University of Arkansas, Fayetteville

ScholarWorks@UARK

Graduate Theses and Dissertations

5-2022

Introduction of Human Acidic Fibroblast Growth Factor (FGF1) Variant with Increased Stability and Bioactivity

Azadeh Tavousi Tabatabaei
University of Arkansas, Fayetteville

Follow this and additional works at: https://scholarworks.uark.edu/etd

Part of the Materials Chemistry Commons, Organic Chemistry Commons, and the Physical Chemistry Commons

Citation

Tavousi Tabatabaei, A. (2022). Introduction of Human Acidic Fibroblast Growth Factor (FGF1) Variant with Increased Stability and Bioactivity. *Graduate Theses and Dissertations* Retrieved from https://scholarworks.uark.edu/etd/4463

This Thesis is brought to you for free and open access by ScholarWorks@UARK. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of ScholarWorks@UARK. For more information, please contact scholar@uark.edu.

Introduction of Human Acidic Fibroblast Growth Factor (FGF1)
Variant with Increased Stability and Bioactivity

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Chemistry

by

Azadeh Tavousi Tabatabaei
University of Esfahan
Bachelor of Science in Pure Chemistry, 2012
University of Shahid Beheshti
Master of Science in Organic Chemistry, 2015

May 2022
University of Arkansas

This thesis is approved for recommendation to the Graduate Council.

Suresh Kumar Thallapuranam, Ph.D.
Thesis Director

Paul Adams, Ph.D.
Thesis Director

Maggie He, Ph.D.
Committee Member

Abstract

Human acidic Fibroblast Growth Factor 1 (FGF-1) involves in a broad spectrum of biological processes, including cell growth, proliferation, differentiation, migration, angiogenesis, wound healing, and embryonic development. hFGF1 non-selectively binds to cell surface hFGF receptor isoforms to elicit these cell-signaling processes. Since hFGF1 plays a significant role in tissue repair activity, that is a prime candidate for novel wound healing therapeutics. However, hFGF1 has been found to unfold near physiological temperature due to a strong electrostatic repulsion created by a dense cluster of positively charged amino acids near the c-terminus. The problem not only leads to proteolytic degradation of the unfolded protein but also limits the bioavailability of hFGF1 and then restricts application of hFGF1 in pharmacology. To overcome this instability, hFGF1 with the positively charged residues near the c-terminus in the region known as the heparin-binding pocket binds to the heavily sulfated glycosaminoglycan, heparin, which decreases the charge-charge repulsion. However, the use of heparin in hFGF1 wound-healing therapeutics imposes a serious challenge. To address these issues, I have designed a charged reversed single mutation in the heparin-binding pocket of hFGF1 to decrease the repulsion between the closely packed positively charged, enhanced stability and cell proliferation activity of the protein. Study of this mutation is performed by biophysical experiments. The results show that site-directed mutagenesis in hFGF1 can enhance the inherent stability of the growth factor and increase mitogenic activity of hFGF1 in absence of heparin.

Acknowledgments

I would like to express my special thanks of gratitude to my advisors Dr. Kumar and Dr. Adams who gave me the golden opportunity to do this project. I am thankful to them for the continiues support to my research, for their patience, motivation, and immense knowledge. Their guidance helped me all the time of research.

I would like to acknowledge my committee member Dr. He for all her time, helpful suggestion.

I would like to extend my sincere thank to Dr. Fan for helping me to design of non-canonical amino acid.

I would like to thank my mentor Zeina Al-Raawi for guiding me on the right path, sharing her valuable experience and knowledge and helping me when I got stuck with research.

I would like to thank Patience Okoto and Phuc Phan who helped me in any manner and sharing their support, experience and knowledge.

I would like to pay my special regards to my husband Hossein for always being by my side through all moments of my life and for always showing how proud he is of me.

My sincere thanks also go to my great parents and my sisters Ayeh, Bahar and Reyhaneh for their moral encouragement and supporting me spiritually throughout my graduate career.

I thank my colleagues in Dr. Kumar and Dr. Adams labs for creating a friendly environment, treating me with respect and inspiring me with their own hard work.

