

**ENGINEERING THE NK1 FRAGMENT OF THE HUMAN
HEPATOCYTE GROWTH FACTOR FOR DUAL USE AS A POTENT
AGONIST AND A GENE THERAPY DELIVERY VEHICLE**

by

CHRISTOPHER J.P. MATHY

under the supervision of Dr. Jennifer Cochran, Department of Bioengineering,
Stanford University

11 MAY 2016

**ENGINEERING THE NK1 FRAGMENT OF THE HUMAN
HEPATOCYTE GROWTH FACTOR FOR DUAL USE AS A POTENT
AGONIST AND A GENE THERAPY DELIVERY VEHICLE**

by

CHRISTOPHER J.P. MATHY

11 MAY 2016

*An Honors Thesis Submitted to the Department of Bioengineering in partial
fulfillment of the Honors Program.*

Approved:

Jennifer Cochran, Ph.D.

Date:

Research Advisor from the Department of Bioengineering

Approved:

Jan Liphardt, Ph.D.

Date:

Faculty Reader from the Department of Bioengineering

Approved:

Karl Deisseroth, Ph.D., MD.

Date:

Chair for Undergraduate Education in the Department of
Bioengineering

Preface

I have far too many to acknowledge for their contribution to this work, undoubtedly more than I could fit on a single page. Foremost is Dr. Cochran, who graciously welcomed me into her lab and has been a sterling role model in every way I could imagine. Next must be Sungwon, whose diligence and care has been a steady reminder of the thoughtfulness that must go into any successful project. And I would be remiss not to thank the rest of the Cochran lab for giving me a home that has lasted longer than any other during my time at Stanford.

I must also acknowledge those who supported me outside of the lab environment, for their job was likely the most difficult. Ashley, for being a better listener than any friend could hope for, and challenging me to listen more each day. Uncle Ted, for his steady companionship and support. And my father, sister, and brother, for keeping me grounded even thousands of miles away.

Lastly, this work is dedicated in memory to my mother. Her tireless support left gifts that continue to benefit me each and every day. Truly, my work shall be forever hers.

Table of contents

i. List of Figures and Tables	p.06
ii. Abstract	p.08
iii. Introduction	p.09
1. Background	p.10
1.1 Introduction to the Field of Regenerative Medicine	p.10
1.2 The Biology of Growth Factors	p.11
1.3 The Use of Growth Factors in Regenerative Therapies	p.12
1.4 Growth Factor Engineering for Advancing Therapies	p.14
1.5 Gene Therapy: Another Paradigm for Regenerative Medicine	p.18
1.6 Supercharged Proteins as Delivery Vehicles	p.20
1.7 Project Motivation: A Dual-Function Mitogenic Agent	p.22
2. Methods	p.24
2.1 Cell Culture	p.24
2.2 Internalization Assay	p.24
2.3 Internalization Inhibitor Assay.....	p.24
2.4 Cell Surface Receptor Expression Check.....	p.25
2.5 Confocal Microscopy – Sample preparation and imaging	p.26
2.6 Plasmid Construction	p.26
2.7 Protein Purification	p.27
2.8 MDCK Scatter Assay	p.27
2.9 Electrophoretic Mobility Shift Assay.....	p.28
3. Results	p.29
3.1 The NK1 Fragment of HGF	p.29
3.2 NK1 internalizes into cells expressing cMet and HSPGs	p.29
3.3 Mechanism study of NK1 internalization	p.31

3.4 Co-localization of NK1-mCherry to endocytic vesicles	p.33
3.5 Engineering strategies for NK1 variants	p.37
3.6 M2.2-D127N variant production for study of point mutations	p.37
3.7 MDCK Scatter Assay.....	p.44
3.8 Electrophoretic Mobility Shift Assay.....	p.47
4. Discussion	p.48
5. Supplementary Figures	p.51
6. References	p.56

i. List of Figures and Tables

- Figure 1:**p. 12
Overview of HGF structure and associated signaling pathways.
- Figure 2:**p. 29
Electrostatic surface model of NK1.
- Figure 3:**p. 30
mCherry fusion protein design.
- Figure 4:**p. 31
Internalization of NK1 and N domain into HeLa cells.
- Table 1:**p. 32
Endocytic inhibitors and their targets.
- Figure 5:**p. 33
Internalization inhibition study for elucidation of NK1 endocytic pathway mechanism.
- Figure 6:**p. 35
Internalization co-localization of NK1-mCherry in BJ5TA cells.
- Figure 7:**p. 36
Internalization co-localization of NK1-mCherry in SKOV-3 cells.
- Figure 8:**p. 39
FPLC traces for E62K variants.
- Figure 9:**p. 40
FPLC traces for E62/170K variants.
- Figure 10:**p. 42
FPLC traces for E62/170K-HA2, separated using centrifugal size filter units.
- Figure 11:**p. 43
FPLC traces for N132K Variants of M2.2.
- Figure 12:**p. 44
FPLC traces for cysM2.2-D127N-E62K-mCherry.

- Figure 13a:**p. 45
MDCK Scatter Assay in the absence of heparin.
- Figure 13b:**p. 46
MDCK Scatter Assay in the presence of heparin.
- Figure 14:**p. 47
EMSA of plasmid-binding dimeric NK1 variants.
- Supplementary Figure 1:**p. 51
mCherry fusion plasmid constructs.
- Supplementary Figure 2:**p. 52
cMet receptor expression on the surface of mammalian cells.
- Supplementary Figure 3:**p. 53
Summary of common endocytic pathway mechanisms.
- Supplementary Figure 4:**p. 53
Plasmid constructs for expression cysteine dimer and HA2 tagged NK1 variants.
- Supplementary Figure 5:**p. 54
Reducing PAGE gels show dimer formation of well expressed variants.
- Supplementary Figure 6:**p. 55
Reducing PAGE gels shows dimer formation of cysM2.2-D127N-E62K-mCherry.

ii. Abstract

Regenerative medicine and wound healing are critically relevant topics in the current medical field. Estimates have placed the total number of patients impacted with chronic wounds in the US at 6.5 million, resulting in an annual expenditure of US\$25 billion [1]. Furthermore, the regenerative medicine industry has taken off, reaching over US\$3 billion in both gross annual spending and sales [2].

Growth factor-based therapies for use in regenerative medicine have shown initial promise in pre-clinical testing, but numerous obstacles to effective delivery and translation of expected activity have limited growth factors' clinical potential. Accordingly, growth factor engineering techniques have been developed and employed to improve clinically relevant characteristics such as stability, circulation time, expression yield, and delivery methods. However, as is often the case in clinical applications, extremely potent therapeutics must be engineered to enable significant impact. This study integrates design and delivery methodologies from the fields of gene therapy and supercharged protein engineering to investigate the potential for the NK1 fragment of HGF to serve as a dual-use exogenous mitogen and plasmid delivery agent. Successful internalization was shown and characterized in mammalian cell systems. Additionally, rational engineering of covalent dimerization via the introduction of an N-terminal cysteine residue enhanced mitogenic activity in a number of NK1 variants. Finally, initial evidence is put forth for the ability of NK1 variants to complex with DNA, a critical step in preparing nonviral protein-based delivery vehicles for gene therapy applications.

iii. Introduction

Growth factors, a large family of soluble signaling proteins found in humans and many other organisms, are strongly integrated into biological regenerative and healing pathways. Success in identifying and characterizing numerous growth factors led to initial optimism surrounding their eventual translation to the clinic for use in regenerative therapies. However, therapeutic results have been mixed, as numerous biological factors stand in the way of efficient growth factor delivery and activity. The field of growth factor engineering seeks to take on these challenging factors, which include poor protein stability, overly rapid clearance, and more. Initial studies have shown enormous success in leveraging rational and directed evolution to alter growth factors in beneficial ways. Accordingly, the time is ripe for attempting loftier engineering challenges with greater potential therapeutic rewards. The work presented here seeks to engineer a fragment of the well-studied hepatocyte growth factor to operate at the intersection of medicine's best performing therapeutic regenerative agents and the cutting edge of nonviral gene delivery techniques. A diverse range of techniques are employed in this study, including confocal imaging and flow cytometry for probing internalization mechanisms in mammalian cell systems, recombinant DNA cloning for the rational design of improved protein variants, and several functional assays for evaluation of the candidate protein's potential as a dual-functioning regenerative agent and gene delivery vehicle.

1. Background

1.1 Introduction to the Field of Regenerative Medicine

Regeneration and healing are powerful strategies employed by biological systems to overcome harmful degradation, whether from direct environmental stimuli, disease, or as by-products of cellular processes. From DNA repair and error correcting [3] to whole limb regeneration in salamanders, regenerative mechanisms pervade all scales of biology [4]. Indeed, regeneration and healing play many key roles in human health at the level of tissues. Liver regeneration has been reported following >90% tissue loss [5], and wound repair processes are initiated following injury to a variety of cell types, including those in the epidermis, the spinal-cord, and even the heart post-myocardial infarction [6]. These healing mechanisms enable human tissues to respond to a diverse range of destructive stimuli, often recovering full prior functionality.

Despite the abundance of pre-existing physiological repair pathways, tissues can undergo more severe injury and require additional support to ensure effective healing. This challenge has led to the field of regenerative medicine, which seeks out interventions to actively regenerate or replace human tissues for the purpose of restoring function [7]. The history of the field can be traced back to foundational work in regeneration and developmental biology at the turn of the twentieth century [8], and has culminated presently with expanded research interest in stem cell and tissue engineering. Advances in techniques to support tissue growth *in vitro* and *in vivo* have led to promising recovery in animal models exhibiting impacted function in an assortment of tissues [9].

Experimental success has translated to market potential for regenerative medicine, reflected in recent growth trends in the tissue engineering industry. Total industry spending on tissue engineering and stem cell products or services was estimated at \$3.6 billion in 2012, a 1.8-fold increase in spending compared to

2007 [2]. Furthermore, total annual industry sales reached \$3.46 billion (96% of spending) by 2012, suggesting that regenerative medicine industry has the capacity to operate at profit in the very near future.

1.2 The Biology of Growth Factors

The strategy for implementing regenerative therapies can vary from cellular or scaffold implantation to that of entire assembled tissues [10]; yet, standard requisite functional components can be defined. Namely, any successful tissue engineering therapy requires a seed cell source, a bioactive degradable scaffold, a bioreactor environment conducive to cell viability (be it an *in vitro* mimic or the body itself), and lastly an optimized media cocktail to reproducibly direct cell growth towards tissue level organization. As the field of regenerative medicine has grown, soluble growth factors have emerged as critical constituents of healing-inducing solutions, fulfilling this last functional component.

Broadly, soluble growth factors make up a family of cytokines that stimulates proliferation by binding cell surface receptors [11]. Most receptor partners are tyrosine kinases, whose intracellular domains phosphorylate cytoplasmic proteins to initiate cell signal transduction pathways and activate expression of genes controlling proliferation. In addition to their mitogenic capabilities, growth factors are known to stimulate angiogenesis, chemotactic recruitment of inflammatory cells and fibroblasts to a tissue site, remodeling of the extracellular matrix, and accelerated local synthesis of other growth factors and cytokines. These mechanisms make growth factors key agents in the body's physiological regeneration and wound healing processes.

Hepatocyte growth factor (HGF) is a well-characterized mitogen present in humans, and is the focus of the engineering efforts presented in this work [12]. HGF is a dimeric protein of molecular weight 84 kDa, consisting of an α -subunit (69 kDa) and a β -subunit (34 kDa) linked by a disulfide bond (Figure 1a). HGF

primarily binds to cMet, a receptor tyrosine kinase containing a 50 kDa extracellular α -chain and a 145 kDa intracellular β -chain housing the kinase domain (Figure 1b) [12,13]. HGF signaling, initiated via cMet binding, induced dimerization, and tyrosine kinase activation, has been shown to promote angiogenesis and stimulate mitogenic pathways in endothelial cells [14], enable liver regeneration and repair [15], and play critical roles in embryo development by inducing cell motility and morphogenesis.

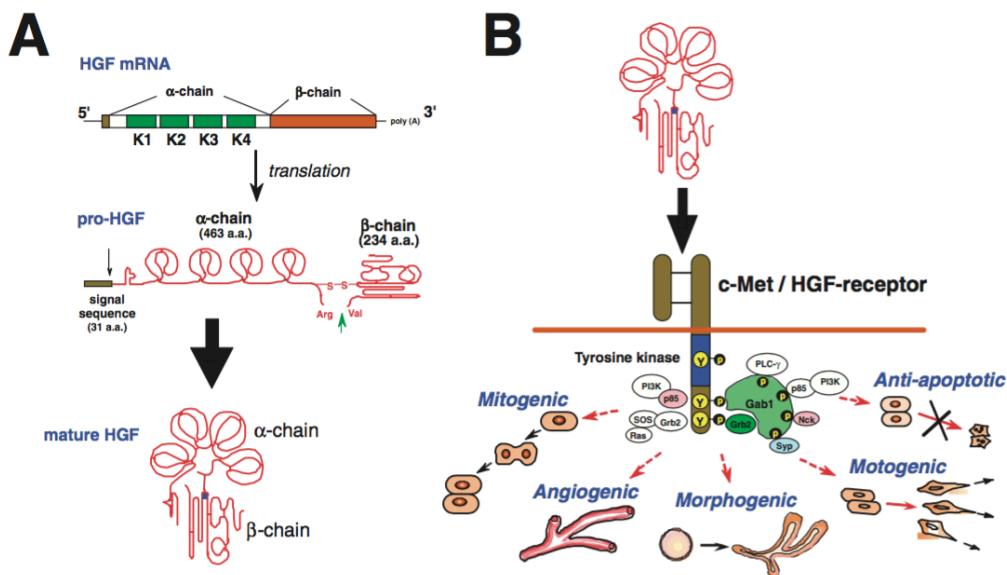


Figure 1: Overview of HGF structure and associated signaling pathways. (A) HGF is translated in its pro-HGF form, containing a signal sequence, the four Kringle domains making up the α -subunit, and the β -subunit. (B) Activation of cMet via HGF binding initiates kinase domain activity and stimulates numerous cell and tissue growth pathways. (Figure taken from Nakamura and Mizuno, 2010) [13].

1.3 The Use of Growth Factors in Regenerative Therapies

Several research efforts have explored the role of growth factors in promoting healing and regeneration. Growth factors have been shown to improve healing in a variety of wound types, both in otherwise healthy animals and in

those impacted by additional impairments, such diabetes, malnutrition, infection, hypoxia, and side-effects of chemotherapy agents, steroids, and radiation [11]. One representative study examined HGF in the context of skin wound healing [17]. Histological results revealed that HGF significantly accelerated wound re-epithelialization in diabetic mice. In another instance, transforming growth factor alpha (TGF α) expression was shown to be up-regulated following liver injury, coinciding with DNA replication [18]. A third study examined the impact of basic fibroblast growth factor (bFGF) in chick embryonic neural retina [19]. Polymer implants allowed for the sustained release of growth factor in chick embryos with surgically neural retina. Embryos receiving implants containing 100 ng bFGF showed complete retinal regeneration at a stage of differentiation appropriate to the embryo. Furthermore, regeneration scaled with the amount of growth factor included, with implants containing 10 ng bFGF leading to partial regeneration in about half of the embryos, and control implants (containing only BSA) resulting in no regeneration. When taken together, this study and those aforementioned establish the critical role of growth factors in initiating and sustaining regeneration and healing in a wide variety of tissue types.

The enormous potential for growth factors to improve wound healing and tissue regeneration has translated to promising results in the clinic. Of note, granulocyte-macrophage colony stimulating factor (GM-CSF), platelet derived growth factor (PDGF), members of the fibroblast growth factor family (FGF) and vascular endothelial growth factor (VEGF) have shown some positive impact in clinical trials for patients with various wound types, including diabetic foot ulcers, chronic venous ulcers, pressure ulcers, and burns [20]. However, significant obstacles to maximizing growth factor efficacy remain. In addition to the optimization of therapeutic protocol (i.e. the formulation of solution used, dosages, and route of administration), issues with the mode of delivery likely contribute to reduced efficacy of growth factor treatments [21]. For example,

rapid degradation of exogenous growth factor at the wound site limits the ability for sustained activity between applications. This degradation can be the result of high protease activity in the wound sites [20], oxidative activity, or general protein denaturation [21]. In addition to these stability challenges, other obstacles related to protein identity -- such as low recombinant expression yield, the difficulty and cost of purification, and off-target activity -- and the need for further optimization of delivery methods have limited the widespread and effective use of growth factors in clinic [22]. These challenges are especially salient in the case of HGF. Despite numerous promising pre-clinical results, issues with ease of manufacturing and sustained delivery have kept exogenous HGF therapies out of clinic [23].

