in disease states, such as PVR, AMD, and proliferative diabetic retinopathy (PDR), there is likely also a role for these growth factors for maintenance of function. Photoreceptor OS express key surface proteins that interact with the RPE in the initial binding phase. Based upon the observation that HGF is required for activation of professional phagocytes in the liver, we hypothesized that the role of HGF in RPE biology was similar. We provide evidence here that increasing HGF concentrations do up-regulate expression of $\alpha v\beta 5$, the key RPE surface protein required for OS binding.[72, 73]

Prolonged exposure to growth factors has been identified as a necessity for cellular development,[200] but can induce unwanted trans-differentiation and morphological anomalies in post-mitotic cells.[201] Since active site mutations of c-Met have been identified as a progenitor of cancer cells, it comes as no surprise that inhibition of this receptor or interference of HGF signaling has become a popular avenue for investigating therapeutic intervention.[202, 203] To date, the effects of long term exposure to HGF have not been studied in the eye during development or otherwise. Since HGF is a key contributor to cellular growth, and since it is synthesized and secreted by RPE throughout

life, our initial experimentation included evaluation of the c-Met receptor following continual exposure to various concentrations of HGF in culture.

Up-regulation of αvβ5 is a particularly useful finding, as this integrin has been identified as a key mediator of POS binding; if HGF does influence phagocytosis, increased expression of αvβ5 may play pivotal role.

CHAPTER III: HGF activates c-Met in ARPE-19, RPE-J cells

Introduction

HGF was the initial member of the plasminogen-related growth factor family, a novel family of high molecular weight growth factors whose domain structure and action resemble blood protease plasminogen.[204] Plasminogen is an inactive enzyme precursor that enters systemic circulation. Upon activation (by way of a variety of enzymes including tissue plasminogen activator, urokinase plasminogen activator, kallikrein, and factor XII)[205-209] these proteases cleave peptide bonds where serine is the active site.[210]

Another member of this family is HGF-like/macrophage stimulation protein (HGFl/MSP), a 78 kDa plasma protein secreted by the liver that causes the phosphorylation of the RON tyrosine kinase receptor (RTK), implicated in the regulation of mononuclear phagocytes.[211, 212] Associated with RON is PI3K, a family of enzymes that participate in regulation of a variety of cellular functions including morphogenesis, differentiation, survival and intracellular trafficking.[213-215] MSP stimulates the motility of murine resident peritoneal macrophages, however, pharmacological inhibition of PI3K abolishes MSP action on cells.[211]

c-Met, another transmembrane RTK (190 kDa) whose ligand is HGF, activates PI3K indirectly through use of adapter molecule Gab1. Upon binding of the ligand, dimerization induces activation of the cytoplasmic multi-dock site found on the C-terminus of c-Met's β subunit (heavy chain) where tyrosine residues Tyr 1349 and Tyr 1356 become phosphorylated. Another important adapter protein, Grb2, is responsible

for activation of the mitogen activated protein kinase (MAPK) pathway, which has been implicated as a key mediator of cellular growth. [204]

After establishing that RPE respond maximally to concentrations of HGF exceeding 15 ng/ml, (the greatest receptor response occurring when cells are treated with 25 ng/ml HGF; findings are consistent with published reports[186]), we sought to reveal the minimum time required to elicit the greatest phosphorylation of c-Met. Multiple RTK's have been linked to phagocytosis, either directly or indirectly. As this process is time sensitive, particularly among RPE that maintain a diurnal rhythm, we believe that intracellular effector activation is regulated in part by the action of HGF and c-Met. Unlike the previous experiment, these results of this time trial will allow us to examine a) the speed of receptor activation and b) the time required for an extracellular signal to be relayed to intracellular targets.

Materials & Methods

ARPE-19 or RPE- J were cultured in Dulbecco's modified Eagle's media (D-MEM), supplemented with antibiotic/antimycotic, 2.5% sodium bicarbonate and 10% fetal bovine serum. Cells seeded at 5000 cells/cm^2 were maintained at 37°C and 5% CO_2. After reaching ~80% confluence, individual cultures were serum starved for 24 hr prior to HGF treatment. Cells were treated at concentrations of 0, 25 ng of HGF per ml in serum free media (SFM) for either 1, 20 or 60 minutes. These concentrations were shown to be effective in the previous study. Following growth factor treatment, cells were washed with PBS and either fixed in 2.5% paraformaldehyde (PFA) in Sorenson's phosphate buffer for 1 hr at room temperature or prepared for western blot analysis

employing established protocols for lysis of epithelial tissue and extraction of membrane bound proteins.

Confocal Laser Scanning Microscopy (CLSM)

Cells were prepared for fluorescent microscopic analysis via immunological detection using a rabbit anti c-Met or phosphorylated-c-Met as the primary antibody (1:250) and goat anti-rabbit IgG conjugated with Alexa Fluor 488 secondary antibody (1:500). For these and subsequent CLSM studies, non-specific binding was reduced by blocking cells in 4% NGS, 2% bovine serum albumin (BSA) and 0.05% Tween-20 in PBS. Cells were imaged using Leica SP2 AOBS confocal microscope.

Western Blot

Cells were harvested by manual dislodgement with use of a rubber policeman, lysed in ice cold RIPA lysis buffer (as described previously) for 15 minutes (total). Extracts were centrifuged at 12,000 RPM for 12 minutes at 4° C, and the resulting supernatant was either analyzed for total protein concentration or diluted with sample buffer as described above. Total proteins were separated by electrophoresis using a 4-20% Tris-Glycine pre-cast poly acrylamide gel as before. Proteins were electrophoresed at 80-120V for 3 hours in tank buffer containing 60 mM Tris-Cl and 2% SDS. Total proteins were then transferred to polyvinylidene fluoride (PVDF) membranes as before; efficiency of transfer was determined using Ponceau S staining (0.1% (w/v) Ponceau S in 5% acetic acid) followed by destaining in 1 M NaOH and distilled H_2O. Non-specific binding sites were blocked in TBS with 4% normal goat serum, 5% bovine serum albumin and 0.01%

Tween-20). Immunodetection employed a 1:250 dilution of rabbit anti phosphorylated-c-Met or rabbit anti c-Met. Protein loading was standardized using a 1:1,000 dilution of rabbit anti-tubulin or 1:10,000 dilution of mouse anti-F-actin. Incubation occurred overnight at 4°C with constant agitation. Secondary staining was performed using a 1:1,000 – 1:2,000 dilution of goat anti-mouse or goat anti rabbit IgG bound to horseradish peroxidase (HRP) for 2 hours at room temperature. Afterwards, membranes were washed with TBST before treatment with chemiluminescent substrate, followed by documentation with the Alpha Innotech FC2 Imager II at various exposure times.

Results

Our findings are consistent with well established hypotheses regarding RTK activation. The fluorescence of cells exposed to 25 ng/ml of HGF for 24 hours is slightly elevated when compared to controls or 50 ng/ml treated groups, though not significantly so when compared statistically (Figure 7). We expected a non-significant result because of receptor inactivation (by way of phosphatase activity or endosomal attenuation) but the increased c-Met expression observed may be due to prolonged availability of HGF.

Results from our temporal studies revealed that cells treated with HGF for 1, 10 or 20 minutes show maximum c-Met phosphorylation after 20 minutes of exposure, consistent with published reports of other receptors.

The data reported above represented the long-term exposure effects for HGF on receptor activation. Growth factors typically are short-lived in biological systems, given the circulation of extracellular fluids surrounding target tissue. To better understand the

temporal pattern of HGF-induced c-Met activation, we examined both cell lines for time of activation when exposed to 25 ng/ml of HGF. We found that in the ARPE-19 cell line, phospho-c-Met activation was significantly increased after 20 min. exposure to HGF ($p < 0.0005$) and that c-Met was still in the phosphorylated (active) state at 60 min. post-exposure (Figure 9). These data are in line with previously reported activation times of other receptor tyrosine kinases.

c-Met phosphorylation increases similarly in RPE-J cells after 20 minutes of exposure to 25 ng/ml HGF (Figure 9). The data indicated that both cell lines, human-derived ARPE-19 and rat-derived RPE-J, responded similarly to HGF treatment. The data also bought us to the next question: is 20 min the minimum time for activation, or did activation occur earlier? Using immunoblot analyses, we confirmed that at 10 min maximum c-Met phosphorylation had still not occurred (Figure 10). Again, one-way analysis of variance (ANOVA) tests were used compare fluorescent intensity values of treated cells. Post-hoc tests (Bonferroni and Tukey) were applied to identify statistical relevance between independent variable groups.

