Affinity release of insulin-like growth factor-1 increases the viability of retinal pigment epithelium cells \textit{in vitro}

By

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ABSTRACT

Poor cell survival in vitro and in vivo is a key challenge in tissue engineering. Pro-survival therapeutic proteins such as insulin-like growth factor-1 (IGF-1) can promote cell viability but require controlled delivery systems due to their brief half-lives. Biocompatible materials are commonly used for drug-delivery platforms or to encapsulate cells for increased viability but no material exists for simultaneous use in both applications. A blend of hyaluronan and methylcellulose known to promote cell survival was modified with Src homology 3 (SH3)-binding peptides and demonstrated tunable, affinity-based release of the pro-survival fusion protein SH3-IGF-1. The material was also shown to significantly increase the viability of retinal pigment epithelium cells (RPE) in anchorage-independent conditions. This novel system is applicable to a broad range of cells and protein therapeutics and is a promising strategy for a drug-delivery/cell transplantation strategy to increase the viability of both exogenous and endogenous cells in tissue engineering applications.
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1. INTRODUCTION

1.1 Retinal Degeneration

The retina is the area of the vertebrate eye primarily responsible for vision. The cells within the retina are responsible for detecting photons that enter the eye and transducing that stimulus into an electrical signal that travels to the brain where it is processed as an image. Dysfunction and death of these retinal cells, in a process known as retinal degeneration, cause visual impairments in millions of people each year. Many of these retinal degenerative diseases such as age-related macular degeneration (AMD), Stargardt’s dystrophy, and retinitis pigmentosa are currently incurable.¹²

Current laboratory approaches to treating, and eventually curing, these diseases have focused on the transplantation of retinal cells to replace dead or dysfunctional cells as well as drug delivery to improve the survival of transplanted or endogenous retinal cells.²⁻⁶ Numerous cell types and drugs have been investigated for their ability to improve retinal degeneration symptoms, with a focus on photoreceptors or retinal pigment epithelium cells (RPE) for cell delivery and protein therapeutics for pro-survival drug delivery.

1.2 Retinal Cell Types

The major cell types in the retina are photoreceptors, RPE, horizontal cells, amacrine cells, bipolar cells, ganglion cells and Muller glia.⁷⁻⁸ Of these cell types, it is problems with the photoreceptors and RPE that are most commonly associated with retinal degenerative diseases.¹ These two cell types provide the crucial first step in vision - the transduction of detected photons into neural impulses - and their death presents a severe, and currently untreatable, problem for many patients with retinal degenerative disease.
1.2.1 Photoreceptors

Photoreceptors are light-sensitive neural cells and are divided into two types, rods and cones. Rods outnumber cones approximately 20 to 1 in most mammalian species and are responsible for vision in dim light, while cones are used for high-acuity and colour vision. The central part of the retina, known as the macula, contains mostly cones and is responsible for the high-acuity vision used for reading and other specialized tasks. It is the affected area in AMD, where dysfunctional RPE cause photoreceptor death, and is one of the most common areas for retinal degeneration to take place.

Figure 1.1 Organization of the eye. (A) Diagrams of the eye; an enlarged diagram of the fovea is shown in the box. Retina forms the inner lining of the most of the posterior part of the eye. The RPE is sandwiched between the retina and choroids, a vascularized and pigmented connective tissue. (B) Diagram of the organization of retinal cells. R, rod; C, cone; B, bipolar cell; H, horizontal cell; A, amacrine cell; G, ganglion cells; M, Müller cell. (C) An H&E-stained transverse section of human retina. Retina has laminated layers. The nuclei of the photoreceptors constitute the outer nuclear layer (ONL). The nuclei of the bipolar cells, amacrine cells, horizontal cells, and Müller glial cells are found in the inner nuclear layer (INL), and the nuclei of ganglion cells form the ganglion cell layer (GCL). The outer plexiform layer (OPL) contains the processes and synaptic terminals of photoreceptors, horizontal cells, and bipolar cells. The inner plexiform layer (IPL) contains the processes and terminals of bipolar cells, amacrine cells, and ganglion cells. The processes of Müller glial cells fill all space in the retina that is not occupied by neurons and blood vessels. Reproduced from Sung et al (2010).
Phototransduction, the process whereby photoreceptors convert photons into electrical signals that are passed to other neural cells, takes place within membranous outer segments which contain 11-cis retinal, a light-sensitive chromophore covalently bound to the pigment opsin. When light interacts with 11-cis-retinal, it isomerizes into all-trans retinal and is released from rhodopsin. The all-trans retinal triggers a signal cascade which decreases nucleotide cyclic guanosine monophosphate (cGMP) levels in the cell, causing $Ca^{2+}$ channels to close and hyperpolarize the cell, reducing the levels of glutamate released at the photoreceptor synapse. This causes a cascade of cell depolarization through the bipolar and retinal ganglion cells (RGCs) that travels through the optic nerve to the brain, where it is turned into a visual image.

Photoreceptors are affected by retinal degenerations either directly, through inherited conditions such as retinitis pigmentosa, or indirectly, such as through inflammation and dysfunction of the supportive RPE layer, as occurs in AMD. Because photoreceptor death and dysfunction is the cause of vision loss in retinal degenerations, preserving their function through drug delivery or transplant of new photoreceptor cells has been one of the primary strategies to treat retinal degenerations.

1.2.2 Retinal pigment epithelium

The RPE layer provides a host of supportive functions and its viability is inextricably linked with photoreceptor health and function. RPE roles include recycling waste products, daily phagocytosis of defunct photoreceptor outer segments that are essential for phototransduction, and supplying photoreceptors with neurotrophic factors. Most importantly, RPE cells regenerate 11-cis-retinal from all-trans-retinal, into which form the cis-isomer is converted during photoreceptors’ transduction of photons into electrical signals that go to the brain. Photoreceptors are unable to re-isomerize cis-retinal from trans-retinal on their own and without 11-cis-retinal supplied by RPE the visual cycle, and vision itself, comes to a halt.
Because of these essential roles in photoreceptor function, the loss of RPE function can have a strong apoptosis-causing effect on photoreceptor cells and is a common cause of retinal degeneration. These RPE losses can often have a considerable effect on photoreceptor function and vision because one RPE cell can phagocytose the outer segments of approximately 30 rod cells. These outer segments are filled daily with many toxic, light-damaged proteins and lipids and without RPE phagocytosis, photoreceptor function and viability are severely inhibited. The ability to prevent RPE death by delivering therapeutics, or even transplanting new RPE cells has therefore drawn strong interest from researchers attempting to treat this group of diseases.

1.2.3 Other Retinal Cell Types

Bipolar cells receive glutamate neurotransmitters from their connected photoreceptors and pass the signal on to RGCs which form the optic nerve and transmit the signal to the brain. This direct pathway is complemented by horizontal and amacrine cells, which form lateral connections between the outer and inner plexiform layers where the synapses between the retinal neurons are located. These lateral connections are important for luminance contrast and many subtypes of amacrine cells serve to relay diverse amounts of information throughout the retina. The final major retinal cell type, Müller glia, maintain retinal homeostasis and integrity. They act as molecular barriers and pathways between retinal cells and change their physiology, morphology and biochemistry in response to retinal damage. This process, known as reactive gliosis, can severely inhibit cell transplant integration and survival.

1.3 Cell Transplantation to treat retinal degenerations

Transplantation to immunoprivileged sites, such as the eye, are thought by many to be the stem cell therapy with the best initial chance of success due to the increased survival of transplanted cells in areas with inhibited immune response. Although stem cells exist in the retina, there are currently no effective methods to stimulate them to renew dysfunctional and dead retinal cells, which are post-mitotic.
and cannot renew themselves. In order to effectively treat and cure the diseases that affect them, new, transplanted cells are required to replace the dead and dysfunctional ones. With the advent of stem cells, which provide a renewable, pluripotent source of retinal cell types, cell therapy has moved to the forefront of strategies to treat retinal degeneration. Since photoreceptors and RPE are the cells most affected by these diseases, the focus of most cell therapy treatments has been the transplantation of these two cell types. RGC, the other main cell type in the visual pathway, is less studied for transplantation because the large axons and specific connectivities required for RGC function make effective transplant integration much more difficult than that for RPE and photoreceptors.

Due to RPE and photoreceptors’ front-line role in vision and well-understood biology, there has been strong progress in the field, culminating in recent human clinical trials. However, many challenges with transplant survival and integration with the host tissue remain.

### 1.3.1 Photoreceptor Transplantation

Photoreceptors are terminally differentiated neurons and once lost they cannot be regenerated. They require no incoming contacts and form only single connections with the established retinal circuitry, making them one of the most feasible neural cell types for transplant repair. Being neural cells, however, they have a more complex integration process than simpler retinal cells, such as RPE. Both rods and cones have been successfully transplanted into animal models, but so far only rods have been transplanted in sufficient numbers to improve electroretinography (ERG) amplitudes, a common measure of broad photoreceptor function, in mice. Cones are rare in the rod-dominant mouse eye, the animal of choice for photoreceptor transplantation studies, and have only shown integration at extremely low (< 1%) efficiency. This currently makes them a much less effective therapeutic transplantation strategy than rod transplantation. This is a problem as transplantation in humans must necessarily focus on the high-acuity cones to achieve the most functional benefit.
Photoreceptor transplantation is also limited by the finding that only post-mitotic cells already committed to the photoreceptor lineage have been shown to integrate into the mature retina. Since photoreceptors cannot proliferate once transplanted, unlike RPE, their successful transplant therefore requires a large amount of the original transplanted cell population (~120,000 in a mouse eye) to integrate for a significant functional benefit to be observed.

While photoreceptor degeneration and dysfunction is the root of retinal degeneration, photoreceptor transplantation alone is unlikely to be an optimal, curative treatment for the most common retinal degeneration, AMD. This is because AMD affects both photoreceptors and RPE and, due to the dependence of photoreceptors on RPE for normal visual function and survival, transplanted photoreceptors are unlikely to survive and integrate unless healthy RPE are also introduced to allow their proper functioning.

Many researchers are currently seeking to overcome the challenges inherent in photoreceptor transplantation, such as poor integration and survival, and establishing a viable source for cone cell transplantation. While this is underway, researchers have seen more therapeutic success using RPE transplants, a simpler cell type with advantages such as improved integration efficiency and an ability to proliferate post-transplantation.

1.3.2 Retinal Pigment Epithelium Transplantation

RPE transplants have had greater success than photoreceptor transplants because they are a simple monolayer that only require close proximity to photoreceptors for effective integration, whereas photoreceptors must form a more complex neural network and synaptic connectivity with surrounding cells. Successful RPE transplantation has been repeatedly shown to increase photoreceptor survival and improve visual function in rat models of AMD. This culminated in recent clinical trials demonstrating safety (18/18 subjects) and significant improvement in visual acuity (8/18 subjects) in...
patients with AMD and Stargardt’s dystrophy, the most common types of adult and pediatric retinal degenerations, respectively \(^{21,27}\). However, there remain many challenges associated with RPE transplantation, not least of which is the limited survival and integration of transplanted RPE cells due to poor adhesion on the aged and damaged Bruch’s membrane, the collagenous tissue upon which the RPE is anchored \(^{36,37}\). In fact, the failure of cells to attach and subsequently proliferate on Bruch’s membrane is the major cause of RPE transplant failure \(^{38-40}\).

Strategies to improve RPE survival and transplant success include enhancing their adhesion to Bruch’s membrane through molecular therapy or genetic engineering \(^{36}\), as well as transplanting RPE as a monolayer using synthetic substrates to overcome problems of functional monolayer formation post-transplantation \(^{41}\). Gullapalli et al. cultured RPE to express higher levels of \(\alpha\)-integrins to improve ECM binding \(^{42}\) while Afshari et al. \(^{43}\) and Fang et al. \(^{44}\) used genetic manipulation to increase RPE expression of \(\alpha9\) integrin and \(\alpha6\beta4\) integrins, respectively, to increase adhesion to Bruch’s membrane and improve transplant survival. Bruch’s membrane substitutes for monolayer formation and transplantation include synthetic polyamide nanofibers \(^{45}\) and polyester membranes \(^{46}\) as well as natural mimics such as Descemet’s membrane \(^{47}\). In a promising study, Diniz et al. found that RPE transplanted in a monolayer on a parylene membrane survived better than cells transplanted in solution. Despite this progress, however, monolayer transplants remain more difficult to perform and have more issues with trauma and biocompatibility than cells transplanted in solution \(^{41}\). Meanwhile, the clinical trials currently underway use cells injected in solution and have shown no significant safety concerns \(^{21,27}\).