1.4 Growth Factor Engineering for Advancing Therapies

Born out of these significant clinical obstacles has been the field of growth factor engineering. By applying protein engineering principles, researchers can improve growth factor stability, expression yield, activity levels, and target specificity, enabling more successful therapeutic interventions in the fields of wound healing and regeneration. Furthermore, the tight linkage between cell growth processes and cancerous phenotypes has placed growth factors as ideal candidates for anti-cancer therapies. Receptor tyrosine kinases are well-known proto-oncogenes, exhibiting aberrant activation profiles when mutated and resulting in mitogenesis and angiogenesis beneficial to tumor progression, invasion, and metastasis formation [24]. By engineering growth factors for inhibitory as opposed to agonistic activities, the clinical impact of growth factor therapies is significantly broadened to include cancer treatment in addition to wound healing and regenerative medicine.

There are abundant examples of successful protein engineering to exhibit greater control over growth factors for therapeutic applications [22,25]. Our group

has previously employed directed evolution strategies to engineer NK1, an HGF fragment consisting of the N terminal amino acids and the first Kringle domain, for enhanced thermal stability (improvement of 15°C in melting temperature) and 40-fold better expression yield over wild-type [26]. Another study introduced combinations of point mutations in fibroblast growth factor 1 (FGF-1) resulted in an improvement of up to 7.8°C in denaturation temperature. In both studies, thermal stability was engineered without loss of biological activity.

Additional strategies to enhance growth factor stability and limit blood clearance include the cyclization of proteins by connecting the N- and C-termini and the conjugation of polyethylene glycol (PEG), which increases circulating half-lives of proteins by increasing their hydrodynamic radius. These strategies were elucidated through the engineering of interferon alpha, a fellow cytokine with similar size to growth factors, and PEGylation has been applied to growth factors in the clinic [22].

Ligand-receptor interactions provide a significant opportunity to engineer the functional impact of growth factor therapies, as receptor binding is the primary means of growth factor activity. That is, adjustment of receptor binding affinity has been shown numerous times to vary the biological activities of growth factors [27]. For example, a study of EGF mutants showed that receptor binding on-rates correlates with receptor activation [28]. However, it should be noted that a stronger binding interaction (which can reflect both enhanced on- and off-rates) does not always correlate with enhanced functional activity, as tighter binding often leads to up-regulation of ligand-receptor complex internalization, effectively reducing the number of available surface receptors to sense ligand signals [27].

In addition to enhancing pre-existing biological activity, engineering growth factor binding can significantly alter the biological activity itself, most notably by transforming proteins that activate pathways (agonists) into those that inhibit (antagonists), or vice-versa. For example, wild-type NK1 agonistically but

the previously mentioned NK1 variants engineered by our group showed antagonism [26]. This altered function was found to be due to mutations disrupting the NK1 homodimerization interface. Typical growth factor receptor activation involves the binding of a symmetrical growth factor dimer to a single receptor, followed by the recruitment of a second receptor to the open binding interface at the opposite pole. The formation of the dimerized receptor complex enables kinase activity, and initiates intracellular signaling. Our group was able to recover agonistic activity in engineered NK1 variants by alternatively introducing point mutations to restore noncovalent homodimerization to NK1, and by introducing N-terminal cysteine residues to allow for covalent dimer formation. Covalent dimer NK1 variants showed enhanced agonistic activity compared to wild-type NK1, almost reaching the level of full-length HGF. In another example, a mutant VEGF was engineered to inhibit proliferative pathway activation [29]. A VEGF heterodimer was designed by substituting loop/turn structures drawn from PDGF, which shares structural homology to VEGF, at one binding interface to knockout receptor activating capabilities. While receptor affinity remained comparable to that of wild-type VEGF, the heterodimer antagonized proliferation by binding but not activating receptors, counteracting the agonistic activity of the wild-type during co-incubation. These studies showcase the potential for binding interaction engineering to significantly alter the functional activity of ligand-receptor interactions. Importantly, the ability to engineer growth factors as antagonists enables growth factor use in cancer therapeutic applications, in which mitogenic pathways are dysregulated.

A final field of growth factor engineering involves the incorporation of delivery methods into design strategies. Polymer matrices, for example, have been identified as ideal platforms for delivery [21]. Growth factors encapsulated in matrices are protected from denaturing mechanisms in the ECM, and their release

kinetics can be extended and finely tuned. Likely, the most successful therapeutics will integrate both growth factor and delivery method engineering.

HGF fragment delivery has been the subject of multiple integrative engineer examples. In one case, a dimeric NK1 variant was delivered in an extracellular matrix-derived hydrogel in a myocardial infarction rat model [23]. The hydrogel successfully prolonged delivery of the protein, and *in vivo* results showed prevention of negative left ventricular remodeling. In another example, 1K1, a non-glycosylated agonistic fragment of HGF, was encapsulated in nanoparticles of poly-lactic acid-glycolic acid (PLGA) copolymer [30]. This study synthesized several engineering principles to optimize the final therapeutic agent. First, removing glycosylation was critical in improving protein structural homogeneity, and using a truncated mutant of HGF reduces protein aggregation. Both of these characteristics allowed for higher expression and yield of the fragment. Second, 1K1 had been rationally engineered to maximize biological activity based on structural analysis of the fragment's binding to cMET, the receptor partner of HGF. Finally, the PLGA nanoparticles were engineered, and impregnated with 1K1 molecules. The resulting therapeutic agent resulted in sustained activation of angiogenic signaling, even enhancing neovascularization in murine and zebrafish models.

Another growth factor study synthesized several engineering strategies to stimulate brain tissue regeneration after stroke in a murine model, a considerable achievement considering the isolation of the brain from systemic circulation by the blood brain barrier [31]. Two growth factors, EGF and erythropoietin (EPO), were used to simulate endogenous neural stem/progenitor cells and induce tissue repair. EGF was PEGylated, both growth factors were encapsulated into PLGA particles with varied release kinetics, and the particles themselves were incorporated into a hyaluronan methylcellulose (HAMC) hydrogel. These designs allowed for sequential release of EGF-PEG followed by EPO over a period of 2

weeks, resulting in tissue repair. Critically, the sustained and sequential release strategy enabled regeneration whereas previous attempts using EGF-PEG and EPO with only the HAMC gel did not. This study and those preceding display the essentiality of diverse and advanced engineering strategies in enabling clinical-grade efficacy of growth factor-based therapies.

1.5 Gene Therapy: Another Paradigm for Regenerative Medicine

Simultaneous to the development of growth factor interventions, the past 25 years have seen the advent of another significant field in regenerative medicine: gene therapy. Gene therapy refers to those treatments that seek to alter cellular behavior by transferring genetic material directly into cells [32]. The suggestion of implementing gene therapy in patients can be traced to Edward Tatum, who in 1966 proposed “that the first successful genetic engineering will be done with the patient’s own cells, for example, liver cells, grown in culture. The desired new gene will be introduced, by directed mutation, from normal cells of another donor by transduction or by direct DNA transfer. The rare cell with the desired change will then be selected, grown into a mass culture, and reimplanted in the patient’s liver” [33]. The potential to fine-tune cellular phenotypes by directly altering DNA incited an explosion of interest in gene therapy in the later half of the twentieth century. While the history of gene therapy since Tatum has not been without significant setbacks, most notably the tragic death of Jesse Gelsinger during a 1999 clinical trial of adenovirus delivery, by 2013 more than 1800 approved clinical trials had been initiated and gene therapy products had been approved in China and the European Union [34].

Cardiovascular disease is the second most common indication treated by gene therapy, behind only cancer [35]. Growth factors, especially VEGF, have emerged as agents for cardiovascular gene therapy because of their ability to induce angiogenesis, for example in the treatment of ischemic myocardium or

peripheral skeletal muscles [36]. VEGF gene delivery for these conditions was first explored in the late 90s, with local application of naked DNA encoding VEGF resulting in reduced ischemia in the heart and limbs [37-39]. Since then, numerous clinical studies to treat cardiovascular disease with growth factor gene delivery have been undertaken, focusing on VEGF, FGF, and HGF [40,41].

Results have been promising, but some argue that the clinical relevance remains unproven. Proposed areas for improvement include the therapy regime design, delivery method, growth factor choice, and study population composition, as most trials are conducted on elderly diabetic patients that may not be able to respond to pro-angiogenic therapy [42].

Other directions for growth factor gene delivery have included periodontal tissue engineering [43], retinal gene therapy for the treatment of blindness [44], and wound healing [45,46]. Wound healing is of particular interest, given that direct application of exogenous growth factors in clinical testing has proven less effective than pre-clinical models would suggest, as discussed above (see section 1.3). Gene therapy, then, can be considered another means of improving growth factor-based treatments, alongside protein engineering. Specifically, transforming cells to produce growth factors endogenously offers localized and sustained release, overcoming issues with stability, protein loss, and non-optimal release kinetics (in this last case handing over control of growth factor release to the cell's transcriptional and translational regulation) [47]. One representative study transfected the human HGF gene into rats with fatal liver cirrhosis [48]. The hemagglutinating virus of Japan (HVJ) was enlisted as a viral vector, and the complex was delivered by liposomes HGF expression stimulated hepatocyte mitosis, resolving fibrosis and enabling survival.

Viruses, such as the HVJ employed in the aforementioned study, are currently the most common form of delivery agent, having been used in ~70% of gene therapy clinical trials before 2014 [49]. However, numerous factors limit the

potential of viral vectors, including carcinogenesis, immunogenicity, difficulty of production, and bounded DNA packaging capacity. In response, the field of gene therapy has begun exploring non-viral delivery vectors. Notably, direct intramuscular injections of VM202, a non-viral plasmid DNA expressing two isoforms of HGF, for treatment of critical limb ischemia was shown to be safe and tolerable in a phase I clinical trial [50], and efficacious in a phase II clinical trial [51]. The phase III clinical study on chronic non-healing foot ulcers was announced in 2015, and represents a promising step for translation of HGF gene therapies to the clinic [52].

Other growth factors have been successfully delivered with non-viral methods as well. One group transformed plasmid DNA encoding human EGF into a porcine wound model by coating ~2 μm gold beads with DNA [47]. Wound fluid EGF concentrations increased 190-fold and healing occurred 20% faster than controls. Another study evaluated wound healing in rat skin flap models when treated with plasmid DNA encoding recombinant VEGF₁₆₅ [53]. DNA was delivered directly to an ischemic skin flap wound site with or without electroporation. Results showed significant increase in VEGF expression with electroporation as compared to delivery without electroporation and increased recovery of baseline and post-operative perfusion compared to control groups. A third study also effectively delivered minicircle DNA encoding VEGF₁₆₅, employing a cationic, branched polymer with an arginine surface [54]. Use of the minicircle DNA and the arginine-conjugated polymer result in increased delivery efficacy, and both are believed to trigger reduced immune responses.

1.6 Supercharged Proteins as Delivery Vehicles

All cellular delivery vehicles have in common the ability to bypass the cellular membrane. Section 1.5 detailed gene therapies enabled by delivery by a diverse range of strategies, including virus-based, polymer, and particle delivery,

as well as electroporation. Another important subclass of delivery vehicles consists of amino acid chains with high net charge. Short cationic chains (< 30 amino acids) of this sub-class are known as cell-penetrating peptides [55]. These peptides can transport a variety of cargo into cells, including small molecules, other peptides, DNA fragments, plasmid DNA, proteins, phages, liposomes, and magnetic nanoparticles [55-58]. Cell-penetrating peptides internalize into cells by several mechanisms, including receptor-mediated endocytosis, macropinocytosis, and direct penetration [59]. The internalization mechanism varies from one cell-penetrating peptide to another, and has even been shown to depend on the presence, type, and size of cargo. Understanding internalization mechanisms is critical for developing functional delivery vehicles, as many endocytic pathways lead to lysosomal degradation, effectively blocking cargo from entering the cytoplasm (For further elaboration on the biology and importance of cellular internalization mechanisms, see sections 3.3-3.4 and Supplementary Figure 3).

Larger proteins that contain a high net charge have come to be known as supercharged proteins. The study of supercharged proteins has been led by the group of Dr. David R. Liu, and began with the artificial supercharging of green fluorescent protein (GFP) to both a cationic and an anionic net charge above 30 [60]. These supercharged variants exhibited resistance to aggregation and enhanced stability, withstanding heat up to 100°C. Similarly to cell-penetrating peptides, cationic supercharged proteins internalize into cells, and even deliver cargo such as siRNA, plasmid DNA [61,62], and proteins [63,64]. While protein cargo delivery can occur through covalent fusion to a supercharged protein, nucleic acid delivery is enabled by non-covalent charge-based binding of positive surface residues to the anionic nucleic acid backbone. Additionally, exploration into the uptake mechanisms of supercharged GFP suggest that protein-containing vesicles maybe not effectively progress through traditional degradation pathways, allowing time for cargo to exit vesicles [65]. These two characteristics – non-

covalent binding of nucleic acids and non-degradative internalization pathway progression – make supercharged proteins clear candidates as delivery vehicles for gene therapies.

Furthermore, the proposed connection between delivery activity and surface charge suggest that many other proteins – either naturally occurring or recombinantly engineered – could serve as delivery vehicles as well. To this end, the Liu group performed a bioinformatics search to find naturally supercharged human proteins (NSHPs), defined as those proteins in the human proteome whose charge units per kDa ratio is greater than 0.75 [64]. Importantly, by restricting the search to human proteins, the immunogenicity of identified proteins should be quite low. Among the identified proteins was N-HGF, an HGF fragment consisting of the amino-terminal domain, whose charge per kDa ratio is 1.23 (calculated using a theoretical net charge of +14 and a molecular weight of 11.4 kDa). N-HGF was shown to functionally deliver a Cre recombinase fusion into cells, establishing its ability to internalize and deliver protein cargo.

1.7 Project Motivation: A Dual-Function Mitogenic Agent

The identification of N-HGF as an NHSP suggests a compelling step forward for growth factors in regenerative medicine: engineer an HGF fragment as a dual function mitogenic activator, operating as both an exogenous agonist and a gene therapy delivery vehicle. The engineered protein would retain high cationic surface density, allowing for DNA plasmid complex formation. Delivery of the exogenous engineered fragment-DNA complex to wound sites or other regenerating tissues would result in receptor activation and proliferative pathway initiation in the short term. Cellular internalization would follow, leading to the release of an HGF-encoding plasmid cargo such as VM202. Sustained endogenous release of encoded HGF would then support longer term healing, constituting an effective gene therapy. Given its status as an NHSP, the

engineered an HGF fragment is hypothesized to exhibit enhanced stability and resistance to aggregation, further demonstrating it to be an optimal molecule for development as a dual-function growth factor for use in advanced regenerative therapies.

2. Methods

2.1 Cell Culture

HeLa cells and MDCK cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher) supplemented with 10% Fetal Bovine Serum (FBS, Gibco®) and 1% penicillin–streptomycin (P/S). BJ5TA cells were cultured in a media containing a 4:1 ratio by volume of DMEM to medium M199 (Thermo Fisher), supplemented with 10%FBS and 1% P/S. SKOV-3 cells were cultured in RPMI-1640 media (Thermo Fisher) supplemented with 10% Fetal Bovine Serum (FBS, Gibco®) and 1% penicillin–streptomycin (P/S). All incubations for mammalian cell culture occurred at 37°C, 5% CO₂ unless otherwise noted.

2.2 Internalization Assay

HeLa cells were seeded onto confocal imaging chambered slides (Millicell EX slide 8-well glass PEXGS0816) for incubation over 1 (3.5×10^4 cells) or 2 (7×10^4 cells) nights. Cells were carefully washed with media, followed by incubation for 4 hours in media containing 100 nM of the desired protein. Cells were then washed 3x with cold PBS + Heparin (20 U/mL) to wash away excess protein, fixed in % formaldehyde, and DAPI stained with Prolong Gold Antifade (Thermo Fisher). After mounting of a coverslip, sealing with nail polish, and letting dry for ten minutes, cells were able to be imaged. Images were taken on a Leica SP2 microscope, using oil immersion. Samples did not typically remain viable for repeat imaging, regardless of storage in a dark, cool environment.

2.3 Internalization Inhibitor Assay

SKOV-3 and BJ5TA cells were seeded in 500 uL media overnight in a 48-well plate at a concentration of 2×10^5 cells per mL. After washing 1x with PBS,

cells were pre-treated for 1 hour with 200 uL of one of the endocytic inhibitors in serum-free media at the following concentrations: mannan – 2 mg/ml; amiloride – 5mM; chlorpromazine – 5 ug/ml; dynasore – 50 uM; nystatin – 25 ug/ml; filipin – 5 ug/ml; and sodium chlorate – 80 mM. Then, NK1-mCh was added to the media at a final concentration of 50 nM. After 3 hours of incubation at 37°C, cells are washed 1x in PBS, trypsinized, and transferred to microcentrifuge tubes. Next, an acidic wash buffer was used to strip away any proteins bound to the cell surface. Acidic wash buffer consists of 50 mM glycine, 100 mM NaCl, and 0.1% polyvinylpyrrolidone. Cells were spun down for five minutes and resuspended in 100 uL of wash buffer. After 30s, cells were rescued with 1 mL of PBS+0.1% BSA (BPBS), spun down, and washed in BPBS buffer another time. At this point, cells were prepared for analysis using flow cytometry. Flow cytometry result analysis as done with the FlowJo software package.