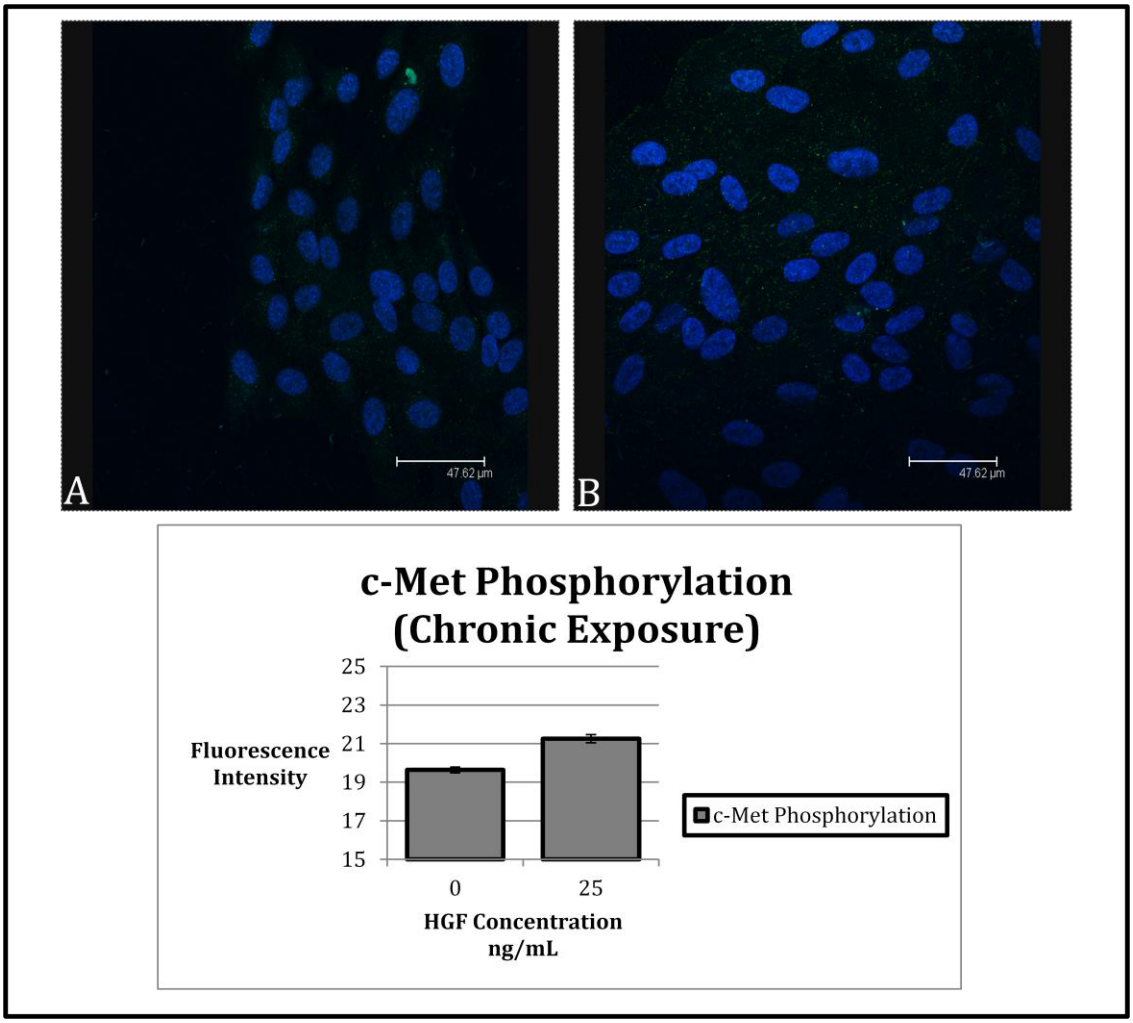

Figure 7. HGF induced c-Met Phosphorylation

c-Met phosphorylation by RPE increased with HGF treatment, although not significantly from controls ($p > 0.05$).

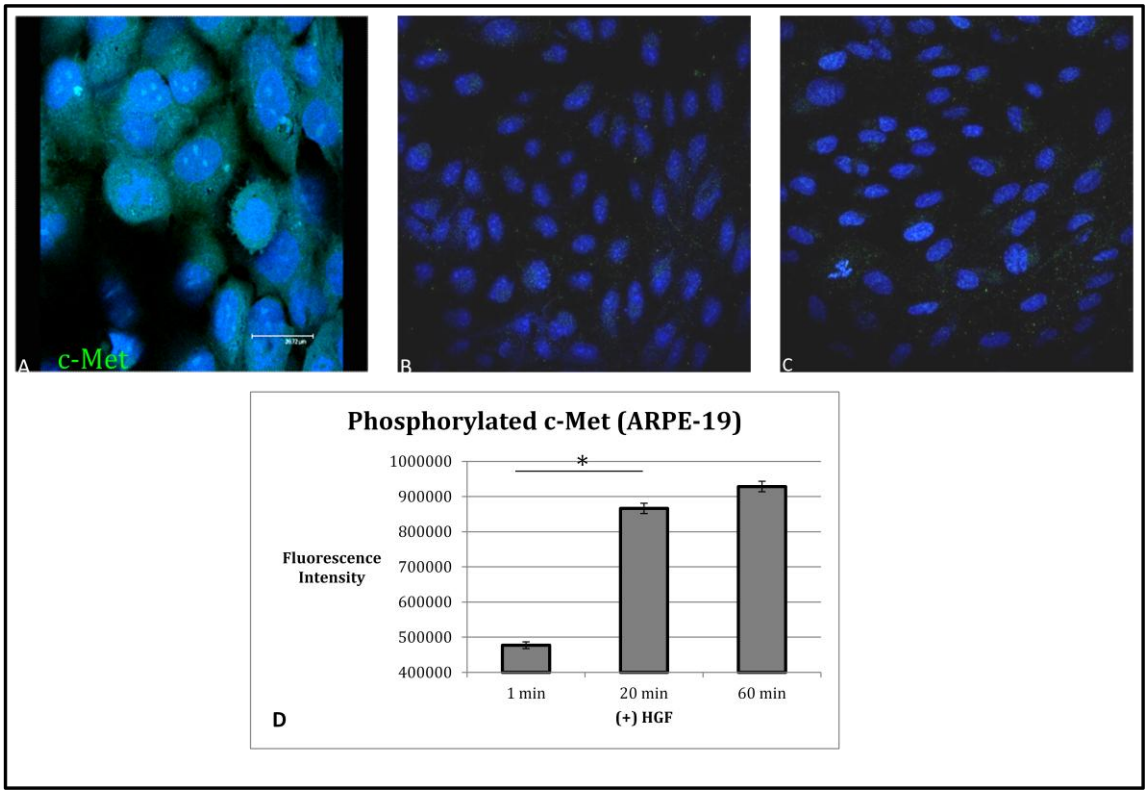

Figure 8. Temporal c-Met Phosphorylation Analysis (ARPE-19).

Cells were treated with 25 ng/ml HGF for A: 1 minute; B: 20 minutes; or C: 60 minutes. D: At 20 minutes, phosphorylation of c-Met was significantly increased compared to cells exposed to HGF for 1 minute ($p < 0.001$); phosphorylation remained significantly increased at 60 minutes ($p< 0.001$) Scale bars: A = 28 μm; B & C = 40 μm.

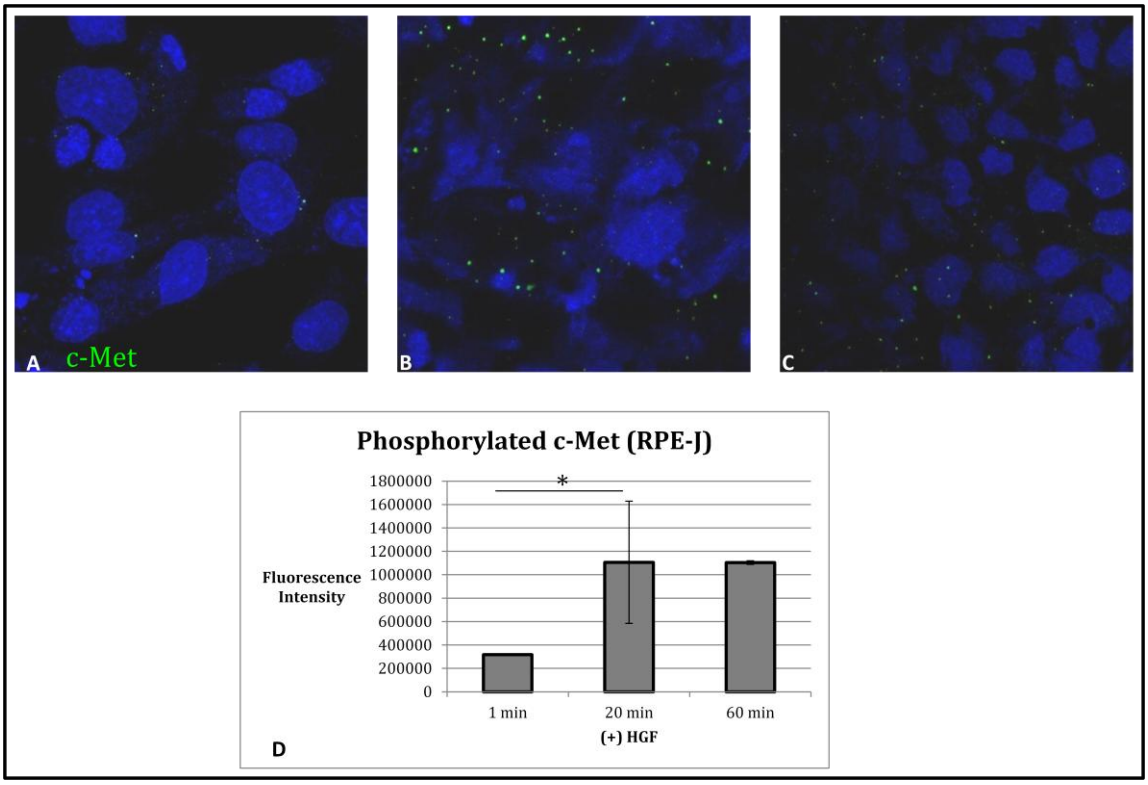

Figure 9. Temporal c-Met Phosphorylation (RPE-J).

Confocal micrographs of RPE-J after short-term exposures to 25 ng/ml HGF. 1, 20 and 60 min exposures were used to determine that c-Met phosphorylation was highest 20 minutes after treatment with HGF ($p < 0.001$) compared to 1 min exposure. Again, no significant changes in phosphorylation were observed at 60 minutes. Scale bars: A, B = 16 μm; C = 20 μm.

