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Characterization of Human Fibroblast Growth Factor 2 Variant to Determine Effects on Structure, Stability, and Cell Proliferation

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Characterization of Human Fibroblast Growth Factor 2 Variant to Determine Effects on Structure, Stability, and Cell Proliferation

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by

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Abstract

Fibroblast growth factors (FGFs) are a family of cell signaling proteins conserved across multiple species. Each individual FGF elicits different cellular functions including, but not limited to, proliferation, migration, differentiation, angiogenesis, and wound healing. One of the most studied members, fibroblast growth factor 2 (FGF2), has demonstrated substantial wound healing capacity in a wide range of tissues including skeletal, muscular, neural, respiratory, epithelial, and cardiovascular. This ability makes FGF2 a potential therapeutic for a wide range of conditions and injuries. However, due to a short half-life at room temperature, therapeutic use of FGF2 is limited. It has been demonstrated that in the structurally similar FGF1, point mutations in the heparin binding region significantly improved the proteins' stability and mitogenic activity. These mutations decreased the overall positive charge in the heparin binding region. Similar mutations in FGF2 may produce similar effects, since there are several positive residues in the heparin binding region of FGF2. Other research has identified lysine 134 as an important residue of the heparin binding region and was thereby chosen for charge reverse mutation. This research designed a K134E mutant of FGF2, and investigated the effects on stability and mitogenic activity. Using various biophysical techniques, proteolytic assay, and cell proliferation assay, we demonstrated that the engineered K134E mutant displayed enhanced bioactivity and thermal stability.

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Abbreviations

FGF - fibroblast growth factor

FGF1 - fibroblast growth factor 1

rhFGF2 – recombinant human fibroblast growth factor 2

FGF2 – fibroblast growth factor 2

FGFR – fibroblast growth factor receptor

RTKs - receptor tyrosine kinases

HSPG - heparan sulfate proteoglycans

RAS - rat sarcoma small GTPase

MAPK - mitogen-activated protein kinase

ERK1/2 - extracellular signal-related kinase 1 and 2

PI3K - phosphatidylinositol-3 kinase

AKT - protein kinase B

PLCγ - phospholipase Cγ

JAK - Janus kinase

STAT - signal transducer and activator of transcription

HMW – high molecular weight

LMW – low molecular weight

VEGF – vascular endothelial growth factor

PDGF – platelet-derived growth factor

PDGF-BB – platelet-derived growth factor composed of B subunits

RUNX – runt-related transcription factor

SOX9 - transcription factor Sry-related HMG box 9

CDK – cyclin-dependent kinase

BBB – blood-brain barrier

CNS – central nervous system

I/R – ischemia/reperfusion

SCI – spinal cord injury

BSCB - blood-spinal cord barrier

TBI – traumatic brain injury

RhoA – transforming protein RhoA, or Ras homolog gene family member A

PEAD - poly(ethylene argininylaspartate diglyceride)

GFAP - glial fibrillary acid protein

NF-200 – neurofilament 200

NGF – nerve growth factor

SP-A, SP-B, and SP-C – pulmonary surfactant proteins

TGFβ1 – transforming growth factor beta 1

αSMA – α-smooth muscle actin

CTGF – connective tissue growth factor

ALI - acute lung injury

BAL – bronchoalveolar lavage

BEAS-2B – human normal lung epithelial cell line

HIF-1α – hypoxia-inducible factor 1 alpha

Chapter 1

Introduction

Multicellular organisms require precise communication between cells in order to survive. This ability to communicate with each other is commonly referred to as cellular signaling. Cells receive some signals externally from the environment or other organisms, such as heat or touch. However, many cell signals are signaling molecules produced within individual organisms. Some of the different chemicals identified as signaling molecules include steroids (i.e. testosterone, estrogen), eicosanoids (i.e. prostaglandins, leukotrienes), monoamines (i.e. dopamine, adrenaline), gases (i.e. nitric oxide, carbon monoxide), amino acids, and proteins/peptides. Proteins represent one of the most intriguing classes of chemical signals, as they can initiate autocrine, paracrine, endocrine, or intracrine signaling. Some types of signaling proteins include antibodies, membrane proteins, peptide hormones, neuropeptides, and growth factors. The term 'growth factor' includes many families such as epidermal growth factors (EGFs), bone morphogenetic proteins (BMPs), interleukins (IL), transforming growth factors (TGFs), and fibroblast growth factors (FGFs).

Fibroblast growth factors are a family of pleiotropic cell signaling proteins found in many multicellular organisms, including invertebrates. The FGF family has 23 identified members in mammals, although only 22 of these have been discovered in humans. They contribute to a wide range of cellular activities, including embryonic development, metabolism, proliferation, differentiation, migration, neurogenesis, angiogenesis, and wound healing (5, 14, 16, 63, 71). FGF activity was first documented in 1939, when O.A. Trowell and E.N. Willmer found bovine brain extracts promoted fibroblast growth *in vitro* (74). Later in 1973 Hugo Armelin cultured 3T3 fibroblasts with bovine pituitary extracts, and observed increased growth compared to controls (3). Thus, the term 'fibroblast growth factor' was coined based on these results. Armelin's collaborator, Denis Gospodarowicz, subsequently purified the first two family members based

on isoelectric points: acidic FGF (aFGF or FGF1) and basic FGF (bFGF or FGF2) (17). Due to their affinity for heparin, other authors also called them “heparin-binding growth factors.”

Since then, an additional 21 FGFs have been discovered in vertebrates, with molecular masses ranging from 17-34 kDa (14, 16). Within the FGF gene family there are seven subfamilies based on phylogenetic analysis. The subfamilies FGF1 (FGF1-2), FGF4 (FGF4-6), FGF7 (FGF3, 7, 10, and 22), FGF8 (FGF8, 17, and 18), and FGF9 (FGF9, 16, and 20) have various autocrine and paracrine functions. The endocrine FGF19 subfamily consists of FGF19, 21, and 23. The intracrine FGF11 subfamily, or the homologous FGFs, is composed of FGF11-14. The only known FGF not found in humans is FGF15, which has been identified as a murine ortholog of FGF19 (80).

Research compiled over decades has shown that FGFs are structurally similar to one another. Crystal structures determined so far (FGF1-2, FGF9-10, FGF20, and FGF23) have verified a homologous core region of 120-130 amino acids (5, 23). When properly folded, this region forms a β-trefoil fold composed of 12 anti-parallel β-strands. The FGF19 subfamily is a slight exception, as these are missing the β11 strand (5). The variations between FGFs occurs in the amino- and carboxy-terminal regions. These regions vary in both length and sequence identity. These are believed to contribute to the regulation of separate cellular functions performed by FGFs (5).

Most FGFs cause pleiotropic effects by binding and activating their primary signaling partner, fibroblast growth factor receptors (FGFRs) (5-6, 14, 16, 23). FGFRs are a family of receptor tyrosine kinases (RTKs) found on the cell membrane. The are only four members within this family, FGFR1-4. Each of these are transcribed from four separate genes in mammals. However, they are highly homologous, sharing between 57-71% sequence identity (9). Each FGFR has a transmembrane helix, an intracellular kinase domain, and three extracellular immunoglobulin(Ig)-like domains (D1, D2, D3). A linker region between D1 and D2

houses an acid box, a highly conserved motif rich in aspartic and glutamic acid residues (9). D1 and the D1-D2 linker have been implicated in receptor autoinhibition (5). The binding site for FGFs is created by the D2 and D3 domains. Each FGFR has unique ligand specificity and is differentially expressed depending on the type of tissue.