Table of contents

Chapter 1 1

References 28

Chapter 2 35

References 62

Chapter 3 65

References 68

Abbreviations

Fibroblast growth factor (FGF), human acidic fibroblast growth factor-1 (hFGF1), wildtype hFGF1 (wtFGF1), site directed mutagenesis (SDM), Sulfotyrosine (sY), circular dichroism (CD), American Type Culture Collection (ATCC), Visual Molecular Dynamics (VMD), Glycosaminoglycan (GAG), Retrovirus-associated DNA sequences (RAS), Mitogen-activated protein kinase (MAPK), Heparin sulfate (HS), Extra cellular matrix (ECM), Heparin binding site (HBS), FGF homologous factors (FHF), Fibroblast growth factor receptor FGFR, phospholipase C (PLC) γ substrate 1 (FRS1), FGFR substrate 2 (FRS2), FGF receptor substrate 2α (FRS2α), phospholipase Cγ (PLCγ), phosphoinositide 3-kinase (PI3K), Protein kinase B (AKT), Growth factor receptor-bound 2 (GRB2), Tuberous sclerosis complex 2 (TSC2), Inositol triphosphate (IP3), Diacylglycerol (DAG), Protein kinase C (PKC), Immunoglobulin (Ig), Src homology2 (SH2), Receptor tyrosine kinase (RTK), Signal Transducer and Activator of Transcription (STAT), Keratinocyte growth factor (KGF), Recombinant human acidic fibroblast growth factor 1(rh-FGF1), Recombinant human acidic fibroblast growth factor 2 (rh-FGF2).

CHAPTER 1

INTRODUCTION

Growth factors are proteins well known for intercommunication between cells through ligand/receptor cell signaling. Cell signaling can be processed by several types of ligands/effector molecules such as proteins. There are three modes for cell signaling known as succession - reception of the signal, transduction through relay molecules, and cellular response by the target cell. Intercommunication between cells is started by an extracellular ligand binding to a specific receptor in an extracellular part of the target cell. A chemical input inside the cell as a transduction signal is triggered by a ligand to detect the reception. The ligand-specific response is created when the chemical signal has reached its target intracellularly. Growth factors are proteins well known for cell growth and differentiation. Therefore, hFGF1 is a good candidate for novel wound healing therapeutics due to its role in tissue repair activity.

Four modes of signaling can occur for the cell signaling process. The first mode known as autocrine secretion, is processed by binding a hormone to autocrine receptors on the same cell. The second mode of signaling, paracrine secretion, is found where neighboring cells release factors onto each other's extracellular receptors. The next one is defined as intracrine secretion that the cell stimulates itself by a cellular product inside the cell. The third mode of secretion is intracrine secretion where in the cell stimulates itself by a cellular product that acts inside the cell. Despite other modes of signaling, endocrine signaling is transported to cells through the bloodstream [1,2]

Classification of the Fibroblast Growth Factor Family

According to sequence homology and phylogeny, the mammalian fibroblast growth factor (FGF) family are divided into 7 subfamilies (Fig. 1); however, they are classified into 6 subfamilies

based on the gene location analysis [1-3]. FGF11 subfamily performs as an intracrine manner, but the FGF19 subfamily is involved in endocrine FGFs, the rest of the FGF subfamilies have an important role in the paracrine manner. Only the member of paracrine subfamily is also found in binding to the heparan sulfate [2].

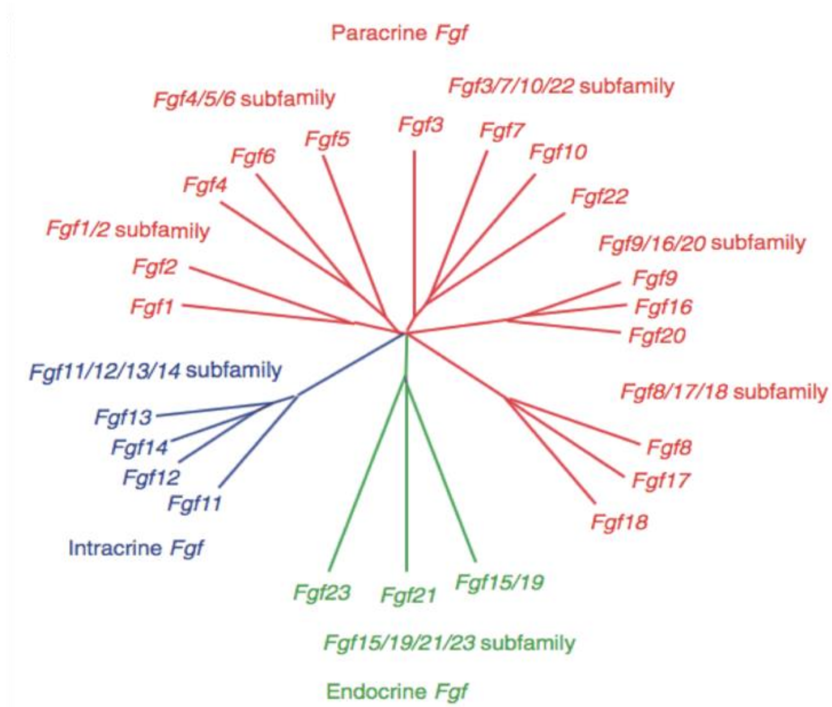


Fig. 1: Classification of FGFs by subfamily and type of signaling [1].

