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The Structural and Functional Properties of a Double Mutant of Human Acidic Fibroblast Growth Factor (hFGF-1)

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The Structural and Functional Properties of a Double Mutant of Human Acidic Fibroblast Growth Factor (hFGF-1)

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by

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Abstract

Human acidic Fibroblast Growth Factor 1 (FGF-1), a member of the FGF superfamily, is a potent mitogen and heparin-binding protein involved in a broad spectrum of biological processes, including angiogenesis, cell proliferation, and wound healing. Design of hFGF-1 with an increased thermal stability and an enhanced cell proliferation activity is highly desired for wound healing applications. Herein, we have designed the variant of FGF-1 by substituting two important amino residues in the heparin-binding pocket. The variant was overexpressed in *Escherichia coli* and was successfully purified to homogeneity using an affinity chromatographic procedure. Far-UV circular dichroism spectroscopic analysis showed that the backbone conformation of the hFGF-1 did not alter due to the introduction of mutations in the heparin-binding pocket. The designed hFGF-1 variant exhibited an increased resistance to limited trypsin digestion. Isothermal titration calorimetry study confirmed that approximately 20-fold decrease in heparin binding affinity (Kd ~90μM) was observed in case of the double mutant compared to that of the wild-type FGF1 (~5μM). Incorporation of positively charged Lys135 adjacent to the negatively charged E136 might have reduced the repulsive effect to heparin. 8-Anilino naphthalene 1-sulfonate (ANS) binding assay revealed that the introduced mutations cause a subtle change in the solvent-accessible non-polar surface of the protein. These results were in concordant with other biophysical data obtained from limited trypsin digestion, 1H 15N HSQC analysis. In addition, no significant change in bioactivity was observed between the mutant and the wild-type FGF1 proteins. This confirms that introduction of positive charge adjacent to E136 nullified the effects of this unique mutation.

Acknowledgment

This work was supported in part by grants from the National Institutes of Health [NCRR COBRE Grant 1 P20 RR15569 (TKSK), P30 GM103450 (TKSK)], the Department of Energy [(Grant DEFG02-01ER15161 (to TKSK)], National Science Foundation [Grant IOS0843397 9TO (to TKSK)] and the Arkansas Biosciences Institute (to TKSK). I would like to express my deepest gratitude to my academic advisor Dr. T.K.S. Kumar for his immense knowledge, constant encouragement, patience, caring, and demanding a high quality of work. Additionally, I would like to thank my outstanding mentor Dr. Srinivas Jayanthi for his hard work and enduring support. I am grateful for the King Abdullah Scholarship Program for giving me the opportunity to complete my higher education abroad. My sincere thanks also goes to my great parents, husband, and daughter for being always by my side and for their constant love and support.

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Introduction

1. Broad Classification of FGFs

Human acidic Fibroblast Growth Factor 1 (FGF-1), a member of the FGF superfamily, is a heparin-binding protein and powerful mitogen for various types of cells, such as hepatocytes, myocytes, uroepithelium cells, endothelial cells, and neural cells. FGFs are involved in a broad spectrum of biological processes, including angiogenesis, cell proliferation, nerve regeneration and embryonic development and wound healing. A total of 22 signaling molecules (FGF-1 to 14 and FGF-16 to 23) have been recognized in humans as members of the FGFs family. These structurally related molecules can be categorized by gene location analysis into seven subfamilies: FGF-1(including orthologs of FGF-1 and 2), FGF-4 (Orthologs of FGF-4, 5, and 6), FGF-7 (Orthologs of FGF-3, 7, 10, and 22), FGF-8 (Orthologs of FGF-8, 17, ad 18), FGF-9 (Orthologs of FGF-9, 16, and 20), FGF-11 (Orthologs of FGF-11, 12, 13, and 14), and the FGF-15/ 19 (Orthologs of FGF-15/ 19, 21, and 23) subfamily (Itoh & Ornitz, 2011). This classification is based on their biological function and structure similarities. 120 to 30 amino acids make up the core region of FGFs, whereas the alteration/diversity of the N- and C-terminal amino acids sequence determines the specificity of the corresponding ligand (Tulin & Stathopoulos, 2010). The expression of these growth factors has been recognized in most tissue, if not all, with almost the same expression pattern but different timing and sites. For instance, some factors appeared only during embryonic development while others are expressed in embryonic cells and continue into adult tissue (FGF-3, 4, 8, 15, 17 and 19) and (FGF-1, 2, 5-7, 9-14, 16, 18, and 20-23) respectively (Ornitz & Itoh, 2001).

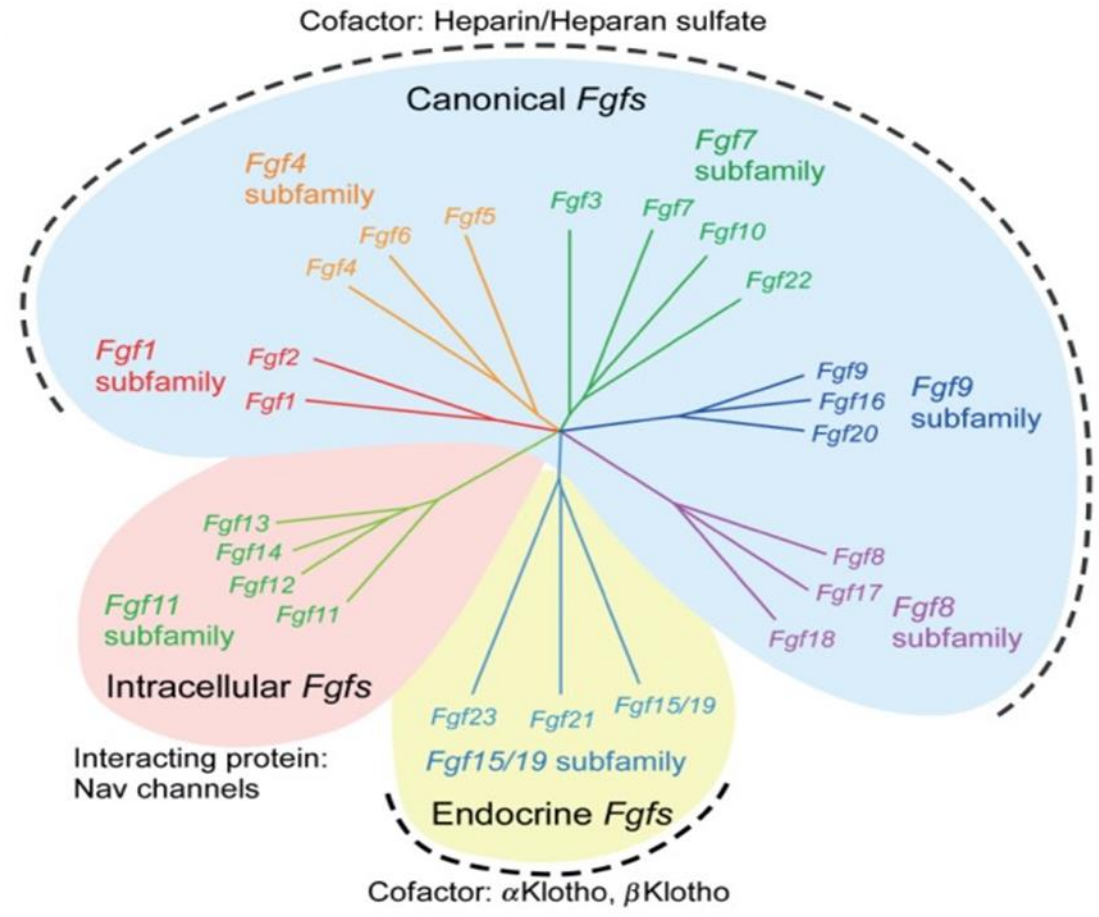


Figure 1. FGF and FGFR families. (a) Phylogenetic analysis suggests that 22 Fgf genes can be arranged into seven subfamilies containing two to four members each. Branch lengths are proportional to the evolutionary distance between each gene. The Fgf1, Fgf4, Fgf7, Fgf8, and Fgf9 subfamily genes encode secreted canonical FGFs, which bind to and activate FGFRs with heparin/HS as a cofactor. The Fgf15/19 subfamily members encode endocrine FGFs, which bind to and activate FGFRs with the Klotho family protein as a cofactor. The Fgf11 subfamily genes encode intracellular FGFs, which are non-signaling proteins serving as cofactors for voltage gated sodium channels and other molecules. Adapted from "The Fibroblast Growth Factor signaling pathway," by D. M. Ornitz, 2015, *Developmental Biology*, 4(3), 215–266. http://doi.org/10.1002/wdev.176 Copyright 2015 by the Authors. WIREs Developmental Biology published by Wiley Periodicals, Inc. Adapted with permission.

2. General introduction about the broad functions of FGF

FGFs are well known as multifunctional proteins as they participate in regulating many endocrine signaling pathways as well as various phases of the wound healing process. They are involved in the wound reparative process by coordinating the intracellular signaling pathways and promoting the migration, differentiation, and proliferation of several cell types. Re-epithelialization, granulation tissue formation and fibroplasia, and matrix deposition are the remarkable stages in the proliferation phase of the healing injury that is highly mediated by fibroblast growth factors. Angiogenesis and formation of new capillaries and blood vessels are generally controlled by autocrine signaling of FGFs proteins, which are considered as potent angiogenesis inducers (Basilico & Moscatelli, 1992). FGFs and FGFRs have a vital role in developing the central nervous system during embryogenesis and adulthood in mammalian growth. For instance, FGF-8 is capable to regulate the boundaries, mass, and function of Brodmann area in the cerebral cortex region of the brain (Fukuchi-Shimogori & Grove, 2001). Furthermore, it has been demonstrated that the malfunction or deficiency of these factors and their receptors would cause birth defects, developmental complications and congenital diseases (Coumoul & Deng, 2003).

3. Chemistry and structure of FGF-1

FGFs are normally produced proteins and are relatively small in size, between 17 and 34 kDa, with a length of 120 to 140 amino acids. 28 highly conserved and six identical amino acid residues build up the core region of this protein (ten of these highly conserved residues determine the specificity of the

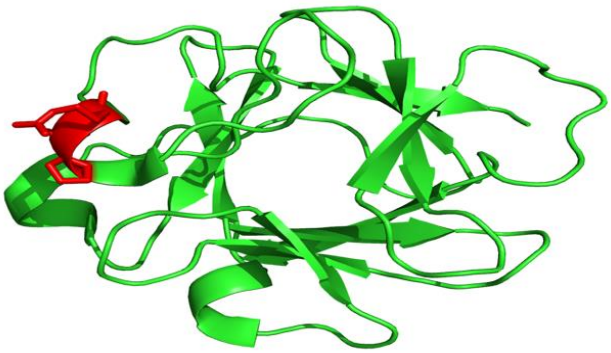


Figure 2. X-ray structure of the wild-type FGF-1. The heparin binding pocket is shown in red.

interaction toward the FGF receptors, and three coding exons makeup the FGF gene (Beenken & Mohammadi, 2009). FGF-1 is found on the top of the cell surface or within the cell matrix and it is usually released by the damaged cells during injury. The crystallography analysis of FGF proteins depicts a β trefoil fold structure containing twelve beta sheets strands organized into the central domain, five of these sheets form a hairpin binding structure. The beta-sheets are arranged in an anti-parallel direction and they are well conserved (Brych, Blaber, Logan & Blader, 2001). FGFs are composed of cell-surface polysaccharide, heparin sulfate proteoglycans (HSPGs), which is essential for the protein regulation especially in signaling transmission and chemical recruitment. The heparin sulfate site that is associated with the FGF protein surface and extracellular matrix interacts with heparin and heparan sulfate glycosaminoglycans (HSGAGs) (Bellosta et al. 2001). This essential step mediates the specific binding to the cell surface tyrosine kinase receptors and as a result activates FGFs' biological response.

4. Role of heparin in FGF activation

Heparin is a naturally formed polysaccharide that releases into the blood circulation from the secretory granules of the white blood cell, mastocyte, during tissue injury. The average molecular weight of this carbohydrate molecule is 3 to 30 Kilo-Daltons (KDa) and it has a significant anticoagulant activity. The linear and polydisaccharide chain of heparin consist of repeated heterogeneous (sulfated and under-sulfated) disaccharide units. The exact composition of these disaccharide units are detailed in the figure below.

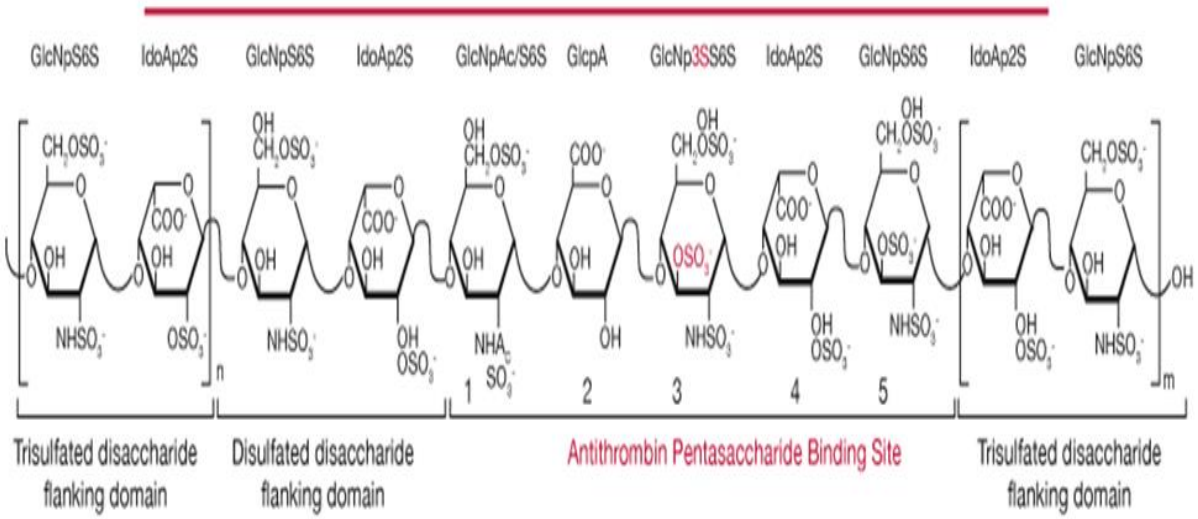


Figure 3. The structure of Heparin. Heparin's Antithrombin III binding site and its structural variants. Reprinted from Heparin Science Retrieved from http://www.heparinscience.com/structure_heparin.html

The high affinity binding of heparin towards FGFs is believed to increase the protein stability by increasing its resistance to proteolysis, heat, and acid as well as enhances its half-life. In addition to the specificity and protective action of heparin to FGFs, it enhances the protein efficiency two to three magnitude orders. The previously mentioned functions clearly explain the dependency of FGFs on heparin in their activation and ligand-receptor complex stabilization (Ornitz et al., 2001).