The paracrine FGFs require heparan sulfate proteoglycans (HSPG) as a signaling cofactor. The glycosaminoglycans of HSPG are heterogeneously sulfated, which helps stabilize FGFs. The HSPG binding site in FGFs is comprised of the β1-β2 loop and the β10-β12 region (23, 56). Between paracrine FGFs there exists some primary sequence variation of the HSPG binding site, which causes differing affinity for HSPG. However, a common topology has been observed in these HSPG binding sites. This can be attributed to a conserved **G**XXXX**G**XXS/T motif (16), known as the glycine box. Some researchers have proposed that the HSPG specificity of FGFs is determined by the 'canyon' formed by the dimer of two FGF-FGFR complexes (45). The primary heparin binding region of FGF2 is located at β-strands 10 and 11 (28,56). Site-directed mutagenesis studies identified K35, N36, R90, K128, R129, T130, Q132, K134, K138, Q143, and K144 as residues significant to the heparin binding region (36,72). Crystal structures of FGF2 complexed with heparin-derived tetra- or hexasaccharides revealed that residues N36, R129, K134, and E143 were involved in heparin binding (12).

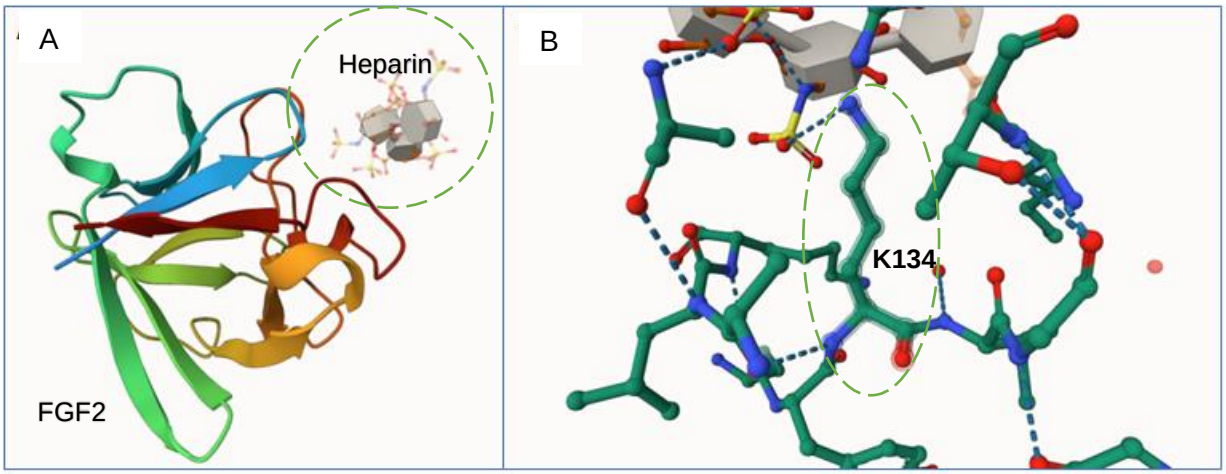

Figure 1: Panel A: Crystal structure of FGF2 complexed with heparin tetramer fragment (PDB 1BFB); Panel B: Lysine 134 shown to contribute to heparin binding.

Once the HSPG-FGF-FGFR complex forms, a homodimer results as a 2:2:2 multicomplex (5, 9, 14, 16, 23). The cytosolic kinase domain becomes autophosphorylated, triggering the activation of downstream signaling pathways. Some of the known signaling pathways associated with FGF-FGFR complexes includes rat sarcoma (RAS)/mitogen-activated protein kinase (MAPK)/extracellular signal-related kinase 1 and 2 (ERK1/2), phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT), phospholipase Cγ (PLCγ), and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) (6, 14, 16).

FGF2 is one of the most studied proteins known to humans. Evidence has suggested regulatory roles in cellular proliferation, differentiation, migration, survival, and stemness (2-11, 13-16, 19-20, 35, 49, 53, 57-58, 69, 73, 80, 83). The cumulative body of research has identified positive effects with skeletal, muscular, neural, respiratory, cardiovascular, kidney, skin, eye, and ear tissues. It has been demonstrated to promote wound healing in burns (2, 56, 86), sutured wounds (58), fractures (29), different types of ulcers (25, 66, 76), corneal epithelium (43), corneal vascularization (11), critical limb ischemia (33), periodontal regeneration (33, 49), skin grafts for skin avulsion (42), and wound dehiscence (88). The combined effects of FGF2 are what allows it to promote healing in a variety of tissues.

Since the nature of a wound is damaged tissue, there is inherently a need for cellular proliferation to fill in or replace existing structures. FGF2 has been shown to promote migration, proliferation, and regeneration in many cell and tissue types.

Skeletal

FGFs and FGFRs are expressed during all stages of skeletal development. Associated signaling pathways regulate limb bud formation and mesenchymal condensation (59). FGF2 has roles in fracture healing, bone formation, and skeletal development (54). Multiple pathogenic states have also been associated with either over or under expression (59). Null mice exhibited decreased bone mass and new bone formation (46).

Highly expressed in bones, the low molecular weight (LMW) and high molecular weight (HMW) isoforms of FGF2 seem to perform different functions (54). The LMW 18 kDa gene product localizes in the cytoplasm and can interact with the plasma membrane. It promotes osteoblast differentiation and mineralization by activating the WNT cell signaling pathway, which is known to have roles in cell proliferation and migration (10). This isoform also acts as a positive upstream regulator of BMP-2. HMW isoforms localize in the nucleus and upregulates expression of genes which impair mineralization (22).

A 2015 study examined transgenic mice expressing LMW hFGF2 (24). They exhibited faster fracture healing compared to the control specimen. Better healing rates were associated with increased expression of vascular endothelial growth factor (VEGF), platelet-derived growth factor B, C, and D (PDGF-B, -C, and -D), runt-related transcription factor 2 (RUNX2), sex determining region Y-box 2 (SOX9), and osterix. In a rat calvarium defect model, combination treatment of FGF2/FGF18 lead to better regeneration, demonstrated by increased bone volume and density (27). In a sheep osteochondral defect model, FGF2 gene transfer with recombinant adeno-associated viral vector enhanced expression of FGF2 and healing (48). Another study

investigated the effects of FGF2 on knee articular cartilage in a rabbit defect model (70). That group implanted a collagen membrane loaded with 100ng/mL of FGF2 into a rabbit knee injury. They observed increased secretion of collagen I and II in chondrocytes, as well as increased chondrocyte differentiation. This was evidenced by improved filling of defects with cartilage-like cells, and fusion of the regenerated surface with surrounding cartilage. Interestingly, they also discovered that pulsed low-intensity ultrasound further increased the positive effects of FGF2 treatment.

Muscular

Research has also demonstrated that FGF2 impacts the growth, survival, and renewal of satellite cells (62, 64). Proliferating skeletal muscle cells express FGF1, FGF2, FGF4, and FGF7 (20). Following terminal differentiation, both FGF1 and FGF2 are no longer detectable. It's also been shown that terminal differentiation of skeletal muscle cells is inhibited by FGF2 (20). FGF2 prevents muscular differentiation by activating the Ras homolog gene family member A (RhoA), a GTPase which aids in regulation of actomyosin structure and contractility (4, 39).

Aging muscle fibers are known to have a decreased regenerative ability, however FGF2 has been shown to increase proliferation of old muscle stem cells (35). Application of FGF2 induced greater proliferation in old satellite cells compared to younger ones. Both young and old satellite cells had similar mRNA expression, indicating that their myogenic fates are the same. The cell cycle inhibitor cyclin-dependent kinase (CDK) p21 was also found to be upregulated in older muscle cells. However, treatment with FGF2 significantly decreased p21 expression to the same amount produced by younger cells. Those results suggest that healing of older tissue is inhibited by p21 arrest of the cell cycle.