FGFs as mitogens, involve in fetal and embryonic developments, pathological conditions, and some biological processes in adult organisms. Regarding adult organisms, the growth factors participate in angiogenesis, tissue repair, and wound healing.

The FGF family consist of 23 related proteins with a molecular weight ranging 17 to 34 kDa [4]. Metabolic processes are induced by FGF through binding and activating specific tyrosine kinase fibroblast growth factor receptors (FGFRs). In fact, formation of FGF-FGFR complex causes induction in downstream signaling through pathways such as the retrovirus-associated DNA sequences (RAS)/mitogen-activated protein kinase (MAPK), and phospholipase-Cγ (PLCγ)

pathways, phosphoinositide-3-kinase (PI3K) /Protein kinase B (AKT) but specificity of the events triggered by individual members of the FGF family is still not clear [4]. There is a similarity among-most FGFs in terms of amino acid composition [5]. The FGF family consists of structurally related polypeptide growth factors that share high affinity for heparin, interact with FGFRs, and carry out distinct functions. Based on crystallography analysis of FGF proteins, it displays a β trefoil fold structure consisting of 12 beta sheets strands organized into the central domain that five of the sheets are placed in the hairpin binding pocket [6]. FGFs are formed from a cell-surface polysaccharide, heparin sulfate proteoglycans (HSPGs) involved in protein regulation, especially in the activation of transcription, post-transcriptional modifications (capping, splicing, polyadenylation, and mRNA stability), translation initiation, post-translational modifications (O-linked glycosylation, N-linked glycosylation, phosphorylation, acetylation, methylation, and ribosylation), intracellular trafficking, secretion, bioavailability, and ligand-receptor interactions [7].

Structure and Function of the FGF Family

FGF1 subfamily consists of FGF1 and FGF2, proteins that are well known for their cell proliferating activity and expression in both developmental and adult stages. The FGF1 subfamily as a prototypical or archetypal family known as an acidic fibroblast growth factor since its isoelectric point (approximately 6.5) is relatively acidic compared to basic FGF (FGF2) with isoelectric point of 9.6 [8]. FGF1 was the first growth factor isolated and identified. The prototypical FGF genes comprise 3 coding exons, out of which exon 1 contains the initiating codon- methionine. Despite the paracrine function of FGF1, it acts in an intracrine signaling. Not only the FGFs members are secreted by the cell but also, they don't have the N-terminal hydrophobic sequence instead, they are directly translocated to the nucleus [9-12]. As the mRNA

does not include a signal peptide sequence that targets FGFs for secretion through the classic ER-Golgi complex secretory pathway, they structure of this subfamily is unique compared to other FGFs. FGFs employ a non-classical pathway to be secreted [11]. The open reading frame in FGF1 is also edged by the termination codons, whereas FGF2 contains a 5 transcribed sequence that initiates from upstream CUG codons for translation [12].

FGF1 and FGF2 are significantly different in size and sequence, but they have similarities such as core region of homology encompassing 120–130 residues; that shows a common ancestral gene. There is 53% protein sequence homology between the structure of FGF1 and FGF2. Both bind tightly to heparin and share many biochemical and biological properties [13]. While in the FGF1 subfamily, there is a similar preference for N-sulfate and O-sulfate, FGF1 differs in that, and it also binds saccharide structures with 6-O-sulfated heparin. This subfamily included three Heparin Binding Sites (HBS), including the primary HBS1 and the secondary binding sites HBS2 and HBS3 [14].

FGF1 is a single chain positively charged polypeptide with a molecular weight around 5,967 Da and a isoelectric point (pI) of 7.73 [4]. FGF1 was purified from the brain by standard chromatographic techniques such as gel filtration, ion-exchange chromatography, reverse-phase HPLC and isoelectric focusing. In addition, purification of FGF-1 is performed by heparin Sepharose chromatography because of its high heparin affinity. While FGF1 was recognized as a polypeptide containing 140 amino acids, the subsequent sequence analysis exhibited 154 amino acids polypeptide in FGF1 that is expressed in microvascular endothelial cells. FGF1 participates in cell differentiation such as cell growth and survival, adipogenesis, embryonic development, and tissue repair.

FGF2 with a molecular weight ranging from 18-24 kDa is well conserved among the other species. Human FGF2 (hFGF2) consists of four cysteine residues (Cys 33, Cys 77, Cys 95, and Cys 100) without disulfide bonds. Two of these cysteine residues (Cys 33 and cys 100) are conserved in all FGF family members. Based on the site directed mutagenesis studies, substitution of Cys 77, 95, and 100 to serine does not affect the cell proliferation activity of FGF2. However, substitution of Cys 33 to serine causes 60% reduction in the biological activity of hFGF2. Several studies indicated that there is no difference between cell surface receptor-binding and heparin-binding domains of FGF2 [5].