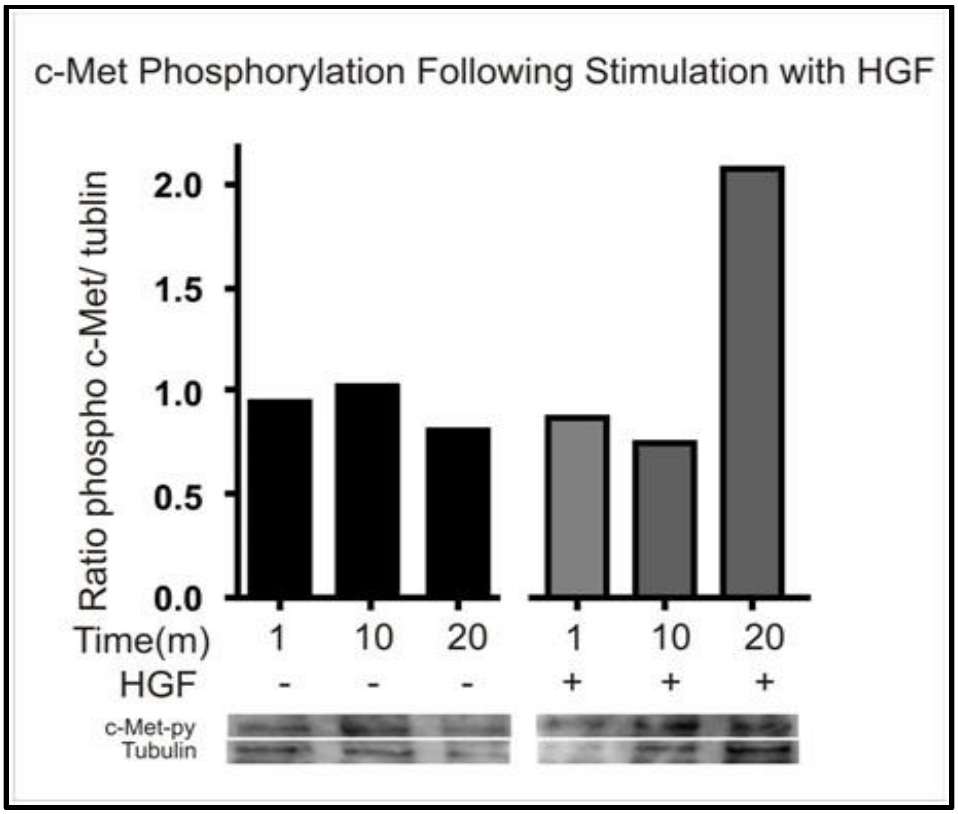

Figure 10. Western Blot analysis of temporal c-Met phosphorylation

Western blot measuring the concentration of phosphorylated c-Met normalized against tubulin. An increase was measured after 20 min, but not 10 min following treatment with 25 ng/ml HGF.

Discussion

With 58 receptor types and 32 non-receptors in the human genome, protein tyrosine kinases represent a diverse group of enzymes capable of mediating a cavalcade of cellular responses with enormous implications, particularly proliferation.[216] While the time required to induce a change in phosphorylation status varies slightly from one receptor to the next, it is generally accepted that RTK activation occurs within 1-30 min,[24] although recent evidence suggests that the temporal order of autophosphorylation among key tyrosine residues involved in various pathways are unique.[217]

In a previous group of experiments we determined that 25 ng/ml of exogenous HGF applied to RPE in culture was sufficient in stimulating a receptor up-regulation. Here we measured receptor phosphorylation in RPE that have withstood exposure to that ligand. We examined the temporal effect by determining the phosphorylation (activation) of c-Met in cells treated with our minimal effective dosage (25 ng/ml) of HGF for 0, 1, 10 or 20 minutes. We determined that RPE respond to HGF between 10 and 20 min post-exposure for maximal activation, and that by 24 hr post-exposure there was an increased, albeit statistically insignificant, activation of c-Met. We conclude that c-Met activation occurs at ~20 min following exposure to HGF, and subsequent to that activation there is an increased expression of the integrins $\alpha v\beta 5$. We then asked whether HGF also activated downstream effectors via c-Met phosphorylation.

CHAPTER IV: c-Met phosphorylation activates downstream targets PI3K, Grb2 & STAT3 in vitro

Introduction

Activation of c-Met subsequently activates a number of substrates via c-Met's kinase activities (Figure 11). One of these substrates is phosphoinositide 3 kinase (PI3K), which is associated with cell survival and cell scattering in the c-Met signaling cascades. In these two events, activation of Akt (protein kinase B) mediates a number of cellular processes that include mitogenesis, survival and differentiation.[218] In the brain, intrinsic pathways that maintain DNA integrity drive cell survival potentiated by Akt. Following Akt activation, there is an increase of transcriptional activity by cyclic AMP response element binding protein (CREB), a member of the Forkhead family of transcription factors.[219] Although there has yet to be described a direct role of Akt/CREB in phagocytosis, it is known that in macrophages, activated Akt is up-regulated in response to an extracellular ligand.[220-224] HGF up-regulates both PI3K and Akt during wound healing in RPE.[176] It is unknown neither if there is an up-regulation of Forkhead transcription factor during wound healing, nor if CREB is involved in RPE phagocytosis of OS. There is evidence that Akt is part of a signaling pathway that begins with c-Mer and is partially responsible for cell survival. This evidence comes from a study of RCS rats in which c-Mer is mutated and OS phagocytosis is diminished via decreased mTOR expression.[225]

Phosphoinositide 3 kinase (PI3K) has been linked to phagocytosis since the discovery that inhibition of this family of cellular regulation enzymes results in strong reduction of

large target internalization by macrophages.[164, 213, 214, 226] Activation of various RTKs by their specific extracellular ligands can lead to transduction of PI3K (which subsequently phosphorylates phosphatidylinositol 4,5 diphosphate (PtdIns-4,5-P_2) to PtdIns-3,4,5-P_3 [PIP_3]).[227] It is relatively unknown whether these processes influences OS phagocytosis by RPE in a fashion similar to their professional phagocyte counterparts where inhibition of PI3K prevents phagosome maturation, but not target binding.[226] Pharmacological pretreatment of RPE with PI3K inhibitors successfully inhibits OS ingestion in a dose-dependent manner, although cells recover their ability to phagocytize OS after a period of recovery,[227] suggesting that PI3K is equally important in OS processing as in apoptotic cell removal or immunological response antigens.

PIP_3, a product of PI3K phosphorylation, is responsible for the recruitment of signaling proteins with pleckstrin homology, including protein serine-threonine kinases and phosphoinositide-dependent kinase 1.[228] It is believed that PIP_3 stimulates actin polymerization through recruitment of Rho GTPases Rac and Cdc42, each known to induce nucleation of actin – an important precursor to polymerization.[229] Cdc42 induces polymerization of actin through Wiskott-Aldrich Syndrome protein (WASP), whose relatives then activate the Arp 2/3 complex.

Since cytoskeletal reorganization is fundamental to the internalization of any target regardless of cell type or species, any pathway that results in actin polymerization may influence phagocytic dynamics. It is well known that a litany of signals can converge to reorganize the actin cytoskeleton.[230] Of particular influence are PI3K and Rac1, as both of these effector molecules have been implicated as

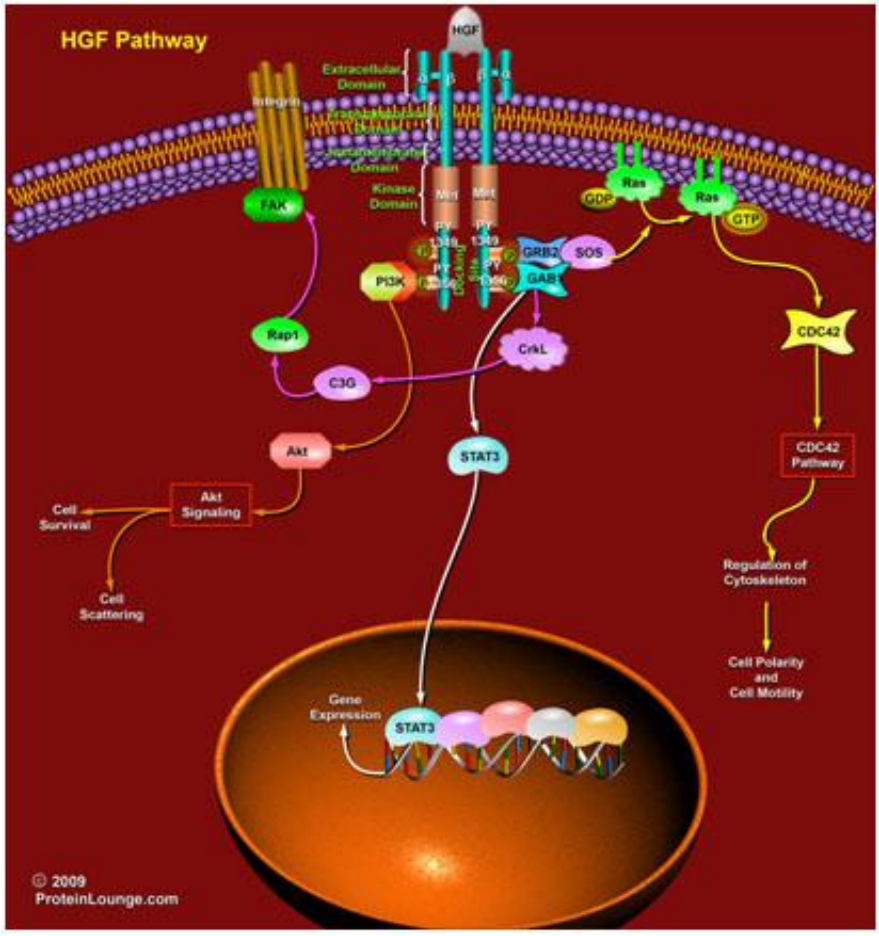

Figure 11. Relevant signaling pathways for c-Met following HGF binding.

We show here that the c-Met substrates PI3K and Grb2 are up-regulated following c-Met activation. Subsequently, the transcription factors CREB and STAT3 are also up-regulated.

mediators of phagocytosis by function of their interaction with actin. While HGF signaling is oft associated with the changes in cellular motility and proliferation, it is unknown how HGF influences phagocytosis (binding or internalization) when PI3K and Rac1 are phosphorylated downstream of c-Met in RPE. We sought to determine the extent of PI3K phosphorylation as a result of c-Met activation in order to a) identify whether our working dosage was sufficient to activate specific downstream targets

(above baseline) and b) determine if PI3K phosphorylation is correlative with the concentration of exogenous HGF and the length of exposure to said ligand.

Materials & Methods

ARPE-19 or RPE- J were cultured in Dulbecco's modified Eagle's media (D-MEM), supplemented with antibiotic/antimycotic, 2.5% sodium bicarbonate and 10% fetal bovine serum. Cells were seeded at 5,000 cells/cm^2 and were maintained at 37°C and 5% CO_2. After reaching ~80% confluence, individual cultures were serum starved for 24 hr prior to HGF treatment. For these studies, we used either 0 or 25 ng/ml HGF in serum free media (SFM) for 20 minutes. The HGF concentration and time of incubations were previously found to be efficacious in eliciting activation of the c-Met receptor. Following growth factor treatment, cells were washed with PBS and either fixed in 2.5% paraformaldehyde (PFA) in PBS for 1 hr at room temperature or prepared for western blot analysis employing established protocols (previous chapters).