Neural

After brain ischemia, platelet-derived growth factor receptor β (PDGFRβ) expression is increased to help maintain the blood-brain barrier (BBB), as well as induce growth and migration of pericytes into an infarct area. Cultured human central nervous system (CNS) pericytes treated with FGF2 displayed significantly higher expression of PDGFRβ, and improved both cell growth and PDGF-BB-induced migratory activity (52). In the presence of FGF2, PDGF-BB also synergistically promotes neovascularization and stabilization of new vasculature (7, 53, 87). A murine brain ischemia model revealed that both FGF2 and FGFR1 are upregulated in peri-infarct tissue (52). Immunofluorescent staining also showed that both were co-localized with PDGFRβ. Furthermore, an FGFR inhibitor eliminated effects of FGF2 on pericyte cultures. Rat astrocytes in an *in vitro* ischemia/reperfusion model had decreased viability with FGF2 knockdown, and improved survival with exogenous FGF2 treatment (40). Intranasal application of nanoliposomes containing FGF2 were able to pass the BBB and directly treat ischemia/reperfusion(I/R) injuries in rats (89). Three days of this treatment caused improvements in neurologic deficit score and spontaneous locomotor activity, as well as reducing the infarct volume by half.

A number of studies have also investigated the effect of FGF2 on recovery of spinal cord injury (SCI). Subcutaneous injection of FGF2 in a rat contusion SCI model resulted in improved motor function and BSCB recovery (84). Another group designed a liposome to deliver FGF2 to the site of SCI, as FGF2 is unable to pass the blood-spinal cord barrier (BSCB) by itself (81). Their liposome is grafted with two targeting peptides: a R2KC peptide which allows BSCB penetration, and a CAQK peptide which targets injury lesions. A rat SCI model demonstrated that these liposomes could effectively cross the BSCB as well as accumulate at the injured area. When FGF2 was incorporated into these liposomes for treatments, SCI rats had decreased neuronal apoptosis and axonal atrophy. Motor function was significantly improved

with continuous treatments. FGF2 also has potential to treat traumatic brain injury (TBI). An *in vivo* murine model of TBI, FGF2 protected BBB from breakdown by suppressing RhoA and upregulating tight junction proteins (79).

A significant body of evidence supports a positive effect of FGF2 on peripheral nerve regeneration. One clinical study implanted an alginate/heparin gel loaded with FGF2 into the gaps of damaged digital nerves (67). Both patients achieved sensory recovery in the fingertip after 6 months. When treated with recombinant human FGF2, crush-injured mental nerves in Sprague-Dawley rats exhibited both improved regeneration of axon density and sensory recovery (34). Interestingly, regeneration was enhanced greater by a 10ug/mL dose of recombinant human FGF2 (rhFGF2) than a 50ug/mL dose.

Composite biomaterials provide novel solutions to drug and growth factor delivery. One group developed a biocompatible coacervate able to load and preserve the bioactivity of heparin-binding compounds (8). The coacervate is composed of the polycation poly(ethylene argininylaspartate diglyceride) (PEAD) and heparin. Following an initial 10% release burst of FGF2, linear rate of diffusion was observed for 42 days with 60% FGF2 remaining coacervate bound. This FGF2-coacervate was to treat sciatic nerve crush injury in rats (38). A single intramuscular orthotopic injection of the coacervate greater recovery of motor and sensory function compared to subjects regularly administered FGF2. Coacervate injection also improved regeneration of myelinated fibers and remyelination. Histological analysis showed denser, more compact and uniform nerve fibers and thicker myelin sheaths in the FGF2-coacervate group. Injury to peripheral nerves causes dedifferentiation and proliferation of Schwann cells, marked by expression of glial fibrillary acid protein (GFAP). The FGF2-coacervate enhanced GFAP signaling, as well as expression of NF-200, in comparison to empty vehicle or exogenous FGF2 treatments. These data suggest that FGF2 promotes regeneration of injured peripheral nerves. Similar results were observed in crushed sciatic nerves of diabetic rats using a thermosensitive

heparin-poloaxamer hydrogel loaded with FGF2 and NGF (37). They reported increased Schwann cell proliferation, increased expression of nerve associated structural proteins, improved axonal regeneration, remyelination, and recovery of motor function.

Respiratory

FGF2 has also been shown to affect lung regeneration and epithelium maintenance. In fetal rat lung epithelial cells, FGF2 treatment both stimulated proliferation and induced expression of lung epithelial cell-specific surfactant proteins SP-A, SP-B, and SP-C (41). It should also be noted that different effects were observed for inhibition of MAPK and PI3K signaling, two well-known pathways associated with FGF2 signaling. Inhibiting MAPK halted cell proliferation and increased the expression of SP-A,-B, and -C. When PI3K was inhibited instead, expression of SP-C was inhibited. Another group tested two models of lung injury in mice, either by intranasal bleomycin or intraperitoneal injection of naphthalene (19). In both models, FGF2 knockout mice had deficient recovery of respiratory-related endothelium, reduced epithelial integrity, and prolonged inflammation. Their results support FGF2 is required for lung epithelial regeneration and epithelial integrity maintenance. Another bleomycin-induced lung injury model in transgenic mice designed to overexpress FGF2 had decreased pulmonary fibrosis compared to controls (32). *In vitro* experiments on human and murine primary lung fibroblasts showed that FGF2 inhibited transforming growth factor beta 1 (TGFβ1) induced expression of α-smooth muscle actin (αSMA), collagen, connective tissue growth factor (CTGF), and stress fiber formation. Importantly, epithelial gene expression was unaffected by FGF2 treatments. This is important because fibroblast-to-myofibroblast differentiation is a key event in pulmonary fibrosis pathogenesis. Treatment of acute lung injury (ALI) with FGF2 has also been investigated (69). Affected mice models treated with FGF2 had decreased cellularity and inflammatory factors in bronchoalveolar lavage (BAL) fluid, as well as reduced levels of oxidative stress in lung tissue. An *in vitro* assay was also performed on human normal lung

epithelial cells (BEAS-2B). FGF2 administration caused decreased inflammation, apoptosis, and increased PI3K/AKT signaling. Recovery from sepsis-induced ALI in mice was also positively affected by FGF2 treatment, resulting in decreased inflammation, pulmonary capillary leakage, and improved survival (61).

Cardiovascular

Evidence also suggests FGF2 has roles in cardiovascular maintenance and repair. FGF2 was shown to reduce oxidative stress, conferring cardiovascular protection in myocardial infarction and I/R injury (73). Another study examined effects of FGF2 on myocardial infarction in mice. They observed decreased apoptosis of cardiomyocytes, as well as increased angiogenesis (65). These effects were attributed to a mechanism mediated by hypoxia-inducible factor 1 alpha (HIF-1α), which had increased expression in presence of FGF2. FGF2 has also been shown to aid in cardiac reprogramming (83). Mouse embryonic fibroblasts were successfully differentiated into spontaneously beating, cardiomyocyte-like cells using a combination treatment of FGF2, FGF10, and VEGF. However, this effect was also observed in FGF2 only treatments, albeit 100-fold less than the combination treatment.

Numerous studies have explored the synergistic effects of FGF2 in combination treatments. One group in 2015 questioned how adipose-derived stem cells (ADSCs) would affect efficacy of FGF2 in cardiac regeneration following myocardial infarction (77). Using a mouse model of induced myocardial infarction, ADSCs and FGF2 were co-injected along the edge of the infarcted tissue. In comparison to injections of either FGF2 or ADSCs with phosphate buffered saline (PBS), the combination treatment showed significantly improved regeneration of cardiac tissue. They observed decreased fibrotic area, as well as increased arteriole density and graft growth. Their results also revealed that both FGF2 and ADSCs reduced both MMP-2 and MMP-9. Another study, using the same induced murine model of myocardial infarction, tested the effects of injecting a FGF2-releasing hydrogel along the infarction border (13). FGF2 led to

significantly improved healing compared to empty hydrogels. This was demonstrated by decreased densities of macrophages and myofibroblasts, reduced collagen content, increased blood vessel density, and improved survival/proliferation of myocardiocytes. Another study has also shown that FGF2 reduces left ventricular remodeling following myocardial infarction (50). Supportive observations included increased vascular density, higher systolic function, and lowered left ventricular end-diastolic pressure.