FGF1 and FGF2, as prototype members of the FGF family, play a critical play in controlling of biological functions such as developmental processes during embryogenesis and various physiological roles in the adult state, including the regulation of angiogenesis, wound healing, organ development (eye, skin, brain, lung, limb, muscle, bone, blood, and heart), and metabolism. FGF1 and FGF2 also exhibit broad mitogenic and cell survival activities, such as cell growth, cell differentiation, tissue repair, tumor growth, and invasion. Moreover, FGF1 and FGF2 participate in neurogenesis. The application of FGF1 and FGF2 in wound healing is more than FGF7 and FGF10. In addition, recombination of human FGF1 (rh-FGF1) and human FGF2 (rh-FGF2) played an important role in the treatment of ulcers and diabetic foot ulcers. Both rh-FGF1 and rh-FGF2 have a positive effect on curing ulcers, diabetic foot abscess, and second-degree burns [15].

FGF4 Subfamily

The FGF4 subfamily includes FGF4, FGF5 and FGF6. FGF4 consists of 206 amino acids protein expressed only during embryonic stages that included both an amino signaling sequence for classical secretion as well as an N-glycosylation site [16, 17].

Several studies suggested that FGF4 and FGF6 are more stable than FGF3 and FGF17 in absence of heparin [18]. Based on data obtained from crystal structure, FGF4 and FGF6 possess 2 heparin binding sites (HBS). The first heparin binding site is located between β6 and β7, β9 and β10; β10 and β12 strands which included Lys 142, 144, 147, 183, 186, 188, and 189, respectively. The second heparin binding site involves three lysine residues (Lys 65, 81, and 158). For FGF6, HBS1 is located the region between β10 and β12 strand and includes Lys 144, 185, and 194. HBS2 includes Lys 83 (found near the N-terminal of β1 strand) and Lys 158 on the β8 strand. Human FGF4 involves in the embryonic development regulation, cell proliferation, and cell differentiation.

FGF5 consists of a highly conserved core region included of 12 β strands and a signal peptide at the N terminus [19]. Human FGF4 involves in the of cell proliferation regulation and cell differentiation. FGF5 plays a critical role in determining hair length in mammals. Based on reported studies in 2007, mutation of proline to histidine in the FGF5 gene causes longhair phenotypes in cats [14].

FGF6 with 25kDa is found in varying lengtheparin (208, 198, or 175 amino acids). Human FGF6 involves in the regulation of cell proliferation, cell differentiation, angiogenesis, myogenesis, and regeneration of calf muscles [20].

FGF7 Subfamily

The FGF7 subfamily includes FGF3, FGF7, FGF10, and FGF22. The subfamily incorporated in various diseases such as congenital deafness, lacrimo-auriculo-dento-digital syndrome, inflammatory bowel disease, Apert syndrome, and prostate cancer [21].

Human FGF3 is a 239 amino acid protein expressed during embryonic stages and is secreted through the classical secretion pathway because of an amino-terminal signal sequence. FGF7 as a

Keratinocyte Growth Factor (KGF) with 28 kDa consists of 194 amino acids and a 31 amino acid N-terminus sequence. It is known as 10 beta strands, which form five double stranded anti-parallel beta sheets with single beta-sheet hydrogen bond between residues 137 and 141. FGF7 subfamily members play an important role in organogenesis and tissue patterning in the embryo, tissue homeostasis in adult mammals, and accelerate wound healing [22]. FGF7 is critical for the regulation of embryonic development, cell proliferation and cell differentiation and normal branching morphogenesis.

Moreover, this growth factor is expressed with FGFR2b in hepatocytes. In vivo and in vitro studies indicate that FGF7 and its related receptor accelerate the proliferation of hepatocytes following liver damage, disease, and partial hepatectomy [23].

FGF10 with 20 kDa consists of 209 amino acids with a hydrophobic 35 amino acid signal The sequence at its N-terminus. The protein is essentially expressed in different cells such as fibroblasts, adult pre-adipocytes, lung mesenchyme, posterior limb mesoderm and mesenchyme. FGF10 is expressed at both embryonic and adult stages and is reported to mediate limb, lung, and brain development. Because of impartial lung development of FGF10, its knockout mice died at birth [16, 24]. FGF10 is also involves in liver development by maintaining the survival of hepatoblasts [23].

FGF22 with 209 amino acids and a molecular weight of 23 kDa has a 22 amino acid signal sequence and is expressed in cerebellar granule cells [25]. Vivo studies indicate that FGF22 is a presynaptic organizing molecule and neutralization of FGF22 inhibits differentiation of mossy fibers involved in signal transmission at sites of contact with granule cells. FGF22 has also been shown in the inner root sheath of hair follicles, which depicts a role in hair development [20]. In addition, this FGF member has shown an important pro-oncogenic role in the developing skin

cancer, as FGF22 knockout mice were found to develop fewer papillomas than control mice in a carcinogenesis challenge study [20].