Confocal Laser Scanning Microscopy (CLSM)

Cells were prepared for confocal microscopy as described above. For these studies, we employed three different primary antibodies, along with the appropriate conjugated secondary antibodies. The primary antibodies included: rabbit anti phosphorylated-PI3K, rabbit anti-Grb2 or mouse anti-STAT3 (each at 1:100). For secondary antibodies we employed either a goat anti-rabbit IgG conjugated with Alexa Fluor 633 to detect PI3K, goat anti-rabbit IgG conjugated with Alexa Fluor 488 to detect Grb2, and goat anti-mouse IgG conjugated with Alexa Fluor 633 to detect STAT3 (each at 1:500). As with

our previous CLSM studies, non-specific binding was reduced by blocking in 5% nonfat dry milk, 0.05% Tween-20 and 4% normal goat serum in PBS. Antibodies were diluted in an antibody dilution buffer consisting of 2.5% nonfat dry milk and 2% NGS in PBS. Cells were imaged using Leica SP2 AOBS confocal microscope.

Western Blot

Cells were harvested by manual dislodgement with use of a rubber policeman, lysed in ice-cold RIPA lysis buffer (as described previously) for 15 minutes (total). Extracts were centrifuged at 16,000 x *g* for 12 minutes at 4° C, and the supernatant was either analyzed for total protein concentration or diluted with sample buffer as described above. Total protein were separated by electrophoresis using a 4-20% Tris-Glycine pre-cast poly acrylamide gel as before. Proteins were electrophoresed at 80-120V for 3 hours in tank buffer containing 60 mM Tris-Cl and 2% SDS. Total proteins were then transferred to polyvinylidene fluoride (PVDF) membranes as before; efficiency of transfer was determined using Ponceau S staining (0.1% (w/v) Ponceau S in 5% acetic acid) followed by destaining in 1M NaOH and distilled H_2O. Non-specific binding sites were blocked in TBS with 4% normal goat serum, 5% bovine serum albumin and 0.01% Tween-20). Immunodetection employed a 1:250 dilution of rabbit anti-STAT3 or rabbit anti-CREB. Protein loading was standardized using a 1:1,000 dilution of rabbit anti-tubulin or 1:10,000 dilution of mouse anti-F-actin. Incubation occurred overnight at 4°C with constant agitation. Secondary staining was performed using a 1:1,000 – 1:2,000 dilution of goat anti-mouse or goat anti-rabbit IgG bound to horseradish peroxidase (HRP) for 2 hours at room temperature. Afterwards, membranes were washed with TBST before

treatment with chemiluminescent substrate, followed by documentation with the Alpha Innotech FC2 Imager II at various exposure times.

Results

We found that the amount of phosphorylated PI3K significantly increased ($p < 0.05$) within 20 min treatment with 25 ng/ml HGF (Figure 12), similar to the increased in c-Met phosphorylation previously observed (Figure 7). There was no additional increase in PI3K activation when cells were treated with concentrations of HGF exceeding 25 ng/ml (data not shown). Under conditions where HGF concentration exceeds 25 ng/ml of HGF, there was no significant increase in the amount of bioparticles bound to the RPE. This suggested that either the activation of PI3K and c-Met preceded the initiation of binding or that binding to the bioparticles required the activation of cell surface proteins, potentially those involved during FcγR mediated phagocytosis.

Activation of c-Met via autophosphorylation may subsequently activate the substrate PI3K, important for cell survival and cell scattering. Other substrates for c-Met include Grb2 (required for activation of Ras-dependent regulation of the cytoskeleton) and STAT3 (for nuclear activation of transcription). We tested here also the ability of HGF to activate or influence expression of two additional c-Met substrate proteins. We found a bimodal effect of c-Met activation with regards to these two proteins. First, at 25 ng/ml, both Grb2 and STAT3 immunofluoresence levels were significantly increased compared to controls (Figure 13; $p < 0.00001$ for Grb2; $p < 0.05$ for STAT3). Second, when treated for 20 min with 50 ng/ml, there was a significant reduction in both Grb2 and STAT3 when compared to 25 ng/ml HGF (not shown). At the higher dose, STAT3

levels were still significantly higher than controls (Figure 13 C, D). Expression of both STAT3 and Grb2 decrease at concentrations exceeding 25 ng/ml (though STAT3 not significantly so). This reduction may indicate a threshold at which RPE prevent expression or activation of molecules involved in proliferative behavior. While speculative, we hypothesize that this preventative measure would reveal a way by which RPE can avoid undirected or misguided growth if a single proto-oncogene were to become mutated. With the increased activation of PI3K and Grb2, we then sought to determine if transcription factor levels were also increased. In particular, we sought to examine the potential up-regulation of CREB (via PI3K activation) and confirm the up-regulation STAT3 (via Grb2 activation) using a western blot assay. In these assays, we confirmed that STAT3 levels were increased following treatment with HGF 25 ng/ml concentrations (Figure 14). For the first time, we also have demonstrated a significant increase in CREB expression in RPE stimulated with 25 ng/ml HGF (Figure 14). Unlike STAT3, however, 50 ng/ml HGF had no effect in increasing CREB levels in the RPE.

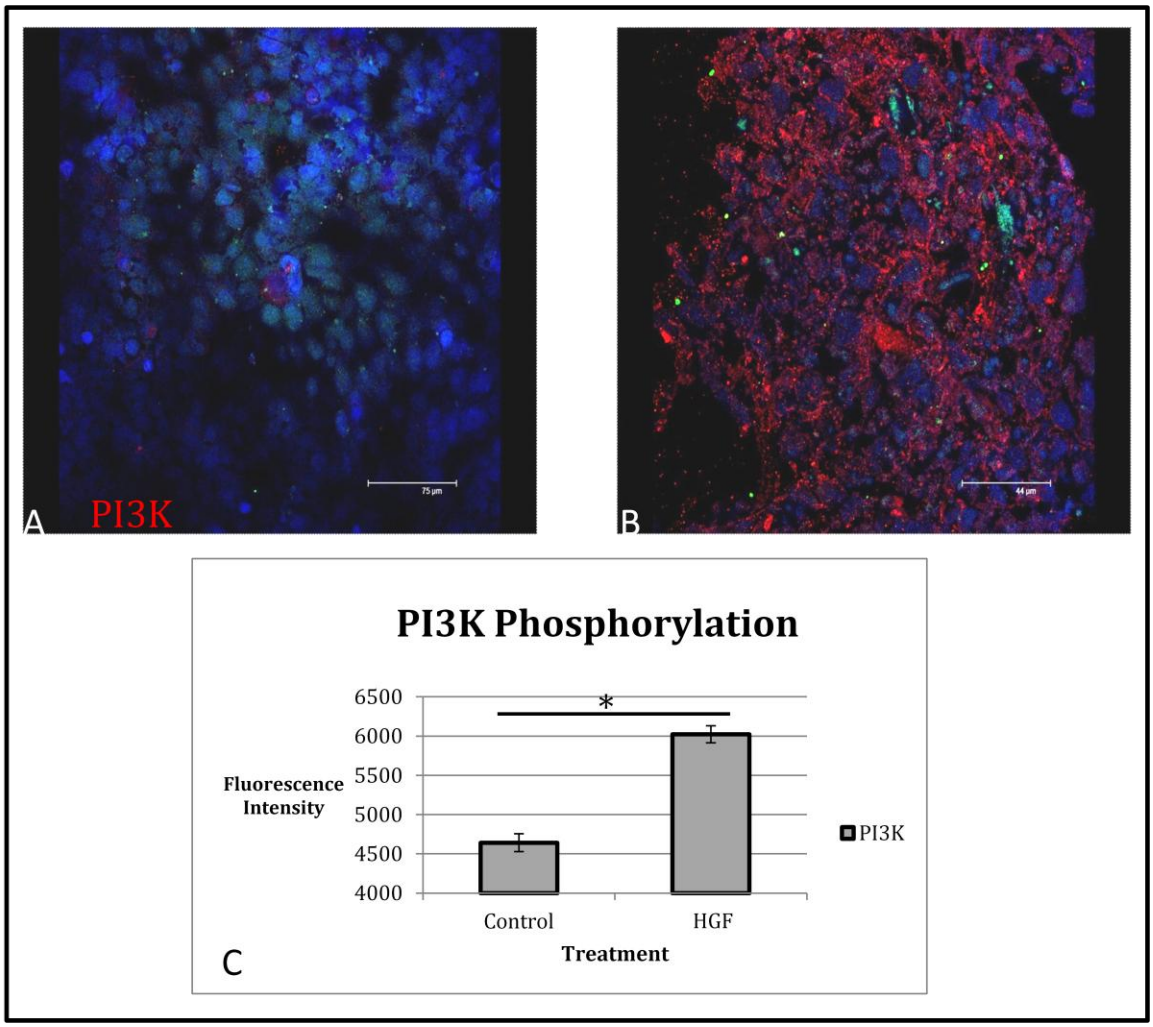

Figure 12. Phosphorylation of PI3K in RPE following exposure to HGF.