Skin

Skin is commonly known to be the largest organ of the human body and confers protection from the environment while also retaining fluids and nutrients. The two major layers are the epidermis and dermis. The epidermis is composed of a multilayered structure of squamous epithelial cells. Components of the dermis include connective tissues, blood vessels, hair follicles, sweat glands, and fibroblasts.

Injury to skin layers initiates a signaling cascade for the healing process. This process can be divided into four phases: hemostasis, inflammation, proliferation, and tissue remodeling. Several FGFs are known to affect these physiological processes, including FGF2. Multiple studies have proven that chitosan hydrogels loaded with FGF2 improve healing by stimulating angiogenesis and fibroblast proliferation (46, 57). Injections of platelet-rich plasma with FGF2 improved wrinkles and areas of skin depression (26). However, since that study had no control or FGF2 only groups, it can be argued that FGF2 alone might not be as effective. Murine skin wounds treated with FGF2 showed increased epithelium-mesenchyme transition of keratinocytes, which was accompanied by more rapid wound closure than controls (31). Wound edge keratinocytes were observed doing two things: 1) they generated thicker, multilayered epithelia, and 2) migrated towards the center of the wound. One study investigated how FGF2 alters effects of UV radiation on a nude mouse model. Application of dalteparin/protamine

nanoparticles loaded with FGF2 significantly decreased the apoptosis of dermal fibroblasts and epidermal keratinocytes (68). FGF2 treated mice also demonstrated greater skin elasticity.

Overall, most FGF2 studies have been performed *in vitro* or in animal models, however recent decades have generated data for FGF2 in clinical settings. One such clinical trial examined the effects of FGF2 treatment on chronic skin ulcers (47). Treated ulcers had been persistent for at least four weeks prior to treatment. FGF2 was released into the wound using a pre-loaded collagen/gelatin sponge. Approximately 94% of treated wounds saw a significant improvement in the wound bed. Interestingly, there was not a significant difference between low or high dosages.

Due to the inherent instability of FGF2, it's use as a therapeutic is limited. Although delivery systems have improved in recent decades to combat this issue, design of a stable form of FGF2 would allow for more clinical uses. Other studies have investigated both single- and multiple-point mutations to the amino acid sequence in order to increase stability.

In our investigations, we chose to mutate a single residue (K134) within the HSPG binding pocket of FGF2. We mutated the positively charged lysine into a negatively charged glutamic acid, which may also affect the structural characteristics, proteolytic stability, and proliferative ability of FGF2.

Chapter 2

Materials and methods

Construction and Purification of FGF2 and the K134E variant

Agilent supplied the site directed mutagenesis kit and competent cells (BL21plysS) used for bacterial transformation. The plasmid isolation kit was obtained from Qiagen Inc., USA. Lysogeny broth was purchased from IBI Scientific, USA. Heparin-sepharose resin was obtained from GE Healthcare, USA. VWR Scientific, USA supplied the buffer components (Na2HPO4, NaH2PO4, NaCl). NIH 3T3 cells were obtained from ATCC. All cell culture reagents including DMEM media, fetal bovine serum (FBS) and penicillin streptomycin were purchased from Thermo Fisher Scientific (Waltham, MA). All other chemicals and materials were of high-quality analytical grade. Unless otherwise stated, samples were made in 10 mM phosphate buffer saline (pH 7.2) and incubated at 37°C. Site-directed mutagenesis was employed to insert the gene for either FGF2 or K134E mutant into the plasmid vector pET20b. Bacterial transformation for each plasmid was achieved by mixing 3μL of the plasmid with 100μL of competent Bl21pLysS cells, then subjecting the cells to a 45 second heat shock. The cells were incubated with 800μL of sterile lysogeny broth (LB) on a shaker at 250 rpm and 37°C for 45 minutes. 100μL of this culture was spread on a sterile LB-agar plate containing ampicillin. Since the gene for ampicillin resistance is contained in the pET20b plasmid, only transformed bacteria will be able to grow on these plates. Plates were incubated overnight at 37°C. For expression of FGF2 and K134E mutant, BL21pLysS cells were grown to an optical density between 0.6–8.0 absorbance at 600nm and incubated with 1 mM isopropyl β-D-thiogalactoside for 2.5 hours. Cells were harvested and resuspended in 10 mM phosphate buffer(PB). Cells were sonicated for 20 minutes with 10 second on/off pulses. Proteins were separated from the cell debris using centrifugation at 19,000 rpm for 30 minutes. Resulting supernatant was decanted from solid pellet, and then passed thru a glutathione-sepharose affinity column. Non-binding particles were

then washed out with equilibration buffer (10 mM phosphate buffer) until baseline was achieved. To cleave FGF2 from the GST tag, thrombin was added to the column and allowed to incubate overnight on a shaker. FGF2 was collected from the column the next day, and the GST tag was recovered by disrupting binding to the resin with reduced glutathione buffer, pH 7.2. All collected fractions were evaluated by 15% sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie brilliant blue. Protein fractions containing the K134E mutant were further purified by passage through a heparin-sepharose affinity column. Non-binding particles were then washed out with equilibration buffer (10 mM phosphate buffer) until baseline was achieved. The proteins were purified by applying an increasing sodium chloride gradient to the column. Fractions were collected and the purity of proteins were verified using SDS-PAGE. The concentration of proteins was quantified using the Bradford method.

Circular dichroism and fluorescence spectroscopy

Using a combination circular dichroism/fluorescence Jasco J-1500 Spectrophotometer dual results of the CD and fluorescence spectra were obtained to determine the secondary and tertiary structural changes of FGF2 and K134E mutant. Circular dichroism is used to determine the secondary structural changes in the protein. For analysis, 10μM of protein was added to 10 mM PB + 100 mM NaCl and loaded into a 0.1 cm path length quartz cell. The wavelength of the spectrophotometer was set in a range of 190 - 250nm at 25ºC, and the scanning speed was 20 nm/min. The data of CD was collected as an average of three scans. The resultant CD data was expressed in terms of molar ellipticity.

Intrinsic fluorescence spectroscopy was used to examine any alternation in the tertiary structure and folding of protein by combining 10 μM protein with 10 mM phosphate buffer (PB). The excitation wavelength for the fluorescence measurements was 280 nm and the emission were recorded from 300 – 450 nm. Intrinsic fluorescence was done at these wavelengths to

study if there were any changes among tyrosine and tryptophan residues, which respectively fluoresce at 308 and 340 nm. A buffer subtraction was made to correct background noise.

8-Anilino-1-napthalenesulfonic acid (ANS) binding assay

ANS binding assay measurements were made using a Fluorescence Spectrophotometer F-2500 (Hitachi) with a slit width set to 2.5nm. Protein concentrations of 28 μM in phosphate buffer containing 100 mM NaCl, pH 7.2 were placed in a quartz cuvette. Titrations using an ANS stock were made by addition of 10 μM increments of ANS followed with mixing and incubation for 2 minutes preceding each reading at 25ºC. Fluorescence intensity was determined with an excitation at 380nm and emission intensity was recorded at 510nm.

Limited trypsin digestion

Limited trypsin digestion of FGF2 and K134E mutant was performed in PB containing 250 mM NaCl, pH 7.2. The initial reaction tube contained 38 μM of protein and 0.5 μg of enzyme. The trypsin control and trypsin containing samples were respectively incubated at room temperature (25 °C) and 37°C. Digested samples were removed at specific intervals as noted in the results section and then the reaction was stopped by the addition of 10% trichloroacetic acid. Proteins were separated using SDS-PAGE as previously described. The percentage of enzymatic digestion was identified from the band intensity, on the SDS PAGE gel, using UN-ScanIT densiometric software (Silk Scientific Inc.). Samples not subjected to the enzymatic digestion were considered as the controls representing 100% undigested FGF2 or K134E mutant.