2.4 Cell Surface Receptor Expression Check

Mammalian cells were prepared by aliquoting 2×10^5 cells per microcentrifuge tube. All steps were performed on ice. Cells were washed in PBS+0.1% BSA (BPBS), spun down, and re-suspended in 100 uL of 20 ng/ml AF276 stock (R&D Systems #P08581), except for control cells, which were resuspended in BPBS. Cells were incubated for 1.5 hours at 4°C, with rotation. Washing was repeated with BPBS, followed by re-suspension of all cells in 50 uL of antiGoat IgG PE. Cells were incubated for 1 hour at 4°C, with rotation and in the dark. Cells were washed once more and run through flow cytometry analysis to measure surface fluorescence.

2.5 Confocal Microscopy – Sample preparation and imaging

Cells were seeded in 500 uL complete media overnight in 48-well plates at a concentration of 10^4 cells per well. Cells were then washed 3x with PBS, followed by up to 4 hours incubation with protein in serum-free media (Images in section 3.4 show experiments pertaining to 30 min of protein incubation). The following concentrations were used: NK1-mCh – 100 nM; Dextran-TR – 500 ug/ml. When Lysotracker Green DND-26 (Life Tech.) was being used, it was added to a final concentration of 50 nM with 30 minutes remaining. Cells were then washed 3x with PBS and fixed with 4% formaldehyde. For EEA-1 stainings, a permeabilization protocol was used to allow for antibody penetration into the cytosol. 200 uL of 0.1% Tween-20 in PBS was added to the cells, which were then incubated for 10 minutes on ice. The cells were then washed 5x with PBS. Next, non-specific binding was blocked by incubating cells in 250 ul of 2% BSA in PBS (Binding Buffer, BB) for 45 minutes on ice. The cells were then washed 5x with 0.5% BSA in PBS (Wash Buffer, WB). Primary antibody incubation occurred for 1 hour in 200 uL BB at a dilution of 1:200 antiEEA-1 (mouse, anti-human, BD 610456). Following 5 washes with WB, secondary antibody incubation occurred for 1 hour using 1:200 (10 ug/ml) antiMouse goat-488. Cells were washed 5 final times with WB, DAPI stained, mounted, and imaged using ZenBlack 2011 software and a Leica SP8 confocal microscope.

2.6 Plasmid Construction

Plasmid constructs were prepared in pPIC9k, a plasmid vector used in *Pichia pastoris* expression systems. DNA constructs were confirmed throughout cloning on 1% agarose gels, and cloning was enabled by Thermo Fisher GeneJET Gel Extraction and Plasmid Miniprep Kits. Sequence verified construct-containing plasmids were transformed into Top10 *E. coli* cells, grown up, and extracted by midiprep. Plasmids were linearized for yeast transformation through

a SacI digestion, and electrocompetent yeast were transformed via electroporation. Successful transformation was selected for on YPD+Geneticin plates.

2.7 Protein Purification

Yeast colonies were picked and grown up in BMGY media at 30°C. Small-scale tests for expression were conducted with 5 ml cultures, but all purification efforts required a scale up of at least 50 mL BMGY (enough for 500 mL of induction culture). Protein production and secretion was induced by switching to BMMY media and incubating over 3 days at 20°C with once daily addition of methanol at a final concentration of 0.5%. 10 mM Imidazole was used to precipitate expressed protein at pH ~ 7.8 for 1 hour. Immediately after, filter supernatant containing precipitated protein was run through columns packed with Ni-NTA beads and eluted in PBS + 500 mM NaCl (PBS500) + 500 mM imidazole. Amicon® Ultra-4 Centrifugal Filter tubes were then used to dilute out imidazole. Samples were then run through FPLC, using either a Superdex S75 size exclusion column or a HiTrap Heparin trap column (both GE Life Sciences). Purified proteins were run on NuPAGE Protein Gels, using 4-12% or 4-20% gels, for size and dimerization confirmation.

2.8 MDCK Scatter Assay

MDCK cells were seeded in 100 uL growth media overnight in a 96-well plate at 10^3 cells per well. Proteins were added to a concentration of 5 uM and incubated in media for ~24 hours. 100 uL of 0.5% crystal violet in 50% ethanol, 50% water was added to cells, and incubation occurred for 10 min at room temperature. Wells were then washed 3x with water, allowed to air dry for >1 hr, and imaged under a microscope.

2.9 Electrophoretic Mobility Shift Assay

Plasmid DNA and protein samples were mixed in water for 30 minutes at room temperature to allow for complex formation. TrackIt Cyan/Yellow Loading Buffer (Thermo Fisher) was added to the samples, which were then loaded onto a 1% agarose gel (with TAE). The gel was run at 55 V for ~4 hours, until the bands had appeared to reach the bottom of the gel. The gel was stained with 75 ml TAE + 7.5 uL SYBR gold (Thermo Fisher) while rocking for 30 min, and then imaged using UV.

3. Results

3.1 The NK1 Fragment of HGF

Previous insights into HGF biology were leveraged when selecting a candidate growth factor structure for this study. Specifically, previous studies conducted by our group and others showed that the NK1 fragment of HGF was highly amenable to engineering for enhanced yield, stability, and both agonistic and antagonistic activity [23,26,66]. NK1, shown in Figure 2, has a molecular weight of 22 kDa [67] and an estimated surface charge of +13, based on a counting of charged surface residues using crystal structure visualization in PyMol [68]. The charge to molecular weight ratio of wild type NK1 is therefore ~0.6, making it close to the cutoff of 0.75 defined by Cronican, et al (2011).

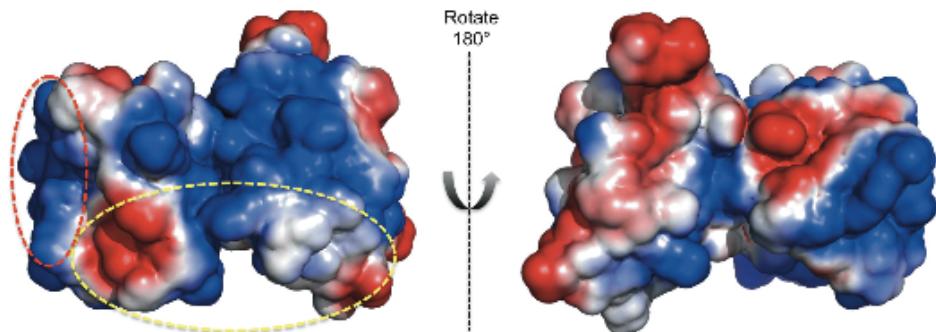


Figure 2: Electrostatic surface model of NK1. Positively and negatively charged residues are shown in blue and red, respectively. Dotted ovals show binding sites to cMet (yellow) and HSPGs (red). The HSPG binding site sits almost exclusively on the N domain, while the cMet binding site spans the junction between the N and K1 domains.

3.2 NK1 internalizes into cells expressing cMet and HSPGs

To initially characterize the potential of NK1 to internalize into cells, as suggested by its highly cationic surface charge density, fluorescent protein fusions with mCherry were produced for confocal imaging (Figure 3, Supplementary

Figure 1). Importantly, heparan sulfate proteoglycans (HSPGs) were implicated as critical to internalization of supercharged GFP [69] and the NK1 fragment contains binding sites to HSPGs in addition to cMet. In NK1 binding, HSPGs are known to be important co-receptors that can induce homodimerization and partial agonistic activity in NK1 [70,71]. Conversely, the N fragment of HGF binds HSPGs, but not cMet [68] To investigate whether HSPGs can enable internalization into cells in the absence of cMet binding, an N-mCherry fusion was constructed as well.

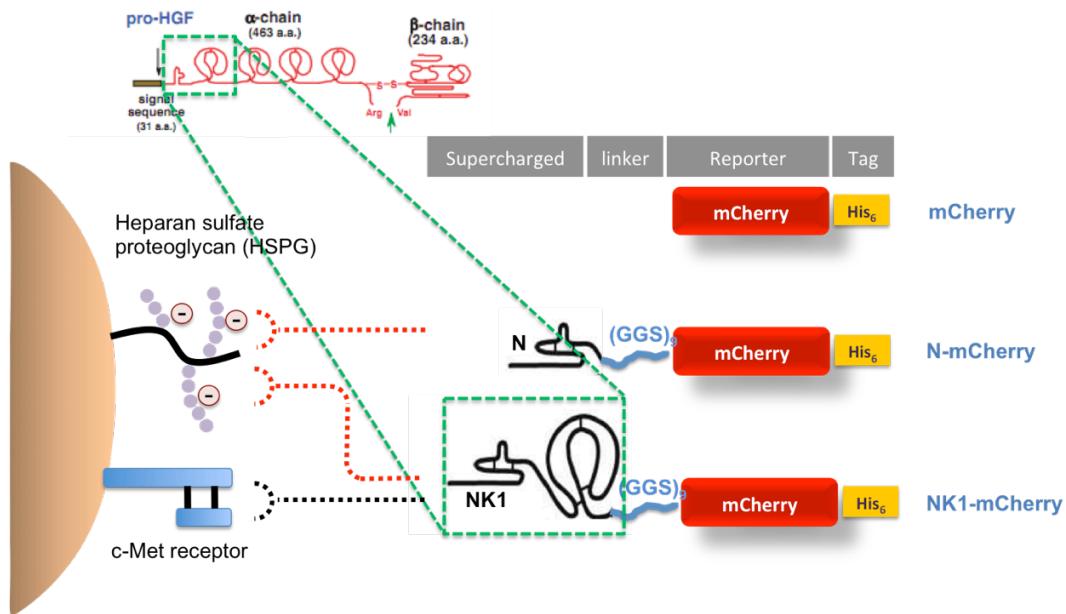


Figure 3: mCherry fusion protein design. Fluorescent fusion proteins were constructed by connecting an mCherry domain to either NK1 or N with a (GGS)₉ linker. A His₆ tag was included for purification of expressed protein. NK1 and N are capable of binding HSPGs (dotted red line) and NK1 is additionally known to bind cMet.

HeLa cells were used to assess internalization of NK1- and N-mCherry, using mCherry as a negative control (Figure 4). The cell surface expression profiles of HeLa cells are positive for both cMet and HSPGs (Supplementary Figure 2). Imaging results showed successful internalization of N-mCherry and

NK1-mCherry, but not mCherry, exhibiting the dependency of these constructs on receptor interaction to enable cell penetration (Figure 4).

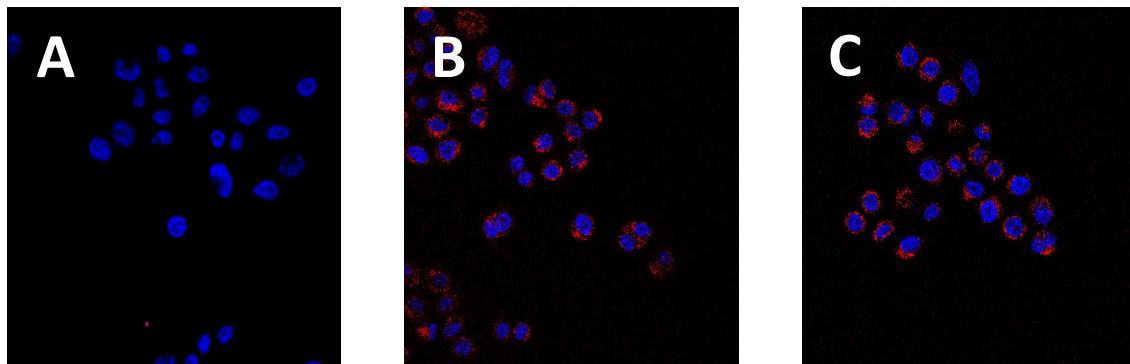


Figure 4: Internalization of NK1 and N domain into HeLa cells. Imaging was used to evaluate internalization of the NK1-mCherry and N-mCherry constructs into HeLa cells after 4 hours of incubation. Cells were fixed and imaged on a Leica SP2 confocal microscope using oil immersion. Red signal corresponds to mCherry fluorescence, and blue signal corresponds to a DAPI nuclear stain. (A) Incubation with mCherry resulted in no cellular mCherry fluorescence. (B) Incubation with N-mCherry, which binds HSPGs but not cMet, resulted in internalization. (C) Incubation with NK1-mCherry, which binds both HSPGs and cMet, resulted in internalization.

3.3 Mechanism study of NK1 internalization

After establishing the ability for NK1 to internalize into mammalian cells, elucidation of the endocytic pathway mechanism was pursued. Knowledge of those proteins involved in NK1 internalization can prove useful in understanding protein localization before post-endocytosis, and potentially enabling better engineering of cargo delivery systems. Fluid-phase endocytosis, also known as pinocytosis, is the major form of internalization observed in mammalian cells, followed by phagocytosis of larger particles [72]. All forms of endocytic internalization begin with accumulation of extracellular particles at the cell surface, followed by remodeling of the plasma membrane to encapsulate particles

into endocytic vesicles. Remodeling is mediated by a variety of proteins, and can occur via invagination or actin-dependent branching of the membrane (Supplementary Figure 3).

Internalization of cMet after activation by HGF is known to occur via a clathrin-dependent pathway. However, the observation that internalization of N-mCherry can occur despite lacking a cMet binding interface suggests that other internalization pathways may exist. However, there remained the possibility that N-mCherry was still binding to cMet, perhaps via an unknown interaction site. Therefore, to more thoroughly investigate the internalization mechanism of NK1, an internalization study was performed in parallel with BJ5ta and SKOV-3 cells. Both cell lines are mammalian and express HSPGs, but BJ5ta cells are noncancerous and do not express cMet, whereas SKOV-3 cells are cancerous and do express cMet (Supplementary Figure 2). Investigating these cell lines in parallel enables the distinction between endocytic pathways unique to HSPGs and those involving cMet.

Cell cultures were incubated with chemical inhibitors to knock down activity of proteins critical to various endocytic pathways, one-by-one. The pathway targeted by each chemical inhibitor is summarized in Table 1. NK1-mCherry was subsequently added to the cell media, and co-incubated with the chemical inhibitors. At the end of incubation, an acidic wash cleared out

remaining surface bound proteins, and cellular mCherry fluorescence was measured by flow cytometry to evaluate internalization success (Figure 5). Internalization was almost completely inhibited by knockdown of HSPG synthesis by incubation with sodium chlorate in both cell lines, revealing that HSPGs are critical to NK1 internalization in both the presence and absence of cMet. Amiloride and dynasore also significantly inhibited NK1 internalization in

Inhibitor	Target
Mannan	Mannose receptor [73]
Amiloride	Macropinocytosis [74]
Chlorpromazine	Clathrin [75]
Dynasore	Dynamin [76]
Nystatin	Calveolin [77]
Filipin	Calveolin [77]
Sodium Chlorate	HSPGs [78]

Table 1: Endocytic inhibitors and their targets.

BJ5TA cells, suggesting that HSPG-mediated internalization is macropinocytotic and dynamin-dependent. Finally, mannan successfully reduced internalization of NK1 20% in BJ5TA cells. Mannan is known to outcompete other binders of the mannose receptor, suggesting that mannose receptor is a potential binding partner for NK1.

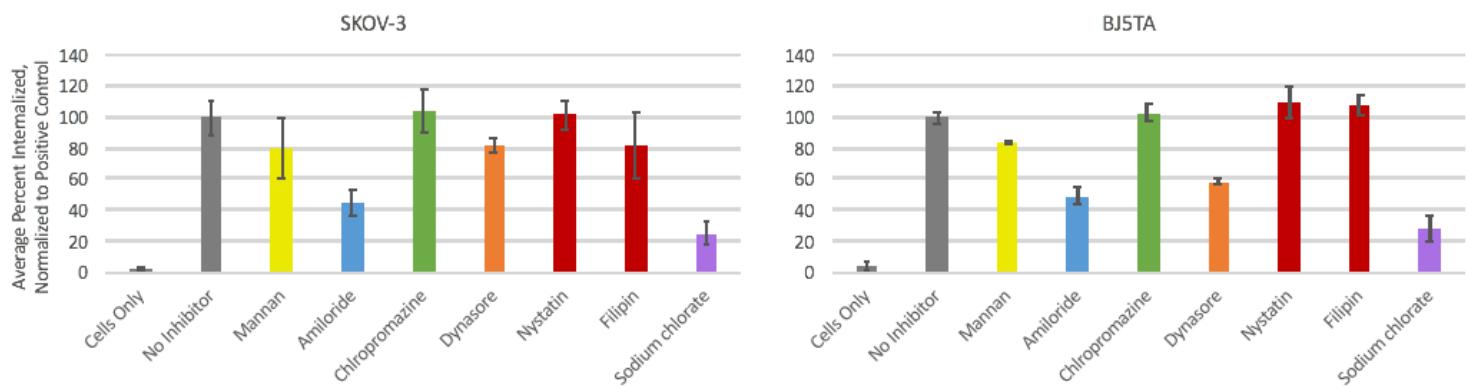


Figure 5: Internalization inhibition study for elucidation of NK1 endocytic pathway mechanism. Co-incubation of NK1-mCherry with several endocytic inhibitors was followed by flow cytometry analysis to evaluate the importance of each pathway in the internalization of NK1 in SKOV-3 and BJ5TA cells.