5. FGF-receptors Structure

FGFs proteins mediate the biological response and trigger many signaling pathways by binding to their high affinity ligands, fibroblast growth factor receptors FGFRs. The high binding affinity of heparin or heparan sulfate proteoglycan (HSPG) toward heparin pocket located in FGFs' surface will facilitate the protein/ligand interaction and as a result stimulate the protein.

The activation of specific cell-surface tyrosine kinase receptors, FGFR1, FGFR2, FGFR3, and FGFR4, results into activation of intracellular signaling pathways. The RAS/mitogen-activated protein kinase (MAPK), PI3K-AKT, and PLCγ cascades are some examples of the pathways that are induced by the FGF/FGFR signaling system. The physical composition of FGFR is an N-terminal extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic domain (Abbass, Asa & Ezzat, 1997). The first domain consists of three immunoglobulin-like domains (D1, D2, and D3). The D2 and D3 components of the extracellular ligand-binding domain have been shown to be the minimal units essential for the ligand-receptor binding interaction (Olsen et al., 2004). The inhibition of the receptors' activation in the absence of the protein is controlled by the acid box found between the D1 and D2 domains. When the FGF/HSPG compound binds to the receptor's extracellular domain, the dimerization of two tyrosine kinase domains in the plasma membrane occurs. The biochemical signals of these adjacent kinases induce the activation and autophosphorlyation of the receptors in their cytoplasmic domain. The activation of these key events results into endocrine, autocrine, and paracrine effects on the cell and subsequently stimulates downstream signaling cascades (Sarabipour & Hristova, 2016).

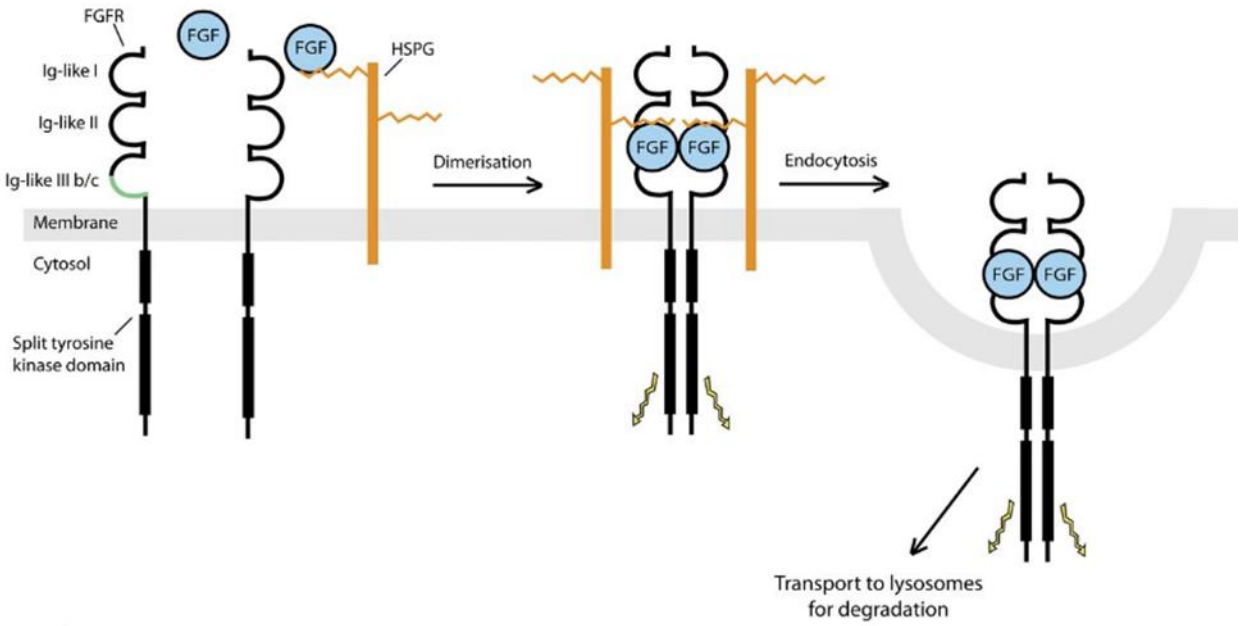


Figure 4. Complex formation between FGF, FGFR and HSPG initiates signalling The basic structure of an FGFR is shown on the left-hand side. FGFRs are single-pass transmembrane RTKs with an extracellular part composed of three Ig-like domains (I–III), and an intracellular part containing a split tyrosine kinase domain. The complex formed by two FGFs, two heparan sulfate chains and two FGFRs causes dimerization and transphosphorylation by the kinases on several tyrosine residues in the intracellular part of the FGFRs, causing activation of downstream signalling cascades. After activation, the complex is internalized by endocytosis and transported to lysosomes for degradation. Adapted from "Fibroblast growth factors and their receptors in cancer" by J. Wesche, K.Haglund, and E. Margrethe, 2011, *Haugsten Biochemical Journal*, 437 (2) 199-213; DOI: 10.1042/BJ20101603. Adapted with permission.

6. Biomedical application of FGF

Wound healing is a complex systematic process of repairing damaged cells and injured tissue layers after severe trauma. This procedure has been one of the most challenging clinical problems since ancient times. It has a significant advantage in protecting vital internal organs by developing an effective defensive barrier against aggressive pathogens' invasion. The capabilities of curing the damaged part and capturing its critical functions are essential for all

multicellular organisms. Although not all tissue and organs can function exactly as they were before the injury, the wound healing mechanism is considered to be conserved in all biological systems (Martin & Nunan, 2015). When you get injured, the wound healing process can take between a few minutes to more than six months depending on the wound type, chronic or acute. The internal bleeding of a vital organ cannot be equated to a skin scratch. In addition, there are different factors that can delay the healing process, such as smoking, alcohol consumption, infections, age, and obesity. Time is the most crucial component in this process. For example, a small superficial wound would heal much faster than a jagged deep wound. The treatment of both acute and chronic wound healing share a huge market of up to $20 billion in the United States alone (Sen, et al., 2009). According to recent reports from WebMed Professional, the treatment of chronic wound healing is expensive and also prevalent. The complete healing of diabetic, venous, and other types of ulcers appears to consume longer time if the traditional synthetic bandages are used.

A. Current wound healing therapy using diverse growth factors

Platelet-Derived Growth Factor (PDGF), Transforming Growth Factor (TGF), Interleukins, Epidermal Growth Factor (EGF), and Fibroblast Growth Factor (FGF) are some examples of growth factors involved in the wound reparative process by coordinating the intracellular signaling pathways and promoting the cell migration, differentiation, and proliferation. In this section, we will concentrate on growth factors that improved healing efficiency in several studies conducted in animal models or in vitro. The safety and the efficacy of topically applied recombinant growth factors were evaluated in some research. The clinical trials that will be discussed include skin and diabetic ulcers, burns, and wounds developed after surgeries. As has been mentioned in previous sections, the first mechanism triggered during the

healing process is the coagulation cascade. Platelets are known as the storage pool for a massive number of growth factors and that is why they are highly involved in promoting the epithelial and internal wounds reparation. An example of platelets wound repair therapy is the autologous platelet-rich plasma that has been shown to stimulate the healing progression and reduce inflammation. Activated platelets and macrophages produce the key mediators in wound healing,

PDGF and TGF-beta 1. The dimeric disulfide-bond growth factor PDGF progresses the healing process by enhancing the collagen and proteoglycan synthesis and driving inflammatory cells to the injury area. It is no wonder that this crucial element being the first approved topical application as a recombinant therapy for accelerating the healing process. On the other hand, the application of the topical platelet-derived growth factor on diabetic foot ulcers seems to be unsuitable (Christine et al., 20015). For example, the PDGF formulation (Regranex®, becaplermin INN) has been used to treat diabetic foot ulcers in the United States and Europe since 1997 and 1999, respectively (Koveker, 2000). However, in 2012, this product was withdrawn because of its weak benefit to risk ratio.

The vascular endothelial growth factors (VEGFs) are responsible for maintaining the healing process especially during tissue hypoxia by encouraging angiogenesis and vascular permeability. VEGFs are capable in halting the bleeding and induce the vascular branching particularly during embryogenesis and to some extent in adult tissue. Preserving the neovascularization status in the form of providing essential nutrition by vessels' capillaries to the wound site, especially in the serious clinical conditions, such as vascular diseases and diabetes, is critical in chronically unhealed wounds. However, this is not the typical scenario in chronically unhealed injuries as insufficient quantities of nutrient are supplied, therefore, failing in maintaining the timely and orderly sequence of the healing process. The VEGFs expression was

detected early after a couple of days, during the first two to five days, of a skin wound injury. Although there are several studies that supported the advantages of the topical application of VEGF therapy in improving the tissue restoration process after injuries and burns (Bao et al., 2009), other evidence suggested that antibodies against VEGFs would decrease the amount of wound fluid, angiogenesis, and granulation tissue formation. In addition, it has been demonstrated that high levels of VEGFs in burn injuries cause further complications, including swelling and edema (Infanger et al., 2004).

The tolerability of applying telbermin (the pharmaceutical name of recombinant human vascular endothelial growth factor rh-VEGF) topically was examined by the Hanft group in a study conducted in 2008. Telbermin was applied weekly three times on neuropathic foot ulcers of diabetic patients over a period of six weeks. The results suggested that Telbermin is well tolerated by the patients and compliance to use in such cases; however, more studies need to be conducted on Telbermin to comprehensively investigate the safety and to validate its clinical efficiency in humans, to test if it works.

Epidermal growth factors (EGFs) family is another mitogen that has been extensively studied for the wound repairing process. These factors have their important role in wound closure by stimulating the collagen secretion from fibroblasts to promote the matrix degradation during the maturation phase. Also, it has been shown that they promote the keratinocyte and fibroblast proliferation as well as accelerate re-epithelialization and the wound healing in general (Alemdaroglu et al., 2006). In burn wounds, elevated levels of epidermal growth factor binding heparin (HB-EGF) and transforming growth factor alfa (TGF- a), members of the EGF family, were detected during the healing phases (Boo & Dagnino. 2013). The Tuyet group accomplished their study to evaluate the efficiency of the recombinant human epidermal growth factor (rh-

EGF) to accelerate the healing of moderate to severe diabetic foot ulcers. This therapy was effectively successful in healing foot ulcers of 28 patients, as well as being safe for topical application. Complete healing was observed within eight weeks in 13 patients, and regardless of the wound severity, 84.8% of the wound was closed by week 8.

Transforming Growth Factor -beta (TGF -β) family consists of several members, with isoforms having diverse functions. The normal adult healing includes collagen remodeling and scar formation; however, the embryo tissue repair is a more regenerative process with almost no scar formation. As the fetal healing seems to be the optimal healing response, studying the factors involved in scarless healing can provide guides for developing new treatments to accelerate the healing. TGF -beta 1 and 2 are known to stimulate the fibroblasts proliferation and the collagen production as well as promote inflammatory cells chemo-attraction. In contrast, their antagonist TGF –beta 3 has been detected in elevated levels in embryonic scarless wound healing and partially in adults' wounds with less prone skin scarring, in the case of TGF -beta 1 and 2 deficiencies (Finnson, Arany &Philip, 2013). TGF-beta 3 knockout mice seem to have a slow progression of healing with less granulation and more scar formation, which indicates the important role of TGF –beta 3 in restoring tissue. According to a study conducted by Branch group in Manchester, UK, an enhancement in cutaneous surgical scar was observed after applying recombinant human TGF-beta 3 therapy. Improvement in the wound closure with fewer scars was detected after injecting neutralizing antibodies to TGF-beta 1 and TGF-beta 2 (MacCornick et al., 2014). On the other hand, the recent findings supported that TGF-beta 1 and TGFbeta 2 can negatively influence the wound contraction by slowing re-epithelization. Excessive amounts of TGF-beta that appear in inflammatory and infectious conditions lead to develop the keloid (reddish nodules that develop after minor injuries) or hypertrophic (more

common, easer to treat, and smaller in size compared with keloids) scarring formation (Palazzo et al., 2012). Another study to evaluate Avotermin, the TGF-beta 3 anti-scarring therapy, potential effect on surgical scars was conducted on sixty patients. Compared to the placebo, Avotermin significantly reduces scars' surface area and improves the appearance (Ferguson et al., 2009).

Fibroblast growth factors (FGFs), specifically FGF-1, have been used as a potential therapy for various cases with damaged or diseased cells that require tissue regeneration such as skin, nerves, blood vessels, and bone tissue. These skin wound healing proteins found in vast quantities around the injury site and produced by the damaged macrophages and endothelial cells. FGFs promote the migration of fibroblast and keratinocyte cells to the wound zone and accelerate the rate of angiogenesis, granulation, and epithelialization processes. Using immunohistochemical methods, the expression of the FGF protein was undetectable in the intact skin; however, it was mainly found in large amounts toward the wound center and started to reduce near the edges (Zheng et al., 2014). The critical function that these fibroblast growth factors play in the progression of the reparation stages has been demonstrated in both vitro and in vivo. Trafermin INN, Beifushu, Beifuxin and Beifuji are some examples of recombinant human FGF pharmaceutical medications that have been tested in clinical trials (Uemura T, Watanabe H and Uemura, 2006; Luca Spaccapelo, 2016). An improvement in deep burn wound along with acceleration in the healing time have been reported by Ma and his group after applying human recombinant FGF-1on the injured site. In another study conducted by Tsuboi and Rifkin, the application of bFGF (FGF-2) accelerates the regeneration of damaged tissue in a diabetic mouse. Shi and his group proved the role of bFGF in facilitating the healing of rat dermal wounds and rabbit hypertrophic scars.

There are several factors that can obstruct the smooth progress through the healing phases of the wound to be cured, such as diabetes, infection, and poor diet. In addition to these elements that would delay the progression of the healing, FGF proteins are thermos-unstable. Even though the effective role of FGFs in tissue regeneration have been demonstrated in vitro, the fact that FGFs can denature at room temperature (Wilson, Groves, Jain, Park & Hamilton, 2003), makes the final therapeutic product ineffectual to apply topically on wounds, in vivo. Another obstacle that can delay the healing process is the presence of both FGF-1 and thrombin around the wound site at the same time. The repair process of a wound is initiated immediately after injury as various coagulation factors are released along with growth factors. However, the FGF1 sequence has a cleavage site, which can be cut by thrombin; thus disabling the FGF-1's function (Chang, Alkan, Hilschmann & Braun, 1985; Naudin et al., 2015). These limitations of FGFs would impede their use as therapeutic applicants.