Thermal stability

Intrinsic fluorescence using the Jasco J-1500 Spectrophotometer was used to monitor the unfolding nature of FGF2 and K134E mutant at increasing temperature intervals. The protein concentration used was 50 μM in 10 mM phosphate buffer and 250 mM NaCl (pH 7.2). A

temperature probe was inserted into the sample cell as a heat source. Fraction of protein unfolded was calculated from the ratio of the wavelength of emission maxima (305/350 nm) observed during chemical denaturant titration. Thermal denaturation as probed by fluorescence was carried by heating samples at 5-degree increments.

Bioactivity assay

3T3 fibroblast cells obtained from ATCC (Manassas, VA) were cultured in media consisting of DMEM supplemented with 10% bovine calf serum. Cells were grown and incubated overnight at 37°C. The bioactivities of FGF2 and K134E mutant were determined by quantifying the cell number increase after the cells were incubated with one of these proteins. Starved 3T3 fibroblasts were collected and seeded in a well plate at a seeding density of 10,000 cells/well. The bioactivity assays were performed in triplicate under the same condition. 3T3 cell proliferation was assessed by the Cell Titer-Glo (Promega, Madison, WI) cell proliferation assay after 24 hours.

Results and discussion

FGF2 is known to have roles in cellular processes such as proliferation, differentiation, and angiogenesis. It's believed that binding of heparin to FGF2 increases both the protein structural stability and cell proliferation activity. Therefore, it was of interest to investigate a charge reversal mutation within the heparin-binding pocket of FGF2. The K134E mutation in the FGF2 amino acid sequence was designed to achieve this, replacing a positively charged lysine residue with a negatively charged glutamic acid residue.

FGF2

MAAGSITTLPALPEDGGSGAFPPGHFKDPKRLYCKNGGFFLRIHPDGRVDGVREKSDPHI KLQLQAEERGVVSIKGVCANRYLAMKEDGRLLASKCVTDECFFFERLESNNYNTYRSRKY TSWYVALKRTGQYKLGSKTGPGQKAILFLPMSAKS

FGF2 K134E

MAAGSITTLPALPEDGGSGAFPPGHFKDPKRLYCKNGGFFLRIHPDGRVDGVREKSDPHI KLQLQAEERGVVSIKGVCANRYLAMKEDGRLLASKCVTDECFFFERLESNNYNTYRSRKY TSWYVALKRTGQYELGSKTGPGQKAILFLPMSAKS

Figure 2: Amino acid sequence of FGF2 and FGF2 K134E mutation. The residue chosen for charge reversal mutation is highlighted in red.

The K134E mutation was chosen based on previous results in Kumar's lab with human FGF1. It was previously found that in FGF1, several lysine residues contribute to heparin binding (1). Results demonstrated that reversing the positively charged residue R136 in FGF1 decreased heparin-binding ability while mitogenic activity was unaffected. Therefore, it was desired to determine if a similar charge reversal in FGF2 could confer similar effects. Sequence alignment between FGF1 and FGF2 was used to select an analogous residue to R136. We chose K134 in FGF2 for mutation because it is also positively charged and shows structural alignment with FGF1.

Substituting a positively charged lysine with a negatively charged glutamic acid in the heparin-binding pocket decreases electrostatic repulsion caused by multiple positively charged residues. Theoretically, this should cause the pocket to "tighten," or be less accessible by heparin. It may also further stabilize the protein, in a manner similar to how heparin neutralizes the positive charges present in the FGF2. Since mutating an amino acid sequence can have downstream effects on secondary and tertiary protein structures, it was critical to compare structural analyses of FGF2 and K134E mutant.

Site directed mutagenesis was used to construct the plasmid containing FGF2 K134E mutant. The resulting plasmid was used for bacterial transformation of BL21pLysS cells.

Untransformed bacteria will not survive on LB-agar containing ampicillin, so colony growth indicates successful transformation with the plasmid containing gene for either FGF2 or K134E mutant.

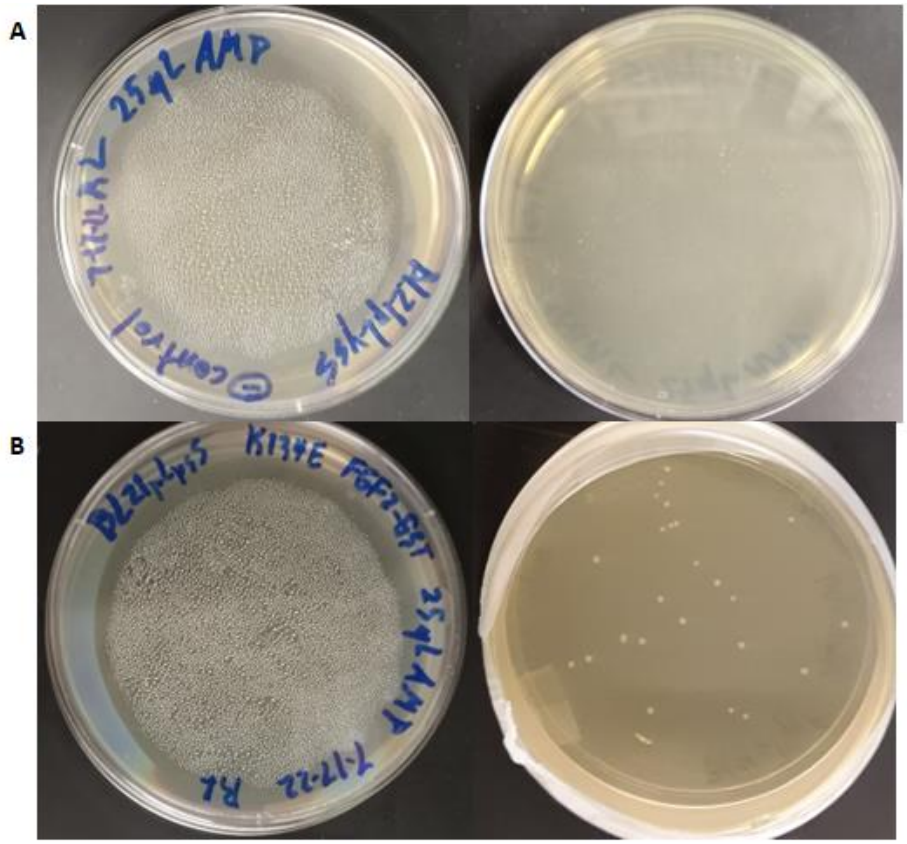

Figure 3: Top and bottom view of LB-agar bacterial transformation plates. Row A – control plate using untransformed BL21pLysS cells; Row B – FGF2 K134E transformation plate.

Both FGF2 and K134E mutant were purified as described in the materials and methods. Pure protein was achieved for both proteins, as demonstrated by the SDS-PAGE results (Fig. 4 and 5). Pure FGF2 was achieved in one step with glutathione-sepharose purification. However, the K134E mutant repeatedly eluted with significant contamination. Therefore, it was further purified using a heparin-sepharose affinity column (Fig. 5).