3.4 Co-localization of NK1-mCherry to endocytic vesicles

Internalization of NK1 is only useful for gene delivery if plasmid cargo can be successfully delivered after the ligand-plasmid complex is encapsulated in endocytic vesicles. Internalization studies performed by the Liu group tracked supercharged GFP after internalization, and found that it avoided perinuclear acidic vesicles for several hours after internalization, suggesting slowing of the maturation of encapsulating endosomes [69]. Heightened ability to avoid lysosomal degradation (the endpoint of endosomal maturation) has been suggested to confer greater delivery potency to supercharged proteins and CPPs.

The co-localization of NK1-mCherry with endocytic vesicle markers was studied to evaluate whether the relatively high charge to weight ratio of NK1

allowed for similar slowing of endosomal maturation. LysoTracker-Green and anti-EEA1 antibodies were used to label lysosomal and endosomal compartments, respectively. LysoTracker-Green particles can accumulate and fluoresce in lysosomes due to the altered pH of those vesicles, whereas anti-EEA1 antibodies label EEA1 a RAB5A effector protein that localizes to early endosomes. EEA1 labeling, therefore, requires a secondary antibody for fluorescent labeling. Additionally, Dextran-TexasRed, a fluid-phase endocytosis marker that localizes to lysosomes, was used as a control. Confocal images of fixed, labeled samples are shown in Figures 6 and 7. Results showed co-localization of NK1-mCherry signal with both EEA1 and with lysosomes, though a significant portion of NK1-mCherry was often not co-localized to lysosomes.

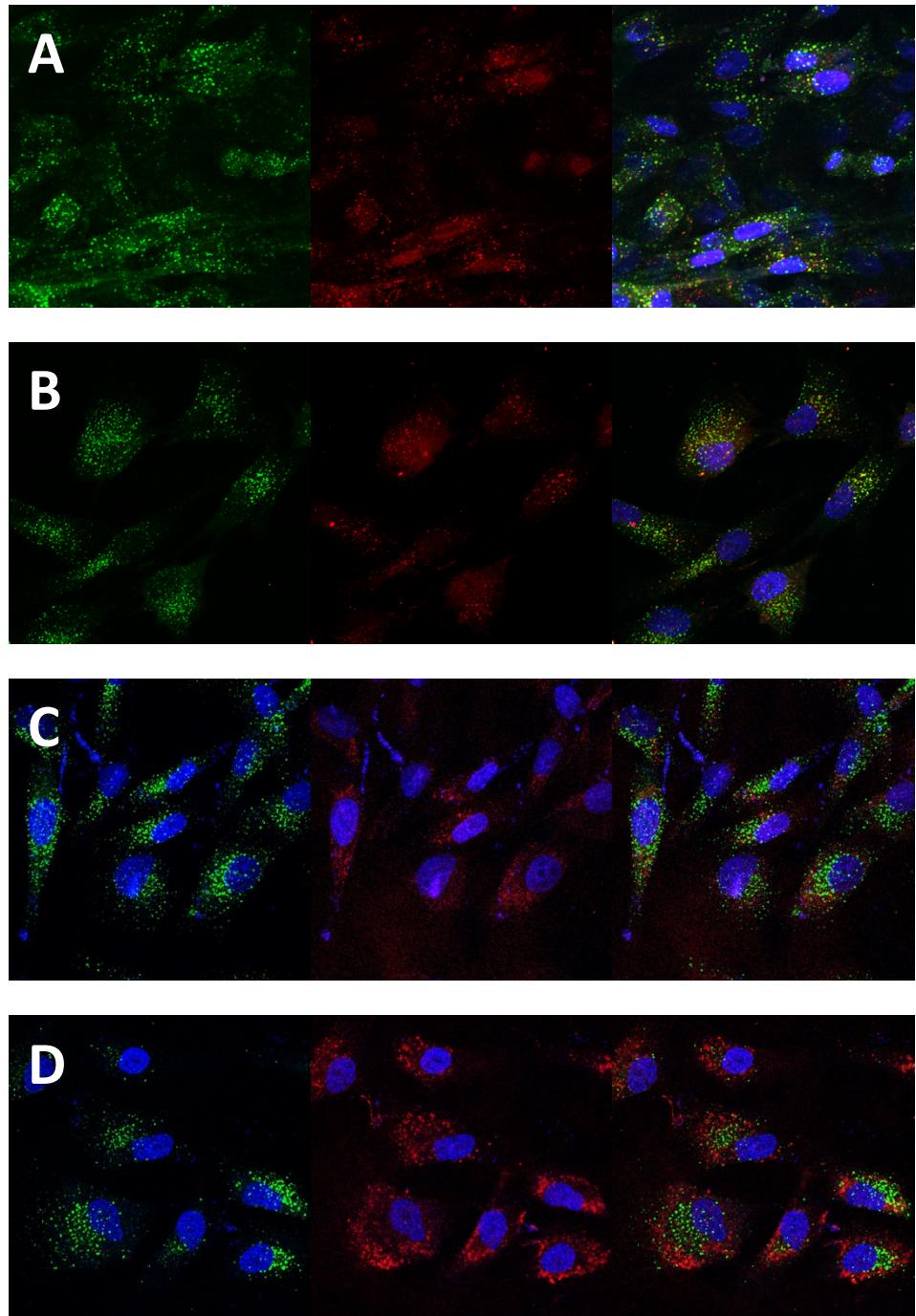


Figure 6: Internalization co-localization of NK1-mCherry in BJ5TA cells. (A) NK1-mCherry and Lysotracker-Green. (B) Dextran-TexasRed and Lysotracker-Green. (C) NK1-mCherry and EEA-1. (D) Dextran-TexasRed and EEA-1.

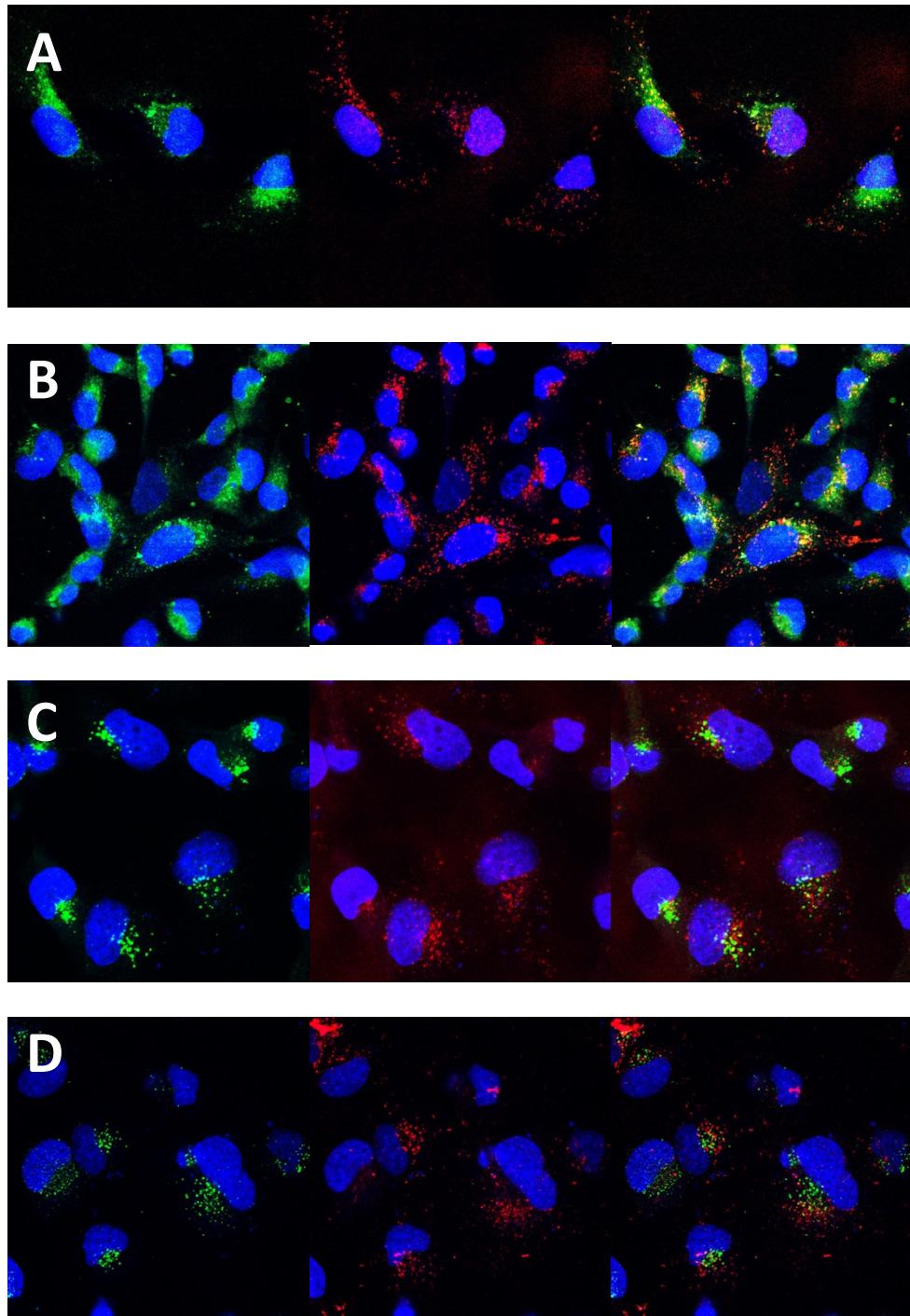


Figure 7: Internalization co-localization of NK1-mCherry in SKOV-3 cells. (A) NK1-mCherry and LysoTracker-Green. (B) Dextran-TexasRed and LysoTracker-Green. (C) NK1-mCherry and EEA-1. (D) Dextran-TexasRed and EEA-1.

3.5 Engineering strategies for NK1 variants

With internalization capabilities of NK1 established, design of engineered variants was pursued to allow for optimized protein expression yield, agonistic behavior, and DNA plasmid delivery, three identified characteristics important for clinical success as both an exogenous mitogen and gene therapy delivery vehicle.

As mentioned above (see section 1.4) dimer formation and dual-receptor activation is a common characteristic of ligand-receptor binding in growth factors. Our group has previously engineered a dimerized NK1 variant by directed evolution and the introduction of a cysteine residue at the N-terminus (forming a covalent dimer, as opposed to a homodimer) [26,80]. This variant, M2.2-D127N, exhibits agonistic activity approaching that of HGF, while offering advantages in terms of stability and expression yield. However, the presence of seven mutations compared to wild-type NK1 suggests that M2.2-D127N could show increased immunogenicity in clinical applications. Accordingly, our engineering efforts began by exploring whether a covalent cysteine dimer could be formed using wild-type NK1. Plasmids were constructed for the production the following four variants: NK1, cysNK1, NK1-HA2, and cysNK1-HA2 (Supplementary Figure 4). The inclusion of an HA2 tag represents a rational engineering effort towards greater plasmid delivery. The HA2 tag, known as an endosomal escape peptide, conferred plasmid delivery transfection ability on supercharged GFP approaching the level of Lipofectamine 2000 [61]. Addition of an HA2 tag to NK1 variants is hypothesized to help improve endosomal stalling and escape, allowing for enhanced plasmid delivery.

3.6 M2.2-D127N variant production for study of point mutations

Unfortunately, after successful DNA cloning of the constructs and transformation into *P. pastoris*, protein expression yield proved too low to continue with subsequent experiments. Therefore, engineering efforts turned back

towards optimizing M2.2-D127N for therapy. At this point, simultaneously conducted protein expression and internalization studies in our group had identified several candidate mutations for improving the therapeutic potential of M2.2-D127N. Three mutated residues in M2.2 had been chosen for reversion to wild-type (lysine) in an effort to increase positive surface charge density: E62K, N132K, and E170K. All three residues are located near the heparin binding site, and were believed to have arisen during directed evolution of M2.2, since selection pressures for stability could have energetically favored the removal of highly charged residues from a surface charge cluster.

Using the same pPIC9K plasmid vector as the cysNK1-HA2 cloning, plasmids encoding the following four M2.2 variants were constructed, transformed into yeast, and purified: cysM2.2-D127N-E62K (“E62K”), cysM2.2-D127N-E62K-HA2 (“E62K-HA2”), cysM2.2-D127N-E62K-E170K (“E62K/170K”), and cysM2.2-D127N-E62K-E170K-HA2 (“E62K/170K-HA2”). Size exclusion FPLC traces are shown in Figures 8 and 9.

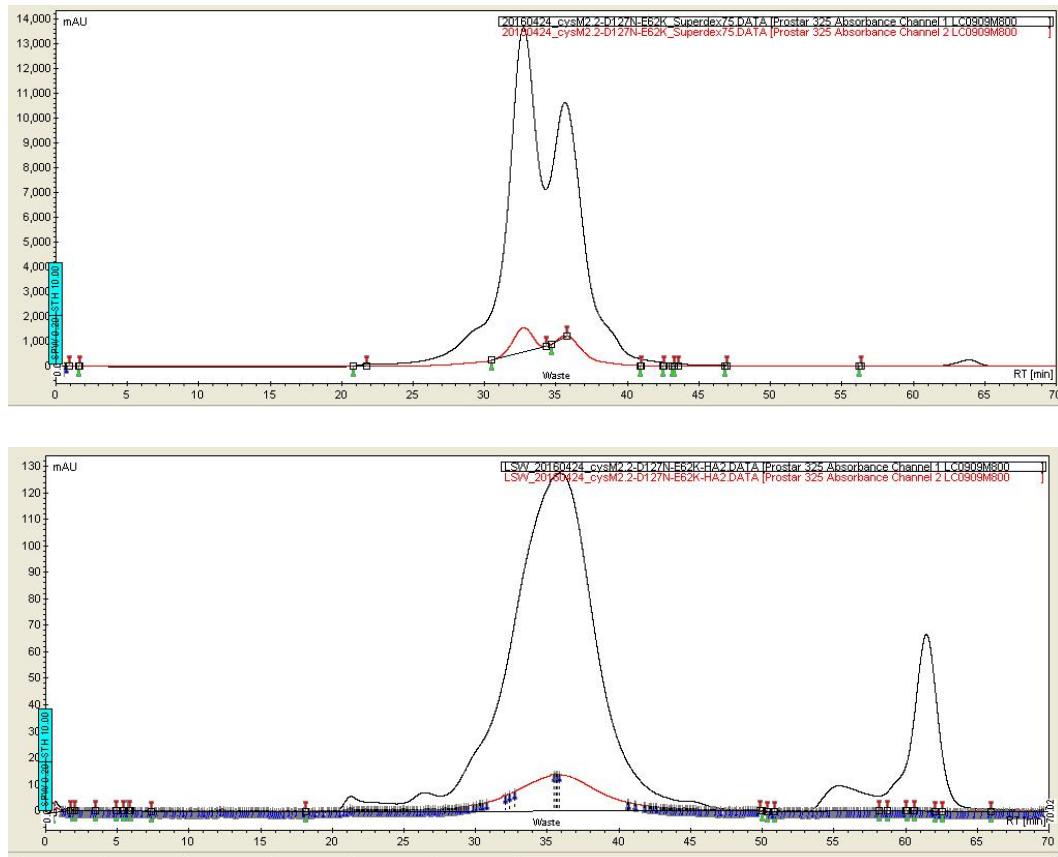


Figure 8: FPLC traces for E62K variants. Purification of cysM2.2-D127N-E62K (above) and cysM2.2-D127N-E62K-HA2 (below) using size Superdex 75 size exclusion columns for FPLC. Smaller dimer peaks can be seen between 25 and 30 minutes. Rightmost peaks correspond to imidazole, an organic compound used to crudely purify proteins on Ni-NTA resin columns.