Based on the studies have done by Terauchi, FGF22 was used as a target-derived presynaptic organizer by hippocampus region of the mouse brain uses [26]. There is also minimal or no heparin binding affinity in FGF3 and FGF2, and the difference causes distinct biological properties and functions [27]. Amino acid sequence alignment shows that FGF3 was stabilized through unmodified heparin and any of the singly desulfated heparins [27]. In the case of FGF10, there was no obvious difference in the stabilizing effect of heparin and the singly desulfated heparins. Therefore, there are similar binding preferences between FGF10 and FGF3 but are not equal.

FGF8 Subfamily

The FGF8 subfamily as a paracrine signaling molecule includes FGF8, FGF17, and FGF18. Multiple human isoforms in Human FGF8 and FGF17 members distinct this subfamily [28]. The variation is formed from alternative splicing within the first exon, which causes length changing of the N-terminus sequence from 62 to 156 amino acids [29]. Regulation of biological activity of these FGFs in midbrain and hindbrain patterning and its development have been determined by the splicing variation [29]. Furthermore, the crystal structure of FGF8-FGFR2c and FGF17-FGFR2c complexes implies that the N-terminus sequence discrepancy modulates receptor binding such that FGF17 binds the receptor with stronger affinity than FGF8 [30]. FGF8 and FGF17 play an important role in regulation of neuroepithelial proliferation in the brain at a junction in the mid-hind region [16, 31]. In addition, FGF8 involves in development of embryonic limb and central nervous system [16].

FGF18 comprised 207 amino acids and an N-terminus 26 amino acid secretion signal

sequence [31]. FGF18 expression is found in muscles, pancreas, midbrain, lung, and the intestinal tract during embryonic development. Furthermore, FGF18 involves in cell proliferation, cell differentiation, cell migration cartilage, formation, and osteogenesis of skeletal development [31].

FGF9 Subfamily

The FGF9 subfamily includes FGF9, FGF16, and FGF20, which are secreted outside of the cell and are paracrine signaling. FGF9, with 208 amino acids and 30 kDa, is a potent mitogen which is produced by neurons [32]. Although it does contain a distinct N-terminus signal sequence, it does not have an unusual hydrophobic sequence as a compulsory part of its secretion [16]. FGF9 expression has been found in high levels throughout kidney, lung, and prostatic stromal cells, and multiple cell types in the central nervous system. Due to sex reversal of knockout mice male to female, FGF9 plays a critical role in role in male sex determination [33]. Furthermore, FGF9 has a vital role in the proliferation of lung mesenchyme, produceing other FGFs, including FGF3, FGF7, FGF10, and FGF22. FGF16 with 207 amino acids and 26 kDa is not classically secreted due to lack of an amino terminal signal sequence. FGF16 expression has been reported during embryonic development through cardiac myocytes and in brown adipose tissue. Severe cardiac abnormalities are found in FGF16 knockout mice due to reduction of FGF16 in adult stages [33, 34].

FGF20 with 211 amino acids and 23 kDa has no signal sequence. FGF20 is expressed in a region of the midbrain known as the substantia nigra pars compacta, where it preserves dopaminergic neurons, which prevents onset of Parkinson's disease. Stimulation of MAPK pathway is happened during interaction of FGF20 with FGFR1c in the midbrain region which ultimately sustains the dopaminergic neurons and prevents Parkinson's disease [35].

FGF11 Subfamily

The FGF11 subfamily includes FGF11, FGF12, FFG13, and FGF14. These FGFs are known as FGF homologous factors (FHFs) and display high levels of sequence homology [16]. Members of the FGF11 subfamily share between 58-71% sequence identity with each other and only up to 30% sequence identity with other members of the FGF family [29]. These members not only secreted from cells but also are intracrine signaling proteins that function independently of cell surface FGFRs [37]. Furthermore, this subfamily interacts with the cytoplasmic C-terminal tails of voltage-gated sodium channels to regulate channel gating and intracellular signal trafficking [36].

FGF19 Subfamily

FGF19 subfamily includes FGF19, FGF21, and FGF23 with endocrine signaling. Studies that have been reported about FGF15 (the mouse ortholog of FGF19), indicates that FGF19 has an important role in the regulation of bile acid homeostasis in the liver through interaction with FGFR4 expressed on hepatocytes [38]. FGF19 consists of 216 amino acids and a 22 amino acid N-terminal signal sequence. According to reports on FGF19 transgenic mice, FGF19 increases fatty acid oxidation and reduces level of liver triglycerides, glucose and enhances sensitivity to insulin [39]. In addition, FGF19 plays an important role in the treatment of diabetes through the reduction of brown adipose tissue [39]. FGF19 signals are not only via binding with FGFR4 in the liver but can also through interaction with other FGFRs in the presence of the single-pass transmembrane protein, β-Klotho [1, 39].