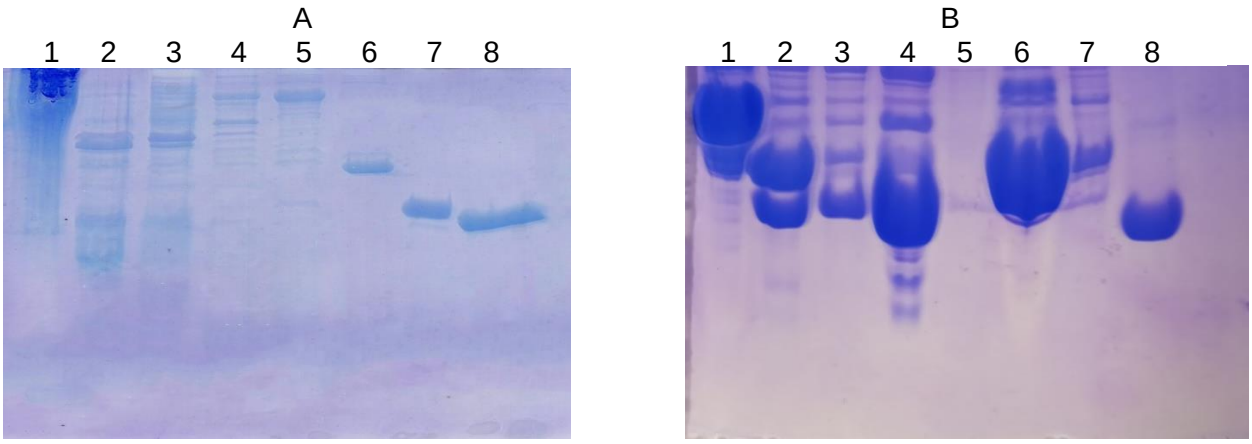

Figure 4: SDS-PAGE analysis of glutathione-sepharose purifications of FGF2 (A) and K134E mutant (B). A: Lane 1 – Pellet produced from sonication and centrifugation of bacteria with FGF2; Lane 2 – supernatant collected after centrifugation; Lane 3-5 – buffer washes with PB + 250mM NaCl; Lane 6 – GST tag eluted with buffer + reduced glutathione; Lane 7 – FGF2; Lane 8 – FGF1 (used as MW marker). B: Lane 1 – Pre-cleavage sample of GST-FGF2 K134E mutant; Lane 2 – Post-cleavage sample of GST + FGF2 K134E mutant; Lane 3-5 – buffer washes with PB + 250mM NaCl; Lane 6 – GST tag eluted with buffer + reduced glutathione; Lane 8 – FGF1 (used as MW marker).

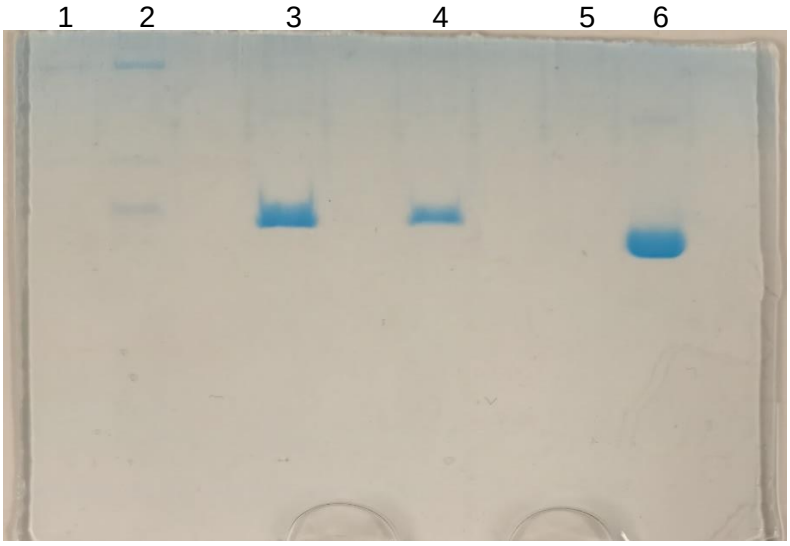

Figure 5: SDS-PAGE analysis of heparin-sepharose purification of FGF2 K134E. Lanes 1-2 – buffer washes with PB + 300mM NaCl; Lanes 3-4 – buffer washes with PB + 500mM NaCl; Lane 5 – buffer wash with PB + 800mM NaCl; Lane 6 – FGF1 (used as MW marker).

Fluorescence spectroscopy was used to examine the intrinsic fluorescence of FGF2 and K134E mutant. The FGF2 has one tryptophan residue and seven tyrosine residues, which are the amino acids primarily responsible for intrinsic fluorescence of proteins due to their aromatic characteristics. A properly folded protein in its' native state will exhibit tyrosine fluorescence, with an emission maximum at 308nm. This is because tryptophan fluorescence is quenched by amine/imine groups of surrounding residues. Denaturation exposes tryptophan to the surface, exhibited by a shift of fluorescence emission maximum from 308nm to 350nm. Intrinsic fluorescence spectra of FGF2 and K134E mutant displayed emission maxima at 308nm. This suggests that the point mutation did not significantly alter the tertiary structure of FGF2.

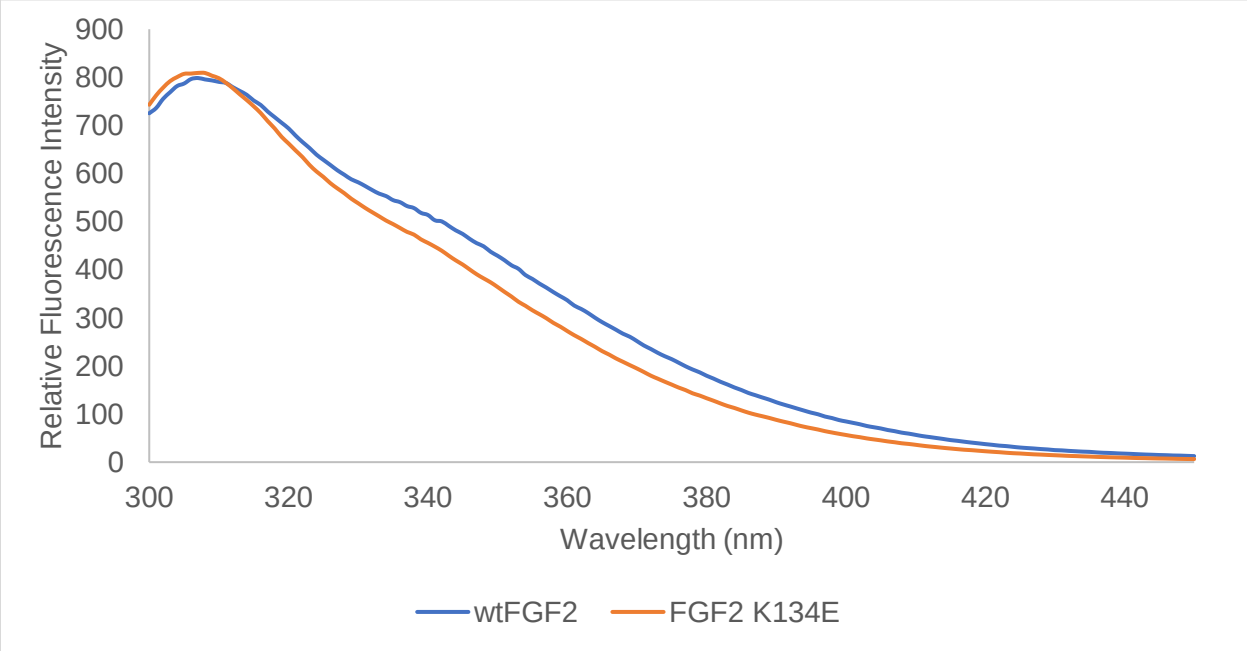

Figure 6: Overlay of fluorescence spectra of FGF2 (blue) and K134E mutant (orange).

In order to determine any structural changes between FGF2 and K134E mutant, far-UV CD spectroscopy was used to examine the secondary structures of both proteins. Spectra from FGF2 and K134E mutant overlay with each other. Both showed a negative peak near 207nm and a positive peak near 228nm, which are typical of β-trefoil structures. These results indicate

that the secondary structure of FGF2 was not significantly affected by the K134E mutation (Fig. 7).