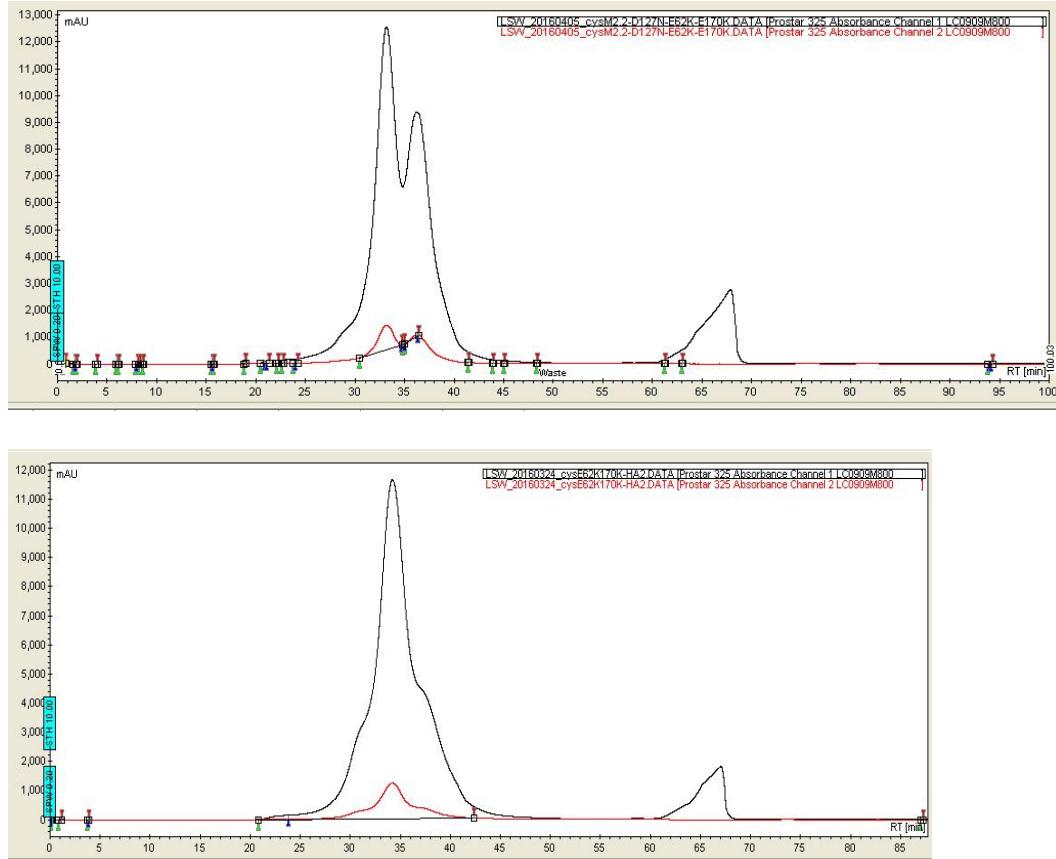


Figure 9: FPLC traces for E62/170K variants. Purification of cysM2.2-D127N-E62K-E170K (above) and cysM2.2-D127N-E62K-E170K-HA2 (below) using size Superdex 75 size exclusion columns for FPLC. Smaller dimer peaks can be seen between 25 and 30 minutes. Rightmost peaks correspond to imidazole, an organic compound used to crudely purify proteins on Ni-NTA resin columns.

Three of the four proteins expressed very well. In the case of E62K-HA2, which did not show good expression yield, multiple *P. pastoris* colonies were picked from plates after two separate transformations, yet none of the clones seemed to express well. Further subsequent repeat transformations could potentially identify a better expressing clone. However, given that E62K/170K variants exhibited high expression yield and represent a reduction in the total number mutations away from wild-type, continuing to create mutants with both

E62K and E170K is promising. Cysteine dimer formation was implicated by small bumps in the FPLC traces during the 25-30 minute time period. Reducing PAGE gels of the purified constructs confirmed cysteine dimer formation was occurring for the three well-expressed constructs, though dimer levels were significantly lower than that of monomers and complete separation of the two subtypes was not achieved (Supplementary Figure 5).

To seek better separation of subtypes, E62K/170K-HA2 was run through different sized Amicon® Ultra-4 Centrifugal Filter Units. Separation of the dimerized proteins with a 30 kDa filter was followed by collection of the monomeric flow-through with a 10 kDa filter, and led to two distinct peaks during FPLC (Figure 10). However, this method resulted in significant protein loss, and must be further optimized for effective use.

Constructs incorporating the N132K mutation were also engineered to assess their ability to be expressed. Specifically, plasmids encoding M2.2-D127N-E62K-N132K-mCherry (“N132K-mCh”) and M2.2-D127N-E62K-N132K-E170K-mCherry (“N132K-E170K-mCh”) were produced, and protein was purified from yeast. FPLC traces using a heparin trap column showed collection of a clean, large peak (Figure 11). Additionally, a cysteine dimer containing mCherry, cysM2.2-D127N-E62K-mCherry, was constructed and expressed to assess impact of an mCherry domain on dimer formation and more effectively compare internalization of NK1 monomers and dimers using fluorescence. FPLC was run twice using a heparin trap column to collect correctly folded protein, followed by a size exclusion column to separate monomeric proteins from dimers (Figure 12). A reducing PAGE gel confirmed dimer formation and correct protein size, as well as correct size for the N132K variants (Supplementary Figure 6).

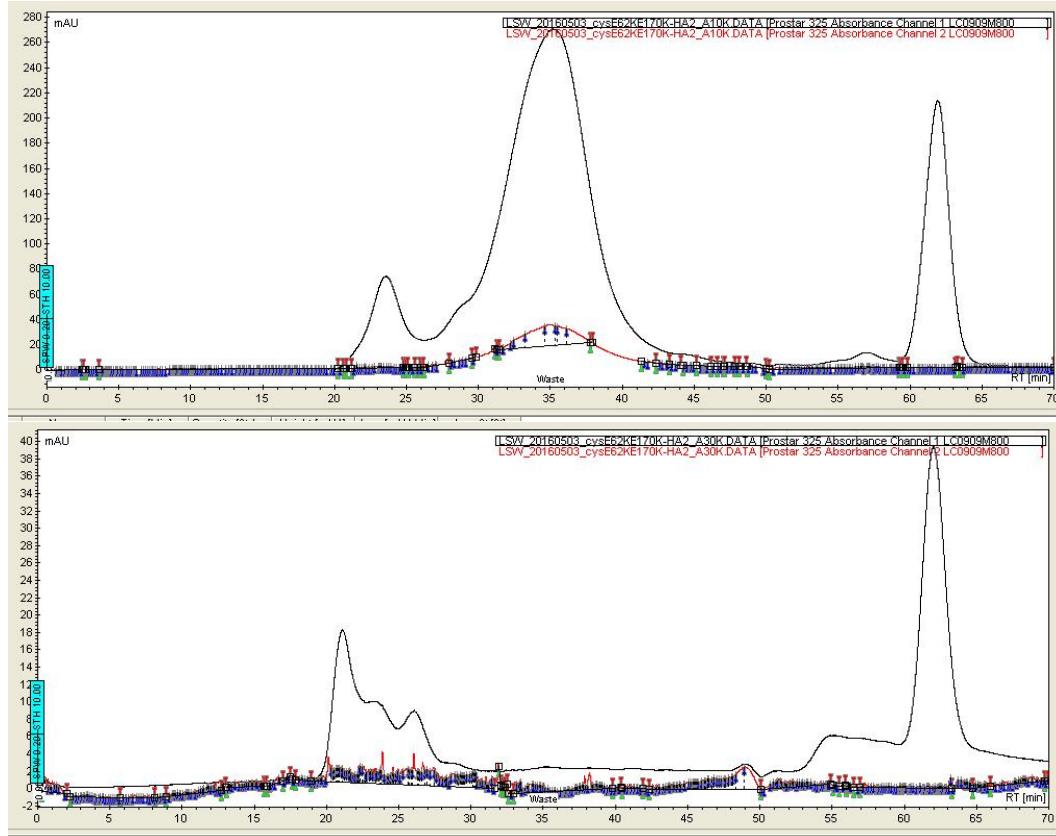


Figure 10: FPLC traces for E62/170K-HA2, separated using centrifugal size filter units. Purified E52/170K-HA2 was run through a 30K Amicon® Filter column to separate out the dimer fraction (top FPLC trace). The flow-through, presumably containing the monomer fraction, was collected using a 10K Amicon® Filter column (bottom FPLC trace). Both fractions were then run through a Superdex S75 size exclusion column for FPLC.

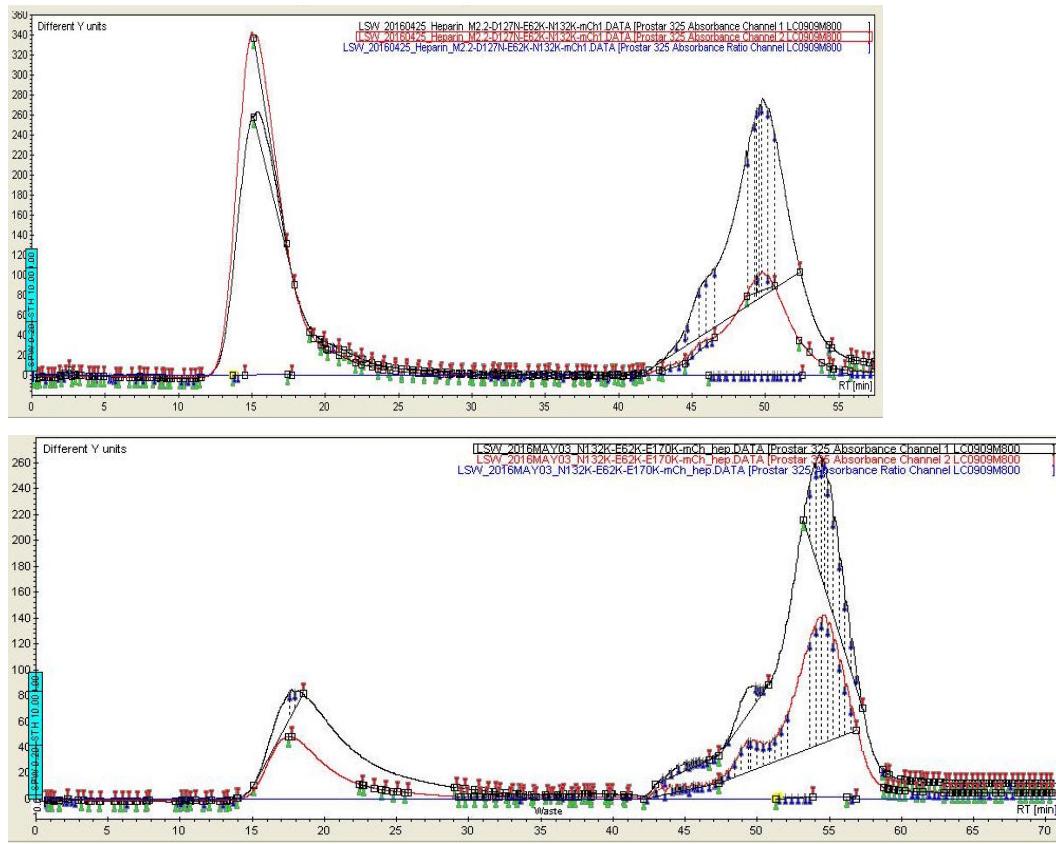


Figure 11: FPLC traces for N132K Variants of M2.2. N132K-mCh (above) and N132K-E170K-mCh (below) variants of M2.2 were successfully cloned, expressed in yeast, and purified using a heparin trap column.

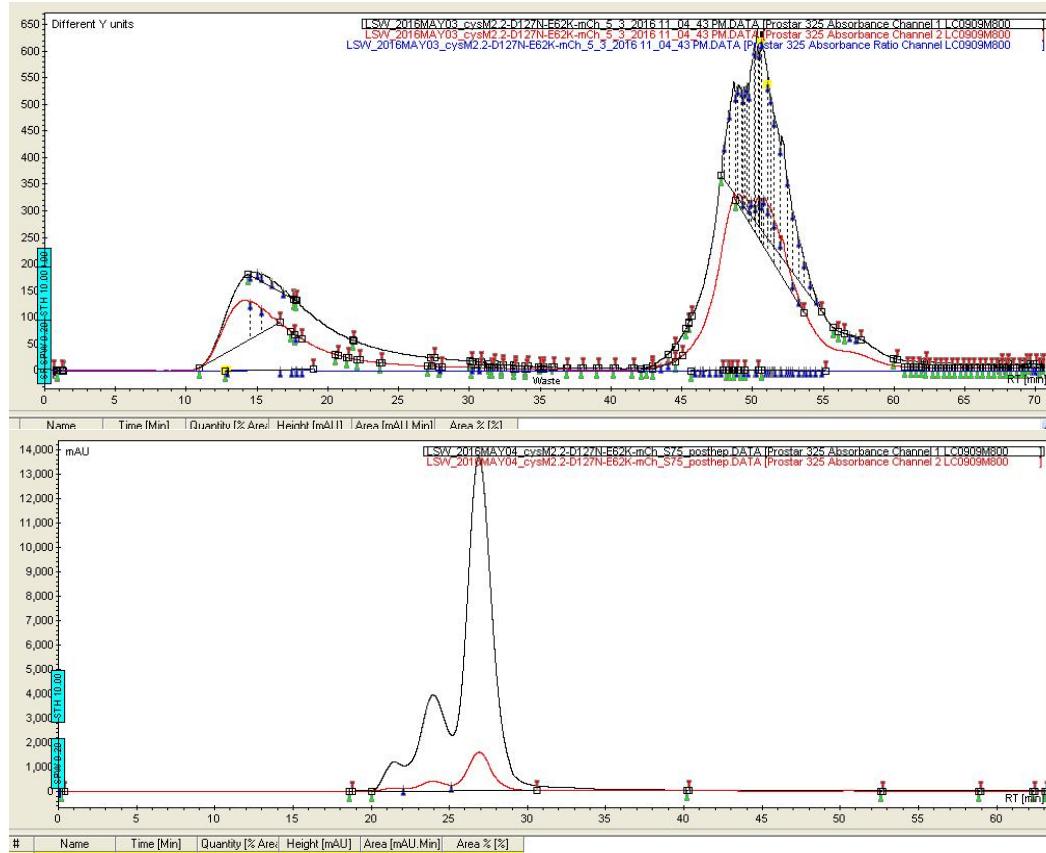


Figure 12: FPLC traces for cysM2.2-D127N-E62K-mCherry. A cysteine dimer containing mCherry domains was constructed and purified by running FPLC two times. First, bound protein was collected using a heparin trap column (above). Second, a Superdex S75 size exclusion column resulted in excellent separation of dimeric and monomeric peaks (below).

3.7 MDCK Scatter Assay

Agonistic activity of the monomers and dimers of all six well-purified constructs (excluding E62K-HA2) was tested using a Madin Darby canine kidney (MDCK) cell scatter assay [80]. MDCK cells are prone to clumping when cultured in standard growth media, but addition of agonists such as HGF result in a distinct scattered morphology. The purified constructs were tested against numerous other NK1 variants engineered in our group, both in the presence and

absence of heparin, to assess agonistic ability (Figure 13a and b). As expected, incubation with HGF and cysteine dimers resulted in significant scattering in both with or without heparin, while monomeric NK1 variants showed limited scattering when heparin was absent. Nonetheless, some variation in scatter profile was seen among monomers, including those extracted from cysteine dimer constructs. Generally, M2.2 monomers containing the E62K mutation seem to show partial agonism, but this effect is lost when combined with the N132K or E170K mutations.

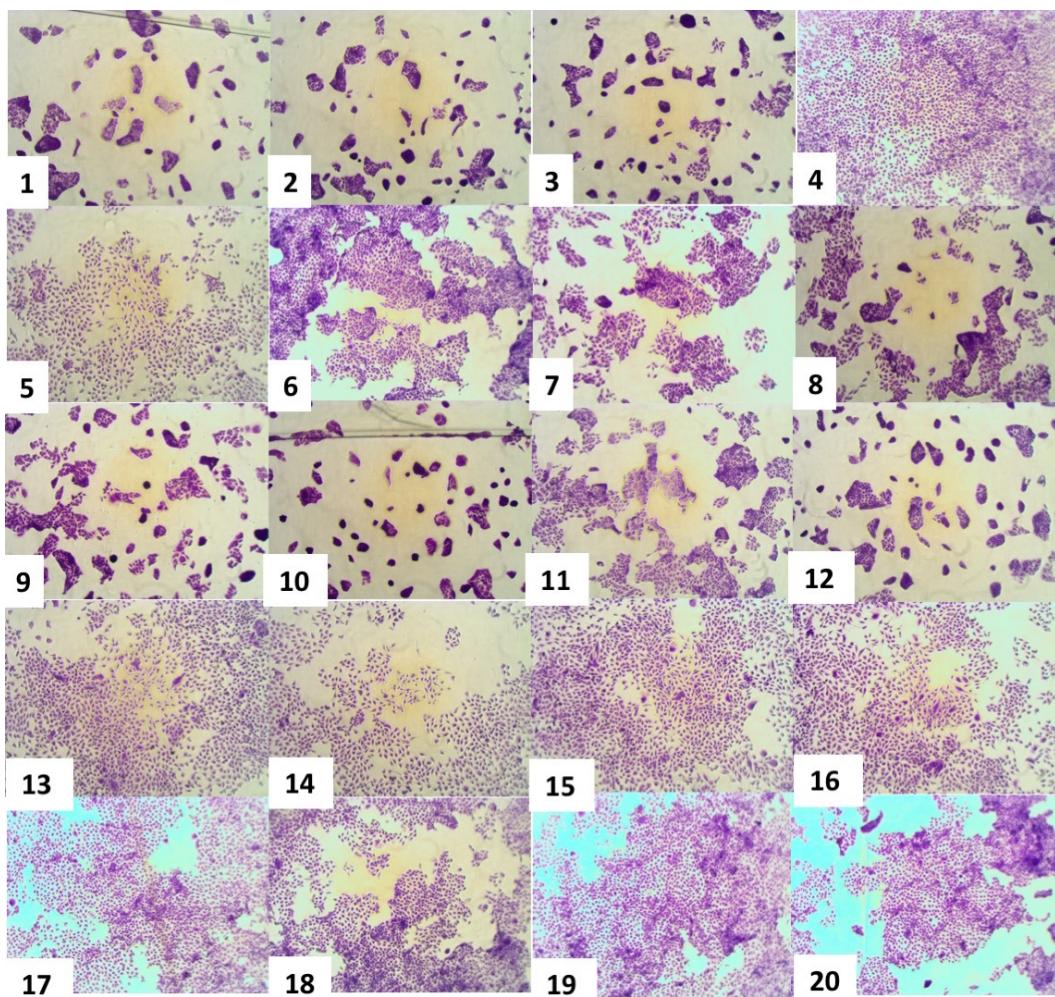


Figure 13a: MDCK Scatter Assay in the absence of heparin. Legend on following page.