1.3.3 Stem cells as a source of retinal cells

Since they were first discovered in 1998 human embryonic stem cells (hESCs) have been a promising source of new cells to replace those lost through injury and disease. \(^{48}\). They have been investigated as a source of cells to repair a diverse range of degenerated tissues including cardiac, bone,
cartilage, and nervous system tissues due to their pluripotency and ability to be cultured for extended periods of time. Once differentiated into relevant cell types, such as neural progenitor cells (NPCs), they are also prized for their ability to rescue endogenous cells by secreting neurotrophic and neuroprotective factors. This has been shown to protect neural cells and improve visual performance in several models of retinal degeneration and glaucoma.

Due to ethical concerns with hESCs, Yamanaka et al. developed a technique to reprogram fibroblasts into stem cells, providing a less controversial renewable source of stem cells termed induced pluripotent stem cells (IPSCs). Both hESCs and IPSCs are currently differentiated to obtain photoreceptors and RPE for cell transplant applications and the field has progressed due to the improved supply of cells for transplant therapy.

1.4 Drugs to improve retinal cell survival in retinal degeneration

Researchers have investigated a range of therapeutic molecules for their abilities to improve outcomes for retinal cells in retinal degenerative models. One of the most promising ways to enhance transplant integration and efficacy is to improve the survival of the transplanted cells and thereby increase their chances to migrate to the site of injury, integrate with the host tissue, and restore function. Most research into prosurvival drugs and growth factors has focused on improving the survival of endogenous retinal cells. However, delivery of factors alone cannot replace the degenerating cells and in order to improve outcomes for patients with retinal degenerative diseases, the knowledge gained from studies on endogenous cells must be used to for therapeutics to improve the outcomes for transplanted cells. There is a wide body of research on prosurvival drugs and an ideal candidate should have multiple effects in addition to survival. Some important beneficial effects are increased proliferation of cells (if the cells are mitotic), as well as pro-migratory effects and anti-inflammatory glial-deactivation to reduce the detrimental impact of glial scar formation on transplant integration success.
1.4.1 Small Molecule Drugs to treat retinal degeneration

Small molecules that improve photoreceptor or RPE survival target the many causes of cell death in retinal degenerative diseases such as oxidative stress, inflammation, lipid-protein aggregates (correlated with AMD), gliosis, and many others. The only current clinical treatment for dry AMD is a small molecule treatment, an antioxidant cocktail of vitamins C and E, β-carotene and zinc that unfortunately only works in a small subgroup of AMD patients and merely serves to delay the progression of the disease.

Prosurvival small-molecule drugs currently under investigation are mostly non-ophthalmologic therapeutics tested in rat and mouse models and have largely focused on improving photoreceptor survival to the detriment of research into RPE prosurvival drugs. Photoreceptor pro-survival molecules such as bromidene and other alpha 2-adrenergic receptor agonists have been shown to protect photoreceptors against retinal ischemia by upregulating the ERK and PI3K/Akt pathways as well as promoting the production of prosurvival factors such as FGF-2, BDNF and CNTF. Other drugs such as calcium antagonists and synthetic progestins have also been shown to reduce photoreceptor degeneration and maintain photoreceptor morphology.

Despite the abundance of potential off-target molecules, no drug strategies currently exist that can be used to reverse the deterioration caused by retinal degeneration. There is a paucity of research on small molecule drugs and a wide range of molecules still need to be screened for their potential to increase photoreceptor and RPE survival. Conversely, a wide range of neurotrophic proteins have already been tested for their ability to promote both photoreceptor and RPE survival in retinal degeneration. Although they present their own drug delivery challenges, these therapeutic proteins provide a very promising strategy to improve cell survival in these diseases.
1.4.2 Therapeutic Proteins to Treat Retinal Degeneration

Therapeutic proteins are naturally produced macromolecules that generally promote survival, proliferation and maturation of cells. In the eye, the main producers of these factors are RPE and Müller glia. A wealth of research into these factors has been performed in the eye due to the low tissue surface area and limited diffusion due to ocular barriers. Some of the most promising, and most investigated factors, are outlined below.

1.4.2.1 Ciliary Neurotrophic Factor (CNTF)

CNTF is one of the most studied protein therapeutics in retinal degenerative diseases. It has been shown to have a protective effect on both rod and cone photoreceptors as well as RGCs and RPE. The safety of long-term CNTF delivery in humans was investigated in a two year study of CNTF-expressing transplanted cells, although the dose was too low to observe any significant functional benefits. This may cast doubt on the accuracy of the safety data with CNTF at therapeutic doses, as CNTF has several noted side effects listed below.

While CNTF can interact directly with RPE, its effects are mostly mediated by Müller glia cells. It is also not known if it can interact directly with human photoreceptors. Photoreceptors do express CNTFRα, one of three parts of the CNTF receptor complex, but more research is required to define its mode of action. Current studies in rodents do not show a direct effect of CNTF on photoreceptors.

Despite its established prosurvival effects, CNTF may not be suitable for use with transplanted cells as it can cause gliosis and increase the size of the glial scar. This was shown to severely reduce the ability of exogenous cells to integrate into the native retinal tissue in comparison to control eyes that received no CNTF treatment. CNTF also has many known side effects and complications. High doses have been associated with decreased optokinetic response, ERG amplitudes, and visual acuity. Other side effects include retinal disorganization and reduced levels of cone opsins essential for...
phototransduction. These side effects, combined with its lack of direct effects on RPE and photoreceptors, bring into question the therapeutic benefit for retinal transplants, although many researchers still use CNTF because it is one of the most well-characterized growth factors.

1.4.2.2 Glial cell line-Derived Neurotrophic Factor (GDNF)

GDNF promotes the differentiation and proliferation of RGCs, photoreceptors and neurons and has been shown to protect photoreceptors and RGCs from cell death in animal models of retinal degeneration. It acts directly on photoreceptors and may also act indirectly on them through Müller glia by increasing the expression of glial L-glutamate/L-aspartate receptor, which reduces glutamate-mediated toxicity, in glial cells located around degenerating photoreceptors. Its effects are significant enough to show increased outer nuclear layer (ONL) thickness, a common measure of photoreceptor survival, and improved photoreceptor function via an increase ERG signal amplitude in a rat model of retinitis pigmentosa. It also decreases Müller cell proliferation, retinal oxidative damage and has been shown to work synergistically with BDNF. Despite the many studies showing its beneficial effects on neural cells in the retina, there is a dearth of research on GDNF’s effects on RPE cells. It has been linked to the regulation of Zn\(^{2+}\) transporters in RPE which may be involved in retinal toxicity in AMD, but more research on its effects on RPE need to be done before it is used as a prosurvival factor in RPE transplants.

1.4.2.3 Brain-Derived Neurotrophic Factor (BDNF)

BDNF promotes survival of photoreceptors and RGCs. It has also been shown to be important for RPE differentiation and survival in *Xenopus laevis*. Its effects on mammalian RPE are not well studied although it has been shown to have no effect on the proliferation and survival of bovine RPE in vitro. However, BDNF does not act directly on photoreceptors because they lack the TrkB receptor, and it can reduce the levels of beneficial GDNF and CNTF in light-damaged retinas. There is also mixed data on its benefits toward retinal cell survival. Gene knockout BDNF +/- mice that only expressed 50%
of BDNF mRNA were actually shown to have increased photoreceptor survival and function when compared to wild-type BDNF +/+ mice 107, leading to some doubts of its use at the high doses usually delivered in prosurvival studies. While a potent factor, BDNF’s lack of direct interaction with photoreceptors and its unexpected poorer pro-survival effects at higher doses do not currently make it an ideal factor to improve retinal transplant survival.

1.4.2.4 Pigment epithelium derived factor (PEDF)

PEDF significantly improves photoreceptor and RGC survival in rat models of retinal degeneration 108-111. Alone among major retinal growth factors studied, it is strongly anti-angiogenic 112 and is under investigation to treat wet AMD and diabetic retinopathy, where uncontrolled blood vessel proliferation causes retinal degeneration and vision loss 113,114. PEDF Receptors (PEDF-R) are located on photoreceptors, RPE and RGC, and may act directly on each cell type 115.

PEDF’s activates its neuroprotective effects through PEDF-R, which in turn activates the anti-apoptotic NF-κB signalling cascade 116 as well as the production of neuroprotectin D1 (NPD-1) in RPE which is secreted and prevents oxidative damage in the retina 117. It is also anti-inflammatory 116. However, like BDNF, little research into its effects on RPE has been performed, and any beneficial effects on RPE transplants are not currently known.

1.4.3 Insulin-like Growth Factor-1:

Insulin-like growth factor-1 (IGF-1) is essential for retinal cell development, signaling and function 37. IGF-1 receptors are expressed in cell types throughout the retina including RGC 118, photoreceptors 119 and RPE 120. It has prosurvival, angiogenic, pro-migration, and mitotic effects on a wide variety of cell types 121 and is being investigated as a treatment to numerous retinal degenerative diseases 122-126.
1.4.3.1 Effects of IGF-1 on Transplanted RPE and endogenous RPE

IGF-1 is a protein that promotes RPE survival, proliferation and migration \(^{127-130}\), key factors in effective integration and visual improvement in RPE transplantation patients. IGF-1 inhibits RPE apoptosis through the PI3K/Akt pathway \(^{131}\) and increases RPE proliferation through the MAPK pathway \(^{128}\). IGF-1-stimulated RPE proliferation occurs in a dose-dependent manner and continuous IGF-1 delivery has a stronger effect than single IGF-1 doses \(^{132}\) as the MAPK pathway is only transiently activated by IGF-1 in neuronal cells \(^{133}\).

1.4.3.2 Bruch’s Membrane, Anoikis, and RPE Survival

Lack of RPE attachment to Bruch’s membrane, due to age-related damage present in retinal degenerations such as AMD, and its subsequent inability to survive and proliferate is the major cause of RPE transplant failure \(^{134,135}\). Recent research has sought to increase RPE attachment and proliferation on Bruch’s membrane by several methods, including modifying RPE integrin expression and signaling to better interact with damaged ECM components on aged Bruch’s membrane \(^{42,44}\).

\(\beta_1\) integrins mediate the majority of RPE interaction with Bruch’s membrane and have been a major focus of RPE modification to increase transplant success \(^{36}\). IGF-1 can take advantage of this interaction without the need for RPE engineering as it can bind many \(\beta_1\) integrin subtypes \(^{136,137}\). It also binds to integrins in lieu of ECM to promote cell proliferation \(^{138}\) and survival \(^{139}\).

There is additional indirect support for IGF-1 as a promising molecule to promote RPE proliferation and survival on damaged Bruch’s membrane. Insulin, a closely related molecule that shares binding partners with IGF-1, can inhibit RPE apoptosis as a result of anoikis \(^{140}\). Additionally, RPE MAPK and PI3K-Akt signaling, which is strongly activated by IGF-1 \(^{128,131}\), has been shown to have a powerful anti-apoptotic effect in anchorage-independent culturing of RPE \(^{141,142}\). Constitutive activation of PI3K-Akt in epithelial cells prevents apoptosis due to anoikis, and the pathway is strongly activated upon attachment to ECM.
These data suggest that IGF-1 could improve RPE survival on damaged Bruch’s membrane and is a strong candidate to improve RPE transplant outcomes.