Panel A (control, 0 ng/ml HGF): Confocal image of RPE expressing phospho-PI3K (red). Panel B: RPE were treated with 25 ng/ml HGF for 20 minutes. This concentration and time is sufficient to induce maximal activation of c-Met. C: Phospho-PI3K was significantly increased over controls ($p < 0.05$)

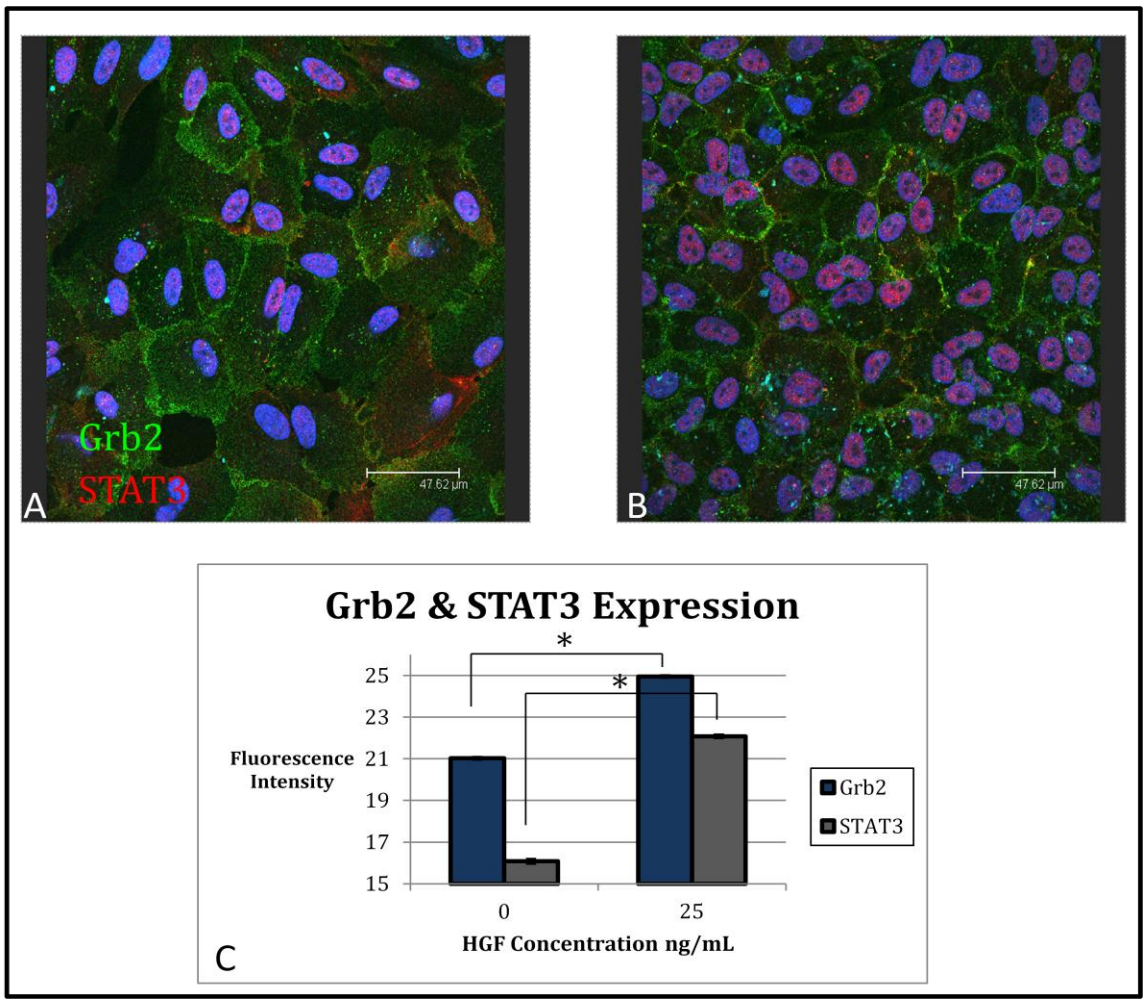

Figure 13. Grb2 & STAT3 expression

A-C: RPE showed a significant up-regulation of transcription factor STAT3 and the adapter protein Grb2 at HGF concentrations of 25 ng/ml ($p<0.001$). Grb2 expression decreased to below control levels when exposed to 50 ng/ml HGF. STAT3 levels decreased at concentrations of 50 ng/ml HGF, but remained significantly higher than controls ($p<0.05$) (not shown).

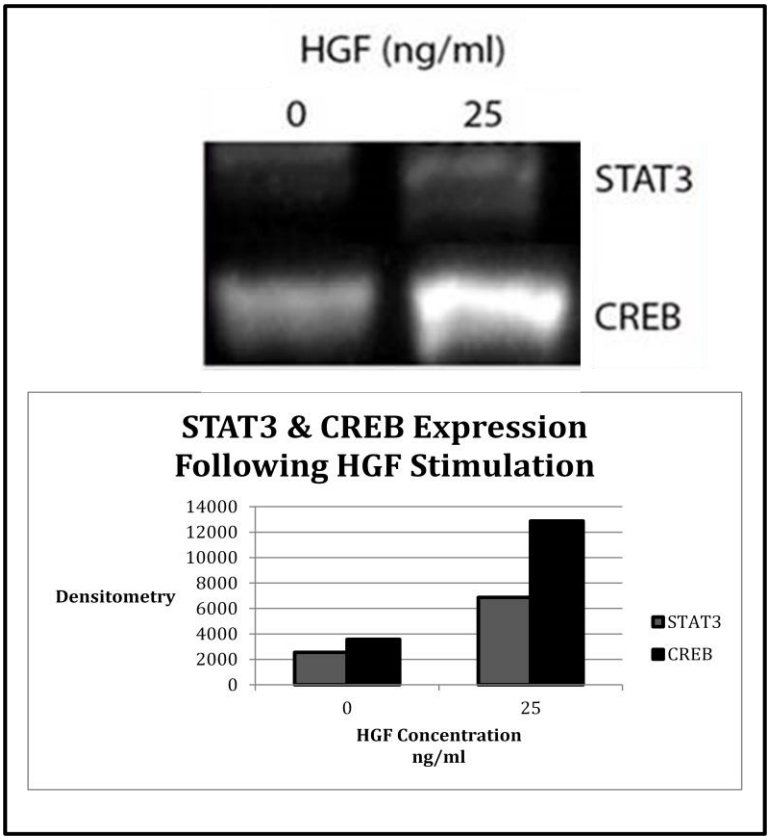

Figure 14. Western Blot analysis of CREB & STAT3

Western blots show an increase of both STAT3 and CREB after RPE are exposed to HGF at concentrations exceeding 25 ng/ml.

Discussion

A plethora of cellular processes begin with, or include the activation of phosphoinositide-3 kinases.[231] These enzymes can be phosphorylated on multiple positions of the inositol head group, which produces seven different species found *in vivo*,[231] each influencing a different aspect of cellular activity. With such versatility, it comes as no surprise that activation of PI3K is involved in phagocytosis. It has been shown that phagosome formation and maturation is regulated in part by activation of class I and III phosphoinositide 3 kinases[226], where the production of PIP_3 from activation of class IA PI3Ks is key among neutrophils, monocytic cells and macrophages [167, 232]. In these cells, phagocytosis is significantly impaired when PI3K is inhibited either chemically or genetically.[165, 233-235] In RPE, phagocytosis of OS is mediated by the PI3K linked tyrosine kinase receptor Mer when stimulated by GAS6[227], and believed to play a role in the activation of Rac1.

Activation of PI3K and ERK1/2 by c-Met are important precursors to invasion and metastasis by way of cytoskeletal reorganization,[236] but there is no data that suggests the c-Met/PI3K tandem influences phagocytosis by RPE. Grb2, which is also recruited by c-Met activation, may also influence phagocytosis in RPE, as it interacts with son of sevenless (SOS) through its Src homology 3 (SH3) domain, promoting the activation of Ras, which is required for the phagocytosis of *E. coli* by insect hemocytes.[237, 238]

CHAPTER V: HGF supplementation results in augmented binding and internalization of non-specific fluorescent targets

Introduction

The intimate association between RPE degeneration and AMD onset has prompted belief among researchers that a cure for this retinopathy may involve cell-based transplantation therapies.[239] AMD is the pathological end to a series of interactions including lipofuscunogenesis, drusenogenesis and inflammation; [240] RPE can contribute to AMD by way of inflammatory response or in the balancing of specific growth factors and their precursors that facilitate neo-vascularization-equally likely is the theory that death of RPE death initiates the cascade of events that result in AMD manifestation.[241] The thought that replacement of compromised RPE with healthy cells may provide full visual recovery[241] has yielded two methods which are currently employed for clinical study: the RPE-suspension technique, where limited amounts of cells on a partially defective basal lamina can be easily delivered through a sub-retinal retinotomies and the RPE-choroid-sheet technique, where the translocation of a full thickness RPE-choroidal sheet provides a polarized RPE and its native basal lamina.[242] Unfortunately, clinical setbacks exist because homologous transplants require long-term combined immunosuppressive therapies that are not ideal for elderly patients. Additionally, basal lamina changes, cell survival post-operation and refinement of surgical technique are variables that alter the outcome of RPE transplant anatomy and function.[242] Long-term observations of vision recovery within RPE transplant recipients does not begin to approach the success of

intravitreal treatments, including the use of anti-VEGF drugs bevacizumab, pegaptanib and ranibizumab.[242]

The use of anti-VEGF therapies for treatment of exudative AMD is unsurprising as its role in angiogenesis and neo-vascularization has long been associated with diseases of the retina.[243] Typically, antibodies against VEGF or as an antagonist to the VEGF receptor have been employed to prevent neo-vascularization, though both side effects and long-term success is unknown.[243] Intravitreal application allows clinicians to maintain a low dosage to maximum effect ratio when attempting to limit choroidal neo-vascularization, though alteration of the blood-retina barrier can facilitate leakage of the drug into systemic circulation.[244] While pharmacological intervention appears to be the most effective, least invasive partial solution to AMD, current treatments addressing VEGF at the cellular surface do not account for downstream targets that facilitate neo-vascularization. It is possible that these effectors are activated by a compensatory mechanism that prevents complete cessation of blood vessel formation.

The aforementioned methods attempt to retard or reverse the damage associated with AMD through a) revival of RPE performance or b) intravitreal application of exogenous drugs. We hypothesize that a potential remedy for AMD may arise from a combinatory approach; intravitreal application of an HGF in attempt to revive RPE ability as a phagocyte.

In the past HGF has been linked to both protection and photoreceptor insult. In the case of the former, glutathione deficiency among RPE leads to apoptosis, a process partially blocked by HGF through up-regulation of cellular redox status and inhibition of caspase-

3 dependent cell death.[245] In the latter, over-expression of HGF in RPE induces profound morphologic changes in the retina choroid consistent with retinal detachment. The dual nature of HGF within RPE suggests that expression levels of HGF and activation of its receptor c-Met can alter RPE behavior, and while sub-threshold concentrations of HGF will not elicit the predicted scatter response, cells may respond by modulation of downstream targets useful for other metabolic and homeostatic tasks.[246] In the previous chapter, we concluded that c-Met activation increases the downstream effects of the PI3K-Akt-CREB and Grb2-STAT3 signaling pathways. An additional pathway that is critical for RPE function in OS processing is the activation of focal adhesion kinase (FAK). In this pathway, c-Met activation of Grb2 activates GAB1 and ultimately FAK. As discussed in the opening chapter, FAK is co-localized with integrins (Figure 11) and is partially responsible for cytoskeletal reorganization and promotes phagocytosis of OS.[71] In order to assess the influence of HGF during phagocytosis by RPE, we employed fluorescently labeled latex beads and fluorescently labeled *E. coli* to serve as extracellular targets.