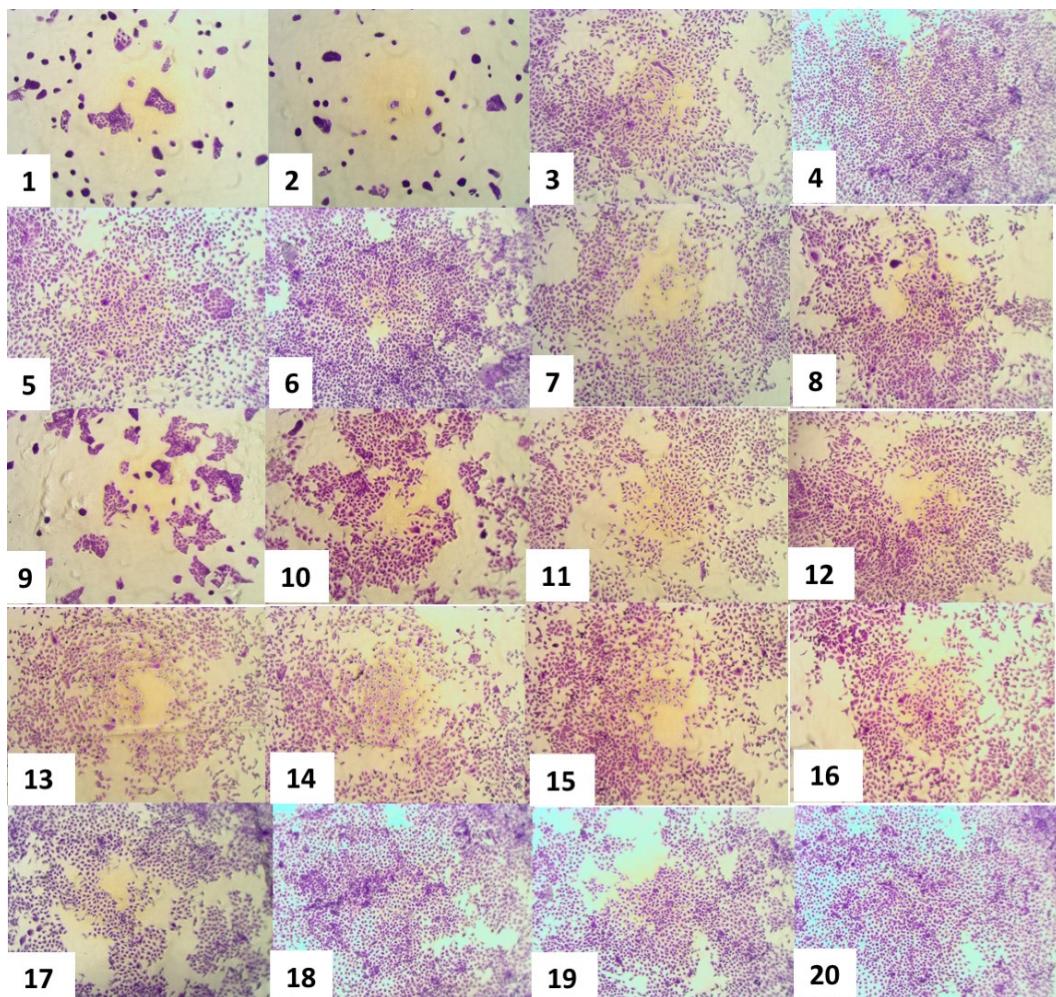


Figure 13b: MDCK Scatter Assay in the presence of heparin.

Legend

- | | |
|------------------------------|---|
| 1 Cells only | 11 M2.2-D127N-E62K-N132K-mCh |
| 2 mCherry (mCh) | 12 M2.2-D127N-E62K-N132K-E170K-mCh |
| 3 NK1-mCh | 13 cysM2.2-D127N-E62K-mCh dimer |
| 4 HGF (discoverX) | 14 cysM2.2-D127N-E62K-mCh monomer |
| 5 M2.2 D127N (1/10 diluted) | 15 cysM2.2-D127-E62K dimer |
| 6 M2.2 D127N-mCh #082113 | 16 cysM2.2-D127-E62K monomer |
| 7 M2.2-D127N-E62K-mCh | 17 cysM2.2-D127N-E62K-E170K dimer |
| 8 M2.2-D127N-N132K-mCh | 18 cysM2.2-D127N-E62K-E170K monomer |
| 9 M2.2-D127N-E170K-mCh | 19 cysM2.2-D127N-E62K-E170K-HA2 dimer |
| 10 M2.2-D127N-E62K-E170K-mCh | 20 cysM2.2-D127N-E62K-E170K-HA2 monomer |

3.8 Electrophoretic Mobility Shift Assay

As a first step towards evaluating the potential of NK1 variants to deliver plasmid DNA for gene therapy, an initial electrophoretic mobility shift assay (EMSA) was performed (Figure 14). EMSAs enable detection of protein-nucleic acid interactions by assessing the distance of nucleic acid travel in agarose gels, making use of the fact that increased molecular weight of DNA-protein complexes inhibits DNA migration [81]. A successful NK1 variant delivery vehicle will be able to form a robust complex with plasmid DNA to remain intact throughout application and endocytosis. cysM2.2-D127N-E62K-mCherry and monomeric cysM2.2-D127N-E62K-E170K-HA2 were allowed to complex with DNA, then run on a 1.5% agarose gel. A molar protein:DNA ratio of 50:1 sufficiently inhibited migration in the case of the mCherry variant, whereas a higher ratio was necessary for the HA2 monomer to exert a noticeable effect. Curtailing the dynamic range tested will establish the ideal ratios for complex formation for these constructs, and the assay can be readily extended to evaluate the DNA binding ability of the other purified candidates.

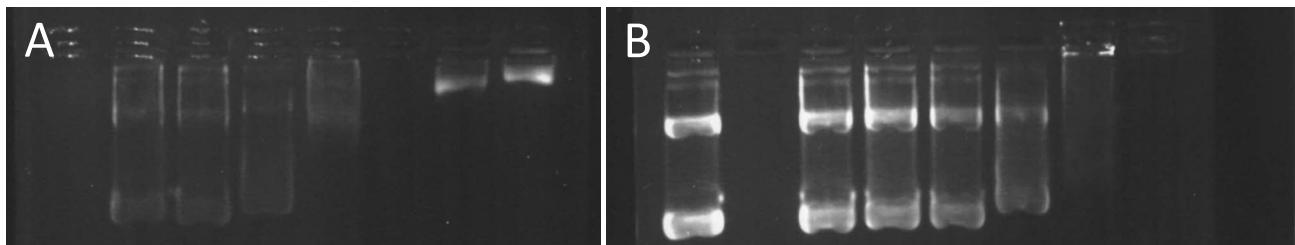


Figure 14: EMSA of plasmid-binding dimeric NK1 variants. DNA-protein binding interaction was shown via EMSA for both cysM2.2-D127N-E62K-mCh (A) and monomeric cysM2.2-D127N-E62K-E170K-HA2 (B). Protein:DNA ratio by lanes: (A1) Protein only; (A2) 1:1; (A3) 10:1; (A4) 50:1; (A5) 200:1; (A6) no sample; (A7) 500:1; (A8) 1000:1; (B1) DNA only; (B2) Protein only; (B3) 1:1; (B4) 10:1; (B5) 50:1; (B6) 200:1; (B7) 1000:1; (B8) 5000:1.

4. Discussion

The work presented here establishes the candidacy of an NK1 variant to serve as a potent regenerative therapeutic via dual-use – both as an exogenous mitogenic factor and as gene therapy delivery vehicle, enabling sustained expression of growth factor release.

First, an in-depth internalization study was undertaken to evaluate the ability for NK1 to internalize into mammalian cells. NK1-mCherry fusion constructs were not only found to successfully internalize into cells, but to do so via numerous pathways. Including an N-mCherry test group in the internalization assessment confirmed that uptake of an HGF fragment could occur without binding to cMet. This point was further exhibited in internalization inhibition studies with BJ5TA cells, which were shown not to express substantial quantities of cMet.

This BJ5TA internalization inhibition study proved especially useful in revealing the unique pathway mechanism of HSPG-mediated entry of HGF fragments into mammalian cells. Flow cytometry results after incubation with inhibitory molecules suggests that HSPG-mediated endocytosis occurs via a macropinocytotic and dynamin-dependent process. Additionally, slight but consistent downregulation in internalization activity was observed during incubation with mannan, suggesting that NK1 and mannose receptor could exhibit some significant binding interaction. Further studies must be undertaken to evaluate whether mannose receptor is indeed a newly discovered binding partner for NK1, or whether its competitive binding by mannan affects NK1 internalization by some less-direct mechanism.

The internalization inhibition study also corroborates pre-existing knowledge on the magnitude of HGF fragment uptake via different endocytic pathways. A previous study proposed that while HSPG-mediated uptake occurs

via a lower-affinity interaction, this pathway is saturated at higher plasma doses than the cMet-mediated pathway. Given that both BJ5TA and SKOV-3 express HSPGs, it is likely that the HSPG-mediated internalization pathway is active in both cell lines. Interestingly, use of chlorpromazine, a clathrin inhibitor, at doses previously tested in mammalian cells did not result in downregulation of NK1 internalization in SKOV-3, even though cMET receptors are highly expressed in SKOV-3 and their internalization is known to require clathrin. One possibility is that the HSPG pathway adjusted to take up a greater percentage of NK1 molecules when SKOV-3 was incubated with chlorpromazine, resulting in sustained internalization levels despite loss of clathrin. If true, the ability to sustain internalization by taking advantage of pathways with higher saturation thresholds holds significant promise for NK1 variant cargo delivery in future clinical applications.

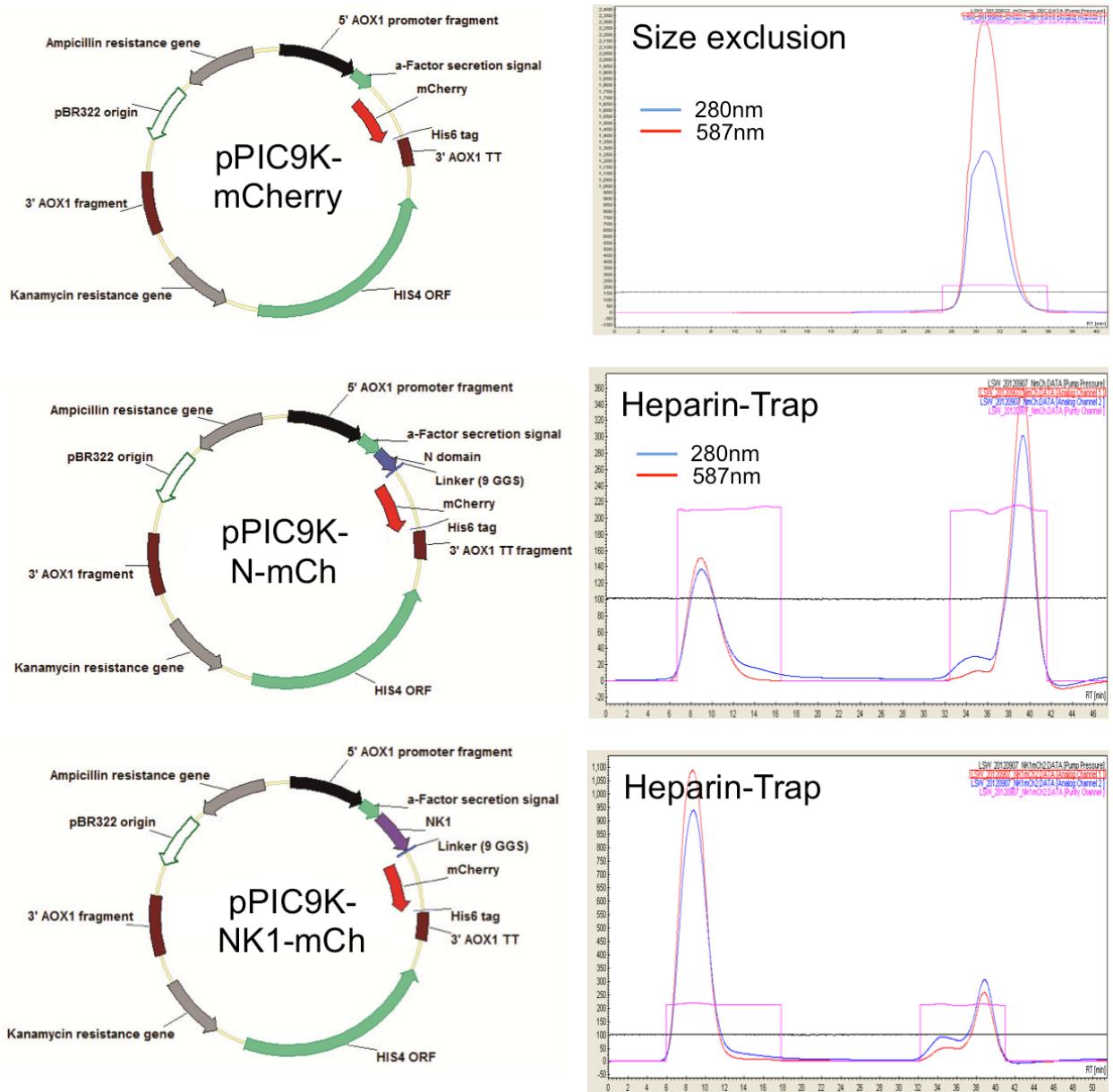
In addition to the internalization study, numerous efforts were undertaken to engineer M2.2 fragments for enhanced potential as an exogenous mitogen and a delivery vehicle. The introduction of a cysteine residue at the N terminus resulted in covalent dimer formation in several variants. Without such a residue, the presence of heparin would be required for dimerization, and therefore receptor agonism, to occur. By engineering covalent dimers, a significant increase in agonistic activity was observed in the absence of heparin, as evidenced in the MDCK scatter assay. Accordingly, most monomeric variants tested were confirmed to require heparin for any significant agonistic activity to occur. However, it was noteworthy that monomeric M2.2-D127N-mCherry and M2.2-D127N-E62K-mCherry showed partial agonism even in the absence of heparin. Similarly, those cysM2.2 proteins that had been separated out as monomers during purification also exhibited partial agonism without heparin. The presence of the D127N and E62K mutations in each aforementioned case suggests that these residues may play a key role in heparin-independent activation of cMet by

NK1, likely by homo-dimerization of the fragment without heparin. Future efforts to engineer M2.2 as a gene delivery vehicle will need to explore the internalization capabilities of these mutants, as well as confirm plasmid binding and delivery via EMSA and functional reporter assays.