1.4.3.3 Effects on retinal cells other than RPE

Insulin-like growth factor (IGF-1) prevents apoptotic retinal cell death, which is the leading cause of cell death, including photoreceptors and RGC, in retinal degenerative models. It also is known to increase the synaptogenesis in neurons, including photoreceptors, which has a positive effect on their survival. These effects were apparent in a study by West et al. that demonstrated that IGF-1 improved the survival and integration of transplanted photoreceptors in C57Bl/6J mice. In addition to its pro-survival effects, IGF-1 is also beneficial to photoreceptor function, accelerating the kinetics and increasing the amplitude of the light response in rod photoreceptors.

IGF-1 is a trophic factor for amacrine neurons and glial cells which are important for ocular function. In an rd10 model of retinitis pigmentosa, IGF-1 caused microglia proliferation, which directly affected photoreceptor survival, amplifying IGF-1 neuroprotective effects. These myriad beneficial direct and indirect effects on all major retinal cell types make IGF-1 a promising candidate to increase retinal transplant survival.

1.4.3.4 Anti-inflammatory effects

Inflammatory responses are involved in the pathogenesis of many retinal degenerative diseases such as diabetic retinopathy, AMD and retinitis pigmentosa. Anti-inflammatory responses are therefore important in secondary effects in pro-survival molecules to treat retinal degenerations. IGF-1 is a well-known anti-inflammatory factor in the CNS that acts through NFkB and also has anti-inflammatory and antioxidant properties on vasculature. Importantly, IGF-1 reduces the inflammatory response of glial cells, which can inhibit the integration of retina transplants. In addition, pro-
inflammatory factors in the CNS reduce endogenous IGF-1 production, causing detrimental effects and demonstrating the need for exogenous IGF-1 upon inflammation-causing retinal transplantation.

1.4.3.5 Benefits of sustained IGF-1 delivery

IGF-1 has a short in vivo half-life of approximately 12 hours and its sustained delivery may be necessary in order to exert its prosurvival and trophic effects. Studies have shown prosurvival benefits of continuous IGF-1 delivery in a variety of retinal cells, including RGC in a mouse glaucoma model. The MAPK pathway, through which IGF-1 acts and an important pathway in photoreceptor resistance to apoptosis, is only transiently activated by IGF-1 in neuronal cells. For RPE, continuous supply of IGF-1 is associated with increased proliferation in comparison with pulsed IGF-1 addition to media. Additionally, constitutive expression of the IGF-1-activated PI3K-Akt pathway inhibits RPE death by anoikis. A continuous supply of IGF-1 may therefore be necessary for maximal survival and proliferative effects of IGF-1 on RPE and other retinal cell types. The sustained delivery of lower doses of IGF-1 may also reduce side effects from high concentrations in tissue by bulk delivery.

Controlled-release systems for IGF-1 have mostly focused on muscle and cartilage using degradation- or nanoparticle-based release. There has not been a strong focus on sustained delivery of IGF-1 to the retina, although there has been a lot work into drug delivery systems (DDS) of therapeutic proteins in general.

1.5 Drug Delivery to Treat Retinal Degeneration

Delivery of pro-survival protein therapeutics to improve endogenous or transplanted cell survival is a major treatment focus for retinal degeneration. Neuroprotective proteins such as BDNF, PEDF, CNTF and IGF-1 have been used to promote cell survival in animal models of retinal degeneration. However, these molecules suffer from relatively short half-lives and subsequently have transient effects on cells.
In order for drugs to promote endogenous or exogenous cell survival in retinal degenerative diseases, they must be delivered to the posterior segment of the eye, where the retina is located, which is traditionally one of the most difficult places in the body in which to deliver proteins. This delivery is typically administered systemically, via intravenous injection, or topically via eyedrops. However, due to limitations with these methods, novel approaches such as intravitreal or subretinal injections, and drug delivery systems are becoming increasingly common.

1.5.1 General Approaches

Topically administered drugs are often the preferred choice for therapeutic delivery to the retina due to the ease of application and excellent patient compliance. However, usually less than 5% of the drug reaches the posterior segment of the eye due to the large diffusion distance, lacrimation and corneal impermeability, making this a poor choice through which to administer the often-expensive neurotrophic factors used to increase cell survival. Systemic delivery, another popular method, also results in a very small percentage of the administered drug reaching the eye due to the blood-retinal-barrier, which prevents the transport of drugs from the circulatory system to the retina through both active and passive mechanisms.

The limitations in delivery efficacy of topical and systemic methods, especially since the majority of effective retinal degenerative drugs are expensive growth factors or DNA aptamers, have driven a rise in the use of intravitreal (IVT) and sub-retinal delivery methods. These methods inject the therapeutic directly into the eye and result in a high percentage of the injected drug reaching the posterior eye. However, despite the delivery benefits, these procedures also present many challenges.

IVT injections effectively deliver the molecules to the vitreous humour of the eye, but injection there is associated with a short retention time and rapid drug clearance. Compensation for this, by
delivering higher doses of drugs intravitreally, can be problematic as higher doses can cause retinal detachment, endophthalmitis or hemorrhage\textsuperscript{170,178}. Subretinal injections are more invasive than IVT but are currently the most effective method with which to deliver drugs to the RPE\textsuperscript{177}. Unfortunately, this method also suffers from rapid clearance of lower molecular weight (MW) molecules. For example, a 10 kDa molecule, greater in molar mass than IGF-1, is eliminated in 30 hours if injected in the subretinal space\textsuperscript{179}.

Because of rapid protein clearance and denaturation, these strategies require repeated injections and can be a substantial burden on both the physician and patient, in addition to increasing the risk of retinal complications\textsuperscript{60}. Taking advantage of the more efficient delivery methods of intravitreal and subretinal injections while reducing the need for repeated injections drives the design of sustained drug-delivery systems to the eye\textsuperscript{57,180}.

1.5.2 Drug Delivery Systems

Implantable drug-delivery methods include encapsulation in particulate based systems such as liposomes or micro- or nanoparticle delivery from hydrogels, as well as affinity-based release\textsuperscript{60,180}.

1.5.2.1 Particulate-Based Systems

In particulate-based systems the therapeutic agent is typically encapsulated within polymers (commonly poly(lactic-co-glycolic acid), PLGA) or inorganic materials, such as silica\textsuperscript{181}. They take the form of microparticles (typically several hundred microns) or nanoparticles (< 1 µm), with the drug either inside the particle or on the surface\textsuperscript{57}. The nanoparticles can be injected in the retina alone\textsuperscript{182,183} or in a scaffold, such as a hydrogel\textsuperscript{184}. While microparticles can stay in the vitreous fluid for over a month\textsuperscript{185}, nanoparticles have the advantage of being able to rapidly diffuse to ocular tissues, including in the posterior segment, where they are internalized\textsuperscript{58,186}.
Liposomes are another encapsulating technology that has been widely used to deliver drugs to the eye due to their ability to lower drug toxicity and increase half-life. They can also protect unstable molecules, such as peptides, from degradation. Their clinical use has been limited, however by difficulties with their sterilization and stability as well as low encapsulation efficiency, incomplete release and poor release kinetics.

While significant advances have been achieved with particulate systems, they generally suffer from low protein loading capacity and lost activity of the released proteins due to exposure to organic solvents and processing. Nanoparticles and liposomes are also hydrophobic by nature and show poor compatibility with hydrophilic biological molecules such as proteins.

1.5.2.3 Affinity Release

To overcome the limitations inherent to particulate DDS, affinity drug release systems have been pursued. These materials delay protein diffusion from polymer networks using transient affinity between binding partners on the therapeutic and polymer network. The most prominent strategy uses heparin with a series of heparin-binding proteins. However, this system is limited to delivering proteins that can naturally bind to heparin.

Many affinity-based DDS involve electrostatic interactions between binding partners. The majority of this research has focused on heparin-based systems which utilize interactions with dissociation constants \( K_D \) between \( 10^{-6} \) M and \( 10^{-9} \) M. The utility of the less invasive processing was shown when heparin-functionalized beads were able to maintain FGF-2 bioactivity while traditional nanoparticle processing resulted in less than 4% of FGF-2 remaining active due to exposure to dichloromethane. Similar systems incorporating heparin have been used to deliver other growth factors, such as VEGF and PDGF.
Single species affinity release involves reversible interactions between the therapeutic protein and a binding ligand bound to a polymer network. A mathematical interpretation of the formation of the temporarily immobile complex is shown below, where $C$ is the equilibrium concentration of each species and $k_{off}$ and $k_{on}$ are the rate of dissociation and association, respectively, of the ligand-therapeutic complex in the equilibrium equation: $\text{Ligand + Therapeutic} \rightleftharpoons \text{Complex}$.

$$K_D = \frac{k_{off}}{k_{on}} = \frac{C_{\text{Ligand}} \cdot C_{\text{Therapeutic}}}{C_{\text{Complex}}}$$

Figure 1.2: Three regimes of release describe affinity-based release of therapeutic protein from a polymeric matrix immobilized with an affinity-binding ligand (peptide). The timescale for each phase of release is shown on the x-axis. $L$ is the thickness of the delivery matrix (usually a hydrogel), $D$ is the diffusivity of the protein through the delivery matrix, $C_{\text{pep,T}}$ is the total concentration of the peptide bound to the delivery matrix, $C_{\text{pro}}$ is the equilibrium protein concentration, $C_{\text{pro,0}}$ is the initial protein concentration, $K_D$ is the dissociation constant of the affinity pair, and $k_{on}$ and $k_{off}$ are the association and dissociation rates for the affinity complex respectively. From Vulic et al. (2015) 197.
In this system, therapeutic release can be modelled by three different regimes, depending on the starting conditions. In Regime 1, protein release occurs over a single timescale, diffusion is the rate-limiting step to therapeutic release, and a large proportion of ligand remains free at the beginning of the experiment. Under this regime $C_{\text{Ligand}}$ must be greater than $K_D$ in order for therapeutic release to be sufficiently attenuated by the affinity interaction. If it is not, the complexation equilibrium will shift toward free ligand and therapeutic and give diffusive release. Regime 2 is similar to Regime 1 except that a large proportion of ligand is bound to protein at the start of the experiment. This gives a two-stage release profile as a fast diffusional release occurs until protein concentration is equal to $K_D$, at which point its release is determined by $K_D$. In Regime 3, unbinding of the ligand-therapeutic complex, not diffusion, is the rate-determining step. This regime also follows a two-stage release as nearly all uncomplexed protein is released before bound protein is released on the timescale $\frac{1}{k_{off}}$.

Multiple species affinity release systems also exist. Sakiyama-Elbert et al. have designed a multicomponent system based on heparin-binding peptides (HBP) that have been immobilized to a fibrin matrix and bind to heparin (HP) which then binds heparin-binding therapeutic proteins (HBPro). The system is noticeably more complex than single-species affinity and involves four reversible equilibrium equations.

\[
\begin{align*}
HBP + HP & \rightleftharpoons HBP \cdot HP_{\text{immobilized}} \\
HBP \cdot HP + HBPro & \rightleftharpoons HBP \cdot HP \cdot HPro_{\text{immobilized}} \\
HP + HPro & \rightleftharpoons HP \cdot HPro_{\text{diffusible}} \\
HBP + HP \cdot HBPro & \rightleftharpoons HBP \cdot HP \cdot HPro_{\text{immobilized}}
\end{align*}
\]
In order to expand the range of therapeutics able to be delivered by affinity release, many researchers have looked into electrostatic interactions other than heparin binding pairs. An example is the use of iminodiacetic acid in the presence of transition metal ions to bind Histidine tags on recombinant proteins to delay their release from hydrogels. However, this system does not have a strong delayed release (i.e. 8 h extended to 48 h) because \( K_d \) only ranges from \( \sim 10^{-4} \) to \( 10^{-6} \) \(^{198} \). Other systems include positively charged peptide hydrogels releasing negatively charged proteins \(^{199} \) and hydroxyapatite-coated porous \( \beta \)-tricalcium phosphate granules that can bind several different proteins \(^{200} \). Other protein-protein interactions such as Src homology 3 (SH3) domains \(^{201,202} \), collagen-binding domains \(^{203,204} \), and specific binding peptides (such as for NGF \(^{205} \) and FGF-2 \(^{206} \)) have also been used.