FGF21 includes 171 amino acid protein with a molecular weight of approximately 19 kDa. FGF21 expression is reported in the pancreas, liver, and white adipose tissue. Although this FGF can activate most FGFR isoforms, it preferentially binds to FGFR1c. Moreover, the binding of

FGF21 with FGFRs is cooperated with by β-Klotho [1, 39]. Therefore, FGF21 plays a metabolic role in increasing insulin sensitivity and glucose uptake, stimulating fatty acid oxidation, and inducing the adaptive starvation response [40].

FGF23 consists of 251 amino acids with a 24 amino acid N-terminus signal sequence and has a molecular weight of approximately 28 kDa. FGF23 expression is placed in bone cells to regulate serum phosphate levels and vitamin D levels by preventing reabsorption in the proximal tubule and intestine through the interaction of FGF23 with FGFR1c (complexed with the transmembrane protein α-Klotho) in the kidney [40]. In addition, FGF23 interact with the parathyroid gland to prevent the secretion of the parathyroid hormone, which regulates phosphate uptake from bone [38].

hFGF1 Structure

β-trefoil family is determined for the structure of hFGF1. One of 10 fundamentals protein superfolds known as the β-trefoil which consists of three connected trefoil units (Fig.2) [41, 42]. While the β-trefoil structure has been reported as a monomeric unit for epidermal growth factors, the β-trefoil is determined in dimeric and trimeric units, respectively for certain protease inhibitors as well as in fibroblast growth factors [42]. Therefore, it's predicted the evolution of the β-trefoil structure is derived from sequential gene duplication(s)/fusion(s) [41, 42]. Different types of proteins, including fibroblast growth factors, interleukin-1α and -1β, Kunitz soybean trypsin inhibitors, plant cytotoxins and bacterial toxins such as the ricin-like toxins, as well as some carbohydrate-binding proteins including xylanase, have trefoil structure [41, 43, 44].

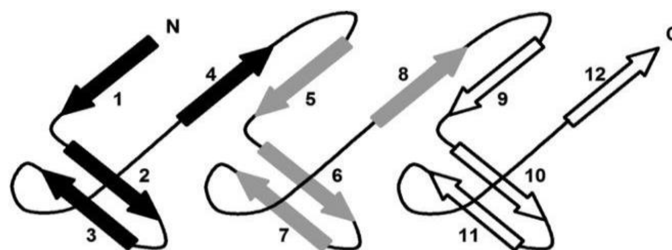


Fig. 2: Secondary structure of hFGF1. Twelve β-strands are folded into β-trefoil structure [41].

Heparin

Chemistry and Structure of Heparin

Heparin is a member of glycosaminoglycan groups (GAGs). These molecules consist of repeating disaccharide units that most frequently include D-glucosamine and L-iduronic acid and less commonly include D-glucuronic acid, N-acetylglucosamine, as well as unsubstituted glucosamine units (Fig. 3) [45, 46]. These sugar units are heavily sulfated and linked via α-(1→4) glycosidic linkages. Sulfate groups are commonly found on positions 2 and 6 on D-glucosamine as well as on position 2 on the L-iduronic acid unit [45, 46].

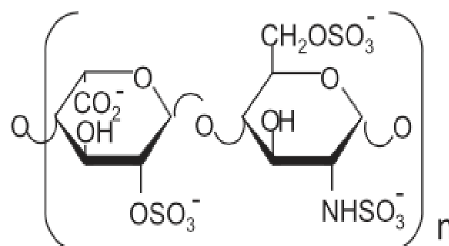


Figure 3. The structure of heparin comprised of repeating disaccharide unit included L-iduronic acid (left) and D-glucosamine (right) linked via α-(1→4) glycosidic linkages [46].

The sugar composition of heparins from various species and tissues creates substantial variation [46]. In addition, the length of heparin-like polysaccharide chains is varied from 3 to 30 kDa.

Heparin and Heparan Sulfate

Another GAG family, known as heparan sulfate (HS), is reported in the extracellular matrix with a similar sequence and backbone structure to heparin. Proportions and arrangements of sugar saccharides and sulfation patterns are completely different in HS compared to heparin [46, 47]. HS expression is placed by all cells throughout the human body. However, heparin is only expressed by mast cells [47]. Moreover, HS and heparin have different degrees of sulfation. Only 30-60 % sulfated with clusters of the polysaccharide sulfate is reported for HS, and other portions are unsulfated, but heparin is 80-90 % sulfated with no clustering of the sulfate groups [47].