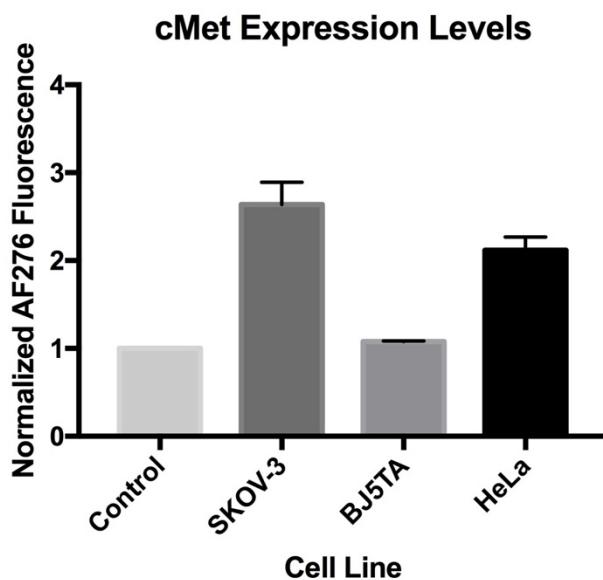
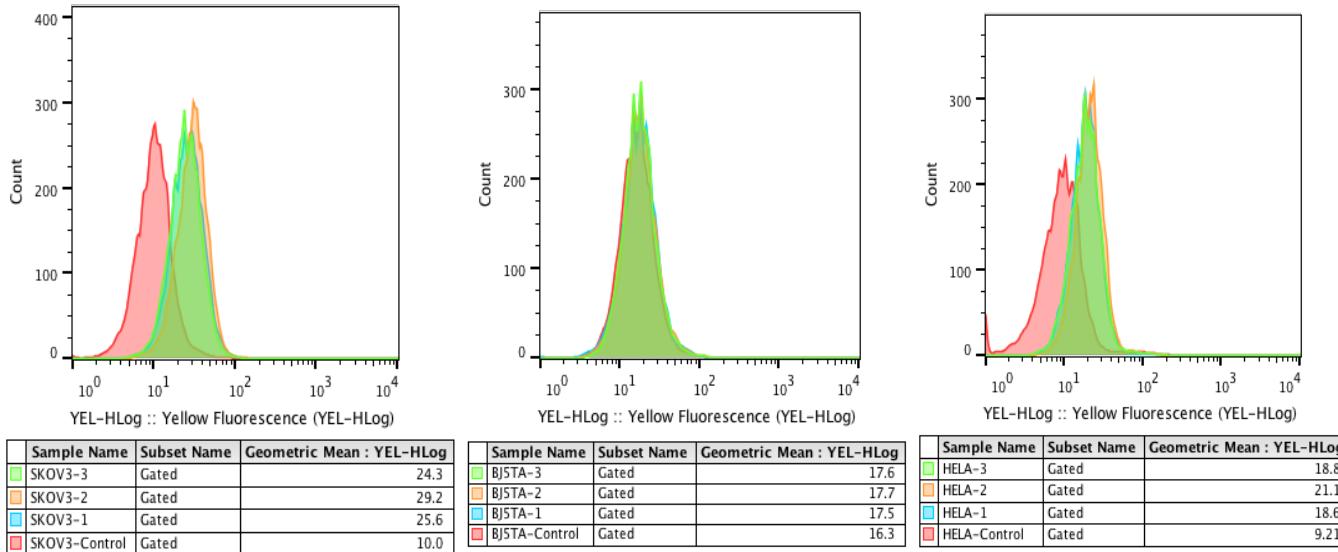
Another critical realm for future work is the continuation of endocytic pathway investigation. The initial imaging studies included here show that NK1-mCherry can be successfully tracked in conjunction with various vesicle markers. Co-localization analysis across a series of incubation time periods, including through live cell imaging, would allow for a better understanding of the intracellular trajectory of NK1 after internalization and answer important questions surrounding cargo delivery.

This work only begins to draw on the vast potential for HGF fragment engineering and characterization to reveal interesting behaviors and suggest promising avenues for development of advanced therapeutics. As gene therapies and engineered growth factor treatments continue their upward trend in terms of clinical use, HGF and its fragments will undoubtedly play a large role in enabling critical breakthroughs in regenerative medicine.

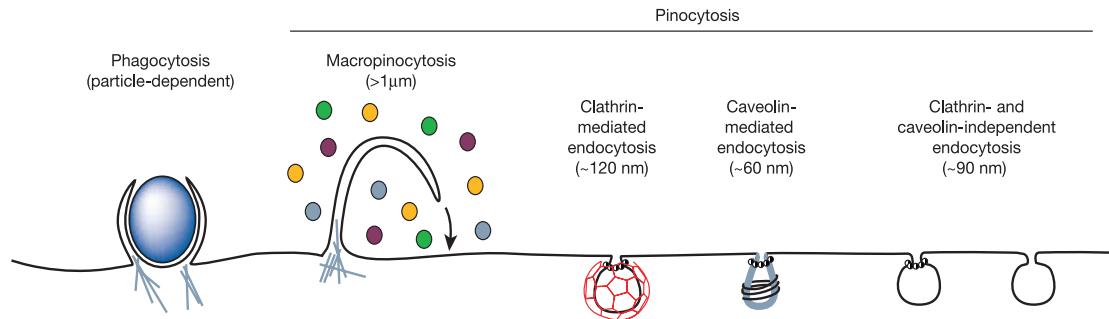
5. Supplementary Figures



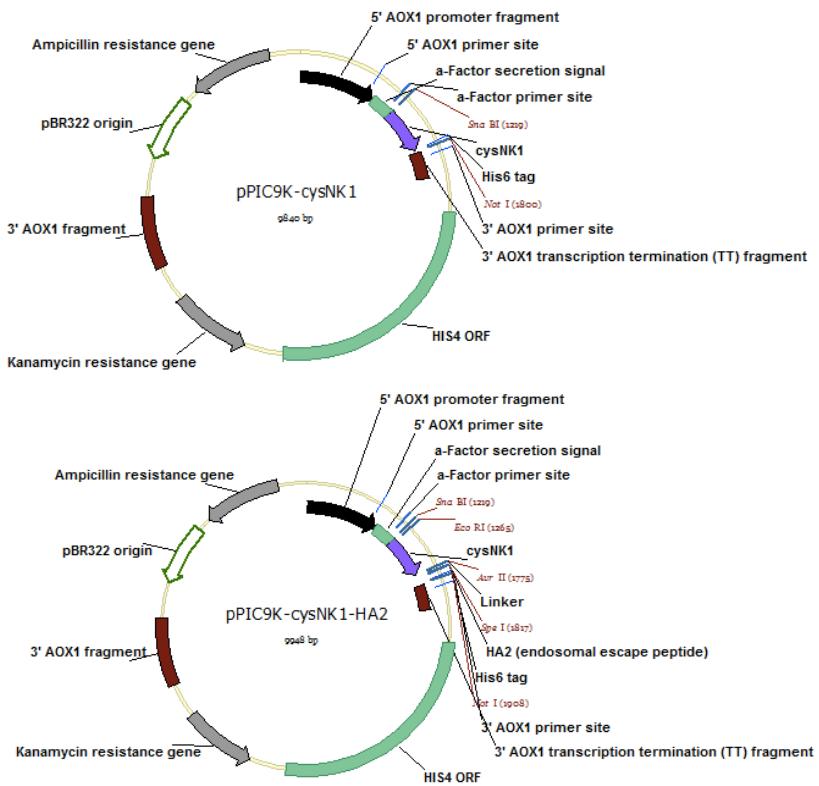
Supplementary Figure 1: mCherry fusion plasmid constructs. mCherry fusion constructs were recombinantly cloned into the pPIC9k vector for eventual expression in *Pichia pastoris*. The inclusion of a His₆ tag allowed for crude purification via a Ni-NTA resin, followed by FPLC using either a size exclusion or heparin-trap column. FPLC trace data courtesy of Sungwon Lim.



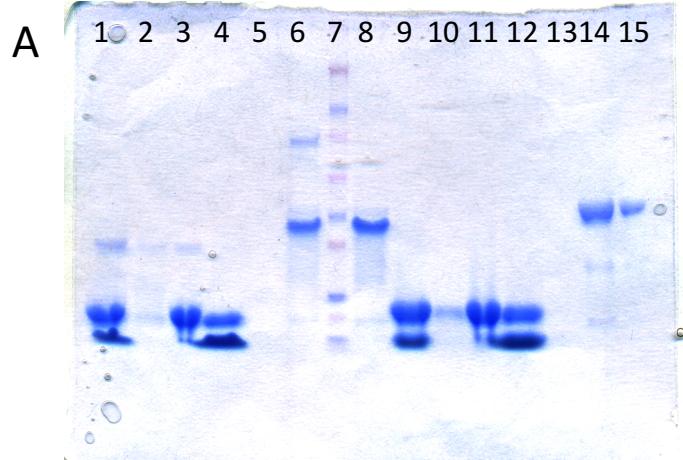
Supplementary Figure 2: cMet receptor expression on the surface of mammalian cells. cMet expression levels were measured via binding assay on the surface of three mammalian cell lines. cMet molecules are first labeled with AF276, an anti-cMet antibody (R&D Systems P08581), followed by labeling with a secondary, phycoerythrin-tagged antibody, enabling yellow fluorescent readout.



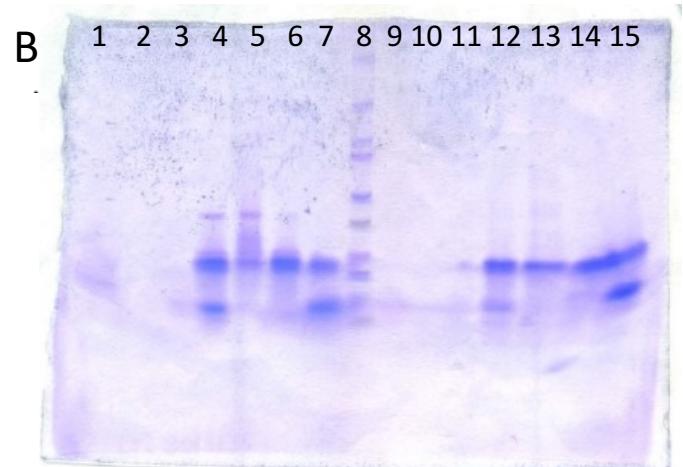
Supplementary Figure 3: Summary of common endocytic pathway mechanisms. Several intracellular proteins mediate membrane remodeling for the formation of endocytic vesicles. These proteins are recruited to the cell membrane upon build-up of extracellular particles that interact with cell surface receptors.



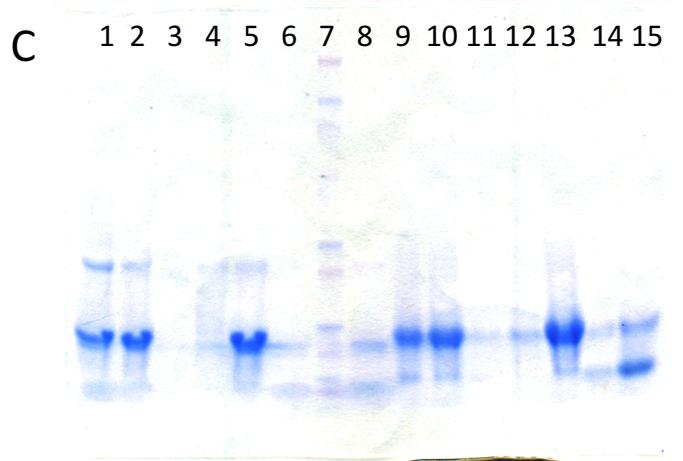
Supplementary Figure 4: Plasmid constructs for expression cysteine dimer and HA2 tagged NK1 variants. NK1 and M2.2 variants were recombinantly cloned into the pPIC9k vector. Inclusion or exclusion of a cysteine residue in one forward primer sequence allowed for the creation of cysteine dimer variants. Each variant was also engineered with and without an HA2 endosomal escape peptide.



1-4: E62K (nr)
 5: E62K-HA2 (nr)
 6: cysE62K-mCh (nr)
 7: Novex Sharp Ladder
 8: N132K-mCh (nr)
 9-12: E62K (r)
 13: E62K-HA2 (r)
 14: cysE62K-mCh (r)
 15: N132K-E62K-mCh (r)

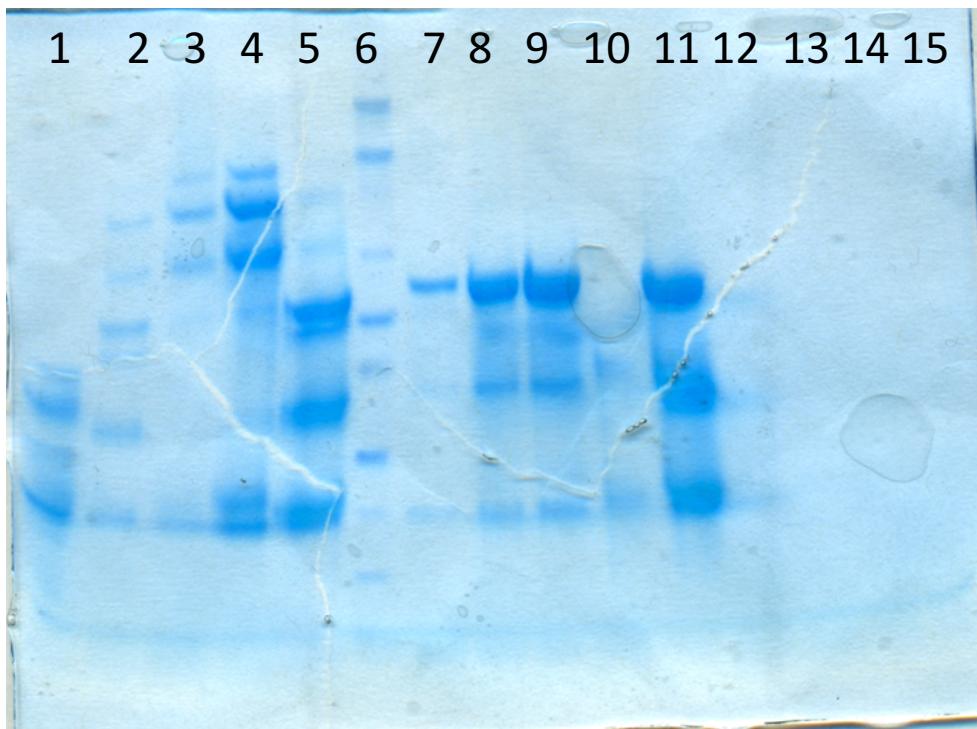


1-3: E62K-HA2 (nr)
 4-7: E62K/170K (nr)
 8: Novex Sharp Ladder
 9-12: E62K-HA2 (r)
 13-15: E62K/170K (r)



1-6, 8: E62/170K-HA2 (nr)
 7: Novex Sharp Ladder
 9-15: E62/170K-HA2 (r)

Supplementary Figure 5: Reducing PAGE gels show dimer formation of well expressed variants. NK1 variants were run on 4-12% reducing PAGE gels. E62K, E62/170K, and E62/170K-HAW all showed dimer bands, which were subsequently lost under reducing conditions, confirming disulfide-mediated dimerization.



Supplementary Figure 6: Reducing PAGE gels shows dimer formation of cysM2.2-D127N-E62K-mCherry. A cysteine dimer M2.2 variant containing mCherry domains was shown to be successfully purified in a dimeric form, as shown in wells 2-4. Reduction of these samples, seen in wells 7-9, resulted in loss of the dimeric band.

6. References

1. Sen, C. K., Gordillo, G. M., Roy, S., Kirsner, R., Lambert, L., Hunt, T. K., ... & Longaker, M. T. (2009). Human skin wounds: a major and snowballing threat to public health and the economy. *Wound Repair and Regeneration*, 17(6), 763-771.
2. Jaklenec, A., Stamp, A., Deweerd, E., Sherwin, A., & Langer, R. (2012). Progress in the tissue engineering and stem cell industry “are we there yet?”. *Tissue Engineering Part B: Reviews*, 18(3), 155-166.
3. Cooper GM. The Cell: A Molecular Approach. 2nd edition. Sunderland (MA): Sinauer Associates; 2000. DNA Repair. Available from: <http://www.ncbi.nlm.nih.gov/books/NBK9900/>
4. Bruce M. Carlson, Chapter 1 - An Introduction to Regeneration, In Principles of Regenerative Biology, Academic Press, Burlington, 2007, Pages 1-29, ISBN 9780123694393, <http://dx.doi.org/10.1016/B978-012369439-3/50003-9>. (<http://www.sciencedirect.com/science/article/pii/B9780123694393500039>)
5. Liver Regeneration George K. Michalopoulos*, Marie C. DeFrances *Science* 04 Apr 1997: Vol. 276, Issue 5309, pp. 60-66 DOI: 10.1126/science.276.5309.60
6. Gurtner, G. C., Werner, S., Barrandon, Y., & Longaker, M. T. (2008). Wound repair and regeneration. *Nature*, 453(7193), 314-321.
7. Mason, C., & Dunnill, P. (2008). A brief definition of regenerative medicine. *Regen. Med.*, 3(1), 1-5.
8. Maienschein, J. (2011). Regenerative medicine's historical roots in regeneration, transplantation, and translation. *Developmental biology*, 358(2), 278-284.
9. Langer, R., & Vacanti, J. (2016). Advances in tissue engineering. *Journal of pediatric surgery*, 51(1), 8-12.
10. Griffith, L. G., & Naughton, G. (2002). Tissue engineering--current challenges and expanding opportunities. *Science (New York, NY)*, 295(5557), 1009-1014.
11. Greenhalgh, D. G. (1996). The role of growth factors in wound healing. *Journal of Trauma and Acute Care Surgery*, 41(1), 159-167.
12. Nakamura, T., & Mizuno, S. (2010). The discovery of hepatocyte growth factor (HGF) and its significance for cell biology, life sciences and clinical medicine. *Proceedings of the Japan Academy, Series B*, 86(6), 588-610.
13. Bottaro, D. P., Rubin, J. S., Faletto, D. L., Chan, A. M., Kmiecik, T. E., Woude, G. V., & Aaronson, S. A. (1991). Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. *Science*, 251(4995), 802-804.
14. Bussolino, F., Di Renzo, M. F., Ziche, M., Bocchietto, E., Olivero, M., Naldini, L., ... & Comoglio, P. M. (1992). Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. *The Journal of cell biology*, 119(3), 629-641.
15. Huh, C. G., Factor, V. M., Sánchez, A., Uchida, K., Conner, E. A., & Thorgeirsson, S. S. (2004). Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair. *Proceedings of the National Academy of Sciences of the United States of America*, 101(13), 4477-4482.
16. Birchmeier, C., & Gherardi, E. (1998). Developmental roles of HGF/SF and its receptor, the c-Met tyrosine kinase. *Trends in cell biology*, 8(10), 404-410.
17. Li, J. F., Duan, H. F., Wu, C. T., Zhang, D. J., Deng, Y., Yin, H. L., ... & Wang, Y. L. (2013). HGF Accelerates Wound Healing by Promoting the Dedifferentiation of Epidermal Cells through-Integrin/ILK Pathway. *BioMed research international*, 2013.