Materials & Methods

ARPE-19 or RPE- J were cultured in Dulbecco's modified Eagle's media (D-MEM), supplemented with antibiotic/antimycotic, 2.5% sodium bicarbonate and 10% fetal bovine serum. Cells were seeded at 5000 cells/cm^2 and were maintained at 37°C and 5% CO_2. After reaching ~80 confluence, individual cultures were serum starved for 24 hr prior to HGF treatment, at initial concentrations of 0, 25, or 50 ng/ml suspended in serum free media (SFM) for either 20 minutes or 24 hrs. Following growth factor treatment, cells were washed with PBS and challenged with 1:1000 latex beads or 1:1000

fluorescent *E.coli* suspended in serum free media. Extracellular challenge persisted for several hours before repeated washes in PBS followed by fixation in either 2.5% paraformaldehyde (PFA) and .05 % gluteraldehyde for 1 hr at room temperature (immunocytochemical assay) or 4% paraformaldehyde and 2.5% gluteraldehyde for 1hr at room temperature (electron microscopy).

Scanning Electron Microscopy

Samples mounted on cover slips were dehydrated in an ascending ethanol series before critical point drying. Samples were then sputter coated in gold-palladium before being imaged with an AMRAY scanning electron microscope.

Confocal Laser Scanning Microscopy (CLSM)

Cells were prepared for fluorescent microscopic analysis via established techniques. Since both latex beads and *E.coli* targets featured fluorescent labeling, there was no need to rely on immunological detection. Cells were however treated as before in an effort to identify nuclei (Dapi with *Slowfade*) and c-Met, phosphorylated c-Met or downstream effectors of c-Met.

Imaris

Imaris software was used to convert three dimensional confocal stacks into fully textured renders. Artificial spheres were added to the final render where latex sphere fluorescence intensity was highest. These artificial volumes were used to a) estimate sphere binding and b) visualize internalization.

Results

Our results show an increase in the number of bound (possibly ingested particles) among both latex bead and *E.coli* challenged cells treated with exogenous HGF for 20 min above

controls. In all images, there appears to be a greater number of fluorescent items attached to cells that have been treated with increased levels of HGF. If binding is increasing, we must conclude that RPE are responding to HGF treatment by increasing the number (perhaps activation) of membranous proteins responsible for recognition of extracellular targets. Aforementioned studies describe an increase in total αvβ5- an integrin known to recognize POS. While it is a possibility that αvβ5 is also involved in recognition of non-specific targets, HGF may also impact membranous proteins involved in traditional phagocytosis.

Three dimensional analysis of latex bead challenged cells provided insight to the extent of internalization; figures 16 and 17 show that A) RPE are capable of internalizing the aforementioned nonspecific targets, suggested by the position of spheres alongside Dapi stained nuclei and B) RPE treated with HGF appears to contain more internalized particles.

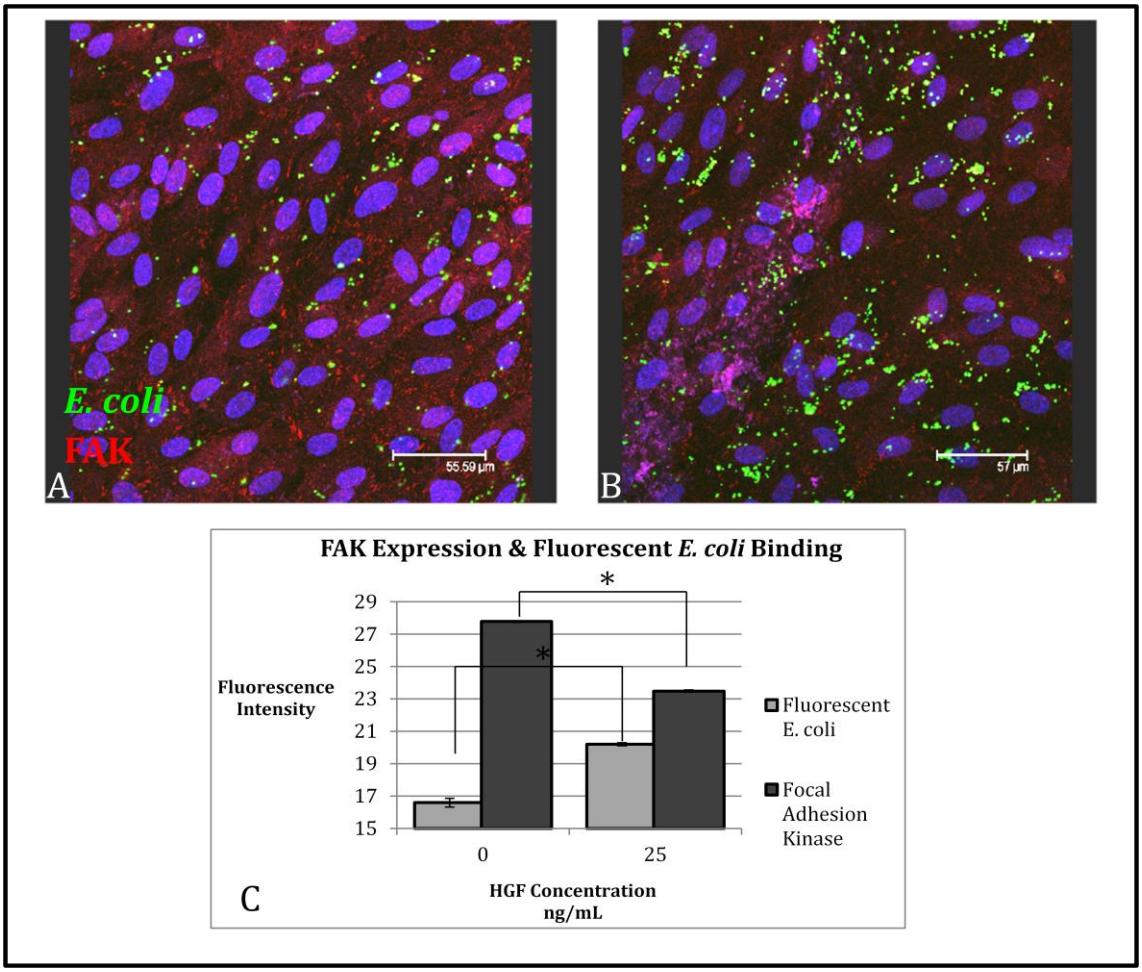

Figure 15. Expression of focal adhesion kinase and binding of non-specific targets

Panels A and B show a significant increase in fluorescent *E.coli* (Green) binding when HGF concentration is increased. Interestingly, FAK (Red), a critical molecular switch is significantly decreased as HGF is increased. (T-Test, $p<0.05$)

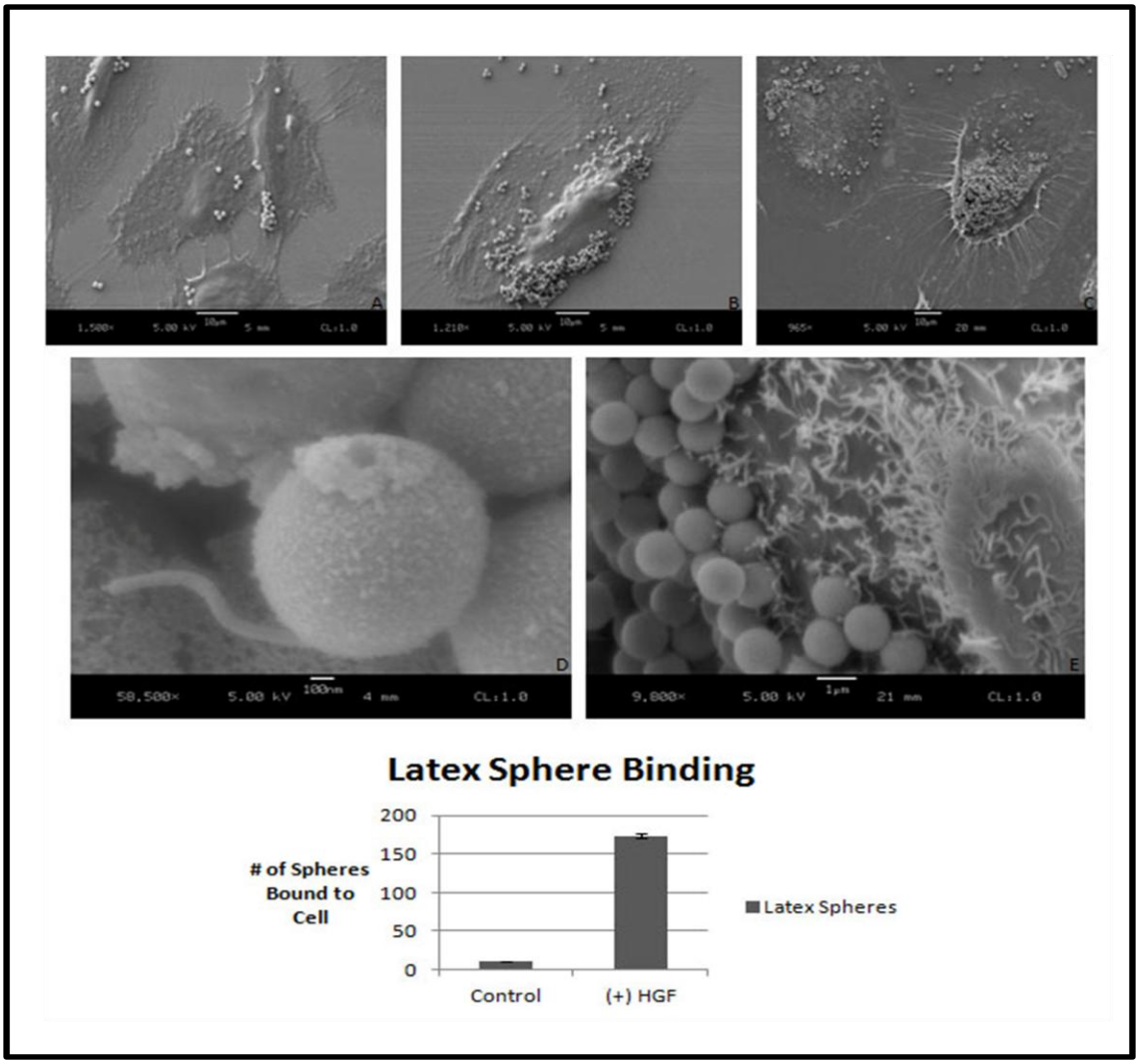

Figure 16. Electron micrographs depicting non-specific target binding and internalization

Scanning electron micrographs of RPE bound latex spheres. Cells were treated with 0, 25 or 50 ng/ml HGF prior to non-specific target challenge. Panels B & C show an increase in bound latex spheres compared to panel A (control). Images D & E show increased expression of apical microvilli and intimate associate with latex beads.