Biosynthesis of Heparin

Heparin is synthesized in the golgi apparatus through several steps by different Enzymes [47]. The first step begins with a serine residue of the core protein, serglycin, followed by forming the non-sulfated tetrasaccharide substrate (-GlcA-Gal-Gal-Xyl-) through the sequential linkage of D-glucuronic acid (GlcA), glacatose (Gal), and xylose (Xyl) by various enzymes [47]. The primary substrate is produced by the elongation process included two steps, the first step involves addition of N-acetyl-D-glucosamine (GlcNAc), and the second step begins with the alternating addition of GlcA and GlcNAc (Fig. 4) [47, 48]. Polymerization of the substrate is then catalyzed by complexed EXT1 and EXT2 polymerases. At the Modification process, GlcNAc N-deacetylase / Nsulfotransferase enzymes are involved to catalyze the substitution of acetyl groups with sulfate groups [49]. Furthermore, uronosyl C5-epimeraser, and O-sulfation are applied to catalyze the epimerization of D-glycuronic acid units into Liduronic acid, that is placed on two positions in Dglucuronic acid and one position in L-iduronic is catalyzed by 2-O, 3-O, and 6-O sulfotransferases (Fig. 4) [48, 49]. Furthermore, a wide variety of polysaccharides as heparin-like molecules are yielded to enhance the biological activity of these GAGs.

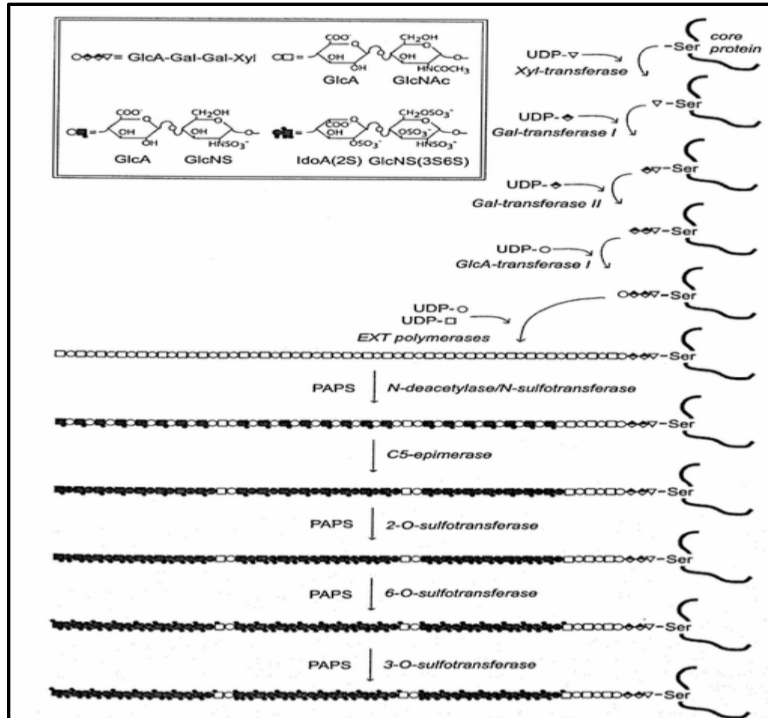


Figure 4. Biosynthesis of heparin [48].

Biological Significance of Heparin

Heparin is an anticoagulant that reveals increasing the inhibitory action of antithrombin. Heparin binds with various serine protease inhibitors; however it has the most dominant interaction with antithrombin. Antithrombin depicts high affinity at a specific pentasaccharide sequence within heparin (αDGlcNAc(6S)-βDGlcA-αDGlcNS(3S, 6S)-αLIdoA(2S)-αDGlcNS(6S)) [50]. Upon binding, a conformational change within antithrombin exposes a reactive loop that mimics the substrate of the serine protease thrombin. The protease becomes locked in an inactive complex, preventing its succeeding action on fibrinogen to initiate the clot forming process when thrombin cleaves the sequence of the reactive loop on antithrombin [50].

Heparin also highly interacts with FGFs to regulate FGF movement and the duration of their biological half-life. Furthermore, heparin plays a critical role in the organization of growth and

development of complex systems and organisms [51]. Due to role of FGFs in tissue repair following injury, heparin is known as a mediator of wound healing processes.

Heparin-hFGF1 Interaction

Electrostatic interactions between the sulfate groups of heparins and the positively charged residues of the heparin-binding pocket of hFGF1 are the basis of the interaction between heparin and hFGF1. The heparin-binding region of FGFs consists of three exposed loops. The loops are included several positively charged residues placed on beta strands 10 and 11 [6]. Site directed mutagenesis studies since 1990 have reported a reduction in the heparin-binding affinity specifically for charge reversal of lysine residues toward the C-terminus (K132, K127, K114, and K115) [52].