18. Fausto, N., Laird, A. D., & Webber, E. M. (1995). Liver regeneration. 2. Role of growth factors and cytokines in hepatic regeneration. *The FASEB Journal*, 9(15), 1527-1536.
19. Park, C. M., & Hollenberg, M. J. (1989). Basic fibroblast growth factor induces retinal regeneration in vivo. *Developmental biology*, 134(1), 201-205.
20. Barrientos, S., Brem, H., Stojadinovic, O., & Tomic-Canic, M. (2014). Clinical application of growth factors and cytokines in wound healing. *Wound Repair and Regeneration*, 22(5), 569-578.
21. Lee, K., Silva, E. A., & Mooney, D. J. (2011). Growth factor delivery-based tissue engineering: general approaches and a review of recent developments. *Journal of the Royal Society Interface*, 8(55), 153-170.
22. Mitchell, A. C., Briquez, P. S., Hubbell, J. A., & Cochran, J. R. (2016). Engineering growth factors for regenerative medicine applications. *Acta biomaterialia*, 30, 1-12.
23. Sonnenberg, S. B., Rane, A. A., Liu, C. J., Rao, N., Agmon, G., Suarez, S., ... & Braden, R. (2015). Delivery of an engineered HGF fragment in an extracellular matrix-derived hydrogel prevents negative LV remodeling post-myocardial infarction. *Biomaterials*, 45, 56-63.
24. Aaronson, S. A. (1991). Growth factors and cancer. *Science*, 254(5035), 1146-1153.
25. Jay, S. M., & Lee, R. T. (2013). Protein Engineering for Cardiovascular Therapeutics Untapped Potential for Cardiac Repair. *Circulation research*, 113(7), 933-943.
26. Jones, D. S., Tsai, P. C., & Cochran, J. R. (2011). Engineering hepatocyte growth factor fragments with high stability and activity as Met receptor agonists and antagonists. *Proceedings of the National Academy of Sciences*, 108(32), 13035-13040.
27. Jones, D. S., Silverman, A. P., & Cochran, J. R. (2008). Developing therapeutic proteins by engineering ligand-receptor interactions. *Trends in biotechnology*, 26(9), 498-505.
28. Lahti, J. L., Lui, B. H., Beck, S. E., Lee, S. S., Ly, D. P., Longaker, M. T., ... & Cochran, J. R. (2011). Engineered epidermal growth factor mutants with faster binding on-rates correlate with enhanced receptor activation. *FEBS letters*, 585(8), 1135-1139.
29. Siemeister, G., Schirner, M., Reusch, P., Barleon, B., Marmé, D., & Martiny-Baron, G. (1998). An antagonistic vascular endothelial growth factor (VEGF) variant inhibits VEGF-stimulated receptor autophosphorylation and proliferation of human endothelial cells. *Proceedings of the National Academy of Sciences*, 95(8), 4625-4629.
30. Roy, R. S., Soni, S., Harfouche, R., Vasudevan, P. R., Holmes, O., de Jonge, H., ... & Blundell, T. L. (2010). Coupling growth-factor engineering with nanotechnology for therapeutic angiogenesis. *Proceedings of the National Academy of Sciences*, 107(31), 13608-13613.
31. Wang, Y., Cooke, M. J., Sachewsky, N., Morshead, C. M., & Shoichet, M. S. (2013). Bioengineered sequential growth factor delivery stimulates brain tissue regeneration after stroke. *Journal of Controlled Release*, 172(1), 1-11.
32. US Food and Drug Administration, Center for Biologics Evaluation and Research. Guidance for industry: gene-therapy clinical trials—observing subjects for delayed adverse events <<http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/ucm078719.pdf>> (November 2006).
33. Tatum, E. L. (1966). Molecular biology, nucleic acids, and the future of medicine. *Perspectives in biology and medicine*, 10(1), 19-32.
34. Wirth, T., Parker, N., & Ylä-Herttuala, S. (2013). History of gene therapy. *Gene*, 525(2), 162-169.

35. Ginn, S. L., Alexander, I. E., Edelstein, M. L., Abedi, M. R., & Wixon, J. (2013). Gene therapy clinical trials worldwide to 2012—an update. *The journal of gene medicine*, 15(2), 65-77.
36. Ylä-Herttuala, S. (2013). Cardiovascular gene therapy with vascular endothelial growth factors. *Gene*, 525(2), 217-219.
37. Isner, J. M., Pieczek, A., Schainfeld, R., Blair, R., Haley, L., Asahara, T., ... & Symes, J. F. (1996). Clinical evidence of angiogenesis after arterial gene transfer of phVEGF 165 in patient with ischaemic limb. *The Lancet*, 348(9024), 370-374.
38. Baumgartner, I., Pieczek, A., Manor, O., Blair, R., Kearney, M., Walsh, K., & Isner, J. M. (1998). Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. *Circulation*, 97(12), 1114-1123.
39. Losordo, D. W., Vale, P. R., Symes, J. F., Dunnington, C. H., Esakof, D. D., Maysky, M., ... & Isner, J. M. (1998). Gene therapy for myocardial angiogenesis initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia. *Circulation*, 98(25), 2800-2804.
40. Giacca, M., & Zacchigna, S. (2012). VEGF gene therapy: therapeutic angiogenesis in the clinic and beyond. *Gene therapy*, 19(6), 622-629.
41. Shimamura, M., Nakagami, H., Koriyama, H., & Morishita, R. (2013). Gene therapy and cell-based therapies for therapeutic angiogenesis in peripheral artery disease. *BioMed research international*, 2013.
42. Ylä-Herttuala, S. (2013). Cardiovascular gene therapy with vascular endothelial growth factors. *Gene*, 525(2), 217-219.
43. Sood, S., Gupta, S., & Mahendra, A. (2012). Gene therapy with growth factors for periodontal tissue engineering—a review. *Med Oral Patol Oral Cir Bucal*, 17(2), 301-10.
44. Boye, S. E., Boye, S. L., Lewin, A. S., & Hauswirth, W. W. (2013). A comprehensive review of retinal gene therapy. *Molecular Therapy*, 21(3), 509-519.
45. Branski, L. K., Pereira, C. T., Herndon, D. N., & Jeschke, M. G. (2007). Gene therapy in wound healing: present status and future directions. *Gene therapy*, 14(1), 1-10.
46. Branski, L. K., Gauglitz, G. G., Herndon, D. N., & Jeschke, M. G. (2009). A review of gene and stem cell therapy in cutaneous wound healing. *Burns*, 35(2), 171-180.
47. Andree, C., Swain, W. F., Page, C. P., Macklin, M. D., Slama, J., Hatzis, D., & Eriksson, E. (1994). In vivo transfer and expression of a human epidermal growth factor gene accelerates wound repair. *Proceedings of the National Academy of Sciences*, 91(25), 12188-12192.
48. Ueki, T., Kaneda, Y., Tsutsui, H., Nakanishi, K., Sawa, Y., Morishita, R., ... & Fujimoto, J. (1999). Hepatocyte growth factor gene therapy of liver cirrhosis in rats. *Nature medicine*, 5(2), 226-230.
49. Yin, H., Kanasty, R. L., Eltoukhy, A. A., Vegas, A. J., Dorkin, J. R., & Anderson, D. G. (2014). Non-viral vectors for gene-based therapy. *Nature Reviews Genetics*, 15(8), 541-555.
50. Henry, T. D., Hirsch, A. T., Goldman, J., Wang, Y. L., Lips, D. L., McMillan, W. D., ... & Keo, H. H. (2011). Safety of a non-viral plasmid-encoding dual isoforms of hepatocyte growth factor in critical limb ischemia patients: a phase I study. *Gene therapy*, 18(8), 788-794.
51. Perin, E. C., Mendelsohn, F., Davies, M., Pham, H., Saucedo, J., Hirsch, A., ... & Comerota, A. (2014). A PHASE 2, DOUBLE-BLIND, RANDOMIZED, PLACEBO-CONTROLLED, MULTICENTER TRIAL OF THE SAFETY AND EFFICACY OF PLASMID DNA EXPRESSING 2 ISOFORMS OF HEPATOCYTE GROWTH

- FACTOR IN PATIENTS WITH CRITICAL LIMB ISCHEMIA. *Journal of the American College of Cardiology*, 63(12_S).
52. ViroMed Co., Ltd. dba VM BioPharma. Safety and Efficacy Study of VM202 in the Treatment of Chronic Non-Healing Foot Ulcers. In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000-2016. Available from: <<https://clinicaltrials.gov/ct2/show/NCT02563522?term=vm202&rank=5>> NLM Identifier: NCT02563522.
 53. Ferraro, B., Cruz, Y. L., Coppola, D., & Heller, R. (2009). Intradermal delivery of plasmid VEGF165 by electroporation promotes wound healing. *Molecular Therapy*, 17(4), 651-657.
 54. Kwon, M. J., An, S., Choi, S., Nam, K., Jung, H. S., Yoon, C. S., ... & Park, J. H. (2012). Effective healing of diabetic skin wounds by using nonviral gene therapy based on minicircle vascular endothelial growth factor DNA and a cationic dendrimer. *The journal of gene medicine*, 14(4), 272-278.
 55. Zorko, M., & Langel, Ü. (2005). Cell-penetrating peptides: mechanism and kinetics of cargo delivery. *Advanced drug delivery reviews*, 57(4), 529-545.
 56. Meade, B. R., & Dowdy, S. F. (2007). Exogenous siRNA delivery using peptide transduction domains/cell penetrating peptides. *Advanced drug delivery reviews*, 59(2), 134-140.
 57. Ignatovich, I. A., Dizhe, E. B., Pavlotskaya, A. V., Akifiev, B. N., Burov, S. V., Orlov, S. V., & Perevozchikov, A. P. (2003). Complexes of plasmid DNA with basic domain 47-57 of the HIV-1 Tat protein are transferred to mammalian cells by endocytosis-mediated pathways. *Journal of Biological Chemistry*, 278(43), 42625-42636.
 58. Mäe, M., & Langel, Ü. (2006). Cell-penetrating peptides as vectors for peptide, protein and oligonucleotide delivery. *Current opinion in pharmacology*, 6(5), 509-514.
 59. Madani, F., Lindberg, S., Langel, Ü., Futaki, S., & Gräslund, A. (2011). Mechanisms of cellular uptake of cell-penetrating peptides. *Journal of Biophysics*, 2011.
 60. Lawrence, M. S., Phillips, K. J., & Liu, D. R. (2007). Supercharging proteins can impart unusual resilience. *Journal of the American Chemical Society*, 129(33), 10110-10112.
 61. McNaughton, B. R., Cronican, J. J., Thompson, D. B., & Liu, D. R. (2009). Mammalian cell penetration, siRNA transfection, and DNA transfection by supercharged proteins. *Proceedings of the National Academy of Sciences*, 106(15), 6111-6116.
 62. Freire, J. M., Veiga, A. S., Conceição, T. M., Kowalczyk, W., Mohana-Borges, R., Andreu, D., ... & Castanho, M. A. (2013). Intracellular nucleic acid delivery by the supercharged dengue virus capsid protein. *PloS one*, 8(12), e81450.
 63. Cronican, J. J., Thompson, D. B., Beier, K. T., McNaughton, B. R., Cepko, C. L., & Liu, D. R. (2010). Potent delivery of functional proteins into Mammalian cells in vitro and in vivo using a supercharged protein. *ACS chemical biology*, 5(8), 747-752.
 64. Cronican, J. J., Beier, K. T., Davis, T. N., Tseng, J. C., Li, W., Thompson, D. B., ... & Zhou, Q. (2011). A class of human proteins that deliver functional proteins into mammalian cells in vitro and in vivo. *Chemistry & biology*, 18(7), 833-838.
 65. Thompson, D. B., Villaseñor, R., Dorrr, B. M., Zerial, M., & Liu, D. R. (2012). Cellular uptake mechanisms and endosomal trafficking of supercharged proteins. *Chemistry & biology*, 19(7), 831-843.
 66. Youles, M., Holmes, O., Petoukhov, M. V., Nessen, M. A., Stivala, S., Svergun, D. I., & Gherardi, E. (2008). Engineering the NK1 fragment of hepatocyte growth factor/scatter factor as a MET receptor antagonist. *Journal of molecular biology*, 377(3), 616-622.

67. Jakubczak, J. L., Larochelle, W. J., & Merlino, G. (1998). NK1, a natural splice variant of hepatocyte growth factor/scatter factor, is a partial agonist in vivo. *Molecular and cellular biology*, 18(3), 1275-1283.
68. Chirgadze, D. Y., Hepple, J. P., Zhou, H., Byrd, R. A., Blundell, T. L., & Gherardi, E. (1999). Crystal structure of the NK1 fragment of HGF/SF suggests a novel mode for growth factor dimerization and receptor binding. *Nature Structural & Molecular Biology*, 6(1), 72-79.
69. Thompson, D. B., Villaseñor, R., Dorr, B. M., Zerial, M., & Liu, D. R. (2012). Cellular uptake mechanisms and endosomal trafficking of supercharged proteins. *Chemistry & biology*, 19(7), 831-843.
70. Lietha, D., Chirgadze, D. Y., Mulloy, B., Blundell, T. L., & Gherardi, E. (2001). Crystal structures of NK1-heparin complexes reveal the basis for NK1 activity and enable engineering of potent agonists of the MET receptor. *The EMBO journal*, 20(20), 5543-5555.
71. Schwall, R. H., Chang, L. Y., Godowski, P. J., Kahn, D. W., Hillan, K. J., Bauer, K. D., & Zioncheck, T. F. (1996). Heparin induces dimerization and confers proliferative activity onto the hepatocyte growth factor antagonists NK1 and NK2. *The Journal of Cell Biology*, 133(3), 709-718.
72. Conner, S. D., & Schmid, S. L. (2003). Regulated portals of entry into the cell. *Nature*, 422(6927), 37-44.
73. Dhami, R., & Schuchman, E. H. (2004). Mannose 6-Phosphate Receptor-mediated Uptake Is Defective in Acid Sphingomyelinase-deficient Macrophages IMPLICATIONS FOR NIEMANN-PICK DISEASE ENZYME REPLACEMENT THERAPY. *Journal of Biological Chemistry*, 279(2), 1526-1532.
74. Koivusalo, M., Welch, C., Hayashi, H., Scott, C. C., Kim, M., Alexander, T., ... & Grinstein, S. (2010). Amiloride inhibits macropinocytosis by lowering submembranous pH and preventing Rac1 and Cdc42 signaling. *The Journal of cell biology*, 188(4), 547-563.
75. Vercauteren, D., Vandebroucke, R. E., Jones, A. T., Rejman, J., Demeester, J., De Smedt, S. C., ... & Braeckmans, K. (2010). The use of inhibitors to study endocytic pathways of gene carriers: optimization and pitfalls. *Molecular Therapy*, 18(3), 561-569.
76. Kirchhausen, T., Macia, E., & Pelish, H. E. (2008). Use of dynasore, the small molecule inhibitor of dynamin, in the regulation of endocytosis. *Methods in enzymology*, 438, 77-93.
77. *Exocytosis and endocytosis*. Totowa, NJ:: Humana Press, 2008.
78. Song, B. H., Lee, G. C., Moon, M. S., Cho, Y. H., & Lee, C. H. (2001). Human cytomegalovirus binding to heparan sulfate proteoglycans on the cell surface and/or entry stimulates the expression of human leukocyte antigen class I. *Journal of General Virology*, 82(10), 2405-2413.
79. Liu, C. J., Jones, D. S., Tsai, P. C., Venkataramana, A., & Cochran, J. R. (2014). An engineered dimeric fragment of hepatocyte growth factor is a potent c-MET agonist. *FEBS letters*, 588(24), 4831-4837.
80. Royal, I., & Park, M. (1995). Hepatocyte growth factor-induced scatter of Madin-Darby canine kidney cells requires phosphatidylinositol 3-kinase. *Journal of Biological Chemistry*, 270(46), 27780-27787.
81. Hellman, L. M., & Fried, M. G. (2007). Electrophoretic mobility shift assay (EMSA) for detecting protein–nucleic acid interactions. *Nature protocols*, 2(8), 1849-1861.