In search of a system for use with an even broader range of protein therapeutics, DNA aptamers, oligonucleotides that can be selected to bind target molecules \textit{in vitro} with high affinity and selectivity \(^{207} \) have also been used to deliver proteins such as PDGF \(^{208} \) and VEGF \(^{209} \). However, these often lack the desired affinity and specificity for the binding proteins they are raised against \(^{210} \).

1.5.2.4 Src Homology 3 (SH3) affinity release system

The Shoichet lab has designed an affinity release system that has the added benefit of allowing cell delivery at the same time. This affinity system modifies a biocompatible hydrogel comprised of hyaluronan-methyl cellulose (HAMC), shown to be beneficial for retinal cell delivery \(^{211,212} \), with SH3 binding peptides for delivery of growth factors containing an SH3 fusion domain. Tunable release of an SH3-FGF-2 fusion protein from HAMC was demonstrated. Both a weak SH3-binding peptide (WBP \( K_d = 2.7 \times 10^{-5} \)) and a strong SH3-binding peptide (SBP, \( K_d = 2.7 \times 10^{-7} \) M) were covalently attached to MC using a Michael-type addition between MC-thiol and WBP- or SBP-maleimide and controlled release was demonstrated for a period of 5 days (HAMC-WBP gels) or 10 days (HAMC-SBP gels) \(^{201} \). The broad
applicability of this system was further demonstrated using Chondroitinase ABC (ChABC) – an enzyme that has shown promise in central nervous system therapy 202.

1.6 Hydrogels for Drug Delivery and/or Cell Delivery

Hydrogels are porous, biocompatible polymers with high water content similar to that found in natural extracellular matrix (ECM) 213. They are generally formed by either physical or chemical crosslinks and have been made from a variety of natural and synthetic materials such as agarose, alginate, gelatin, hyaluronic acid and poly(ethylene glycol) (PEG) 211,214-216. Due to their biocompatibility and injectability, they have been extensively investigated for both cell- and drug-delivery applications 213,217.

Hydrogels are beneficial in cell delivery because they present a 3D microenvironment similar to the cells’ native environment and their porosity allows the diffusion of waste and nutrients needed for proper cell functioning. Some also contain ECM-like binding motifs for cells to attach to through their integrin receptors. These are important as many anchorage-dependent cells (ADCs) will undergo anoikis if their integrins are not interacting with an appropriate substrate or ECM motif 218,219.

Hydrogels have been used to deliver cells for regenerative applications in a variety of tissues, including cartilage 220, cornea 221, liver 222 and nervous system tissues 223. Despite these advances, there remain significant challenges to cellular encapsulation in hydrogels such as poor cellular adaptation to the hydrogel environment and poor control over cellular migration, proliferation and cell/hydrogel interaction 224.

Hydrogels are excellent candidates for drug-delivery materials for the same reasons that they make good cell-delivery materials: their biocompatibility, biodegradability, and tunable porosity. They generally function as a storage depot that gradually releases the drug over time 217. The release can occur by several mechanisms including diffusion, degradation, or swelling and can be modified by fine-tuning the polymer architecture, molecular weight, and other physical properties 189. However hydrogels also
have limitations, including poor injectability, low tensile strength leading to premature dissolution, and rapid drug diffusion due to large pore sizes and high water content. 

There have been many attempts to modify hydrogels for improved drug delivery. One approach is to utilize ionic interactions between charged hydrogels to delay drug release. For example, phosphate-functionalized hydrogels with cationic lysozyme or 4-vinylpyridine and poly(hydroxyethylmethacrylate) hydrogel and anionic non-steroid anti-inflammatory drugs. Another strategy is to covalently conjugate the drug to the hydrogel in a system that allows drug release via chemical or enzymatic cleavage of the drug-hydrogel bond or the polymer backbone.

A hydrogel drug delivery system (DDS) must be biocompatible, biodegradable and non-toxic. It is also beneficial if the hydrogel is crosslinked physically instead of chemically, as physically crosslinked hydrogels are thought to have lower toxicity, in situ gelling ability, stimuli responsiveness and greater drug-loading capacity. Hydrogel DDS generally utilize either nanoparticle-based release, using diffusion limitations of the nanoparticle porosity, or an affinity-based approach usually adapted from heparin binding proteins and motifs, although liposomes have been used as well. Affinity-based release is preferred for proteins because it avoids exposing proteins to harsh organic solvents and shear stresses used in nanoparticle fabrication which can render proteins inactive. A hydrogel system that combines beneficial physical properties for cell- and drug-delivery with an affinity-based drug release system, such as the hyaluronan-methylcellulose system outlined below, presents a promising strategy with which to deliver both drugs and cells to the body.

1.6.1 A Combined Hyaluranan and Methylcellulose Hydrogel (HAMC)

A physical blend of hyaluronan and methylcellulose (HAMC) is an injectable, bioresorbable and fast-gelling hydrogel applicable for both cell and drug delivery.
Hyaluronan is biocompatible and non-immunogenic anionic glycosaminoglycan. It is also anti-inflammatory and reduces scar formation. It is shear-thinning and flows well when injected through a small needle, a necessary condition for use as a material in minimally-invasive implants, such as those needed for the retina and other CNS applications. Such a material for cell delivery is important as minimally-invasive surgeries are associated with lower patient morbidity.

HA also has beneficial properties for encapsulated RPE. The CD44 HA-receptor is found on proliferating and non-proliferating RPE and its activation is associated with cell migration and adhesion, as well as resistance to apoptosis and anoikis. These are all key measures of success in cellular transplants.

Methylcellulose, the other half of the HAMC system provides complementary benefits to using an HA hydrogel alone, the foremost of which is its inverse thermal gelling property. This means that as temperature increases, such as when room temperature MC is injected into 37 °C tissue, hydrogen bonds between MC and water break and are replaced with hydrophobic interactions between MC strands, forming a gel. This allows hydrogels formed from MC to remain at the site of injection and to rapidly gel to encapsulate cells or drugs. This function is applicable to retinal and other CNS injections as MC is known to be biocompatible for scaffold applications in the CNS. However, addition of HA is necessary as a physical property for MC hydrogels as MC does not form a strong enough gel upon injection for many drug- or cell-delivery purposes and requires physical crosslinking with HA to form a strong physical gel necessary for these purposes.

HAMC has been shown to increase retinal stem-progenitor cell (RSPC) survival and proliferation in vitro. In in vivo applications it promotes coverage of Bruch’s membrane, upon which the RPE cells attach, as well as improving the integration of RSPCs and rod photoreceptors in the retina. HAMC alone has been shown to have other beneficial properties such as promoting wound-healing and reducing...
inflammation upon injection to the intrathecal space that surrounds the spinal cord\textsuperscript{244}. It has also been established as a viable DDS \textit{in vivo}, delivering PLGA nanoparticle-encapsulated neurotrophin-3 (NT-3) to the injured spinal cord\textsuperscript{245}. Importantly, HAMC has been modified for the controlled affinity release of several different proteins and does not require harsh solvent conditions\textsuperscript{201,202}, opening up a potential application as both a pro-survival factor and cell delivery material.

Both therapeutic protein delivery and biomaterial scaffolds are strategies that have potential to increase RPE transplant survival. A material that delivers pro-survival factors to, or with, transplanted cells would combine these methods into a dual-delivery strategy to increase RPE survival and integration with the host tissue. Due to its many beneficial properties and established track record for both cellular and drug delivery, HAMC is ideally suited for delivering both drugs and cells to a target site in a combined cell and drug-delivery vehicle.

\textbf{1.6.2 HAMC/SH3-IGF-1 Affinity Release Cell Encapsulation System}

The beneficial effects of both a therapeutic protein and an ECM-mimicking substrate are present in an affinity release system of IGF-1 from HAMC. Together, they provide a promising platform to increase cell viability upon transplantation. This combination cell- and drug-delivery system was investigated for its ability to promote RPE viability under non-adhesive and serum free culture conditions \textit{in vitro}. This material is one of the first of its kind designed for dual delivery of drugs and cells and has broad applicability for the transplantation of many different cell types that interact with IGF-1 and HA.

\textbf{1.7 Hypothesis and Objectives}

The hypothesis governing this body of work is that \textit{a hyaluronan-methylcellulose hydrogel (HAMC) that has been modified to control the release of insulin-like growth factor-1 (IGF-1) will increase the viability of retinal pigment epithelium cells in vitro over cells in HAMC or serum-free media alone.}
Two objectives were set to test this hypothesis:

1. Synthesize a HAMC polymer with covalently attached SH3-binding peptides and demonstrate sustained release of SH3-IGF-1 from the system.

2. Encapsulate RPE in the HAMC-IGF-1 affinity release hydrogel and determine its effects on cell viability in vitro.
2. MATERIALS & METHODS

2.1.1 MATERIALS

3-maleimidopropionic acid was purchased from TCI America (Portland, USA). Modified pET32b vector was a gift from Dr. Karen Maxwell (University of Toronto). Cloning of the SH3-FGF2 and WBP-IGF-1 vectors was done by GenScript (Piscataway, USA). Sodium hyaluronate (1.4 x 10^6 – 1.8 x 10^6 g/mol) was purchased from Kikkoman Biochemifa (Tokyo, Japan). Methyl cellulose (3 x 10^5 g/mol) was purchased from Shin Etsu (Tokyo, Japan). Sandwich ELISA kit for human IGF-1 was purchased from Assaypro (St. Charles, USA). All buffers were made with distilled and deionized water prepared using a Millipore Milli-RO 10 plus and Milli-Q UF Plus at 18 MΩ resistance (Millipore, Bedford, USA). Artificial cerebrospinal fluid (aCSF) was prepared as previously described. Human embryonic stem cells were supplied from Dr. Andras Nagy and differentiated to RPE by Nick Mitrousis.

2.1.2 METHODS

2.1.2.1 Synthesis of carboxylated methylcellulose (MC-CO2H)

Methyl cellulose (2.0 g) was dissolved in 150 mL of dH2O for 16 h at 4 °C and combined with of NaOH (6.0 g) dissolved in 50 mL dH2O and chilled to 4 °C. Bromoacetic acid (6.95 g) was added to the mixture and stirred for 4 h at 4 °C. The polymer was then transferred to 12-14 kDa MWCO dialysis tubing and dialyzed against 0.2 M NaCl for 4 days and dH2O for 48 h.

2.1.2.2 Synthesis of thiolated methylcellulose (MC-SH)

Carboxylated methylcellulose (MC-CO2) (2.0 g) was dissolved in 200 mL of dH2O that had been adjusted to pH 4.5 using 1 M HCl for 16 h at 4 °C. The solution was warmed to room temperature and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI, 1.2 g, 6.25 mmol) and 3,3’-dithiobis(propionic dihydrazide (DTP, 1.5 g, 6.25 mmol) were added and reacted for 3 h, maintaining pH 4.5. After 3 h, the
reaction was stopped by adding 1 M NaOH until the mixture reached pH 7. Dithiothreitol (DTT, 6.2 g, 40.625 mmol) was added, the basicity was raised to pH 8.5 using 1 M NaOH and stirred for 24 h at room temperature. The solution was then acidified to pH 3.5 using 1 M HCl, transferred to 12-14 kDa MWCO dialysis tubing and dialyzed against 0.1 M NaCl, pH 3.5 for 48 h and dH2O for 24 h. The mixture was lyophilized and the extent of thiolation was determined by Ellman’s assay using a standard curve of L-cysteine.