7. Wound healing mechanism

The wound healing restorative response is a dynamic sequence of events involving sophisticated interactions of intracellular and extracellular cascades. This process can be classified into four distinct phases including: hemostasis, inflammation, proliferation, and maturation. Of course the duration of these stages varies depending on wound types, affected regions, and whether or not the healing process is combined with other complications or negative factors such as diabetes or smoking. The injury remedial process is initiated by the hemostasis phase that works to halt the bleeding. The vasoactive modulators that are released by endothelium, such as epinephrine, norepinephrine, prostaglandins, and others, triggers the vasoconstriction of injured blood vessels to reduce the blood flow and, as a result, restricts the amount of blood loss. Exposed collagen and other tissue factors at the damaged subendothelium

layer promote the clot formation by activating the adherence of platelets to the injury site along with the intrinsic and extrinsic coagulation cascade. As aggregated platelets activate, platelets' cytoplasmic granules release chemotactic and growth factors, such as platelet-derived growth factor (PDGF), thromboxane A2, and serotonin that have a role in attracting the inflammatory cells to the injured area, leading to the following phase (Eduardo, Isabel, Bruno, Paquita & Alan, 2004). The family of polypeptide growth factors mainly considers as homeostatic factors because its significant role in tissue regeneration during injuries. After controlling the bleeding in the hemostasis phase, vasodilation occurs in order to increase the vascular permeability. That facilitates the inflammatory cells migration to the injured site. By contributing the necessary immune cells, the infection and foreign debris at the wound area will be cleared out. Neutrophils initially attack bacteria by releasing bactericidal mediators. This is followed by the most critical cells in the healing process, Macrophages.

Macrophages decontaminate the wound by the lysis of foreign organisms and phagocytosis of the waste. They also secrete numerous enzymes, cytokines, necrosis and growth factors that have a role in promoting angiogenesis and stimulating the proliferation of fibroblasts, the subsequent stage (Demidova-Rice, Hamblin & Herman, 2012). Re-epithelialization, angiogenesis, granulation tissue formation and fibroplasia, and matrix deposition are the remarkable stages in the proliferation phase. In order to restore the damaged basement membrane of the wound and connect the wound edges, epidermal growth factors promote the epithelial cells migration and proliferation, forming thin cell layers. As the wound periphery has been re-epithelized, angiogenesis is contributed in developing new blood vessels that help supply the tissue with essential nutrients. This process is controlled by chemical signals and numerous molecules including basic fibroblast growth factors (bFGF), vascular endothelial growth factors

(VEGFs), angiogenin, the angiopoietin-1 to tumor necrosis factor alpha (TNF-alpha), and the transforming growth factor-β (TGF β). Excreting collagen and fibronectin by fibroblasts progress the formation of fibroplasia and granulation tissue at the wounded area. It is believed that fibroblasts provide a dynamic stability to the wound as they are important for the matrix deposition and maturation (Demidova-Rice, Hamblin & Herman, 2012). Collagen and fibroblasts have a major role in the continuous changes in the wound during the maturation and remodeling phase. As the collagen and fibroblasts concentration extends to the maximum abundance, the wound contraction reaches the ultimate tensile strength and damaged layers are repaired and replaced. The time frame of this stage varies from weeks to months and sometimes years (Valentina & Lucas, 2011) depending on the depth of the wound and the affected organ.

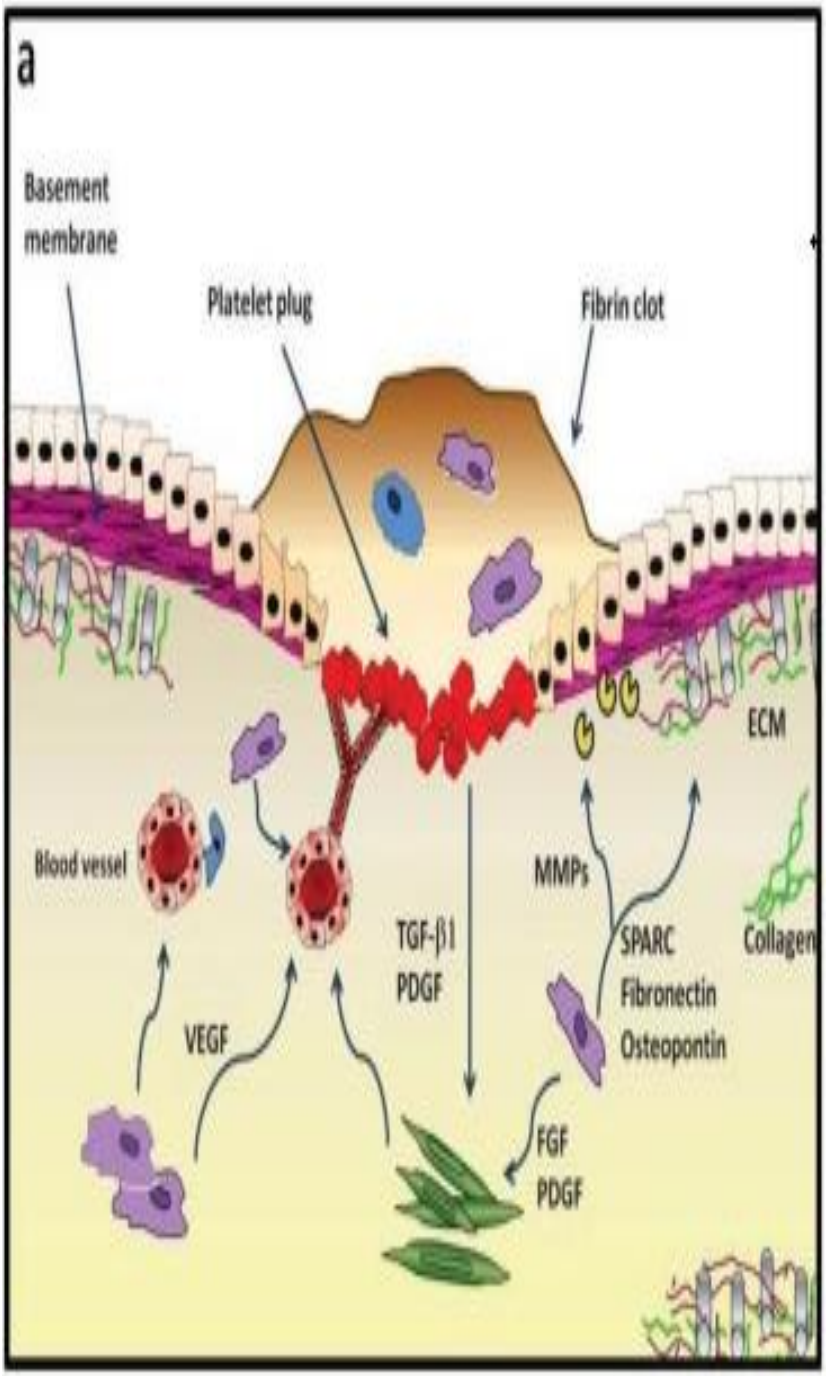
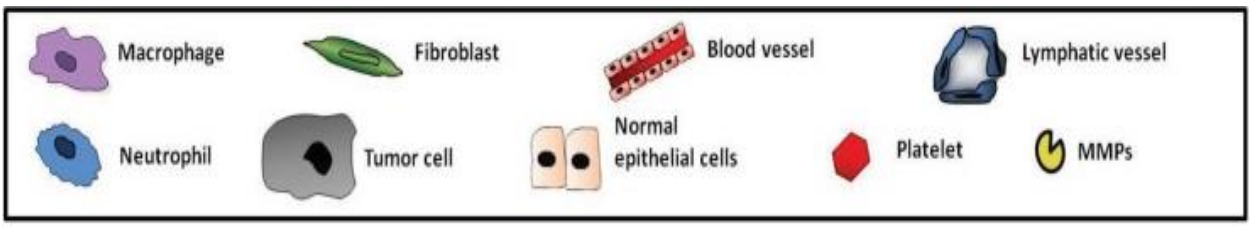


Figure 5. Comparison between wound healing and the reactive tumor microenvironment. Wound repair after tissue injury (a) is characterized by platelet aggregation, migration of leukocytes (neutrophils and macrophages) to the site of injury and by production of growth factors and cytokines involved in neo-angiogenesis and cell proliferation (e.g., PDGF, VEGF, FGF, TGFβ), and in ECM remodeling proteases (e.g., MMPs, SPARC, Fibronectin), thereby promoting wound healing and resolution. Adapted from "Tumor-Associated Macrophages as Incessant Builders and Destroyers of the Cancer Stroma," by M. Liguori and G.Solinas, 2011, *Cancers*, 3(4):3740-61. Copyright 2011 by the authors; licensee MDPI, Basel, Switzerland. Adapted with permission.



A. Types of wounds

There are numerous ways to classify wounds depending on the healing duration, wound origin (site and depth), and whether it is contaminated with infection or not. Acute and chronic wounds differ by the amount of recovery time they consume during the healing process. Acute wounds usually arise after surgery or trauma, such as surgical wounds and burns, and can be healing in the predicted recovery time, usually in weeks to a few months. Chronic wounds, in contrast, fail to achieve the predicted time as they take a longer timeframe throughout the healing stages to produce functionally integrated tissue, a period over twelve weeks (Werdin, Tennenhaus, Schaller & Rennekampff, 2009). Unfortunately, any wound has the potential to turn chronic. Lower extremity ulcers are the most common chronic wound type. Several factors can obstruct the smooth progress through the healing phases of the wound to be cured. Accumulation of coagulation factors and inflammatory mediators at the wound site can have a negative effect on the healing process. For instance, thrombin, a coagulation factor that induces the blood clotting formation, is capable of degrading the Fibroblast growth factor -1 (FGF-1) resulting in delaying the wound restoration (Chang, Alkan, Hilschmann & Braun, 1985; Naudin et al., 2015). Also, contamination of wounds with bacteria that releases degradation enzymes, Matrix Metalloproteinases as an example, can have the same harmful influence on necessary growth factors and fibrin (Wright, Lam, Buret, Olson & Burrell, 2002). Furthermore, tissue hypoxia can promote bacteria proliferation and obstruct collagen and fibroblasts production, which are critical for the healing process (Darby, Laverdet, Bonté & Desmoulière, 2014). Hypoxia appears in patients suffering from chronic illnesses, such as peripheral vascular disease (PVD), cardiovascular disease, and pulmonary diseases. In order to accelerate the progress of the healing process, these serious diseases should be controlled. The body nutrition status also can influence

the wound healing. The catabolic state that results from poor diet consumption reduces the fibroblasts formation and extends the inflammatory phase duration. Adequate nutrition with sufficient calories and enough proteins would help overcome the restriction of the progress through healing phases. The first crucial step toward effective treatment is to Highlight the etiology of chronic wounds, arterial perfusion and diabetes as examples, as well as other factors behind the slowness of the reparation process including infection and poor diet. The ability of distinguishing wound types and considering the reasons contributing to poor healing would definitely assist specialists in providing appropriate management and care for the patients.

B. Significant:

In the United States, the healthcare system is facing significant biomedical and socioeconomic burdens in treating chronic wounds and associated wound complications. This prevalent epidemic that affects a considerable population in the States is the most terrifying nightmare to public health and economy. It has been estimated that up to 6.5 million patients are suffering from chronic wounds in the United States alone (Sen et al., 2009). This significant number is subjected to a rapid increase as diabetes and obesity cases are growing quickly. With more than US$25 billion annual expenses of treatment for chronic wounds, healthcare costs still increase (Brem et al., 2007). According to the American Diabetes Association, one fourth of diabetic patients' chronic wounds are in the form of ulcers including the venous leg ulcer, diabetic foot wound, and pressure ulcer. The annual healthcare costs spent on treating patients suffering from foot ulceration is $45,000 per patient (Wu, Driver, Wrobel & Armstrong, 2007). In 2010, the annual cost of wound care products extended to $15.3 billion and the market costs continued to grow as additional technologies and advanced treatments of wound management become available (Sen et al., 2009). The immense number of surgeries are performed each year

in the United States, a total of 71.5 million inpatient and outpatient surgeries in 2000 as an example according to the National Center for Health Statistics, maximizes the cost of the post-surgical wound care dramatically. Of course, other emergency cases need urgent care and cost similarly or sometimes more, such as natural disasters, terrorism attacks, and war wounded soldiers. Previous statistics emphasize the demand in developing the wounds reparation care in a cost effective manner and investing a greater attention in wounds management in order to fit the healthcare system economy.

8. Mutations which increase the stability of FGF1

The fact that Fibroblast growth factors (FGF) have a crucial role in many biological processes especially in wound healing focuses the interest to use them as bio-therapeutic proteins. However, there are some major limitations that obstruct the application of FGF1 as biopharmaceuticals for treating serious diseases, such as chronic wounds and ulcers and gastrointestinal syndrome. FGF1 proteins, as previously mentioned, are heparin dependent; yet the coagulation factor—thrombin—that presents at the wound site and has ability to cleave FGF1 binds also to heparin. In this case, FGF1 will be more susceptible to thrombin cleavage and become inactive. Another problem that leads to loss of the activity in FGF1 is that this protein is thermounstable, which means it can be degraded easily at room temperature. In order to overcome these obstacles and create highly stable variants of the protein, mutations on the heparin binding pocket of FGF1 have been designed. Zakrzewska and her group (2004 & 2005) demonstrated that substituting these specific amino acid residues (Q40P/ S47I/H93G) showed increase in the stability as well as mitogenic activity of FGF1 protein. Another study conducted by the same group confirmed that by introducing single mutation (K112N), the variant FGF1 protein can be mitogenic active and show higher stability at the same time it is heparin

independent (Zakrzewska et al., 2009). One of the most interesting findings in modifying the FGF1 protein among those reported so far is the study conducted by Rebecca Kerr (former PhD student) in Dr.Kumar's lab. She proved that substituting single amino acid in the heparin pocket of FGF1 (R136E) can cause the variant FGF1 to be extremely biologically active as well as thermostable. Also, this modified FGF1 has heparin independent mitogenic activity and higher proteolytic resistance against thrombin cleavage. This ideal human acidic fibroblast growth factor can be use as topical or injectable application for accelerating the healing process of chronic ulcers and wounds and improve patients' quality of life. Kerr's findings will be explained in additional detail in the results and discussion section and compared to the results of this study, (P135KR136E-FGF1).

- **Amino acid sequence of wtFGF-1**(140)

FNLPPGNYKKPKLLYCSNGGHFLRILPDGTVDGTRDRSDQHIQLQLSAESVGEVYIKS TETGQYLAMDTDGLLYGSQTPNEECLFLERLEENHYNTYISKKHAEKNWFVGLKKNGS CKRGPRTHYGQKAILFLPLPVSSD

9. Scope of the present work

Dr. Kumar's group long-standing interest is to understand the structural and functional aspects of the FGF-1 protein, which is one of the vital growth factor involved in the various key cellular processes, especially in wound healing. Over the last few decades, several studies have focused on developing a potential therapeutic agent to benefit the wound management. For this purpose, engineering of growth factors and cytokines has lately gained considerable interest in the field of medicine. Although, there are numerous growth factors that are commercially used to accelerate the wound healing in today's market, such as PDGF, TGF, EGF, FGF, the development of a novel cure continues to be a clinical challenge. In this research, we aim to

understand the role of individual residues in the heparin binding pocket region of FGF-1, create a heparin independent protein, increase the half-life, and biological stability of the protein. In order to achieve that, we designed double mutant hFGF-1 by substituting two positively charged residues in the heparin-binding pocket (G**PR**THY) Proline (**P**) and Arginine (**R**) at position 135 and 136 respectively, which are known to be critical for the heparin binding property of this protein. This mutations will reveal a clear picture about the heparin independent activity of FGF-1 as well as overcome the burdens and challenges of wild-type FGF-1. Our results will highlight the future guides in developing compatible biomaterials for topical and internal applications for wound healing. These novel biomaterials must achieve high efficient delivery with limited loss of activity.