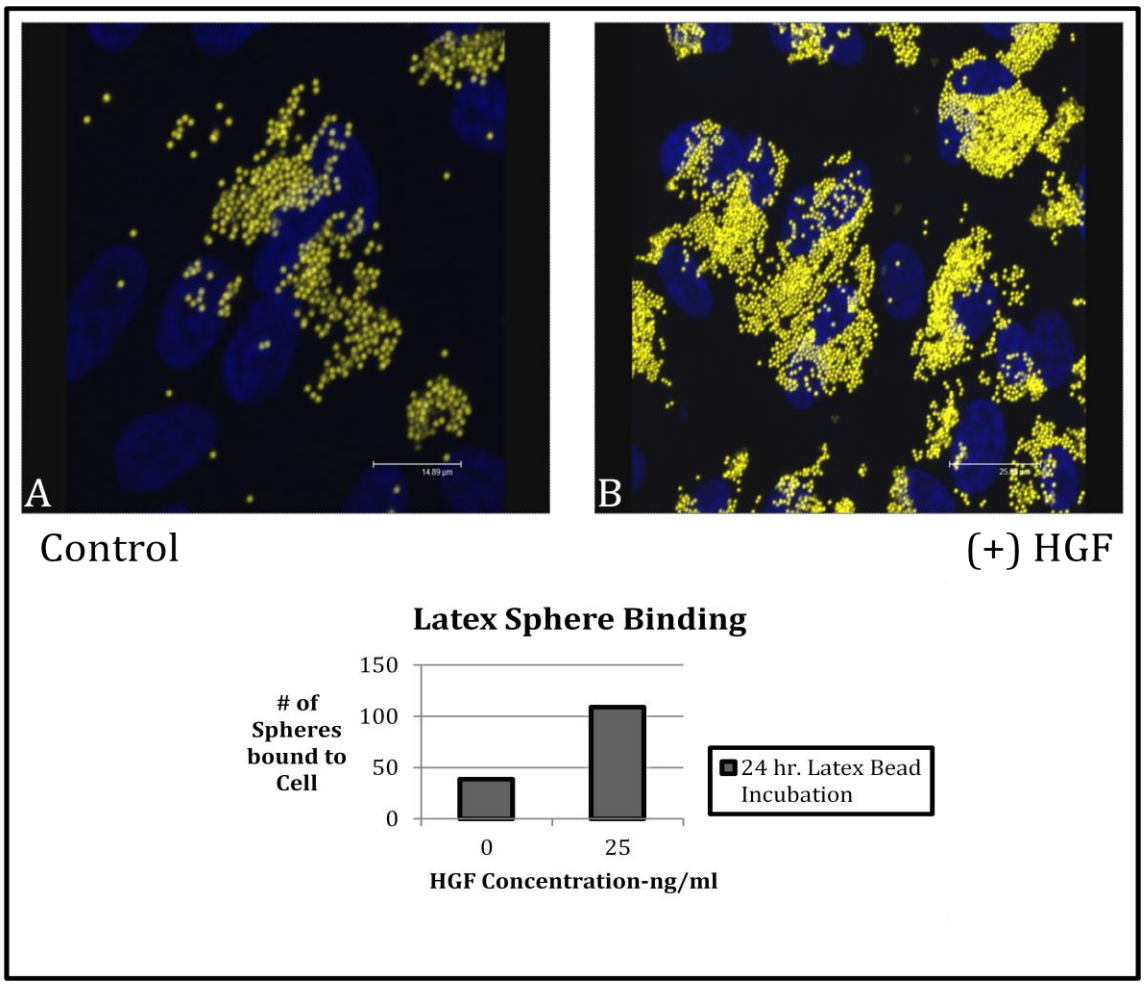

Figure 17. Three-dimensional stack image of RPE challenged with fluorescent latex spheres.

Fluorescent maximum projection capturing the phagocytosis of latex beads by RPE. Panels A & B represent control and treated cells respectively.

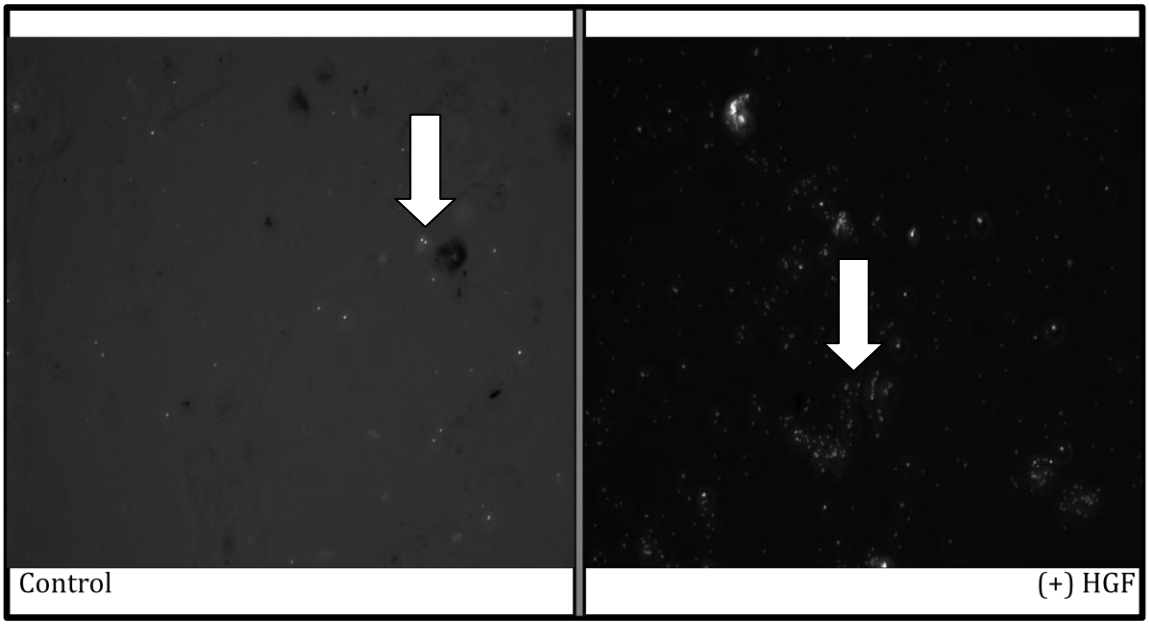

Figure 18. **Live cell imaging stills depicting binding of non-specific targets**

Snapshots taken from live video capture of RPE phagocytosis of latex beads. Arrows indicate fluorescent beads. Images were captured every 30 minutes over a period of 10 hours.

Discussion

Fluorescent particles have been used for decades to characterize binding and ingestion by RPE in controlled environments. While Philip and Bernstein provided suggestive evidence that the specific phagocytosis of ROS and nonspecific phagocytosis of latex beads by RPE cells involve two different mechanisms,[247, 248] recent evidence has shown that both specific and nonspecific phagocytosis by RPE share pathways when undergoing cytoskeletal reorganization needed for internalization of extracellular targets.[1, 2] The pathways that govern either specific or nonspecific phagocytosis have not been completely elucidated in any cellular system to date; however, many of the messenger molecules involved in said cascades have been fully characterized, and associated with other cellular behaviors. In RPE, activation of VEGFR has been linked to rapid angiogenesis[249]. c-Met activation has been linked to cell proliferation, migration and metastasis, while PI3K, FAK and Grb2 have been shown to influence phagocytosis.

Our current line of experiments sought to determine if there are any significant changes among binding and internalization of external targets when cells have been exposed to elevated concentrations of HGF, and when phosphorylation of both c-Met and downstream targets are highest. This study attempts to evaluate binding of non-specific targets, facilitated by challenging RPE with latex beads and fluorescent *E.coli*.

CHAPTER VI: Inhibition of PI3K phosphorylation results in decreased binding and internalization of non-specific fluorescent targets

Introduction

Actin polymerization of the developing phagosome is thought to be under the control of Rho family GTPases, with specific stimulation of Rac1 and Cdc42 occurring downstream of $F_c\gamma$ receptor phosphorylation.[250] Subsequent activation of PI3K, local membrane accumulation of PIP_3 and actin assembly then allows for proper extension of phagocytic cups and synching of contractile mechanisms that facilitate closure of phagosomes and subsequent trafficking to intracellular organelles.[251, 252] Of particular importance here are the myosin family of motor proteins that play various roles during each stage of phagocytosis in addition to other membrane reorganizing programs including cytokinesis, migration and vesicle trafficking.[87] PI3K inhibition of myosin II maintains cytoskeletal organization in a state that promotes protrusive activity during axonal growth.[253]

In RPE, MerTK driven myosin II redistribution to the site of OS engulfment represents an interface facilitating cellular recognition of an external target and a mechanism for clearance.[87] Since PI3K is involved in redistribution as well as ligation of cell surface receptors and surface ruffling,[164] it is likely that inhibition will result in incomplete internalization of the target. Studies have shown that pharmacological blockage of PI3K results in diminished phagocytosis of non specific debris. Similarly the use of small-interfering RNA targeting the p85α and p110α catalytic subunits of PI3K has been used to characterize cellular responses to a loss of this ubiquitous protein kinase family.[254] Clinically, inhibition of PI3K results in abnormal regenerative response of the liver after

resection, likely induced by inhibition of macrophage infiltration and cytokine elaboration [254]; an interesting finding considering the necessity for HGF/c-Met signaling required for efficient liver regeneration and repair.[3]

We hypothesize that chemical or translational inhibition of PI3K is sufficient to reduce RPE phagocytosis of non-specific debris *in vitro*. While other studies have evaluated the role of PI3K during RPE particle binding and engulfment, none have considered c-Met as the initiator of this cascade. Additionally, if PI3K signaling is perturbed and there is no detectable change in phagocytosis, we can begin to explore the theory that HGF signaling allows for compensatory action by RPE *in vivo* during the failure of certain messenger systems.