2.1.2.3 Synthesis of 3-maleimidopropionic-SH3-binding peptides

Weak SH3-binding peptide (WBP, sequence: GGGKPPVVKKPHYLS) and strong SH3-binding peptide (SBP, sequence: GGGKKTKPTPPPKPSHLKPK) were synthesized (0.25 mmol scale) using solid-phase peptide synthesis on a Liberty 1 automatic microwave peptide synthesizer (CEM). The resin-bound peptide was washed with dichloromethane (DCM) using an ISOLUTE column and dried under vacuum. 3-maleimidopropionic acid (406 mg, 2.5 mmol) was dissolved in 20 mL DCM with 2 mL of N-methyl-2-pyrrolidone (NMP) under N2 (g) after which N,N'-diisopropylcarbodiimide (2.5 mL, 10 mmol) was added and the mixture was stirred for 30 min under N2 (g). The mixture was sterile filtered (0.2 µm filter) and added to the peptide resin. The resulting mixture was stirred for 2 h under N2 (g) after which it was washed with DCM, methanol and isopropanol sequentially and dried under a vacuum. The peptide was deprotected and cleaved from the resin by adding 20 mL of a 95% trifluoroacetic acid (TFA), 5% ddH2O solution and reacting under N2 (g) with stirring for 2 h. The solution was filtered through an ISOLUTE column to isolate the filtrate and the TFA was removed by roto-evaporation. The peptide was precipitated with 10 mL of cold diethyl ether, which was evaporated with N2 (g). Crude peptide was purified using reverse-phase HPLC (Shimadzu, Japan) with a Phenomenex C18 250 x 10 mm column. The purified peptide was lyophilized to yield a final, solid product and peptide purity was confirmed using mass spectrometry (ABI/Sciex Qstar mass spectrometer).
2.1.2.4 Synthesis of SH3-binding peptide modified methyl cellulose (MC-WBP and MC-SBP)

Thiolated methylcellulose (MC-SH) (100 mg) was dissolved for 4 h in 10 mL of PBS, pH 6.8 at 4 °C. The flask was covered in tinfoil to prevent light oxidation of the thiol from reducing the freely available groups to add binding peptides. A 5-fold molar excess of WBP or SBP to thiol groups (the concentration of thiol/mg MC as determined by Ellman’s assay) was added and reacted for 24 h at 4 °C. The mixture was dialyzed in 8 kDa dialysis membrane in PBS for 48 h at 4 °C, then dH2O for 24 h at 4 °C. The mixture was then sterile-filtered in a biosafety cabinet and lyophilized. Quantification of peptide loading was determined by amino acid analysis at the SPARC Biocentre at Sick Kids Hospital.

2.1.2.5 SH3-IGF-1 Gene synthesis

IGF-1 gene with 5’ overlap with the SH3-linker gene and 3’ overlap with pET32b plasmid was prepared for PCR amplification by combining IGF-1-WBP GenScript plasmid (200 ng) with forward primer CTGGCGGTGCACCGATGCGCCCGGGGAAAAC (1.0 μL of 25 μM), reverse primer GTGCGGCCGCAAGCTTTTATCACGCGATTTCCGGG (1.0 μL of 25 μM), 10x pfx buffer (5.0 μL), 10 mM dNTP (1.5 μL), 50 mM MgSO4(1.5 μL), Pfx platinum polymerase (0.5 μL) and sterile, ddH2O (38.5 μL). The amplification program was 96 °C – 2 min, (96 °C – 30 sec, 60 °C – 30 sec, 72 °C – 1 min) repeated 30x, 72 °C – 10 min. The gene was then PCR-purified using QiaGen PCR purification kit.

The SH3-linker gene with 5’ overlap with pET32b plasmid and 3’ overlap with IGF-1 gene was prepared for PCR amplification by combining SH3-linker-FGF2 Genscript plasmid (200 ng) with forward primer TTCCAGGGCGCCCATGGCCCGGCCCAGAG (1.0 μL of 25 μM), reverse primer TTCCGGGCGCCCATCGGTGCACCGCCAGAG (1.0 μL of 25 μM), 10x pfx buffer (5.0 μL), 10 mM dNTP (1.5 μL), 50 mM MgSO4(1.5 μL), Pfx platinum polymerase (0.5 μL) and sterile, ddH2O (38.5 μL). The amplification program was 96 °C – 2 min, (96 °C – 30 sec, 60 °C – 30 sec, 72 °C – 1 min) repeated 30x, 72 °C – 10 min. The gene was then PCR-purified using QiaGen PCR purification kit.
The SH3-IGF gene was prepared by combining IGF-1 (3 μL of 159 ng/μL) and SH3-linker (3 μL of 124 ng/μL) genes with SH3-linker forward primer (1.0 μL of 25 μM) and IGF-1 reverse primer (1.0 μL of 25 μM), 10x pfx buffer (5.0 μL), 10 mM dNTP (1.5 μL), 50 mM MgSO₄ (1.5 μL), Pfx platinum polymerase (0.5 μL) and sterile, ddH₂O (32.5 μL). The amplification program was 96 °C – 2 min, (96 °C – 30 sec, 60 °C – 30 sec, 72 °C – 1 min) repeated 30x, 72 °C – 10 min. The gene was then PCR-purified using QiaGen PCR purification kit.

The pET32b plasmid was cut with NcoI-HF (200 ng plasmid, 5 μL CutSmart Buffer, 1.5 μL NcoI-HF, 43.5 μL sterile ddH₂O) at 37 °C for 4 h and PCR purified using QiaGen PCR purification kit. The NcoI-cut plasmid was cut with HindIII-HF (43.5 μL of PCR-purified NcoI-cut plasmid, 5 μL CutSmart Buffer, 1.5 μL HindIII-HF) at 37 °C for 4 h. The plasmid was PCR-purified using QiaGen PCR purification kit and concentrated using a centrifuge evaporator to 24.5 ng/μL.

The SH3-IGF gene (80 ng) and double-digested plasmid (100 ng) were combined in a total volume of 10 μL ddH₂O. The mixture (10 μL) was added to LIC InFusion pellet and pipetted up and down to mix. It was then incubated at 37 °C for 15 min and 50 °C for 15 min then incubated on ice for 5 min. The solution (4 μL) was added to chemically competent BL21 E. coli cells (50 μL) which were incubated on ice for 30 min., heat shocked at 42 °C for 90 sec, put on ice for 5 min. and recovered in 1 mL LB at 37 °C for 50 min. The cells were spun down, resuspended in LB (50 μL) and plated on an agar plate containing ampicillin. The plate was incubated at 37 °C for 18 h.

2.1.2.6 Trx-SH3-IGF-1 Expression

Colonies were selected and grown in 2 mL LB media with ampicillin for 16 h after which they were diluted 1:1 (v/v) in 50% glycerol and stored at -80 °C. Cultures in LB (20 mL) with ampicillin were grown for 18 h and used to inoculate 1.8 L LB flasks. These were grown at 37 °C until OD₆₀₀ = 0.8, induced with a final concentration of 0.8 mM IPTG and grown for 24 h at 16 °C.
2.1.2.7 SH3-IGF-1 Purification and Refolding

The cultures were centrifuged at 7 000 rpm for 10 min and resuspended in 20 mL of 6 M guanidine hydrochloride, 0.1 M NaH2PO4, 10 mM Tris, 10 mM imidazole, pH 8.0 (Buffer A). These were incubated on a rotator at 4 °C for 16 h after which they were centrifuged at 45 000 x g for 15 min and the supernatant collected. The supernatant was incubated with 2 mL of Ni-NTA agarose for 25 min, then centrifuged at 5000 rpm for 1 min and the supernatant removed. The Ni-NTA agarose containing the bound SH3-IGF-1 was resuspended in 20 mL of fresh Buffer A and added to a gravity filtration column. It was washed 5 times with 20 mL of Buffer A. The protein was eluted by adding 10 mL of 6 M guanidine hydrochloride containing 0.2 M acetic acid (Buffer F) and incubating for 10 min before eluting. The column was then washed with 40 mL of Buffer F to dilute the protein.

Purified SH3-IGF-1 was dialyzed in 8 000 MWCO dialysis tubing against 4 L of 50 mM Tris, 125 mM Arginine, 5 mM Cysteine for 36 h to refold, changing 3 times. It was then dialyzed against 4 L of 50 mM Tris, 0.5 mM EDTA, pH 8.0 (TEV cleavage buffer) for 36 h. The protein was concentrated to 10-14 mL of solution and 3 mM GSH and 0.3 mM GSSG and then TEV at 6:100 TEV:SH3-IGF-1 ratio (based on nanodrop spectrophotometry readings of SH3-IGF-1 and TEV) were added. The mixture was incubated 24 h at room temperature. The cleaved protein was incubated for 30 min with 2 mL of Ni-NTA and filtered through a gravity filtration column. The eluent was collected, passed through again and collected as pure SH3-IGF-1. This was dialyzed in 3 500 MWCO dialysis tubing against 50 mM Tris, 100 mM NaCl, pH 8.0 buffer for 48 h. The protein was then sterile-filtered and aliquotted in 50 μL fractions and stored at -80 °C for future use.

2.1.2.8 SH3-IGF-1 Bioactivity

MCF-7 Cells were cultured in DMEM/F-12 with 10% FBS, 1% Penicillin/Streptomycin (P/S) and 1% insulin grown to confluence. 2 x 10^6 cells were seeded on a T25 flask and grown for 24 h. 4 mL of Trypsin-
EDTA was added and the cells were incubated for 7 min at 37 °C, at which point the trypsin was neutralized with 9 mL of media. The cells were diluted to 2 x 10^4 cells/mL in media and 200 μL were added per well in a 96-well plate. The cells were incubated for 24 h at 37 °C, at which point the media was removed and serum-free (SF) DMEM/F-12 was added, and the cells were incubated for a further 24 h at 37 °C. The media was removed and either 200 μL of DMEM/F-12 with 10% FBS, 1% P/S and 1% insulin (positive control), SF DMEM/F-12 with 50 ng/mL of commercial IGF-1 (as determined by nanodrop spectrophotometry), SF DMEM/F-12 with 50 ng/mL of SH3-IGF-1 or SF DMEM/F-12 was added. The cells were incubated for 48 h at 37 °C at which time 40 μL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent was added and the absorbance at 490 nm was read.

2.1.2.9 In vitro SH3-IGF-1 Release from HAMC, HAMC-SBP and HAMC-WBP

310 μg/mL SH3-IGF-1 (46.8 μL, 890 pmol) and sterile-filtered artificial cerebral spinal fluid (aCSF, 46.8 μL) were added in sterile, 2 mL Eppendorf tubes to: i) 1.4 mg of HA, 5 mg of MC (HAMC gel); ii) 1.4 mg of HA, 2.09 mg MC-WBP (115 nmol WBP/mg MC, WBP:SH3-IGF-1 = 270:1, HAMC-WBP gel); iii) 1.4 mg of HA, 0.51 mg of MC-SBP (88 nmol SBP/mg MC, SBP:SH3-IGF-1 = 50:1, HAMC-SBP gel). The samples were vortexed and incubated at 4°C for 1 h. These were then speed-mixed at 3 500 rpm for 1 min and centrifuge at 16 162 g for 1 min and incubated at 4°C for 1 h. This process was followed by 2 more centrifuges at 16 162 g for 1 min and incubations at 4°C for 1 h, and then a final centrifuge at 16 162 g and an incubation at 4°C for 18 h.

After 18 h, the tubes were put vertically on a rotator plate at 37 °C for 7 min, after which 900 μL of sterile, 37 °C aCSF was added and the gels were put back on the rotator plate at 37 °C. At the specified time points (1 h, 3 h, 5 h, 8 h, 24 h, 48 h, 96 h, 168 h, 240 h), 900 μL of aCSF was removed and replaced with new, sterile 37°C aCSF. The collected aCSF (180 μL) was added to 0.5% BSA (20 μL) in four aliquots
and flash frozen in liquid nitrogen and stored at -20 °C. The concentration of SH3-IGF-1 was measured by human IGF-1 ELISA from Assaypro.

2.1.2.10 Effects of HAMC-SBP and HAMC-WBP with SH3-IGF-1 on RPE survival and proliferation in vitro

Poly(2-hydroxyethylmethacrylate) (poly-HEMA) stock solution was prepared by dissolving 600 mg of PHEMA in 5 mL of 95% ethanol for 18 h. Poly-HEMA working solution was prepared by dissolving 2 mL of stock solution in 22 mL of 95% ethanol. 60 µL poly-HEMA working solution was added to each well of a 96 well plate under sterile conditions. The plates were incubated at room temperature for 48 h with the lids on and then another 60 µL of poly-HEMA working solution was added to each well. The plates were incubated for 96 h at room temperature with the lids on, then 1 h with the lids off, sealed with parafilm and stored at 4 °C for 24 h before using.