Methods and Materials

The buffer that has been used with protein in all experiments is 10 mM phosphate buffer containing 25 mM ammonium sulfate and 137 mM NaCl (pH ~7.2).

1. Bacterial Transformation

The P135KR136E mutation was created using the site-directed mutagenesis technique (QuikChangeKit^{TM}). The number of the designed mutations was amplified by PCR then confirmed by DNA sequencing. The BL21 (DE3) super competent *Escherichia coli* cell-line was used because of its high efficiency in transformation as well as its lack of proteolytic enzymes that would degrade the desired mutation. BL21 (DE3) super competent *E. coli* cells were placed on ice for 30 minutes after being inoculated with 1µL of the P135KR136E plasmid. After 30 minutes incubation, the tube was set in 42ºC water bath for 45 seconds and then immediately returned to ice for 3 minutes. This heat shock helps the *E. coli* cells to uptake the plasmid. The tube was incubated in a shaker at 250 rpm for 45 minutes at 37ºC after the addition of 800µL

sterile lysogeny broth (LB), rich medium. After spreading 100μL of the incubated sample on the agar containing the antibiotic ampicillin (Amp), transformed bacterial cells were selected. Only bacterial cells containing the plasmid were able to grow as the plasmid has ampicillin resistant genes. The agar plate was incubated at 37ºC overnight (12 – 14 hours). Glycerol stocks of P135KR136E were prepared and stored in a -80ºC freezer.

To generate the mutations, the following bases were substituted in the wtFGF1 sequence:

5'TGCAAACGCGGTCCTCGGACTCACTATGGCCAG 3' - wtFGF
5'TGCAAACGCGGTAAAGAGACTCACTATGGCCAG 3' – P135KR136E

2. Overexpression of P135KR136E -FGF1

The purpose of the overexpression is to produce large quantities of the P135KR136E -FGF1 protein in order to be purified and characterized using biophysical techniques. This procedure was performed as a subsequent step of the small-scale expression to ensure the presence of the protein. A 1mL glycerol stock and 150μL of Amp were added to 150 mL of sterile autoclaved LB media and then the solution was incubated overnight in a shaker at 37ºC and 180rpm. The culture was divided into six 2L flasks containing 500mL of LB and 500μL of Amp (25mL of the overnight starter culture per 2L flask). The optical density of the medium was checked after approximately two hours incubation (in a 37ºC shaker at 250 rpm) using UV-visible spectroscopy at a wavelength of 600 nm. When the bacteria reached the log phase (an OD between 0.4-0.6), all flasks were induced with 1mM (500μL) Isopropyl $\beta\beta$-D-1-thiogalactopyranoside (IPTG). This would trigger the induction *Lac operon* and as a result quicker protein expression. After four hours incubation at the same conditions, the culture was harvested by centrifugation at 6000 rpm for 20 minutes. The supernatant was discarded and

pellet was resuspended in 1xPBS pH 7.2 buffer then centrifuged again. The resultant pellets were stored in a -20ºC freezer for purification.

3. Purification of P135KR136E -FGF1

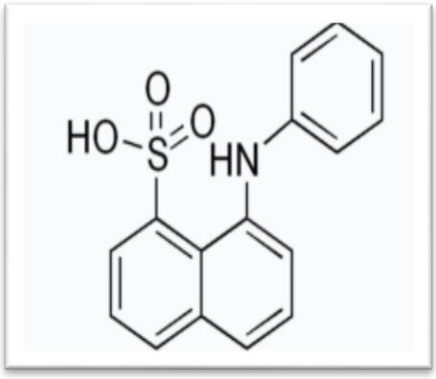
In order to purify the P135KR136E -FGF1 protein, the affinity chromatography separation method was accomplished using a heparin-Sepharose column. The bacterial pellet containing the P135KR136E -FGF1 protein that obtains from the previous step was resuspended in 10 mM phosphate buffer containing 25 mM ammonium sulfate and 10 mM NaCl at pH 7.2, and then cells were ruptured using ultra sonication. To separate the protein in the supernatant without extra contamination from the pellet, centrifugation at 19,000 rpm for 20 minutes was performed. The resultant clear supernatant containing P135KR136E -FGF1 was loaded to the heparin-Sepharose column, washed with the same buffer used to resuspended the pellet, and then exposed to a gradient of different salt concentrations (100, 200, 300, 500, 800, and 1500 mM NaCl). Fractions were collected and SDS-PAGE analysis was performed for each sample to define the salt concentration where the protein eluted. The desired fractions (800 mM NaCl and 1500 mM NaCl) were buffer exchanged to 100 mM NaCl and concentrated down to 5 ml using a 10kDa MW cutoff Millipore centrifugal concentrator. After measuring the concentration of the pure P135KR136E -FGF1 protein using NanoDrop at A280 nm, the protein was aliquoted into 1 ml eppendorf tubes and stored in a -80 ºC freezer for further experiments.

4. Limited Proteolytic Trypsin Digestion

To highlight the changes that may occur in the proteolytic degradation resistance of the FGF1 protein after inducing the mutations (P135KR136E) compared with the wild-type FGF1, the limited trypsin digestion experiment was performed for both proteins, the wild type and the variant FGF-1, in the presence and absence of the carbohydrate, heparin. First, the appropriate

trypsin concentration was determined by preparing different titrations of the enzyme (0.1, 1, and 10 mg/ml) and incubating different volumes of trypsin with the protein for the same period of time and under the same conditions. After selecting the desired trypsin concentration by examining the SDS-PAGE gel, 0.5 mgs/ml of the protein was incubated with trypsin in 37°C water bath and 110 µl of the sample was removed from the mixture every 10 minutes for 80 minutes. The reaction was stopped in the removed samples by adding 11ul of 10% trichloroacetic acid (TCA). After running SDS-PAGE for all samples, the band intensity of the protein was analyzed using densitometric scanning analysis to evaluate the enzyme action of the protein.

5. 8-Anilinonaphthalin-1-sulfonsäure (ANS) Binding Assay



The 8-Anilinonaphthalin-1-sulfonsäure binding assay was accomplished to study the folding mechanism and conformational changes that may occur in the P135KR136E -FGF1 protein compared with wtFGF1. The ANS compound serves as a fluorescent molecular probe as it binds to the hydrophobic regions on the surface of the protein. The protein sample was diluted to 15 µM and the ANS stock was prepared in a way that the addition of 1 µL from the stock to the variant FGF-1 sample would increase the ANS concentration by 20 µM. The experiment was performed in a Hitachi F-2500 spectrofluorometer with 380 nm excitation wavelength and 450 to 600 nm emission wavelength. The relative fluorescent intensity (RFI) was recorded at 500nm, the wavelength at which ANS fluoresce, until the ANS final concentration became 400 µM. This was done twice for the P135KR136E -FGF1 protein in presence and absence of heparin.

6. Thermal Denaturation

This assay was performed on JASCO 1500 CD spectrometer to measure the thermodynamic and conformational stability of the variant FGF-1. The folding mechanism of the protein as a function of temperature was evaluated in the presence and absence of heparin. 100 μL of the P135KR136E -FGF1 protein sample was loaded in a 0.2 mm path-length cell with a protein concentration of ∼19 μM. Both CD and fluorescence data were collected at wavelength of 228nm after smoothing the obtained spectra. Curves were plotted by overlaying the wild type and variant FGF-1 values versus the temperature. The van't Hoff enthalpy (ΔH), entropy (ΔS), and the free energy (ΔG) of unfolding as well as the unfolding transition midpoint (TM) were calculated from the graphs.

7. Chemical Denaturation

Using JASCO 1500 CD spectrometer, a 3 ml sample with protein concentration of 12.5 μM was prepared and loaded into 1cm path length cuvette to analyze the folding and unfolding properties for both the wild type and variant FGF-1 in the presence and absence of heparin. A stock of 8M urea concentration was used in this experiment to estimate the proteins' conformational changes that were induced by the denaturant. The data was scanned in wavelength range of 190-250nm and scan speed of 50nm/min. Values were obtained at 308 and 350nm after smoothing the data using the spectra analysis software. The fraction unfolded was calculated and plotted against the denaturant concentration.

8. Bioactivity Assay

This experiment was performed to evaluate the cell proliferation activity of the P135KR136E -FGF1 protein, in the presence and absence of heparin, compared to the wild-type FGF-1. NIH/3T3 mouse embryonic fibroblast cells have been used in this assay with

specific incubation conditions. The cells were conserved in tissue culture under 37°C temperature in presence of CO_2 as well as other growth factors and antibiotics that allow the favorable environment for best cell growth. Protein samples were prepared with concentration of 1 mg/ml and 200µL volume. The turbidity of cell growth was evaluated using a Biotek EL808 microplate reader and the absorbance was measured at 450 nm.

9. Isothermal Titration Calorimetry (ITC)

To assess the changes in the binding affinity of P135KR136E -FGF1 toward heparin compared with the wild-type, two solutions of 0.05mM P135KR136E -FGF1 and 0.5mM heparin were prepared. The MicroCal ITC200 instrument and Origin software were used to perform the experiment and analyze the data. From the resulted K_d (dissociation constant) value, we were able to determine the heparin binding affinity of the P135KR136E -FGF1 protein.

10. Nuclear Magnetic Resonance (NMR) Spectroscopy

1H-15N HSQC experiments were accomplished using Bruker Avance 700 mHz NMR spectroscopy. The three dimensional structure of the 15N isotope P135KR136E -FGF1 protein was studied alone as well as protein-ligand complex. The sucrose octasulfate (SOS)-mediated dimer experiment was conducted with 1:10 protein to SOS ratio. The concentration of the P135KR136E -FGF1 protein was 300µM. All experiments were performed at 298°K.

Results and Discussion

Optimizing fibroblast growth factors using recombinant protein technology is a critical strategy in accelerating wound healing and tissue regeneration. FGF1 has been the focus for developing tissue regeneration therapeutic agents among all other kinds of existing healing applications. This is because the unique capability of FGF1 can activate all the four sub-types of

FGFR. It is true that this universal ligand plays a critical role in various cell survival activities; yet it is prone to thermal denaturation and proteolytic degradation, as 50% of the protein tends to be completely unfolded at physiological temperature (Culajay et al., 2000; Zakrzewska et al., 2004). Additionally, thrombin, which is the heparin binding protein and serine protease, is present at the same time with FGF1 and within a close proximity at the wound site. This situation facilitates the access of thrombin to slice FGF1 at the cleavage site at residue position R136 in the heparin-binding pocket (Erzurum et al., 2003; Duarte 2006). The high degradation susceptibility and poor stability of native FGF1 limit the use of this powerful mitogen in treating serious chronic wound cases.

Heparin is known to effectively stabilize FGF1 by reducing the charge repulsions within the heparin-binding pocket of the folded protein; however, the exact role of this carbohydrate within FGF signaling is still ambiguous. It has been claimed that heparin is necessary for FGF ligand-receptors interaction and activation (Ornitz et al., 2001; DePaz et al., 2001). On the other hand, several studies were conducted using site directed mutagenesis to demonstrate the heparin independent activation of the receptors. The identified mutations have not only stabilized the protein in the absence of heparin, but have also increased the mitogenic activity of the protein with an increase in the cellular proliferation activity (Szlachcic et al., 2009). Another study demonstrated that the combination of thermodynamically stabilizing mutations with these non-heparin-binding FGF1 mutants that lose their mitogenic activity can result in complete retrieval of the biological activity of the protein (Zakrzewska et al., 2009). The previous findings conclude that heparin is not necessary to mediate the FGF1 protein signaling through its receptors (Arunkumar, 2002); but the debate about the role of heparin as a cofactor of FGF1 signaling is still ongoing (Ornitz, 2015; Brown, 2013). Further studies of a charge-reversal

mutation at heparin binding pocket at putative thrombin cleavage site on FGF1 at position 136 were accomplished. One of the most interesting findings of R136E mutation is that the substitution of only one amino acid at the heparin-binding pocket, replacing arginine with glutamic acid, resulted in a dramatic enhancement of the mitogenic activity and overall stability of the protein while the binding affinity to heparin was decreased significantly. (Data unpublished)

To generalize the picture of the previous findings and gain more insight for a better understanding about heparin's role in activating FGF1-receptor and downstream signaling, and to examine whether the observations of the charge reversal mutation at 136 are unique to this position can be achieved by examining the adjacent residue, P135 has been considered.

A previous study that used P135K to investigate the role of introducing a positively charged residue in the neighboring position of R136 indicated that the loss of proline significantly increases the susceptibility of the mutant protein to the proteolytic degradation with almost 80% of the protein digested after the first 10 minutes of trypsin exposure. The fluorescence data from the ANS binding assay revealed that the mutant FGF's hydrophobic regions were exposed more as the introduced mutation lead to greater influence on tertiary structure, which made the protein more flexible. A possible explanation for this is the increase in the protein's flexibility that resulted from replacing proline, which is known to effect the structural rigidity of the protein. The thermal stability data indicated that the protein tends to be less stable and the reduction of the heparin binding affinity was due to the introduction of a positive charge residue. In conclusion, the introduction of lysine near to R136 altered the thermodynamic balance and decreased the overall stability of the variant protein (Data unpublished). With respect to these preliminary studies, P135ER136E mutation has been rationally designed. This study has been

designed to evaluate the hypothesis that introduction of positive charge, in a close vicinity to R136E, adjacent to the negative charge will nullify the stabilizing effects of R136E.

Overexpression and Purification of P135KR136E -FGF1 and wild type FGF-1

The transformed bacterial cells containing the P135KR136E -FGF1 protein were overexpressed in LB medium (after ensuring the presence of the protein using the small-scale expression technique) according to the protocol that has been detailed in the Materials and Methods section. The corresponding band matching the size of the control protein in the SDS-PAGE gel determined the success of the expression.