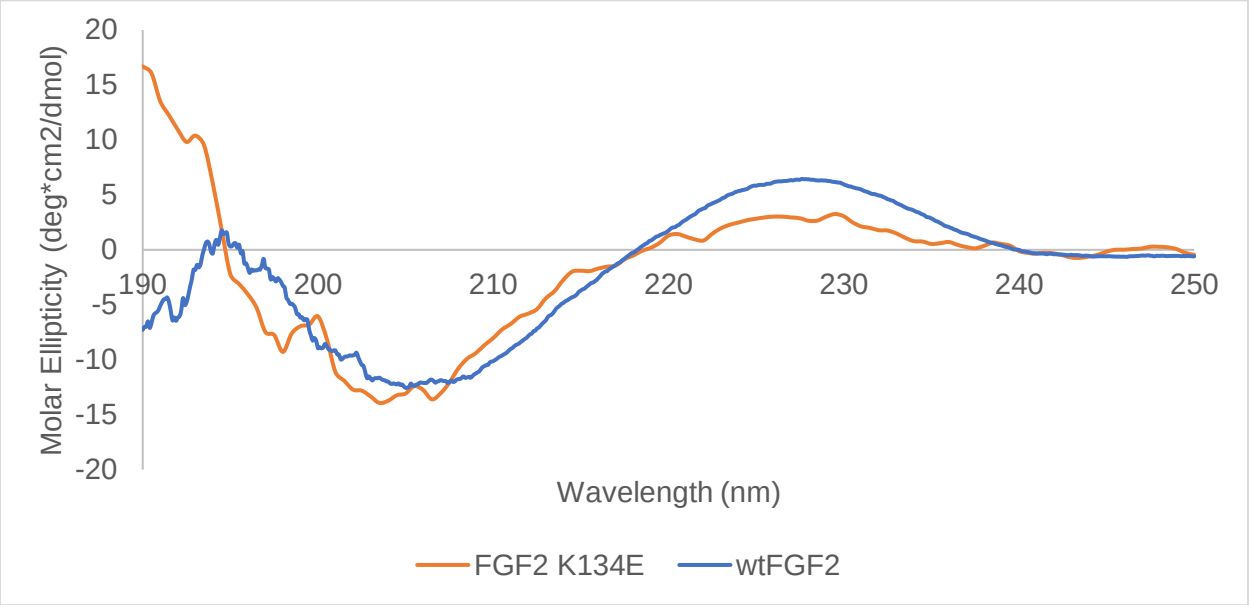

Figure 7: Overlay of far-UV circular dichroism spectra of FGF2 (blue) and K134E mutant (orange).

8-anilinoanphthalene-1-sulfonate (ANS) is a non-polar, fluorescent dye that has been used to detect the presence of solvent-exposed hydrophobic surfaces of proteins (15). Relative fluorescence intensity of a protein is directly proportional to the number of hydrophobic residues which are solvent accessible. Typically, hydrophobic residues are buried within tertiary structures. Therefore, a higher relative fluorescence from ANS binding to protein indicates there are more solvent-accessible hydrophobic surfaces.

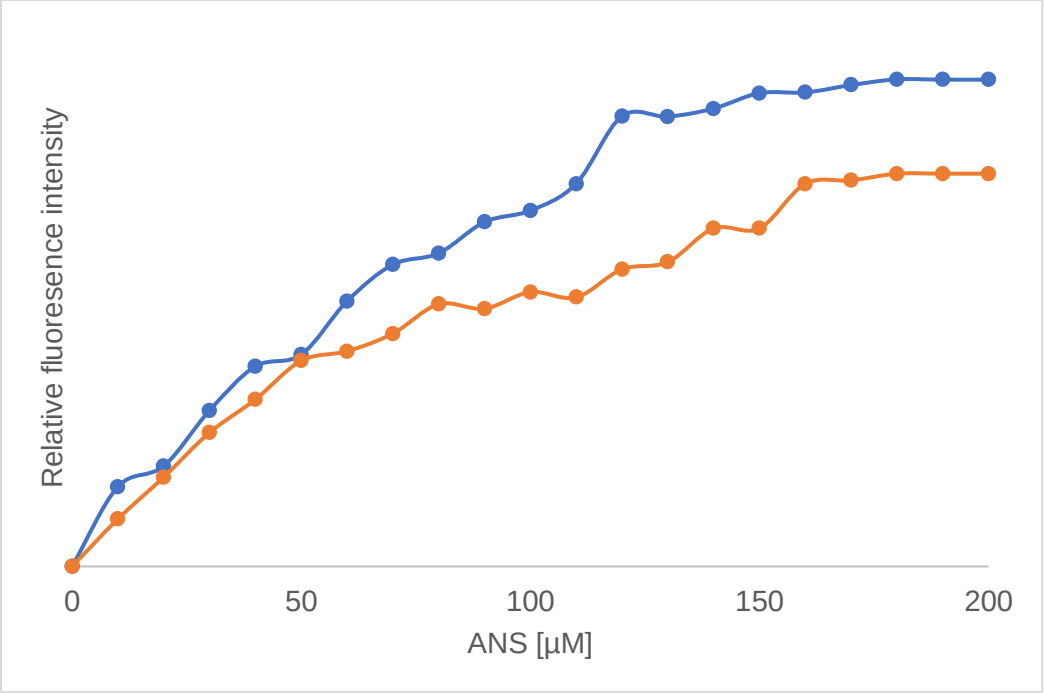

Figure 8: ANS binding assay of FGF2 (blue) and K134E mutant (orange).

Limited trypsin digestion was performed, in order to determine the effect of K134E mutation on the conformational flexibility of FGF2. It was predicted that enzymatic digestion also wouldn't be as strong, since the K134E mutant has one less cleavage site compared to FGF2. Following digestion, protein samples were separated using SDS-PAGE. The amount of protein digested for each time interval was determined by densitometric analysis of the 18 kDa band corresponding to FGF2. The rate of digestion was slightly increased for FGF2, however their digestion curves are similar. The slightly increased digestion of FGF2 can be explained by the lysine residue not present in the K134E mutant. It also suggests that the K134E mutation does not significantly change the conformational flexibility.

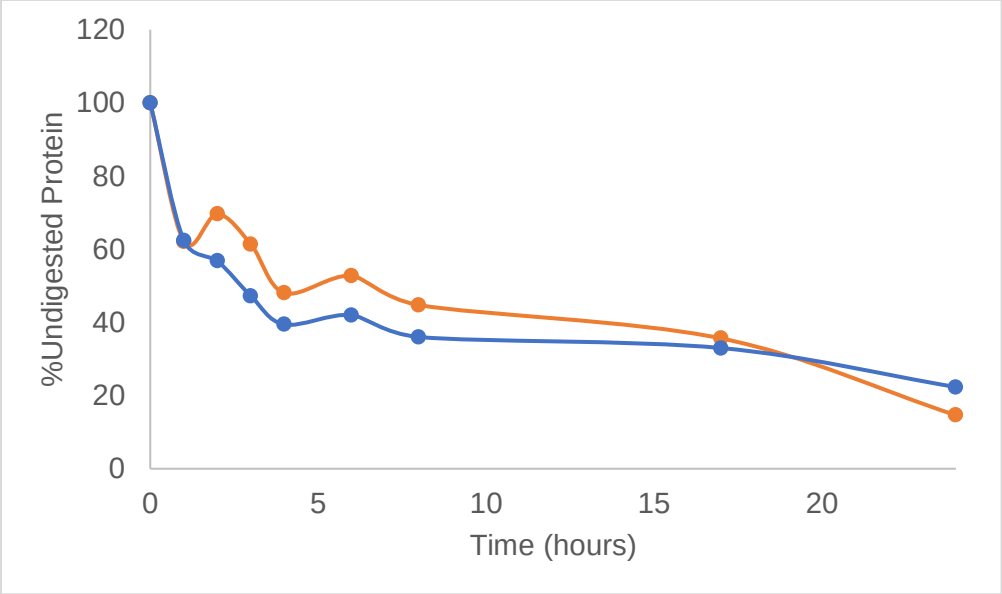

Figure 9: Time dependent trypsin digestion of FGF2 (blue) and K134E mutant (orange).

Thermal stability of FGF2 and the K134E mutant were investigated by monitoring changes in the ratio of intrinsic fluorescence intensities at 308nm and 350nm. The K134E mutant displayed significantly higher thermal stability, with ~20°C increase in T_m. This suggests that the single point mutation improves the thermal stability similar to protective action of heparin.