HAMC gels (0.9% HA/0.9% MC (w/v)) were made in 1.25 mL bulk gels of HAMC alone (11.25 mg HA, 11.25 mg MC), HAMC-WBP (11.25 mg HA, 8.25 mg MC, 3 mg MC-WBP) or MC-SBP (11.25 mg HA, 10.13 mg MC, 1.12 mg MC-SBP) by adding 614 µL of serum-free DMEM/F-12 and 614 µL of 50 mM Tris, 100 mM NaCl, pH 8 (HAMC gels) or 614 µL of 53.4 µg/mL SH3-IGF-1 in 50 mM Tris, 100 mM NaCl, pH 8 (HAMC-WBP and HAMC-SBP gels). The samples were vortexed and incubated at 4°C for 1 h. These were then speed-mixed at 3 500 rpm for 1 min and centrifuged at 16 162 g for 1 min and incubated at 4°C for 1 h. This was repeated once and then followed by 2 more centrifuges at 16 162 g for 1 min and incubations at 4°C for 1 h, and then a final centrifuge at 16 162g and an incubation at 4°C for 18 h.

RPE cells (1.6 x 10^5 cells/mL) were diluted 1:6 in Serum-Free DMEM/F-12, DMEM/F-12 + 10% FBS or 0.9% HA/0.9% (w/v) MC HAMC, HAMC-WBP or HAMC-SBP gels (final composition 0.75% HA/0.75% MC w/v). The cells in gels or media were plated on the PHEMA-coated plates at 75 µL per well and incubated 10 minutes at 37 °C. 125 µL of Serum-free or full media was added (Final cell concentration of 1 x 10^4
cells/mL). Prestoblue (22 μL) was added to each well at 0 h, 48 h, 96 h and 148 h and incubated at 37 °C for 6 h. The fluorescence at 590 nm was measured using a Tecan plate reader with a gain of 83.
3. RESULTS

3.1 Synthesis and characterization of HAMC/S3-IGF-1 affinity system

3.1.1 SH3-IGF-1 Gene Synthesis, Expression and Purification

The SH3-linker gene (228 base pairs (bp)) and IGF-1 gene (216 bp) were PCR-amplified from their source vectors and ligated together to create the SH3-IGF-1 gene (444 bp) as detected by agarose gel electrophoresis (Figure 3.1A). The SH3-IGF-1 gene was successfully ligated into a modified pET32b vector and transformed into DH5α E. coli for gene storage and BL21 E. coli for protein expression. Four colonies were screened for the plasmid containing the SH3-IGF-1 gene by PCR amplification. The transformation was confirmed in colonies 1, 3, and 4 by detection of the gene using gel electrophoresis (Figure 3.1B).

![Figure 3.1: SH3-IGF-1 gene synthesis and bacterial transformation. A) Agarose gel showing ligation of SH3-linker gene with IGF-1 gene: Lane 1) SH3 gene (228 bp), Lane 2) IGF-1 gene (216 bp), Lane 3) SH3-linker-IGF-1 fusion gene (444 bp) B) PCR colony screen of transformed BL21 E. coli.](image)
Trx-SH3-IGF-1 was expressed at high levels in BL21 *E. coli* and purified to a suitable level, with no other protein bands than the target protein detected by Coomassie blue staining in SDS-PAGE (≥ 95% purity) (Figure 3.2A,B). The Trx-SH3-IGF-1 protein was successfully solubilized in aqueous buffer and the

![Figure 3.2: SDS-PAGE characterization of Trx-SH3-IGF-1 expression and purification. A) Expression of Trx-SH3-IGF-1 in insoluble fraction of BL21 *E. coli* lysate, 16 °C, 24 h B) Trx-SH3-IGF-1 after Ni-NTA purification](image)
Trx domain was subsequently cleaved by TEV protease. The resulting SH3-IGF-1 protein (16 318 g/mol)

Figure 3.3: SDS-PAGE characterization of purified of SH3-IGF-1 after TEV cleavage of Trx domain. Lane 1) Trx domain (17313 g/mol); Lane 2) purified SH3-IGF-1 (16 318 g/mol) after elution from Ni-NTA resin

Figure 3.4: ESI protein mass spectrum of SH3-IGF-1 (measured mass 16 317.5 g/mol, theoretical mass 16 318 g/mol)

Trx domain was subsequently cleaved by TEV protease. The resulting SH3-IGF-1 protein (16 318 zg/mol)
was purified from both TEV protease and Trx by Ni-NTA chromatography (Figure 3.3). The final product’s mass (16 318 g/mol) was confirmed by mass spectrometry (Figure 3.4).

3.1.2 SH3-IGF-1 Bioactivity

SH3-IGF-1 was found to stimulate MCF-7 proliferation to the same degree as commercially available IGF-1 as determined by an MTS metabolic assay (Figure 3.5). There was a significant difference ($p < 0.05$) between the absorbance signal of formazan product, which is directly proportional to the number of living cells in culture, between serum-free conditions and both commercially available IGF-1 and SH3-IGF-1. There was no significant difference ($p > 0.05$) in the formazan absorbance signal between commercial IGF-1 and SH3-IGF-1.

![Figure 3.5: SH3-IGF-1 has equivalent bioactivity to commercially available IGF-1. MCF-7 cells were cultured on 96 well plates in DMEM/F-12 with 10% FBS and 1% P/S for 24 h, then serum-starved for 24 h. The cells were incubated in serum-free media containing SH3-IGF-1 (50 ng/mL) or commercial IGF-1 (50 ng/mL) for 48 h. Proliferation was measured using an MTS colourimetric assay. Both SH3-IGF-1 and commercial IGF-1 caused significantly different proliferation from the negative control but were not significantly different between each other ($n = 3$ mean ± standard deviation plotted). * indicates $p < 0.05$.](image)
3.1.3 Synthesis of SH3-binding peptide modified methyl cellulose

SBP-maleimide and WBP-maleimide peptides were purified by HPLC and their mass confirmed by mass spectrometry (Figure 3.6A,B). Synthesis of MC-SH was confirmed by Ellman’s assay, with an

Figure 3.6: ESI protein mass spectrum confirming conjugation and purity of HPLC-purified maleimide-conjugated SH3-binding peptides A) WBP-maleimide: observed mass = 1713.9 g/mol (theoretical mass = 1714 g/mol) B) SBP-maleimide: observed mass 2231.3 g/mol (theoretical mass = 2231 g/mol)
Figure 3.7: Amino acid analysis confirming successful immobilization of WBP and SBP to MC-SH to form MC-WBP and MC-SBP. The lack of amino acid detected in adsorption controls demonstrates that there was no non-specific adsorption of WBP and SBP to MC-SH. MC-SH reacted with A) 3-maleimidopropionic-GGGKPPVKKPHYLS (mal-WBP) (substitution 115 nmol WBP/mg MC) B) WBP (adsorption control, 0 nmol WBP/mg MC) C) 3-maleimidopropionic-GGGKKTKTPPPKPSHLKPK (mal-SBP) (88 nmol SBP/mg MC) and D) mal-SBP (adsorption control, 0 nmol SBP/mg MC).
average thiol concentration across multiple syntheses of 111 µM SH/g MC. Synthesis of the final MC-SBP and MC-WBP products was confirmed and quantified by amino acid analysis chromatography and photometry (Figure 3.7). Substitution was on average 130 nmol for WBP/mg MC and 89 nmol for SBP/mg MC with no detectable protein adsorption on dialysis controls of maleimide-peptide with non-thiolated MC (Table 3.1). There was a discrepancy between the thiol substitution (125 nmol SH/mg MC) and the WBP substitution on the second MC-WBP synthesis (154 nmol WBP/mg MC), which was likely caused by an inaccurate standardization of thiol substitution using an old bottle of L-cysteine.

Table 3.1: Quantification of amino acid substitution by amino acid analysis of 2 syntheses of MC-WBP and MC-SBP. Non-specific adsorption of peptide to MC-SH was also measured after reacting WBP or SBP without maleimide with MC-SH.

<table>
<thead>
<tr>
<th>Synthesis</th>
<th>Peptide Substitution (nmol/mg MC)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MC-SBP</td>
<td>MC-SBP Adsorption Control</td>
<td>MC-WBP</td>
<td>MC-SBP Adsorption Control</td>
</tr>
<tr>
<td>1</td>
<td>88</td>
<td>0</td>
<td>115</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>90</td>
<td>0</td>
<td>154</td>
<td>0</td>
</tr>
</tbody>
</table>

3.1.4 Characterization of SH3-IGF-1 release from HAMC-SH3 system

SH3-IGF-1 (890 pmol) was added to HA and MC polymer to form 1.4% HA/5% MC (w/v) gels. This was done with unmodified HA and MC for HAMC gels, or HA and MC with MC-WBP or MC-SBP for HAMC-WBP and HAMC-SBP gels. MC-WBP and MC-SBP were added in molar ratios of 270:1 (WBP:SH3-IGF-1) or 50:1 (SBP:SH3-IGF-1) using the peptide substitution per mg MC as determined by amino acid analysis. These ratios were based on those used in release experiments of SH3-FGF-2 and SH3-ChABC from the HAMC-SH3 system. The ratios were slightly modified from those done previously to better demonstrate the different release rates achievable with SH3-IGF-1 from the HAMC affinity system. The samples were speed-mixed and the system was allowed to reach equilibrium overnight. SH3-IGF-1 release
from the gels was initiated by incubating the mixtures at 37 °C to form gels and adding aCSF as a release medium. The aCSF was removed and replaced at the indicated timepoints and the amount of SH3-IGF-1 in each sample was quantified to characterize the release profile from each gel.

The fastest rate of SH3-IGF-1 release was obtained from HAMC, followed by HAMC-WBP and then HAMC-SBP. HAMC sustained SH3-IGF-1 release over 4 days, HAMC-WBP sustained release of SH3-IGF-1 over 10 days, and HAMC-SBP sustained SH3-IGF-1 release over 24 h, with non-significant amounts being released after 24 h (Figure 3.8). Over 90% of IGF-1 was detected as released from both HAMC and HAMC-WBP, and 20% was detected from HAMC-SBP.

Figure 3.8: In vitro cumulative release profile of SH3-IGF-1 delivered from HAMC, HAMC-WBP (270:1 molar ratio WBP:SH3-IGF-1) and HAMC-SBP (50:1 molar ratio SBP:SH3-IGF-1) hydrogels. SH3-binding peptides attenuate release such that different release profiles are achieved (n = 3 mean ± standard deviation plotted).

Figure 3.9: Cumulative release of SH3-IGF-1 from HAMC, HAMC-WBP (270:1 molar ratio WBP:SH3-IGF-1) and HAMC-SBP (50:1 molar ratio SBP:SH3-IGF-1) against the square root of time. A) The slope of SH3-IGF-1 release from HAMC, HAMC-WBP and HAMC-SBP hydrogels against the square root of time. The slope is representative of Fickian diffusion coefficient, k, for each gel. B) Graphical interpretation of the slopes of SH3-IGF-1 release from each hydrogel and with significance indicated (** indicates p < 0.01, **** indicates p < 0.0001)
Differences in protein release rates between the gels were compared using a plot of cumulative fractional protein release against the square root of time (Figure 3.9A). The linear fit of the data is indicative of Fickian diffusion, and the slopes of the curves are proportional to the protein diffusivity within the gel \(^{247}\). This was done by assuming unidirectional diffusion from a plane sheet as has been done for this affinity system previously \(^{201,202}\), using the equation

\[
\frac{M_t}{M_\infty} = k t^{1/2}
\]

where \(M_t\) is the mass of drug released at time \(t\), \(M_\infty\) is the mass of drug released as time approaches infinity and \(k\) is the diffusion constant and slope of the line in the graph.