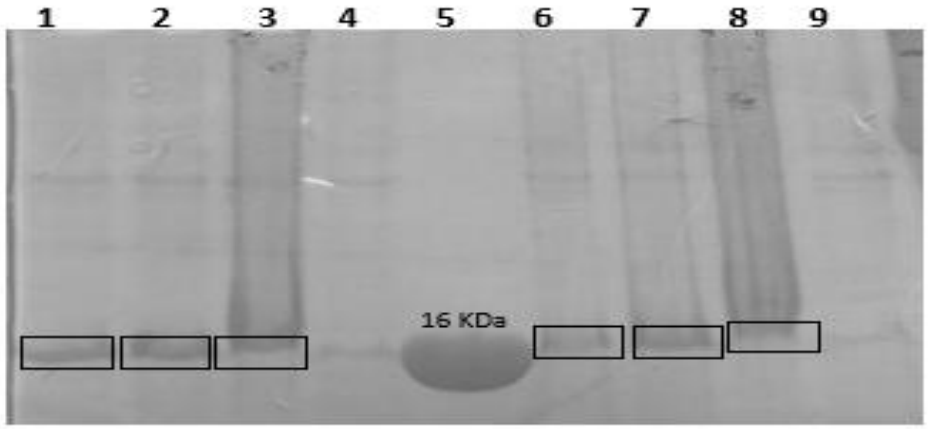


Figure 6: the SDS-PAGE analysis of P135KR136E -FGF1 and wild type FGF-1's small scale expression. The black box indicates the corresponding band of the desired protein with a molecular weight of 16 KDa (confirms the presence of the protein). Lane 1 represents the uninduced sample of wild type FGF-1. Lane 2 is the induced sample of wild type FGF-1. Lane 3 is the supernatant of wild type FGF-1. . Lane 4 is the pellet of wild type FGF-1. Lane 5 is the positive control (wtFGF-1). Lane 4 is the uninduced sample of P135KR136E -FGF1. Lane 5 is the induced sample of P135KR136E -FGF1. Lane 8 is the supernatant of P135KR136E -FGF1. Lane 9 is the pellet of P135KR136E -FGF1.

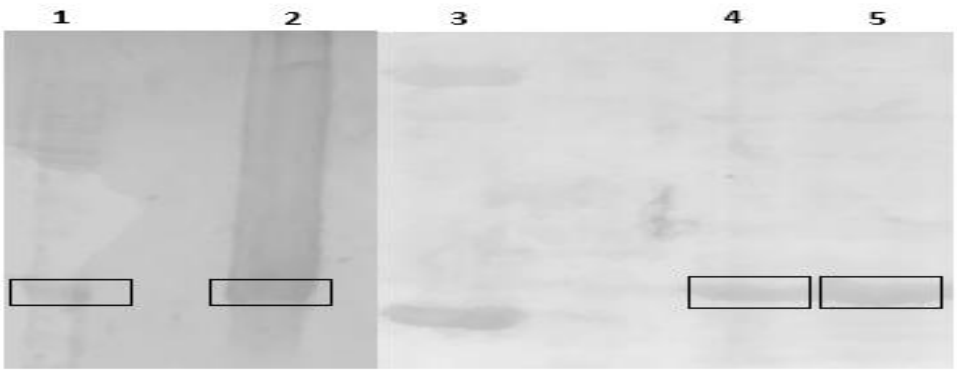


Figure 7: the SDS-PAGE analysis of P135KR136E -FGF1 and wild type FGF-1 overexpression. The black box indicates the corresponding band of the desired protein with a molecular weight of 16 KDa (confirms the presence of the protein). Lane 1 represents the uninduced sample of wild type FGF-1. Lane 2 is the induced sample of wild type FGF-1. Lane 3 is Lysosome. Lane 4 is the uninduced sample of P135KR136E -FGF1. Lane 5 is the induced sample of P135KR136E -FGF1

In order to biophysically characterize the P135KR136E -FGF1 protein and compare the alternation that may occur after introducing the mutations in these specific sites with the wild type, both proteins have been purified using the affinity chromatography method. Pure proteins have been isolated from a heparin-Sepharose column by increasing the salt gradient concentrations. For the variant FGF-1, concentrations were 100 mM NaCl, 300 mM NaCl, 500 mM NaCl, 800 mM NaCl, and 1500 mM NaCl. For the wild type FGF-1 protein, 300 mM NaCl, 800 mM NaCl and 1500 mM NaCl were used as the method is well standard. All collected fractions were analyzed using SDS-PAGE technique. Both the wild type and the variant FGF-1 eluted at high salt concentration of 800 mM NaCl and 1500 mM NaCl,. At this high concentration of salt, the electrostatic interactions between the protein and the heparin-Sepharose resin are broken down, which causes the disassembling of the bound FGF-1 from the column. This result suggested that the introduced mutations did not significantly change the heparin pocket surface of FGF-1 in the way that conserved the property of binding affinity toward

heparin. As the majority of the double mutant FGF1 eluted at lower salt concentration (800 mM), the high heparin binding property of the protein could be slightly decreased compared with the wild type.

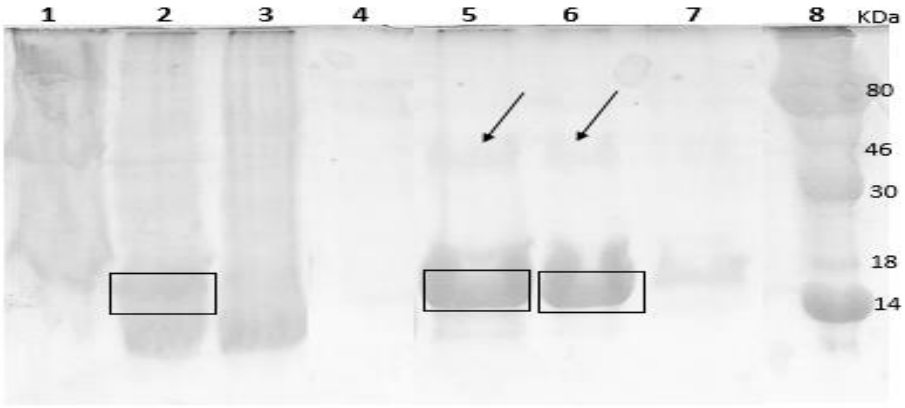


Figure 8: the SDS-PAGE analysis of wild type -FGF1 purification using heparin-Sepharose column. The black box indicates the corresponding band of the desired protein with a molecular weight of 16 KDa (confirms the presence of the protein). Lane 1: pellet. Lane 2: supernatant. Lane 3: flow-through of the loaded sample. Lane 4 :300 mM NaCl. Lane 5: 800 mM NaCl. Lane 6: 1500 mM NaCl. Lane 7: urea wash to clean the rasin. Lane 8: protein marker. The bands in lane 5 and 6 indicated by the black arrows at 46 KDa position could be a TCA artifact

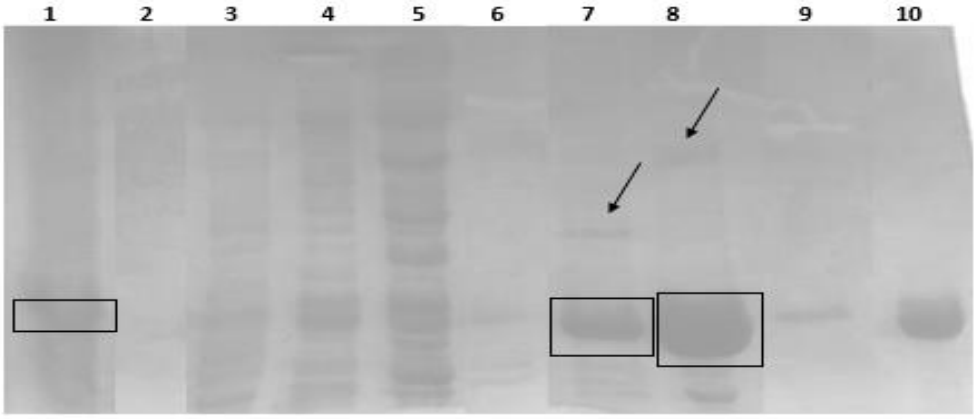


Figure 9: the SDS-PAGE analysis of P135KR136E -FGF1 purification using heparin-Sepharose column. The black box indicates the corresponding band of the desired protein with a molecular weight of 16 KDa (confirms the presence of the protein). Lane 1: supernatant. Lane 2: pellet. Lane 3: flow-through of the loaded sample. Lane 4 :100 mM NaCl. Lane 5: 300 mM NaCl. Lane 6: 500 mM NaCl. Lane 7: 800 mM NaCl. Lane 8: 1500 mM NaCl. Lane 9: urea wash to clean the rasin. The bands in lane 7 and 8 indicated by the black arrows at 25 KDa and 46 KDa position could be either a P135KR136E dimer or a TCA artifact.

After confirming the fractions that contain the protein of interest which are not contaminated, in this context both 800 mM NaCl and 1500 mM NaCl fractions were combined and buffer exchanged. Using a 10kDa MW cutoff Millipore centrifugal concentrator, a total of 25 ml of P135KR136E –FGF1 and 20 ml of wild type FGF-1 were reduced to 5 ml. The final concentrations of the wild type and the variant FGF-1 were 3.5 and 3 mg/ml, respectively.

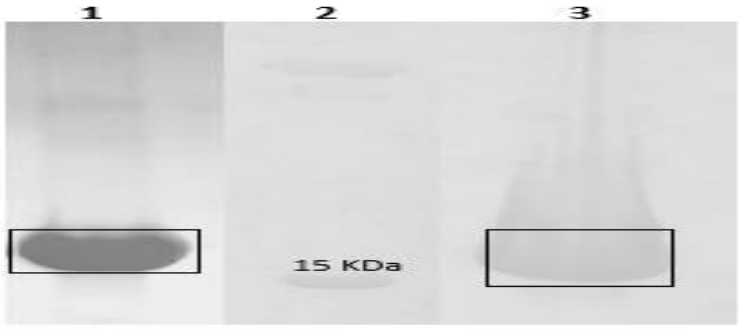


Figure 10: the SDS-PAGE analysis of P135KR136E -FGF1 and wild type FGF-1 post concentration. The black box indicates the corresponding band of the desired protein with a molecular weight of 16 KDa (confirms the presence of the protein). Lane 1: concentrated sample of wild type FGF-1. Lane 2: protein marker (Lysosome). Lane 3: concentrated sample of P135KR136E -FGF1.

Biophysical Characterization of P135KR136E FGF-1

- **Assessment of the surface hydrophobicity of P135KR136E mutant**

ANS binding assay was performed to measure the variations in the surface hydrophobicity of P135KR136E FGF-1 compared with the wild type. The intensity of the fluorescence emission of the bound ANS molecules is directly proportional to the exposed hydrophobic residues on the protein surface and as a result reveals the flexibility and the folding mechanism of the protein. The increase in the ANS fluorescence indicates that the hydrophobic surface of the protein is being more accessible, as the hydrophobic residues are naturally buried in the protein core. By comparing the saturation curve in the absence of heparin in figure 11, we can see that the wild type and the mutant protein follow almost the same pattern with a little

increase in the fluorescence in the case of the variant FGF-1. This means the structural confirmation and folding mechanism is nearly the same in both proteins as they are packed in a similar fashion. Additionally, no significant changes have been noticed when heparin was added to the protein, as shown in figure 12. The minor increase in the saturation curve of P135KR136E suggests that the introduction of the positively charged residue—lysine—at position 135 slightly influences tertiary structure of the protein, which causes the protein to be more relaxed and increases the exposure of hydrophobic regions in the surface of P135KR136E FGF-1 protein.

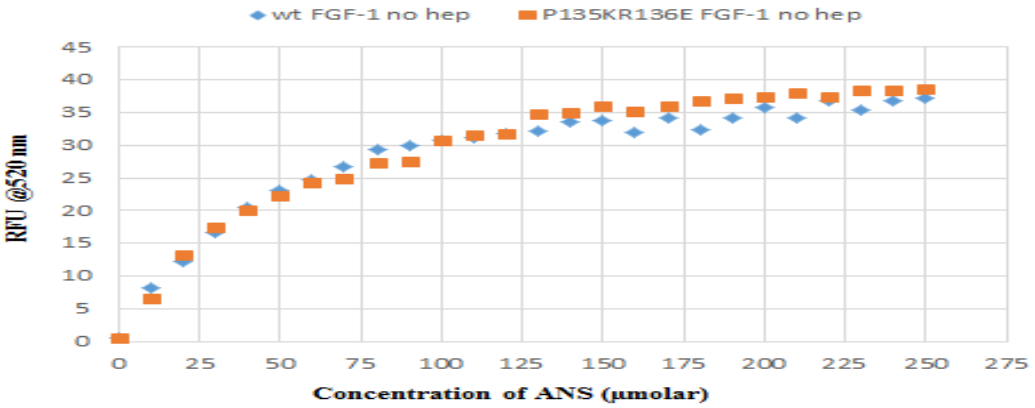


Figure 11: ANS titration of wild type FGF-1 and P135KR136E FGF-1 in the absence of Heparin

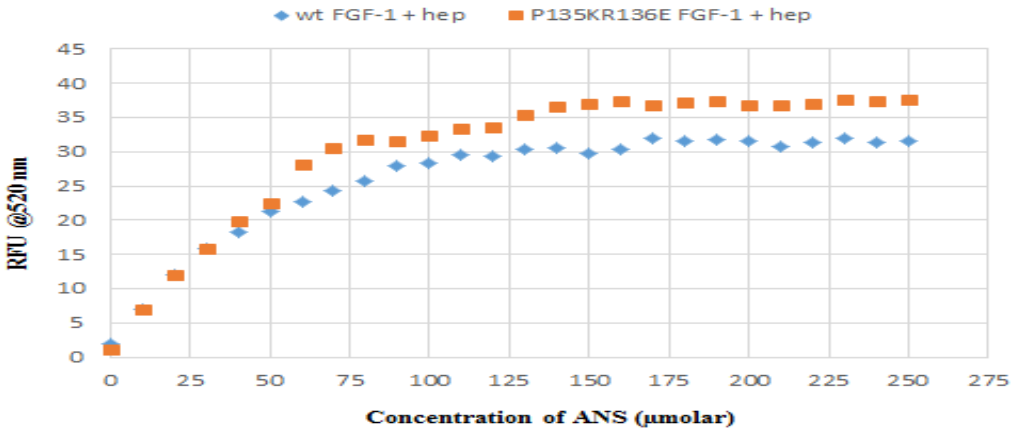


Figure 12: ANS titration of wild type FGF-1 and P135KR136E FGF-1 in the presence of Heparin

- **Determination of the heparin binding of P135KR136E mutant**

In order to detect the thermodynamic interactions and quantitatively determine changes in the binding affinity of the variant protein toward heparin, Isothermal Titration Calorimetry (ITC) technique was used. The dissociation constant (Kd) of heparin toward the P135KR136E FGF-1 was measured and compared with the wild type. Lower Kd values indicate stronger binding interactions between the molecule (variant FGF-1 protein) and the ligand (Heparin) whereas higher values mean weaker interactions and less binding affinity. These values highlight the changes in the binding affinity of the heparin to the protein that could occur as a result of introducing the desired mutations. The Isothermogram in figure 13 shows the titration of heparin versus FGF1-P135KR136E. Almost 20-fold decrease in binding affinity of the P135KR136E FGF-1 toward heparin (Kd ~90µM) was observed in case of the double mutant compared to wtFGF1 (~5µM).