Materials & Methods

In order to evaluate the role of PI3K during phagocytosis by RPE, ARPE-19 or RPE- J were cultured in Dulbecco's modified Eagle's media (D-MEM), supplemented with antibiotic/antimycotic, 2.5% sodium bicarbonate and 10% fetal bovine serum. Cells were seeded at 5000 cells/cm^2 and were maintained at 37°C and 5% CO_2. Upon reaching ~ 80 confluence, individual cultures were serum starved for 24 hr prior to HGF treatment. Post starvation, cells were treated with 0, 25 ng/ml (HGF exposure never exceeded 20 minutes). Following growth factor treatment, cells were washed with PBS and subsets were treated with the chemical inhibitor of PI3K Wortmannin at 10μM for 1 hour. Upon copious washing, cells were challenged with 1:1000 fluorescent *E.coli* to serum free media. Extracellular challenge persisted for several hours before repeated washes in PBS followed by fixation in either 2.5% paraformaldehyde (PFA) and .05 % gluteraldehyde

for 1 hr at room temperature (immunocytochemical assay) or 4% paraformaldehyde and 2.5% gluteraldehyde for 1hr at room temperature (electron microscopy).

Confocal Laser Scanning Microscopy

Established immunocytochemical techniques were used to prepare cells for confocal analysis. Our primary antibody was a polyclonal rabbit anti phosphorylated-PI3K (1:100), while our secondary antibody was a goat anti-rabbit IgG conjugated with Alexa Fluor 488(1:500). For these and subsequent CLSM studies, non-specific binding was reduced by blocking in 4% NGS, 2% BSA and 0.05% Tween-20 in PBS. Antibodies were diluted in an antibody dilution buffer consisting of 2.5% nonfat dry milk and 2% NGS in PBS. Cells were imaged using Leica SP2 CLSM and fluorescence intensities were compared via statistical analysis (T-Test).

Results

Our data suggest that PI3K plays a key role in phagocytosis, as inhibition of PI3K phosphorylation appears to reduce binding of external targets, thus eliminating internalization. This effect occurs despite the presence of elevated exogenous HGF, compared non-antagonized cells. We conclude that the aforementioned impairment of phagocytosis is a direct result of an inability of PI3K to phosphorylated Rac1, a key mediator of phagosome formation and inability to activate the MAPK pathways.

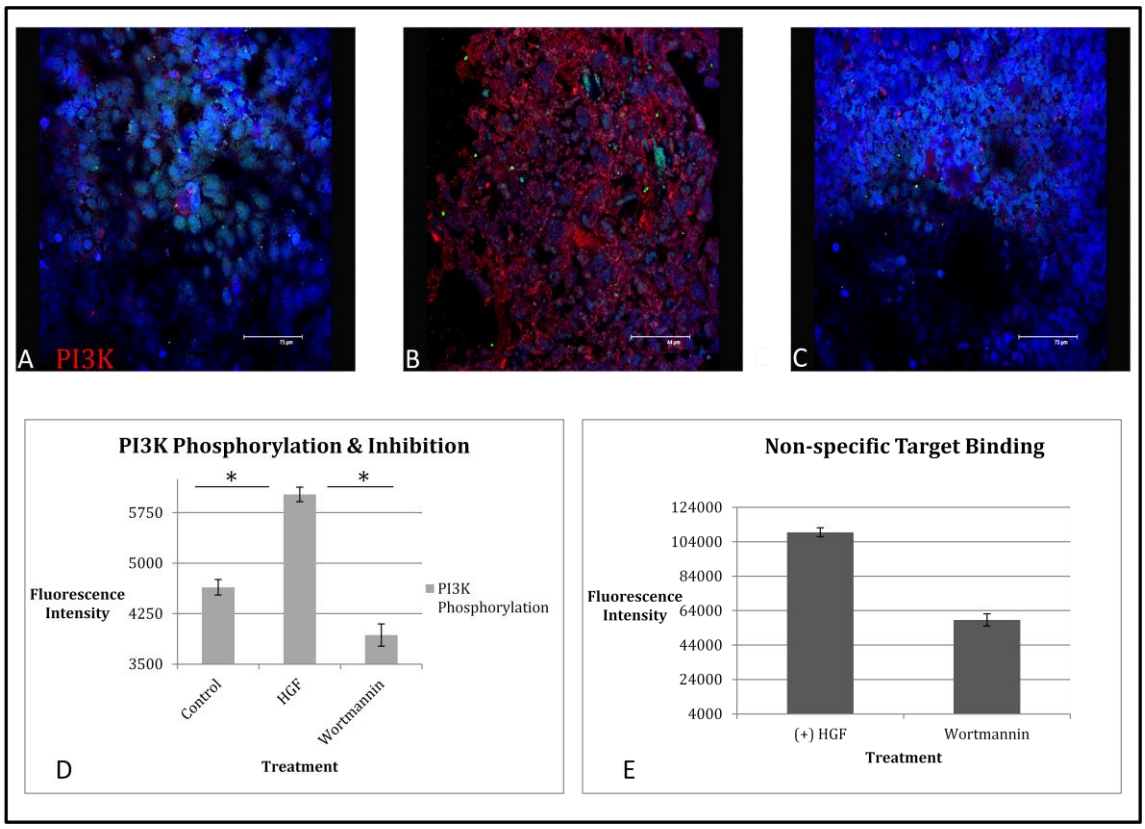

Figure 19. Analysis of labeled non-specific target binding pre- and post PI3K inhibition

Confocal micrographs of RPE with or without HGF exposure, and with or without Wortmannin all while challenged with fluorescent *E. coli*. Panel A (controls) show little target binding or activation of PI3K. Panel B shows an increase in both *E. coli* binding and PI3K phosphorylation. Panel C shows that PI3K activation has been muted and binding of *E. coli* has decreased.

Discussion

RPE respond to prolonged exposure exogenous HGF by up-regulating the c-Met receptor, select downstream targets and possibly integrins αvβ5. In response to acute exposure, c-Met and PI3K become phosphorylated in a predictable sequence that has been demonstrated by others *in vitro*. Our recent experimentation has shown that increases in binding and potentially internalization of nonspecific targets by RPE are consistent with both receptor up-regulation and phosphorylation. We suspect that the phosphorylation of PI3K by c-Met controls phagocytic activity via activation of cytoskeletal reorganization pathways. To evaluate the importance of PI3K during this apparent increase in phagocytic efficiency, we challenged RPE with HGF in addition to Wortmannin, a pharmacological inhibitor of the p85 subunit of PI3K. Our data show a significant decrease in phosphorylated PI3K when Wortmannin is applied to cells ($p<.05$), suggesting successful chemical inhibition of p-PI3K. Furthermore, binding of non-specific targets appear to be reduced between HGF+ and PI3K inhibited cells, though not significantly so.

CHAPTER VII: Discussion and future directions

Exposure to elevated levels of HGF appears to augment nonspecific phagocytosis by RPE *in vitro*. Our data suggests that up-regulation and increased phosphorylation of the c-Met receptor, its downstream targets, and increased expression of select integrins implicated in POS binding all contribute to this accelerated behavior among debris removal. Since each of these factors play a key role in specific and nonspecific phagocytosis, it is likely that the influence of HGF on RPE is greater than initially suspected. As many retinopathies feature compromised RPE, we are hopeful that revival of these fatigued phagocytes is possible by manipulating the HGF/c-Met partnership, and such manipulation will potentially cease or reverse the decay of vision *in vivo*.

Breakthroughs in the study of angiogenesis have proven valuable to those suffering from AMD. There are several angiogenesis inhibitors approved by the FDA that aid arresting the progression of AMD by interfering with VEGF/VEGFR signaling, a key facilitator of choroidal, sub-retinal and retinal vascularization, coupled with photodynamic therapy and laser photocoagulation. While the aforementioned therapies have proven somewhat successful, there is no remedy for enhancing or reviving RPE phagocytosis in aged or injured cells. RPE transplantation, while initially promising, has inherent instability due to the chaotic sub-retinal environment. Our approach would serve as a bridge between pharmacological agents intended to halt the onset of AMD, and tissue transplantation designed to reestablish photoreceptor renewal mechanisms.

Of particular interest to our lab is the increase in microvillus expression when RPE are exposed to HGF for periods exceeding 24 hrs. *In vivo*, apical microvilli emanating from

the surface of RPE ensheath photoreceptors increasing surface area initiating phagocytic behavior and maintaining stability during POS removal. c-Met has been shown to interact with signaling proteins PI3K, PLCγ, and pp60c-sr, resulting in the generation of phosphoinositides which in turn regulate many actin binding proteins including those in the gelsolin family.[4] While it's certain that these down-stream signaling mechanisms facilitate proliferation, they may also activate tubulogenesis involved in endosome formation and trafficking. Furthermore, ezrin, a linker of actin filaments and plasma membrane proteins, is yet another effector of HGF and is found widely in RPE and neuroepithelia in mammals. While much remains unknown, it is believed that ezrin partially facilitates the trafficking of membrane proteins to specific regions affiliated with apical microvilli, in a potential effort to maximize phagocytic efficiency.[5]

Our results, while encouraging, serve only as a foundation for future research. Our next group of experiments will feature POS from freshly enucleated bovine eyes instead of fluorescent *E. coli* and latex spheres, as repeating our phagocytosis evaluations with POS may uncover subtle modifications during binding and uptake. We will also develop an animal model where RPE failure is induced; this method will allow us to observe *in vivo* consequences of c-Met pathway manipulation.

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