All \(k\) values were significantly different between HAMC \((k = 2.59 \times 10^{-3})\), HAMC-WBP \((k = 1.35 \times 10^{-3})\), and HAMC-SBP \((k = 7.92 \times 10^{-3})\) \((p < 0.0001\) between HAMC and HAMC-WBP and HAMC-SBP, \(p < 0.001\) between HAMC-WBP and HAMC-SBP) (Figure 3.9B). HAMC and HAMC-SBP had Fickian diffusion release (applicable for the first 60% of protein release) for the first 8 h, while HAMC-WBP extended Fickian diffusion to 48 h.

3.2 RPE Proliferation and Survival in HAMC hydrogels in vitro

To assess the viability of cells encapsulated in the HAMC/SH3-IGF-1, RPE were encapsulated in HAMC gels and plated on a non-adhesive coating to simulate the non-adhesive nature of diseased Bruch’s membrane in advanced retinal degenerations. The gels were prepared as per the release study but with a 0.75% HA/0.75% MC (w/v) gel instead of 1.4% HA/5% MC (w/v) gel, as was used in the release studies. Stiffer gels were used in the release studies, as previously \(^{201,202}\), to avoid accidentally pipetting the gel when collecting the release samples as occurs when using softer gels, which can be disturbed by gentle pipetting. The 0.75% HA/0.75% MC (w/v) gels were used in the RPE experiments to form a softer gel that
was more conducive to cell viability\textsuperscript{212,248} than the much stiffer gels used in release studies. A Prestoblu
assay was used to quantify cell viability over time. In this assay, cells metabolize resazurin (7-Hydroxy-3\textit{H}-
phenoxazin-3-one 10-oxide) to fluorescent resorufin (7-Hydroxy-3\textit{H}-phenoxazin-3-one), with the intensity
of fluorescence correlated with cell number. The Prestoblu signal was measured at regular timepoints
over a period of 6 days to determine the effects of the gels on RPE viability.
RPE encapsulated in HAMC-SBP/SH3-IGF-1 showed increased viability at day 4 in comparison to RPE in HAMC alone (p<0.05) or SF media (p<0.01) (Figure 3.10). The viability of RPE in all conditions decreased after 6 days, with only RPE in HAMC-WBP/SH3-IGF-1 showing increased viability over RPE in SF conditions. There were no significant differences in viability between RPE in HAMC-SBP/SH3-IGF-1 and HAMC-WBP at any timepoint.
4. Discussion

4.1 SH3-IGF-1 Expression and Bioactivity

IGF-1 exerts its effects through the IGF-1 receptor (IGF-1R), which is essential to human development and growth and is expressed in a wide variety of cells, including RPE. The bioactivity of the SH3-IGF-1 fusion protein was compared to that of commercial IGF-1 using MCF-7, a breast cancer cell line that is commonly used to test IGF-1 bioactivity due to its high sensitivity to IGF-1. The SH3-IGF-1 fusion protein was able to stimulate MCF-7 proliferation to the same degree as commercial IGF-1, demonstrating that the SH3-linker needed for controlled IGF-1 release from the HAMC-binding peptide system did not affect the bioactivity of IGF-1. The level of both SH3-IGF-1 and commercial IGF-1-stimulated MCF-7 was also comparable to that found by other groups. This is consistent with previous studies showing similar negligible effects of an N-terminal SH3-linker group on the bioactivity of SH3-FGF2 and SH3-ChABC fusion proteins expressed for the same HAMC affinity release system. The SH3 fusion to IGF-1 maintained the bioactivity of IGF-1 likely because the linker region (amino acid sequence VSLGNEFPKPSTPPGSSGAP) was designed to allow both distinct protein domains to fold correctly into their native state.

Although the renatured SH3-IGF-1 was bioactive to the same degree as commercial IGF-1, the expression and subsequent purification of the SH3-IGF-1 fusion protein was much different from that of IGF-1 alone. Both proteins were expressed from the pET32 plasmid, which attaches a thioredoxin (Trx) domain to the N-terminus to aid with solubilisation and refolding of the protein. However, the SH3-containing protein was expressed at higher levels in the insoluble fraction of BL21 E. coli lysate than Trx-IGF-1, which was almost entirely expressed in the soluble fraction. Due partly to the loss of protein by precipitation and general purification techniques during insoluble protein refolding and purification, the yield of Trx-SH3-IGF-1 (4-6 mg per L of culture) was much reduced from that obtained by expression of
Trx-IGF-1 alone (420 mg per L of culture). The likely reasons for the insolubility are a change in the isoelectric point (pl) of the protein with the addition of the SH3-linker domain, a result of the intrinsic insolubility of the SH3-linker domain, or an inability of the protein to fold into a soluble state within *E. coli*, possibly due to the SH3 domain interfering with disulfide bond formation.

It is unlikely that SH3-linker makes Trx-SH3-IGF-1 more insoluble than Trx-IGF-1 due to a change in pl. According to theoretical pl calculations, the SH3-linker domain actually changed the protein’s pl from slightly positively charged at a neutral pH to very positively charged at a neutral pH, thereby actually increasing its theoretical solubility. Even though theoretical pl may often be significantly different than actual pl$^{256}$, the pl explanation is still unlikely as Trx-SH3-IGF-1 was able to be refolded and solubilized at high concentrations near physiological pH *in vitro*.

Even though intrinsic properties of proteins are difficult to correlate with protein solubility$^{257}$ it is also unlikely that Trx-SH3-IGF-1 was insoluble when expressed in *E. coli* due to intrinsic insolubility of the SH3-linker. This is because, again, Trx-SH3-IGF-1 was able to be solubilized at high concentrations near physiological pH in *in vitro* refolding, as well as the fact that the two other proteins previously synthesized with the SH3-linker, SH3-FGF-2 and SH3-ChABC, were expressed at high levels and able to be purified from the soluble fraction$^{201,202}$. Since the Trx domain makes IGF-1 expression soluble by aiding in disulfide bond formation,$^{255}$ and the SH3 domain was incorporated between the Trx and IGF-1, domains, it may be that the placement of the SH3 domain interferes with disulfide bond formation of IGF-1. This would explain the high insoluble expression and some precipitation of SH3-IGF-1 during *in vitro* refolding of the protein. The process may also be aggravated in *E. coli* by slow folding kinetics and high expression levels causing aggregation, common reasons for protein deposition in inclusion bodies.$^{258}$
4.2 SH3-IGF-1 release from HAMC, HAMC-WBP and HAMC-SBP

The levels of SBP- and WBP-attachment to MC-SH were consistent with results obtained in previous experiments\(^{201}\). However, the peptide substitution in synthesis 2 (154 nmol WBP/mg) appeared to be greater than the total amount of thiol groups available for substitution (125 nmol SH/mg MC). This is likely because the L-cysteine used to standardize MC-SH substitution in the Ellman’s assay was partially oxidized and was not an accurate measure of thiol substitution of MC-SH\(^-\). Using new L-cysteine, Ellman’s assays on subsequent batches of MC-SH showed thiol concentrations approaching 200 nmol/mg MC and much lower concentrations using the older L-cysteine, supporting this explanation. Release experiments confirmed that the difference between thiol and WBP substitution was not an error with amino acid analysis quantification in the second MC-WBP synthesis, which was performed 4 months after the first. The highly substituted MC-WBP (154 ng WBP/mg MC) gave identical release profiles to the lower-substituted MC-WBP (115 ng WBP/mg MC which had WBP substitution less than thiol substitution), at the same molar concentration and ratio of SH3-IGF-1 (270 nmol WBP: 1 nmol SH3-IGF-1).

Both HAMC-WBP and HAMC-SBP attenuated the rate of SH3-IGF-1 release in comparison to HAMC alone. SH3-IGF-1 in HAMC-WBP and HAMC-SBP both had much slower Fickian diffusion release rates than SH3-IGF-1 in HAMC alone and had significantly different Fickian diffusion release rates from each other. After 24 hours, approximately 67% of SH3-IGF-1 was released from HAMC, 40% from HAMC-WBP and 19% from HAMC-SBP. HAMC and HAMC-WBP released SH3-IGF-1 for 4 days and 10 days, respectively, but HAMC-SBP gels essentially stopped releasing SH3-IGF-1 after 24 h, with only small nanogram quantities of SH3-IGF-1 (0.03% of total) released over subsequent timepoints. This apparent termination of SH3-IGF-1 release is likely due to the following factors: the sequestration of SH3-IGF-1 within the HAMC-SBP hydrogel due to nonspecific interactions between active or unfolded SH3-IGF-1 and HAMC-SBP, and the denaturation of significant amounts of SH3-IGF-1 due to sample freezing and thawing, rendering some protein undetectable by ELISA. The total amount of protein able to be detected in HAMC-
SBP gels (both released and remaining in gels) has previously been shown to be much less than in HAMC or HAMC-WBP gels in previous experiments, supporting this hypothesis.

Electrostatic adsorption of IGF-1 to anionic scaffolds has previously been shown to reach a maximum at 24 hours, but it is unlikely to cause SH3-IGF-1 sequestration in HAMC-SBP gels. Similar release termination was not observed in either HAMC or HAMC-WBP hydrogels, ruling out the hydrogels themselves as the source of the interaction, and non-specific electrostatic adsorption of SH3-IGF-1 to the SBP peptide itself is unlikely since they are both electropositive. An increase in the gels’ negative charge in HAMC-SBP gels due to the lower substitution of MC-SBP vs. MC-WBP is also not a probable cause since the ratio of peptide:SH3-IGF-1 was lower in HAMC-SBP gels (50:1) than HAMC-WBP gels (270:1), and much less MC-SBP (0.51 mg) than MC-WBP (2.09 mg) was used to make them.

There is some evidence that non-electrostatic binding interactions may be involved in IGF-1 adsorption to polymer scaffolds, as high salt concentrations have been found to incompletely abolish IGF-1 adsorption to GAGs. These types of interactions, such as between the hydrophobic surface residues of SH3-IGF-1 and the numerous prolines on SBP, may be partially responsible for the lack of release from HAMC-SBP gels.

Additional sequestration may result from the high concentration of SH3-IGF-1 in HAMC-SBP gels after 24 h causing protein aggregation or denaturation and subsequent adsorption. Any aggregation and denaturation effects, however were not significant in the in vitro studies of RPE viability in HAMC-SBP gels, which showed strong SH3-IGF-1 activity at 4 days, 3 days after the release plateau. This was likely the result of the much lower amounts SH3-IGF-1 (100 pmol/gel vs. 890 pmol/gel for release experiments) used in these experiments being less prone to aggregation.

Freezing and thawing of protein samples has been shown to inhibit protein detection by ELISA and may be partly responsible for the apparent cessation SH3-IGF-1 release from HAMC-SBP gels.
smaller the amount of protein in a release sample, the larger the fraction of it is undetectable by ELISA after freezing and thawing, thereby giving the appearance that much less protein was released than was in actuality. This would have had a greater effect on the release profile of SH3-IGF-1 from HAMC-SBP, which has a slow release rate and therefore less protein per release sample, than from HAMC or HAMC-WBP, which have faster release rates and more protein per release sample. The HAMC-SBP release plateau at 24 h may therefore actually have been a slow diffusive release of SH3-IGF-1 that appeared to release much slower, if at all, due to the majority of the released protein being rendered undetectable by ELISA due to freeze-thaw denaturation.

The levels of SH3-IGF-1 remaining in all hydrogels at the end of the 10 day release were unable to be determined due to polymer interference with the ELISA detection method. Similar absorbance magnitudes were observed for the same sample at a 50-fold or 600-fold dilution, and much greater absorbance magnitudes were observed for a sample diluted 400-fold than the same, undiluted sample. Attempts to dissociate the polymer from the hydrogel for more precise and accurate results using increased salt concentrations or by vortexing the samples were unsuccessful. It therefore remains undetermined how much of the SH3-IGF-1 undetected in HAMC-SBP release remained in the gel at the end of the release and how much was simply undetected because of freeze-thaw denaturation.