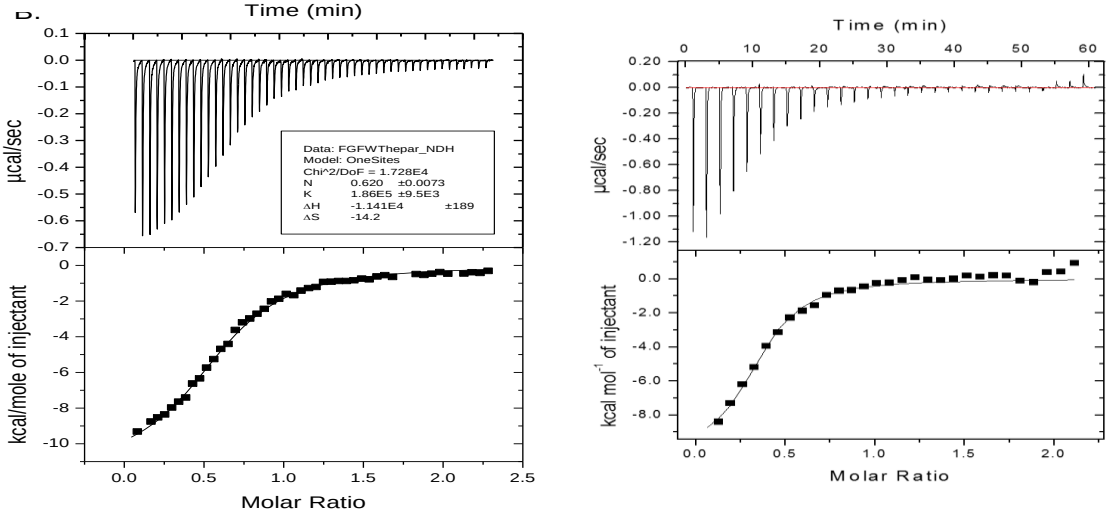


Figure 13: Isothermograms of the wild type FGF-1 (left) and P135KR136E FGF-1 (right) representing their interactions to heparin. The dissociation constants (Kd) values of both proteins were calculated with Kd of ~90µM for the double mutant and ~5µM for the wild type FGF1. The bottom panel shows the best-fit of the raw data.

This can be explained as the incorporation of positively charged Lys135 adjacent to the negatively charged E136 might have a repulsion effect, which eventually led to the reduction of the heparin binding affinity of the variant protein.

Similarly, the single mutation R136E showed almost 15-fold decrease in the binding affinity toward the heparin analog sucrose octasulfate (SOS), and 5-fold decrease in the heparin binding affinity (Kd 72µM and 16.72µM respectively) compared to wtFGF1. These results, shown in figure 14, indicate that the single mutation R136E is capable of showing heparin independent mitogenic activity. Overall, the introduction of a positive charge at position 136 near to R136E seems to have a similar effect on the heparin binding affinity of the protein as the single mutant R136E.

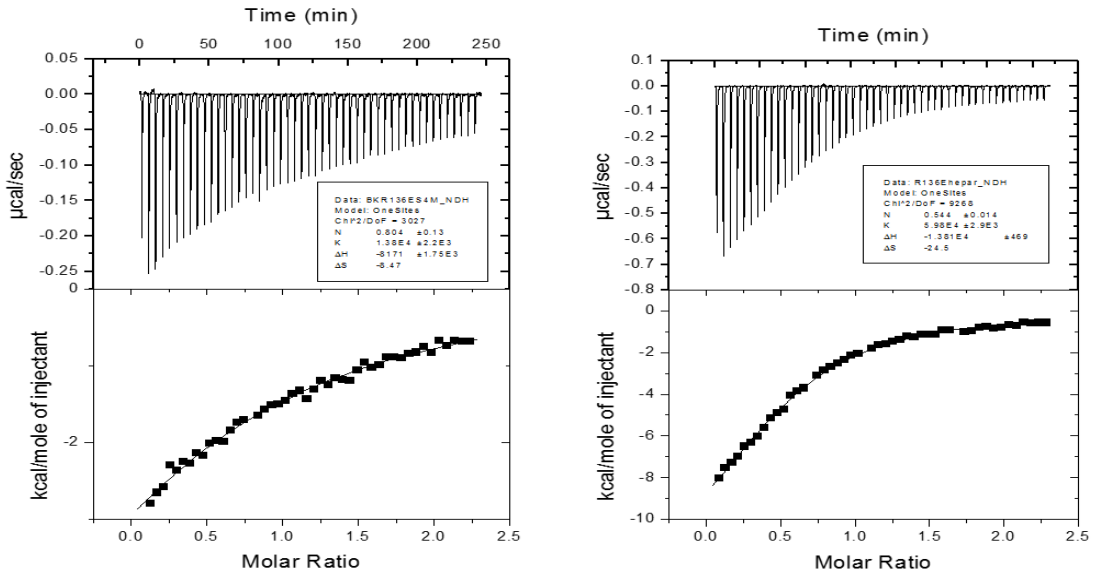


Figure 14: Isothermograms of the R136E mutant titrated with SOS (left) and heparin (right). The dissociation constants (Kd) values were calculated with Kd of ~72.4µM for the mutant with SOS and 16.72µM when R136E titrated with heparin.

- **Evaluation of the stability of P135KR136E**

Trypsin digestion experiment was performed to assess the changes, which may follow the introduction of mutations, in the protein stability against the proteases cleavage. In order to evaluate the stability of the tertiary conformation and intrinsic structure of the desired protein as well as the degradation resistance toward proteases, an enzyme with more uniform proteolytic activity over time has to be used. That is why we chose Trypsin in this experiment. Trypsin cleaves the basic residues (Lysine and Arginine) at the carboxyl end. Both the variant and the wild type proteins were subjected to limited trypsin digestion in the presence and absence of heparin. All SDS-PAGE gels were analyzed using the densitometric scanning.

By comparing the data, we can see that both the wild type and the variant FGF-1 are fairly stable when they bind to heparin. This emphasizes the role of heparin in protecting FGF-1 from the proteolytic degradation by installing the folding confirmation of the protein. Although wild type FGF-1 in the absence of heparin was less stable and more suitable to trypsin degradation compared to when it is bound to heparin (20% of the protein was cleaved by the enzyme after 25 minutes), over 60% of the variant protein was digested by trypsin after the same period of exposure in the absence of heparin. This highlights the fact that the variant FGF-1 is at twice the risk of being digested by trypsin compared with the wild type as the binding affinity of the double mutant toward heparin decreased.

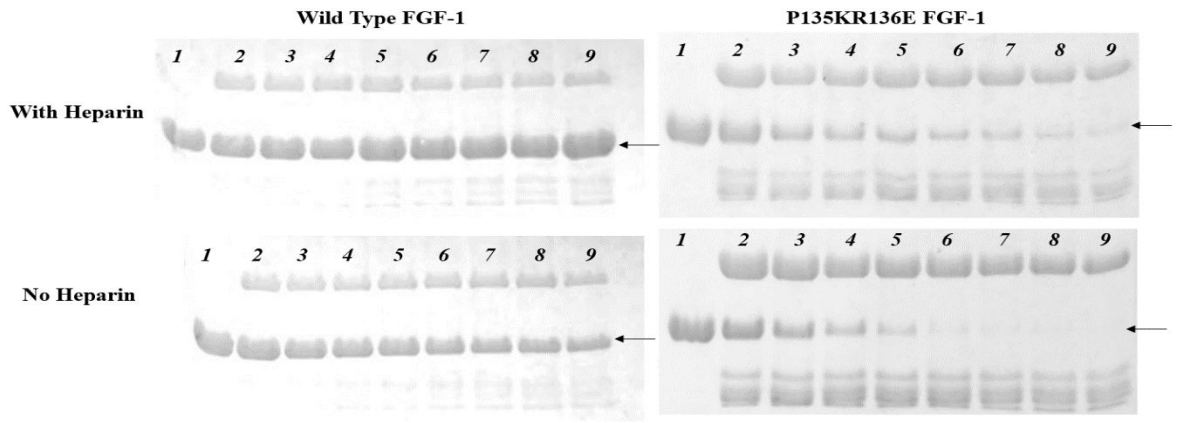


Figure 15: SDS-PAGE gels of the limited trypsin digestion samples of the wild type and P135KR136E FGF-1 in the presence and absence of heparin. The bands with a molecular weight of 23.3 KDa in the first row correspond to the enzyme, trypsin, whereas the bands on the second row pointed with the arrow represents the desired protein with a molecular weight of 16 KDa. Lane 1 is the zero sample before the addition of the enzyme. Each following lane (lanes 2-9) represent the 5 minute intervals samples from the trypsin digestion reaction that collected and halted by adding trichloroacetic acid (TCA). Lane-1: time-zero sample, Lane-2: 5 minutes, Lane-3: 10 minutes, Lane-4: 15 minutes, Lane-5: 20 minutes, Lane-6: 25 minutes, Lane-7: 30 minutes, Lane-8: 35 minutes, Lane-9: 40 minutes.

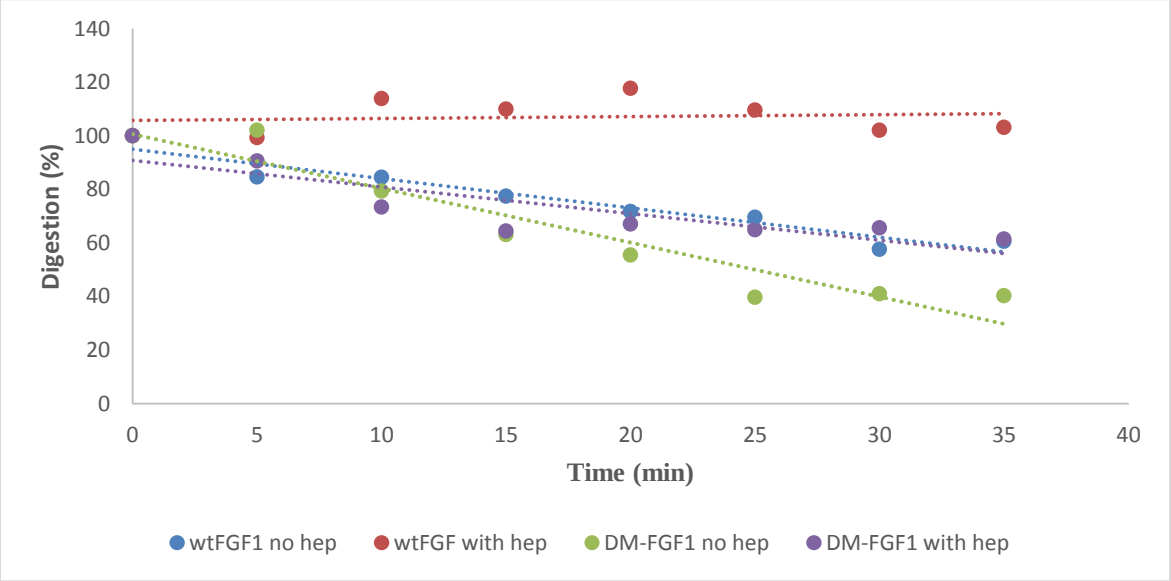


Figure 16: Densitometric scan of the undigested wild type and P135KR136E FGF-1 over time in the presence and absence of heparin after trypsin treatment. The green line represents the wild type FGF-1 in the presence of heparin. The blue line is the wild type FGF-1 in the absence of heparin. The yellow line is the P135KR136E FGF-1 in the presence of heparin. The red line is P135KR136E FGF-1 in the absence of heparin.

Taken altogether, introduction of positively charged residue P135K causes the protein core to be not firmly packed, and as a result the protein folding formation tends to be less stable, which leaves lysine and arginine residues more exposed to trypsin. The previous findings indicate that the increase in the proteolytic resistance of the protein upon the introduction of the single mutant R136E has been reversed by P135K. The enhancement of the structural flexibility of the double mutant is consistent with the obtained data from ITC as well as the ANS experiment.

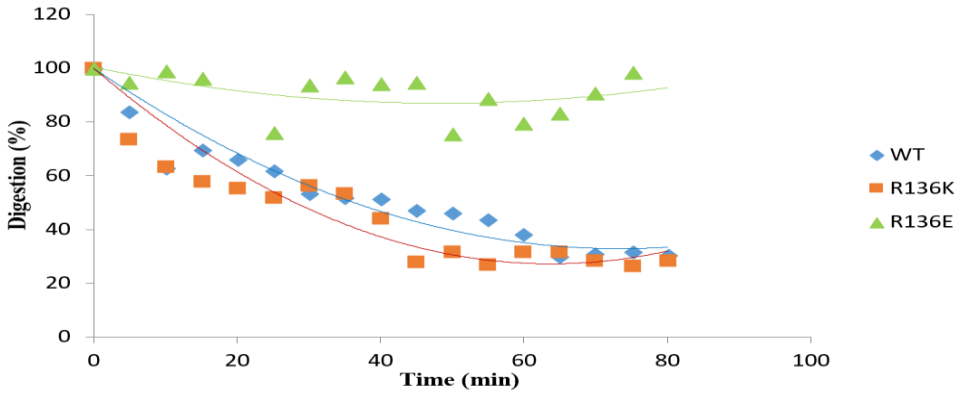


Figure 17: Densitometric scan of the undigested wild type, R136K, and R136E FGF-1 over time in the absence of heparin after trypsin treatment.

- **Studying the effect of the introduced mutations on the secondary structure of FGF-1**

Circular dichroism (CD) is a decent technique to determine the secondary structure of the protein and examining the protein's conformational stability under different stressors, such as high temperatures and strong chemical denaturants. As FGF1 has only one Tryptophan (W121) that located in a close vicinity of the introduced mutation, intrinsic fluorescence is an ideal probe to monitor changes occurring in the secondry structure. This aromatic residue located in the proximity of P135 will provide information on the local changes resulted by the introduction of mutation. To compare the changes that may occur upon introducing the mutations on the secondary structure of the protein, the CD spectra of the wild type and P135KR136E FGF-1 was

collected in the presence and absence of heparin using far-UV Circular Dichroism (CD). The β – trefoil motif of both proteins in the presence and absence of heparin represent by two positive peaks from approximately 190-210 nm and 240-220nm (figure 18). As has been indicated from the graph, there are no significant alternations in the secondary structure of the variant protein compared to that of the wild type. Therefore, P135KR136E mutations did not affect the secondary structure and the antiparallel beta sheets' formation of the protein stayed intact.