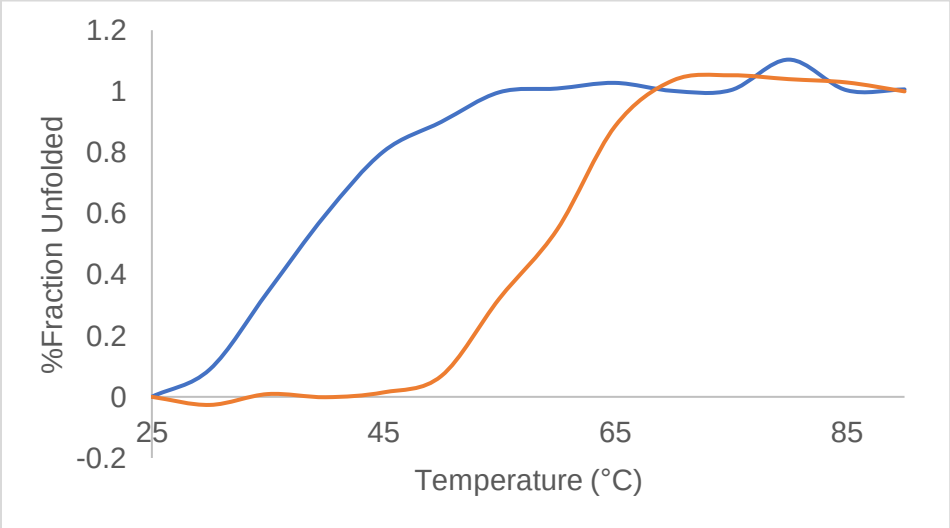

Figure 10: Thermal denaturation of FGF2 (blue) and K134E mutant (orange).

FGF2 is known to stimulate cell proliferation activity. Therefore, the effect of K134E mutation on bioactivity was investigated. NIH 3T3 fibroblasts were cultured with either FGF2 or K134E mutant. It's predicted that by altering the heparin-binding pocket, proliferative activity can be enhanced without addition of heparin. Proliferation was similar in concentrations up to 20ng/mL of each protein, however at higher concentrations the K134E mutant exhibited greater proliferation. This suggests the mutated protein can still bind to FGFR without heparin and elicit a stronger growth response.

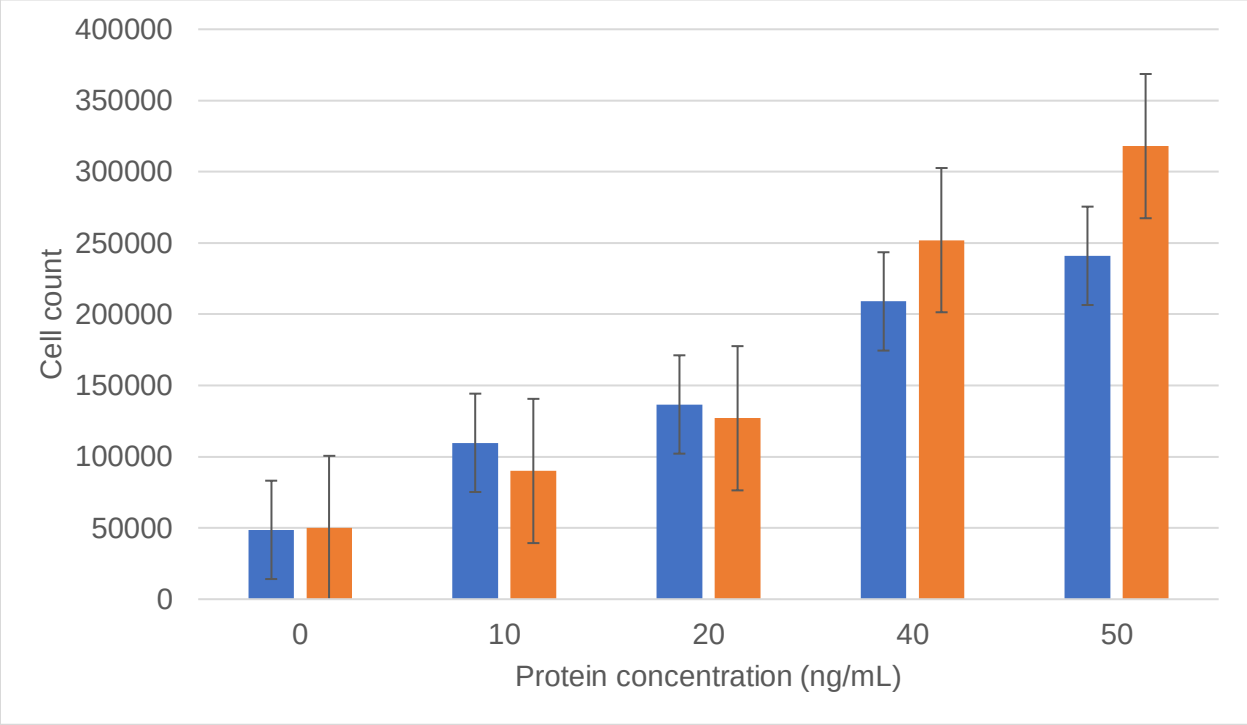

Figure 11: Bioactivity assay of FGF2 (blue) and K134E mutant (orange).

Chapter 3

Conclusion and future scope

As demonstrated across research groups, FGF2 can dramatically affect wound healing in a variety of tissues. However, it is susceptible to quick degradation which makes it difficult to use as a therapeutic. Therefore, it is of interest to engineer a more shelf stable FGF2 variant for wider treatment usage.

Previously, Dr. Kumar's research group identified a FGF1 variant with increased thermal and proteolytic stability compared to wild-type FGF1. This variant was engineered with the point mutation R136E. This replaced a positive charge with a negative charge in the heparin binding pocket, thereby decreasing the electrostatic repulsion caused by several positively charged residues. The heparin binding pocket was 'tightened' as a result, with decreasing dependence on heparin for thermal stability and bioactivity. Sequence alignment revealed that a similarly charged residue in FGF2, lysine 134, corresponded to R136 in FGF1. We chose to create a similar point mutation, K134E, in the primary sequence of FGF2, in order to investigate if the stability of FGF2 could also be improved.

We investigated the structural features, thermal stability, and bioactivity of the K134E FGF2 variant. Studies on intrinsic fluorescence and secondary structure via far-UV spectroscopy revealed that the overall structure was unaffected by the K134E mutation. Spectra demonstrated that the β-trefoil structures remained intact, which are the most prominent secondary structures found in FGF2. The proteolytic stability of K134E to trypsin cleavage is slightly improved. However, since trypsin cleaves at the C-terminus of arginine and lysine residues, this may be explained by the loss of a cleavage site. Greater improvement was seen in the thermal stability, increasing from 38.1°C in FGF2 to 58.9°C in the K134E variant. The bioactivity was significantly enhanced when the concentration of FGF2 was ≥40ng/mL. Overall,

the K134E point mutation seems to improve the thermal stability and bioactivity of FGF2 without altering important structural features.

Future work should investigate this mutation further. It needs to be determined how susceptible the K134E mutant is to chemical denaturants, such as urea or guanidine hydrochloride. Although it eluted from heparin-sepharose at lower NaCl concentrations compared to FGF2, this work did not investigate the heparin binding capability of the K134E mutant. After elucidating these points, the FGF2 sequence should be examined for other important amino acids in the heparin binding region.

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UNIVERSITY OF ARKANSAS

Office of Research Integrity and Compliance

May 8, 2023

MEMORANDUM

TO:	Dr. Suresh Thallapuranam
FROM:	Ines Pinto, Biosafety Committee Chair
RE:	Protocol Renewal
PROTOCOL #:	13004
PROTOCOL TITLE:	Survey of stabilizing mutations of Human Fibroblast Growth Factor-1

The Institutional Biosafety Committee (IBC) has approved your request, dated May 9th 2022, to renew IBC # 13002 "Survey of stabilizing mutations of Human Fibroblast Growth Factor-1".

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

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