4.3 In vitro RPE assay

It is important to note that the gels used in RPE experiments likely did not behave entirely the same as the gels used in the release experiments. Stiffer gels containing more polymer (1.4% HA/5% MC (w/v) for release studies, 0.75% HA/0.75% MC (w/v) for in vitro gels) were used in the release experiments to prevent dissolution from rough handling during the experiment. The gels used in the in vitro experiments therefore likely swelled less and had a smaller diffusion length and a faster release. The in vitro gels, while having the same ratio of binding peptide to SH3-IGF-1 as the gels in the release
experiments, contained less peptide and protein per gel. Lower peptide concentration is correlated with faster release kinetics, which should be independent of SH3-IGF-1 concentration. An estimate of the increase in release rate is difficult without knowing how much the diffusion length changed and how much the weaker gel and media components affected the protein diffusivity and the interaction between the peptides and SH3-IGF-1.

The effects of the HAMC/SH3-IGF-1 system reached on RPE viability a maximum at 4 days and tapered off at 6 days, possibly due to depletion of the nutrients over the course of the experiment. RPE in HAMC-SBP with SH3-IGF-1 showed increased viability in comparison to cells in HAMC (p < 0.05) and SF media (p < 0.01) alone at 4 days but no difference at 6 days. RPE in HAMC-WBP gels with SH3-IGF-1 had significantly increased viability in comparison to RPE in SF media alone at 6 days, but not at any other timepoints. Together, these results demonstrate that the HAMC/SH3-IGF-1 affinity DDS is a promising technology to increase the viability of RPE cells in non-adhesive conditions.

Differences in cell viability between HAMC-SBP/SH3-IGF-1 and HAMC-WBP/SH3-IGF-1 gels were not significant, likely because both gels contained the same amount of SH3-IGF-1 and HA. However the HAMC-SBP/SH3-IGF-1 gels alone showed significance over HAMC and SF conditions at the maximum viability timepoint at day 4. This is possibly due to less SH3-IGF-1 being released between day 2 and day 4 from the HAMC-SBP than the HAMC-WBP gels, allowing more interactions between the growth factor and the encapsulated RPE.

The effects observed in the gels containing SH3-IGF-1 may not be attributable to IGF-1 alone as HAMC itself can promote cell survival and proliferation. CD44 is upregulated in proliferating RPE and the proliferative effects of IGF-1 on RPE may increase the pro-survival and proliferative effects of CD44 on RPE, increasing the beneficial effects of the material. However, the effects are not significant in these experiments between RPE in HAMC alone and HAMC in SF conditions. Experiments with CD44-
knockout RPE, as has been done previously with rod photoreceptors\textsuperscript{212}, would better separate the effects of both HAMC and IGF-1 as both could be beneficial based on these experiments.

5. CONCLUSIONS

HAMC hydrogels with SH3-binding peptides were able to attenuate the release of SH3-IGF-1 fusion protein over several days in comparison to HAMC gels alone as well as increase the proliferation of RPE cells on non-adhesive surfaces \textit{in vitro}. HAMC-SBP gels with SH3-IGF-1 showed a significant increase in RPE proliferation and viability when compared to RPE in SF media alone or HAMC alone. This study further established the applicability of the HAMC-SH3 affinity release system for IGF-1 as well as establishing for the first time its ability to increase encapsulated cells’ viability and proliferation. Further studies using RPE or photoreceptors should be performed to study the effects on transplanted cells \textit{in vivo} as well as the additional effects of SH3-IGF-1 released from the hydrogels on endogenous retinal cells and the retinal environment in general.
6. FUTURE WORK

6.1 In vivo testing of HAMC/SH3-IGF-1 system in the retina

The next step for this system is to test its effects on transplanted and endogenous RPE in vivo. Royal College of Surgeons (RCS) rats, which have a defect in RPE that causes photoreceptor death and retinal degeneration, are a well-established model with which to test RPE transplantation. Transplants of \textit{gfp}-labeled RPE, to differentiate them from endogenous RPE would be injected into the subretinal space at a late stage of the disease to mimic the human retinal environment in clinical trials. The pro-survival, proliferative, migratory and integrative effects of HAMC and IGF-1 on the transplanted RPE could all be observed in the \textit{gfp} labeled RPE and supported by staining for factors such as Ki67 or BrdU for proliferation and propidium iodide (permeable to only dead cells) for cell viability.

Additionally, the effects of IGF-1 and HA beyond their direct effects on the exogenous RPE, such as anti-inflammatory effects for HA and IGF-1 by looking at Müller glial and microglial activation could be observed. IGF-1’s effects on the survival and function of endogenous photoreceptors, RGCs and RPE can also be measured. Photoreceptor survival can be quantified at late stages of retinal degeneration in RCS rats by measuring the thickness of the ONL. RGC survival in RCS rats can be quantified by looking at the density of labeled RGC. An optomotor testing apparatus and electrode-measured luminance thresholds can be used to determine improvements in visual acuity, while the effects of released IGF-1 on endogenous RGC function can be observed by measuring action potential of RGCs in retinal slices as per Chen et al. These broad \textit{in vivo} effects on the retinal environment are a necessary next step to look at the real effects of this material and strategy in improving outcomes for retinal degenerations.

The ability of IGF-1 to promote the survival and proliferation of a wide variety of cell types means that this transplantation system is broadly applicable to many cell types and disease treatments. This is
not limited to transplantation of photoreceptors or RPCs to the eye but could also be used in other cell therapies due to IGF-1’s beneficial effects on cell types such as cardiac 283, cartilage 284, hepatocytes 285 and neurons 286. The effects may be particularly pronounced in photoreceptor transplantations, where the ability of transplanted cells to integrate and improve visual outcome is directly correlated with their ability to survive in the retinal environment 20,55. IGF-1 has pro-survival effects on both cone 287 and rod photoreceptors 126 and effects on photoreceptor or dual photoreceptor/RPE transplants could be studied.

6.2 Simultaneous, multi-factor affinity release

Another avenue of research is the reversal of the SH3-binding peptide system itself to allow the differential release of two or more growth factors. This would involve conjugating the SH3 protein onto MC or HA and expressing fusion proteins of therapeutic factors with SBP, WBP or other SH3-binding peptide tags. This would allow the polymer-protein system to establish different equilibria with the SBP- and WBP-tagged therapeutic factors and release them at different rates. This would be useful for dual-protein therapies where one factor is released before the other for maximum efficacy such as delivering EGF and then EPO to treat stroke injury 288,289. Dr. Katarina Vulic previously attempted to covalently attach SH3-maleimide to MC-SH using the same chemistry as is currently used to attach SBP and WBP peptides to MC. Unfortunately, uncontrolled adsorption limited the conjugation of SH3 to MC to such an extent that it there was not enough SH3 covalently attached to MC to allow the equilibrium to establish itself and control the release of proteins \( C_{\text{ligand}} < K_D \) 197.

A new strategy involves reversing this chemistry. Instead of conjugating the maleimide to SH3 and coupling it with MC-SH, a thiolated SH3 would be coupled to a maleimide-conjugated HA 290, which may avoid some of the adsorption issues apparent in the reaction with MC. This approach is still in the preliminary stages but looks promising. The current undertaking looks at co-delivery of complementary growth factors, such as IGF-1 or GDNF for cell survival and an anti-angiogenic peptide derived from PEDF.
to counteract any unwanted angiogenesis that may result from the delivery of pro-survival factors, which are often pro-angiogenic. The controlled delivery of PEDF, currently one the strongest known anti-angiogenic factors, also has the potential to treat the uncontrolled angiogenesis characteristic of wet AMD.

In this vein, expression of fusion proteins of SBP-IGF-1 and WBP-IGF-1, as well as WBP-FGF2 and SBP-FGF2 have been attempted but have shown drastically decreased expression versus the SH3-IGF-1 and SH3-FGF-2 fusion proteins. This has resulted in difficulty purifying the proteins so they can be used in the system. If this is a signal of a larger trend that fusion proteins containing the binding peptides are difficult to express and purify then chemical conjugation will need to be explored as another means of attaching the binding peptides to their therapeutic protein partners.

The dual delivery strategy should be explored concomitantly with cell delivery of RPE and photoreceptors to the retina. There is also great promise in using this system to deliver cells and growth factors to other difficult-to-access places in the CNS such as the spinal cord and brain where cell replacement and drug delivery strategies are some of the only viable means with which to treat degenerative diseases.
Appendix A: Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>aCSF</td>
<td>Artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>ADC</td>
<td>Anchorage-dependent cells</td>
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<tr>
<td>Akt</td>
<td>Protein kinase B</td>
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<tr>
<td>AMD</td>
<td>Age-related macular degeneration</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
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<tr>
<td>bp</td>
<td>Base pairs</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>cGMP</td>
<td>Nucleotide cyclic guanosine monophosphate</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CNTF</td>
<td>Ciliary neurotrophic factor</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DDS</td>
<td>Drug delivery system</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Deionized water</td>
</tr>
<tr>
<td>DIC</td>
<td>N,N'-diisopropylcarbodiimide</td>
</tr>
<tr>
<td>DMEM/F-12</td>
<td>Dulbecco’s modified Eagle’s medium with F-12</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTP</td>
<td>3,3’-dithiobis(propionic dihydrazide)</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDCI</td>
<td>Ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>EPO</td>
<td>erythrythropoietin</td>
</tr>
<tr>
<td>ERG</td>
<td>electroretinography</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Fibroblast growth factor-2</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell-derived neurotrophic factor</td>
</tr>
<tr>
<td>Gfp</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione (reduced)</td>
</tr>
<tr>
<td>GSSG</td>
<td>Glutathione (oxidized)</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronan</td>
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<tr>
<td>HAMC</td>
<td>Hyaluronan methylcellulose</td>
</tr>
<tr>
<td>HBP</td>
<td>Heparin-binding peptides</td>
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<tr>
<td>HBPro</td>
<td>Heparin-binding therapeutic proteins</td>
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<tr>
<td>hESCs</td>
<td>Human embryonic stem cell</td>
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<tr>
<td>HP</td>
<td>heparin</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
</tr>
<tr>
<td>IGF-1R</td>
<td>Insulin-like growth factor-1 receptor</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cells</td>
</tr>
<tr>
<td>IVT</td>
<td>Intravitreal</td>
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</tbody>
</table>
\( K_d \) Dissociation constant
\( kDa \) Kilodalton
\( k_{off} \) Rate of complex dissociation
\( k_{on} \) Rate of complex association
LB Lennox broth
MAPK Mitogen-activated protein kinases
MC Methylcellulose
MC-CO\(_2^-\) Carboxylated methylcellulose
MC-SH Thiolated methylcellulose
MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MW Molecular weight
MWCO Molecular weight cut-off
NFkB nuclear factor kappa-light-chain-enhancer of activated B cells
NGF Nerve growth factor
Ni-NTA Nitrilotriacetic acid
NMP N-methyl-2-pyrrolidone
NPC Neural progenitor cells
NPD-1 Neuroprotection D1
NT-3 Neurotrophin-3
OD\(_{600}\) Optical density at 600 nm absorbance
ONL Outer nuclear layer
PBS Phosphate buffered saline
PDGF Platelet-derived growth factor
PEDF Pigment epithelium derived factor
PEDF-R Pigment epithelium derived factor receptor
pI Isoelectric point
PI3K Phosphoinositide-3 kinase
PLGA Poly(lactic-co-glycolic acid)
Poly-HEMA Poly(2-hydroxyethylmethacrylate)
P/S Penicillin/streptomycin
RCS Royal College of Surgeons
RGC Retinal ganglion cell
RPC Retinal progenitor cells
RPE Retinal pigment epithelium cells
SBP Strong Src homology 3 binding peptide (KKTKPTPPPKPSHLPK)
SF Serum free
SH3 Src homology 3 protein
SH3-ChABC Fusion protein of Src homology 3 with chondroitinase ABC
SH3-FGF-2 Fusion protein of Src homology 3 with fibroblast growth factor-2
SH3-IGF-1 Fusion protein of Src homology 3 with insulin-like growth factor-1
TEV Tobacco etch virus protease
Trx Thioredoxin
TFA Trifluoroacetic acid
TrkB Tropomyosin receptor kinase B
VEGF Vascular endothelial growth factor
WBP Weak Src homology 3 binding peptide (KPVVKPHYLS)
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