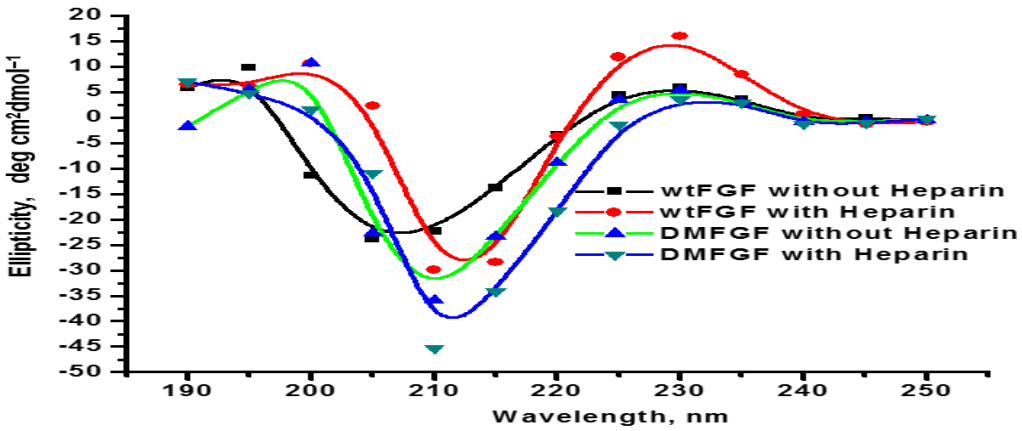


Figure 18: Far UV Cirrcular Dichromism spectra of the wild type and P135KR136E FGF-1 protein in the presence and absence of heparin.

- **Determination of the stability of P135KR136E mutant**

In the chemical denaturation assay, 8 M urea was the denaturant of choice. Both proteins, the wild type and P135KR136E FGF-1, were exposed to different titrations of urea in the presence and absence of heparin to assess the stability as well as folding and unfolding behavior of the protein. In figure 19, both proteins behave similarly with almost the same pattern. The overall trend of the curves indicates that there are no significant changes in the stability of the protein upon the introduced mutations with milting concentration of 1.8M compared to that of the wild

type, 1.75M. However, as the variant protein bound to heparin less tightly than the wild type, the stability of P135KR136E FGF-1 protein increased in the presence of heparin but only by 0.8M.

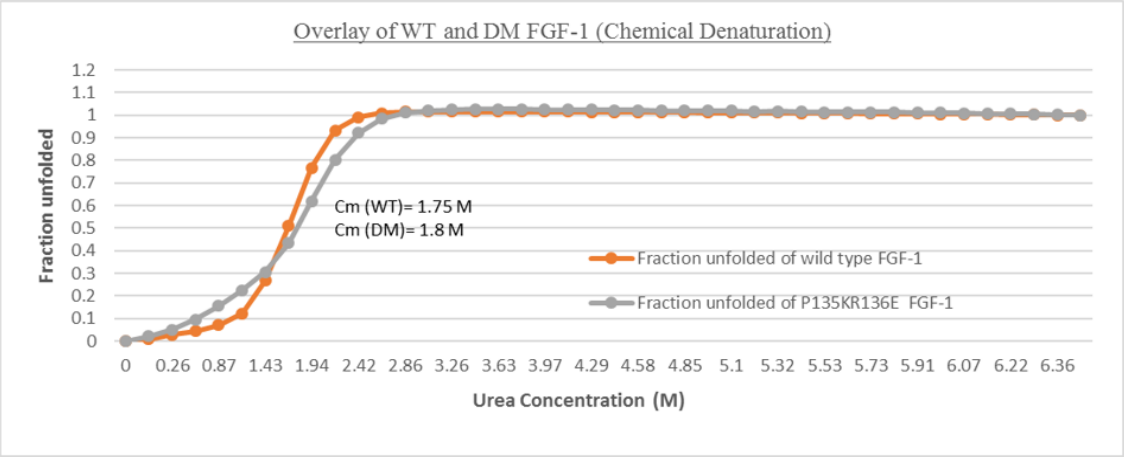


Figure 19: The chemical denaturation data of the wild type and P135KR136E FGF-1 in the absence of heparin using Cirrcular Dichromism.

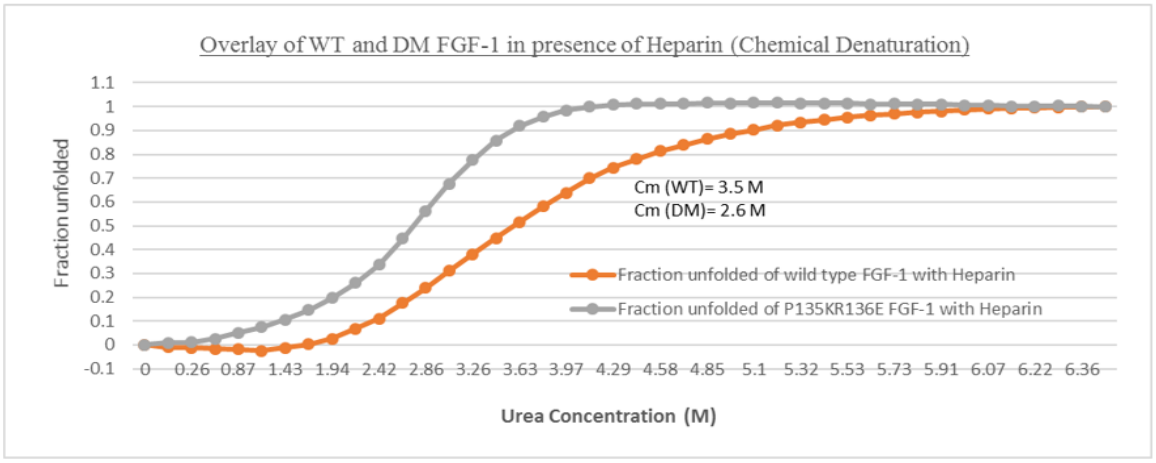


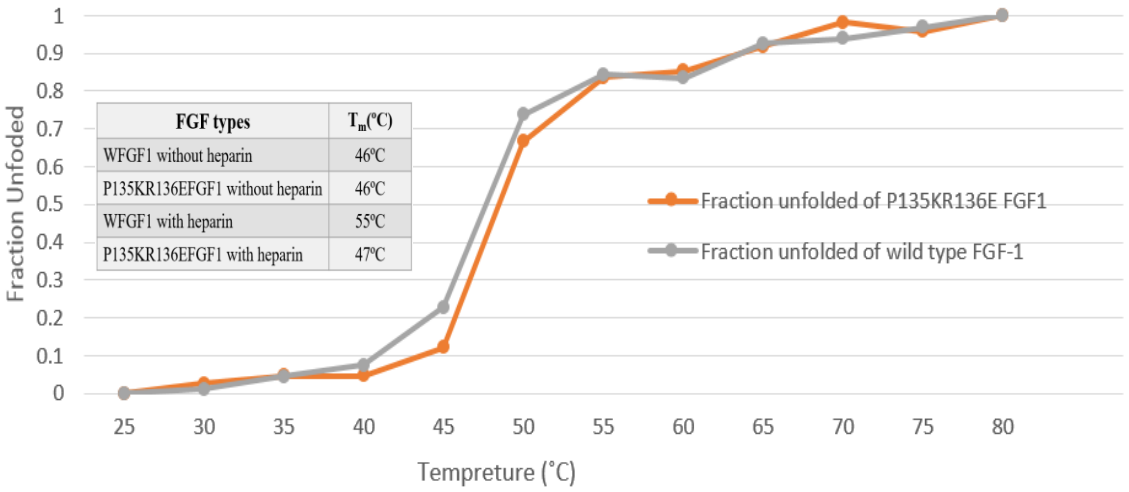
Figure 20: The chemical denaturation data of the wild type and P135KR136E FGF-1 in the presence of heparin using Cirrcular Dichromism.

In the presence of heparin, 50% of the wild type protein needed the double amount of the urea (Cm= 3.5M) to be in the unfolded state compared with the wild type in the absence of heparin. These results indicate the important role of heparin in stabilizing the protein. We can

conclude that the introduced mutations did not affect the overall stability of FGF-1 protein compared with the wild type but slightly decreased the high binding affinity of the protein toward heparin.

The fact that the introduced mutation is located in the heparin-binding pocket and heparin stabilizes the native protein by increasing the thermal denaturation resistance; it was reasonable to hypothesize that the substitution of two positively charged residues would increase the repulsion forces between heparin and the variant protein. This eventually decrease the heparin binding affinity and increase the possibility of making the protein heparin independent.

The analysis of the thermal stability data in of the wtFGF1 and double mutant FGF1 in the absence of heparin indicates there is no change in thermal stability of the variant protein (T_m = 46°C). In the presence of heparin, the double mutant exhibition a lower thermal stability level (T_m = 47°C) compared to the wild type (T_m = 55°C). These results suggest that the introduction of charge reversal mutations did not change the overall thermal stability of the protein but decrease the binding affinity toward heparin.



FGF types	T_m(°C)
WFGF1 without heparin	46°C
P135KR136EFGF1 without heparin	46°C
WFGF1 with heparin	55°C
P135KR136EFGF1 with heparin	47°C

Figure 21: Thermal denaturation data of the wild type and P135KR136E FGF-1 in the absence of heparin using Cirrcular Dichromism

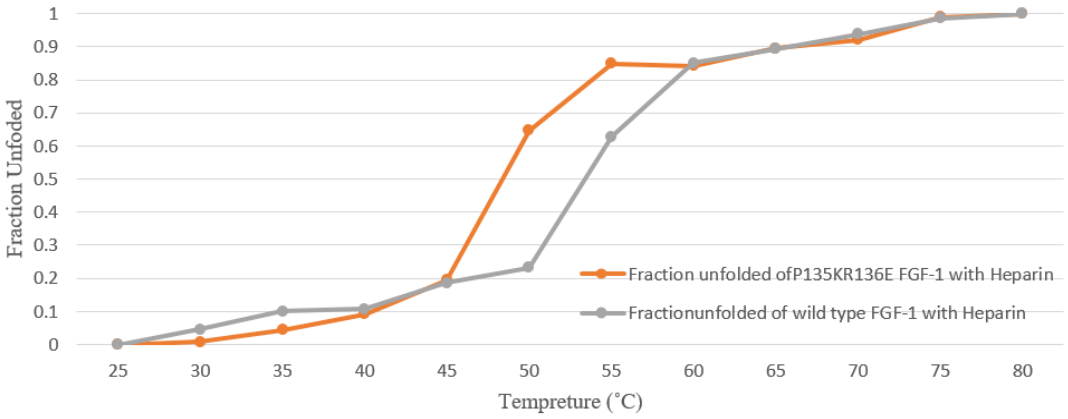
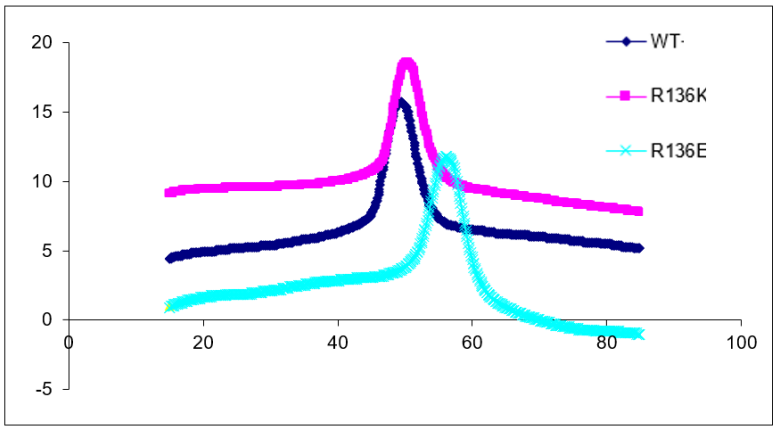


Figure 22: Thermal denaturation data of the wild type and P135KR136E FGF-1 in the presence of heparin using Cirrcular Dichromism

The thermal denaturation data of the single mutant (R136E) in the absence of heparin showed increase in the thermal stability of the protein by 6°C with a Tm of 56.1°C compared with the

wild type FGF1(49.3°C), as shown in figure 23. These results indicate that the thermal stability of the R136E mutant is heparin independent.

The introduction of positively charged residue in the vicinity of R136E dramatically reduces the overall stability of the variant protein with 10°C collapse in the thermal resistance. This drastic drop in stability level of the variant protein compared with the single mutant R136E strongly support our hypothesis.



Mutation	T_m(°C)
WT	49.3
R136K	50.3
R136E	56.1

Figure 23: The thermal denaturation data (DSC profiles) of wild type, R136K & R136E forms of FGF-1 in the absence of heparin

- **Effect of P135KR136E mutation on the structure of FGF1**

To further investigating the structural changes upon the introduced mutation and understand the consequences of this mutation on the conformational alterations of the variant protein, multidimensional NMR experiments were performed. The heteronuclear single quantum coherence (HSQC) spectra of the P135KR136E-FGF1 in the presence and absence of sucrose octasulfate (SOS) were acquired in order to obtain the tertiary structure of the protein in a high resolution. In the two-dimensional 1H-15N HSQC spectrum, each crosspeak represents an amino acid residue and this considers as a finger-print of the protein's backbone conformation. The HSQC spectrum of the P135KR136E-FGF1 overlaid with wtFGF1 and P135KR136E-

FGF1+SOS to calculate the chemical shift perturbation for each residue. This result will provide information about the backbone structural changes at the atomic level, which eventually explains the effect of the introduced mutation on the global structure of the protein.

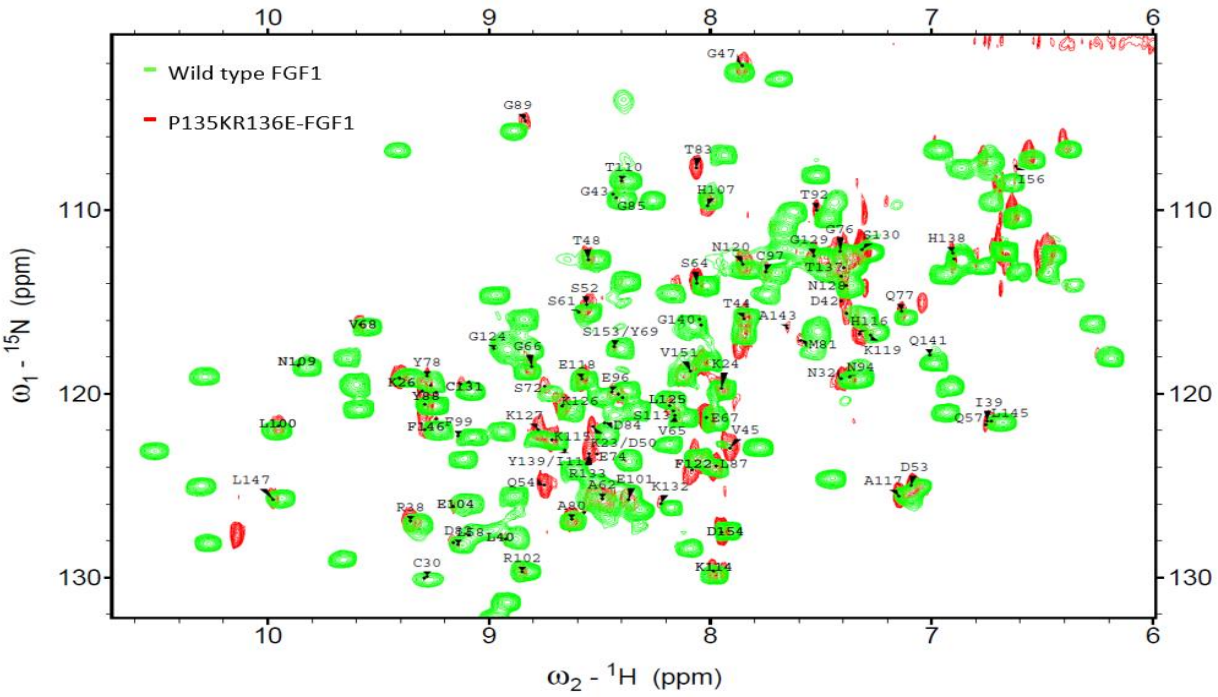


Figure 24: Overlay of 1H 15N HSQC spectra of wild type and P135KR136E FGF1 spectra

The special position of P135 is located on a loop that links two beta strands 11 and 12. This loop makes up the heparin binding region in addition to two loops. The position of P135 reflects its importance as its located at the middle of three positively charged amino acids; two of them known to be critical heparin binding residues (R133 and R136) in addition to K119. The analysis of the HSQC overlay of P135KR136E with wtFGF along with the perturbation plot (figure 24 and 25 respectively) reveal that the introduction of two charged amino acids causes a minor shift only for one residue outside the heparin-binding pocket—Lue 87—which is located in the C-terminal in a close vicinity of the introduced mutation. The distance between the introduced double mutant and the affected residue increased by 2.2 Å compared with the wtFGF, which is

6.2 Å. As Lue 87 is located 8.4 angstroms away from P135KR136E explains the increase in the flexibility of the variant protein. The overall conformation of the protein is still intact and there is no significant alternation in the global structure upon the introduced mutation.

Figure 25: The chemical shift perturbation plot of wild type FGF1/ P135KR136E-FGF1

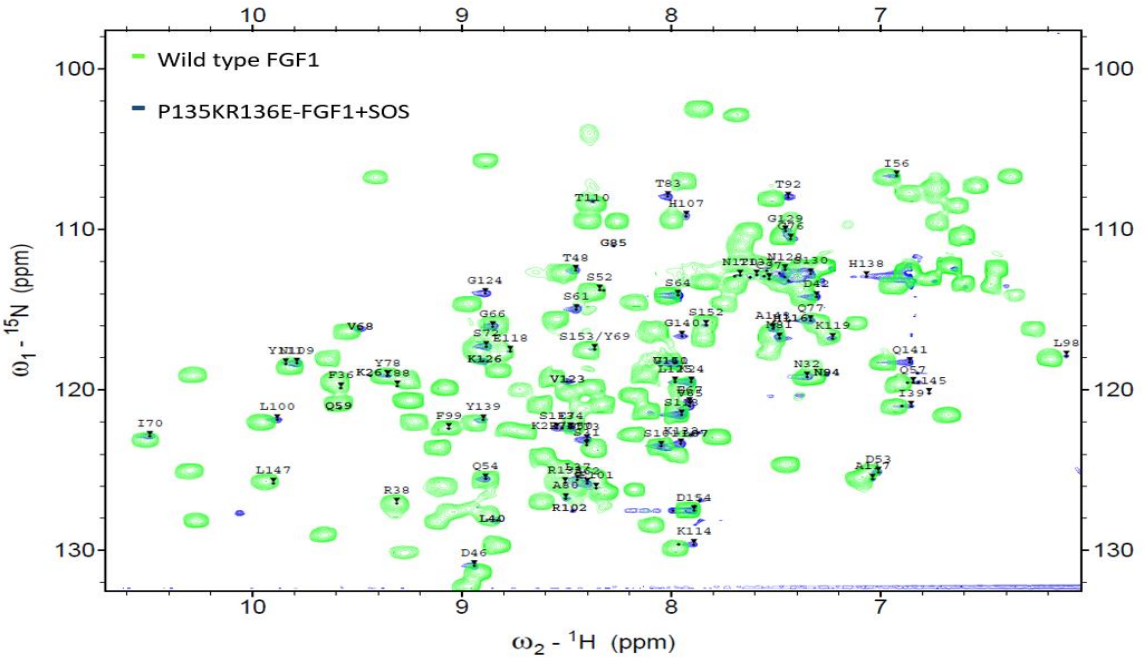


Figure 26: Overlay of 1H 15N HSQC spectra of wild type FGF1 and P135KR136E-FGF1+SOS

More significant shift with additional residues affected observed from the overlay of the HSQC spectra of P135KR136E with the double mutant with SOS (figure 26 and 27). The number of the peaks in the spectrum represents the number of the amide bonds in the protein. The pecks in the HSQC spectra are well dispersed, which means that the protein is well structured, and look almost as the wild type with some peak shifts. The shift in Lue 87 (figure 27) presents as a consequence of the introduced mutation; however, other peaks shift that caused minor conformation changes occurs when the protein binds to SOS. The location of most perturbed residues (T137, Y139, G140, E118 and K132) is neighboring the introduced mutation on the heparin binding pocket. That can be concluded as the introduction of the double mutant did not grisly change the general conformation of the protein but it did change the local structure at the site of mutation.

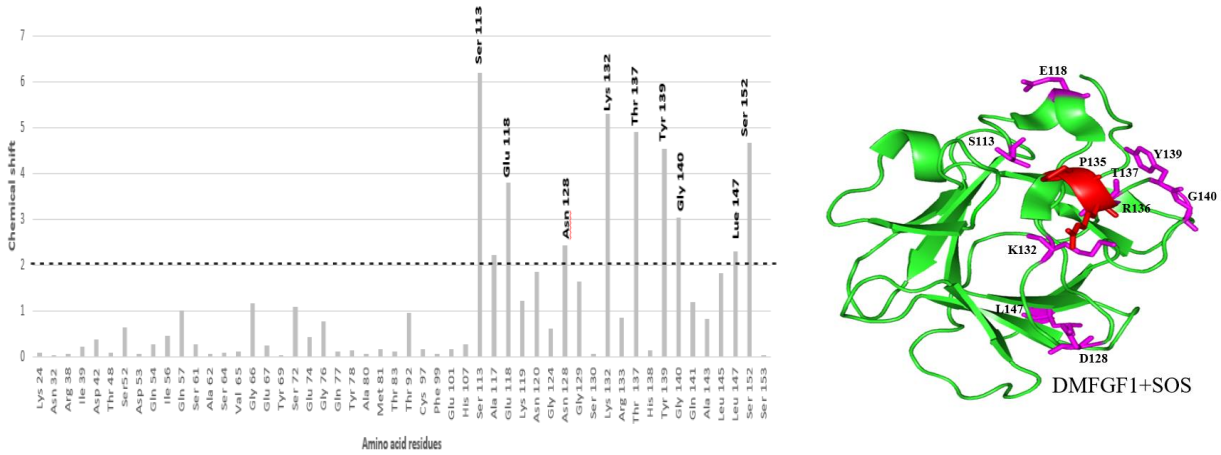


Figure 27: The chemical shift perturbation plot of P135KR136E-FGF1/ P135KR136E-FGF1SOS

- **Effect of the P135KR136E mutation on the cell proliferation activity of FGF1**

This experiment was performed to evaluate the changes in the cell proliferation activity of the protein after introducing the mutations compared to the wild type using different protein concentrations. The overall trend of both proteins is similar, and they act almost the same in the presence and absence of heparin. An increase in the cell production (~20,000 cells) in both proteins was detected when heparin bound to FGF-1 (figure 28). P135KR136E FGF-1 tends to be more biologically active with a slight rise in cell proliferation compared to that of wild type.

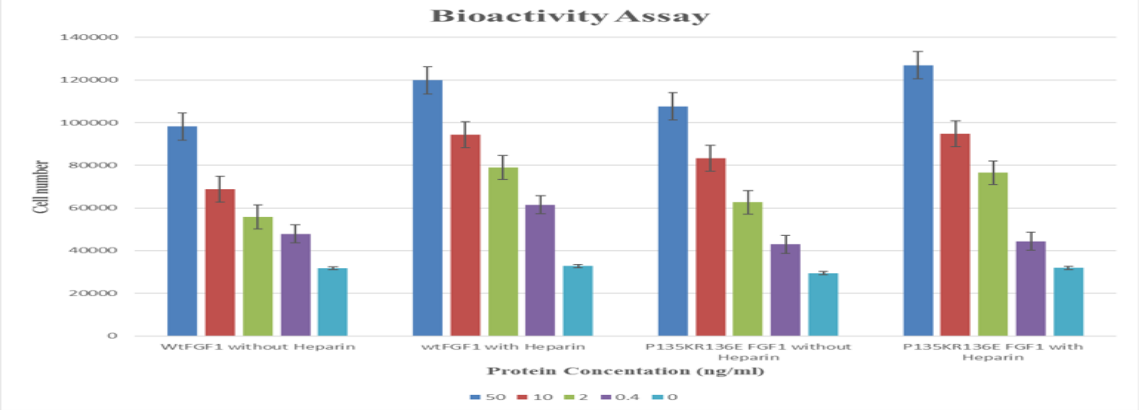


Figure 28: the bioactivity assay of the wild type and P135KR136E FGF-1 in the presence and absence of heparin with different protein concentrations.

In the case of R136E mutant, the mitogenic activity increased approximately two fold compared to the wtFGF-1 and other mutants (figure 29). This signifies that charge reversal especially using glutamic acid in place of arginine at position 136 might actually enhance the overall stability of the FGF-1 molecule. With respect to the proliferation activity of the wild type and double mutant P135KR136E FGF1, R136 showed a considerable increase in the cell division activity with 15,000 cells. Taken altogether with the ITC data, the decrease in the heparin binding affinity toward heparin for the single (R136E) and double (P135KR136E) mutant does

not seem to affect the cell proliferation activity of both proteins. This indicates both proteins regain heparin independent mitogenic activity.

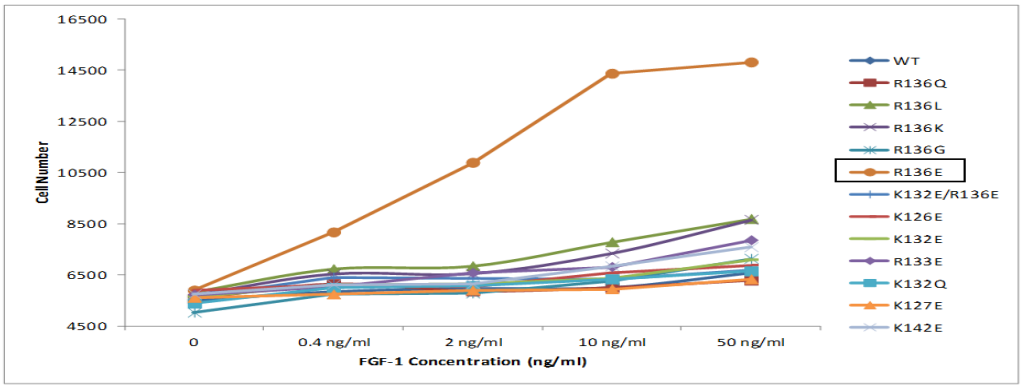


Figure 29: Mitogenic Activity assay of wtFGF-1 and its mutants.

Conclusion and Future Work

The data from this study evidently validated our hypothesis. In a previous study, the R136E mutant has overcome the low biological stability limitation of the native FGF by increasing the thermal and proteolytic resistance as well as the bioactivity. Additionally, the protein showed heparin independent mitogenic activity, which contributes in enhancing the overall stability of the molecule. These findings had to be evaluated to determine whether they are unique to position (R136) and if the effects of this single mutation can be voided by feeding a positive charge in a close vicinity. The introduction of lysine in place of proline did not seem to have a profound effect on the stability of the protein as the stabilizing effect provided by the neighboring amino acid—glutamate—was nullified. However, the decrease in the binding affinity toward heparin tended to be similar to R136E. Another positive consequence that has been demonstrated in this study is that although other prolines in FGF1 protein play an important role in the overall biological activity and structure of the protein, at least P135 is not essentially

responsible for this part, as it does not have a huge role. Furthermore, as there is a conservation of these residues among other family members of the protein, these kinds of mutations might have a similar effect, which lead to a heparin independent property of this molecule. A comparable trend can be observed even in FGF2 protein, which is one of the well-studied prototypical growth factors and has huge implications in the biomedical research.

Additionally, introduction of these kinds of mutations benefit in terms of understanding the tertiary structure because the perturbation that occurs in the residues can facilitate the understanding of local changes. For instance, the aromatic residue, tryptophan, which is an ideal probe to perform various intrinsic fluorescent based experiments, seems to be more solvent exposed as has been observed by the intrinsic fluorescence and NMR data. In the case of wild type and R136E mutant, the quantum fluorescence that emits from tryptophan is quenched by the positively neighboring amino acids which results in only 308 nm peak; however, substituting proline by lysine leads to project this aromatic residue toward the surface and expose it to more solvent. The resulted peaks at 208 and 320 nm in the florescence data as well as the change of the Tryptophan position in the PyMol structural viewer after introducing the double mutation compared with the wild type confirms the previous findings. The understanding of these local changes occurring around the environment of tryptophan can be extrapolated to other groups of proteins, as tryptophan of FGF is well conserved in all FGF group members.

Since 1980s, proteins have been predominantly occupied the majority of pharmaceuticals with around 200 marketed products including therapeutics, diagnostics, and vaccines (walsh, 2010). Advanced protein design techniques and recombinant protein technology have been used to generate novel therapeutic agents by engineering different types of proteins. This molecular evolution should be rational and knowledge based in order to modify the desired protein by

targeting its limitations and overcome the challenges. Our study is opening avenues for better understanding structure-activity relationships of FGFs allowing systematic generation of novel proteins with entirely modified properties to be applicable as topical and injectable treatments for various chronic wounds and ulcers. In fact, unless we have a comprehensive understanding of the protein's structure-activity relationship, we cannot develop the next generation of advanced therapeutic. Such studies are going to benefit as a whole in advancing the field of the protein engineering.

In regard the future work, the Hydrogen Deuterium Exchange (HDX) experiment can be accomplished for the double mutant in order to understand the role of individual residue in the overall stability of the molecule. In addition, further studies based on this research findings are more likely to be performed toward improving the properties of the native FGF1. Charge-reversal mutations in the vicinity of R136E will be explored to enhance the bioactivity of FGF with respect of the neighboring residues without compromising the stability. This can be accomplished through performing molecular dynamics stimulation studies of the neighboring amino acids in the vicinity in order to develop a more knowledge-based approach. Subsequently, the site directed mutagenesis technique would be used to design the desired variant that will be more efficient in terms of both activity and stability.

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