X-ray crystallography and multidimensional nuclear magnetic resonance are used to determine the full characterization of the heparin-hFGF1 interface. One of the first hFGF1-heparin crystal structures displayed the binding of a hFGF1 dimer to a heparin disaccharide (PDB 2AXM) through C-terminal residues 126-142 of hFGF1 [35]. In addition, there is no protein-protein interactions in the crystal structure and each monomer binds the heparin through sulfate groups on opposing sides of the polysaccharide [35]. According to studies performed by DiGabriele and coworkers, heparin binding does not induce any conformational change in hFGF1 structure. An additional crystal structure consisting of two 1:1 hFGF1-FGFR2 dimers indicates that a helical heparin disaccharide chain causes contacts with both hFGF1 monomers (denoted A and B) (PDB 1E0O) [53]. The crystal structure also shows that K126, K127, N128, K132, R133, R136, and K142 positions in the hFGF1 facilitate heparin binding [53].

While there is an interaction between hFGF1 monomer and 6 monosaccharides of heparin, hFGF1 monomer B interacts with only 5 heparin monosaccharides, and it creates distinct

contacts with heparin through a hydrogen bond between Trp121 and GlcN-1 [53]. According to reports performed by Pellegrini and coworkers, there are 34^0 kinks between the second and third disaccharides in heparin chains, and it shows that van der Waals forces significantly contribute to hFGF1-heparin binding as well as electrostatic interactions [53].

Following studies of the positively charged residues in the heparin-binding region of hFGF1 revealed the significance of the conserved lysine at position 132, located in the base of the pocket toward the protein core. Interaction of K132 with critical N-sulfate and 2-O-sulfate groups on the heparin iduronic acid sugar induces a conformational change in the positioning of iduronic acid. The conformational alternation of heparin changes the backbone torsion angles of heparin and induces a "kink" in the ligand structure [54]. Repositioning of heparin induces the formation of additional van der Waals forces that help to achieve optimum binding with hFGF1, as previously noted [53, 54]. Each family has a unique spatial arrangement of these positively charged heparin-binding residues, making FGF with different affinity for different heparin-like GAGs.

Overlay of the Cα traces of residues in the heparin-binding region from five different FGF crystal structures indicated (including hFGF1) the structural arrangement of these loop regions corresponds to and is more rigid in character, as previously mentioned [54]. This implies that the spatial arrangement of these side chains for hFGF1 provides a unique "signature" for its heparin-binding interface [54]. Moreover, biophysical characterization of the protein in the presence and absence of heparin displayed that the binding of heparin with hFGF1 does not conduct any conformational shift within the protein.

Role of Heparin in hFGF1-FGFR Signaling

The debate about the role of in hFGF1-FGFR interaction and subsequent activation is still ongoing. Heparin plays an essential role in protein stability, receptor binding, and activation of hFGF1 [45, 52, 53, 55]. According to the reported crystal structures, heparin involves in the biological function of hFGF1. These structures also imply that both monomers of dimeric hFGF1 are linked to the same heparin chain as well as the FGFR. Thus, the heparin plays an important role in the hFGF1 dimerization and FGFR activation process [53, 56]. In addition, the dimer structure of these crystals reveals that hFGF1 dimerization is mandatory for subsequent receptor binding and activation. In 2002, Kumar and coworkers used sucrose octasulfate (SOS) as a molecular mimic of heparin to assess the biological activity of hFGF1 [57]. Their study indicates that the binding of SOS to hFGF1 retains the protein in a monomeric form. In addition, they indicate that binding of monomeric hFGF1 to SOS involves in cell proliferation activity [57]. In conclusion, hFGF1 oligomerization is not mandatory for its biological activity with corresponding FGFRs. Further study by Angulo and coworkers in 2005 indicates that hFGF1 dimerization is not critical for its biological activity, and the sulfation pattern of heparin-like ligands has an essential impact on the mitogenic activity of hFGF1 [58]. In the study, two synthetics sulfated hexasaccharides (with different sulfation patterns) are utilized to bind and retain hFGF1 in its monomeric form as they cannot facilitate hFGF1 dimerization. The sulfate groups of both ligands expand the length of the ligand molecule; however, there are sulfate groups on both faces of the molecule at the first ligand, and the other ligand shows sulfate groups only on one side [58, 59]. Previous studies indicated that the ligand with sulfate groups oriented toward only one face of the molecule facilitated hFGF1-mediated cell signaling at the same level as natural heparin and that dimerization of hFGF1 is then not mandatory for biological activity [58, 59]. In multiple mutagenesis studies, heparin is an absolute requirement for hFGF1 cell

signaling. Several studies have been reported that heparin is not critical in the biological activity of hFGF1, but that is utilized for stabilizing the inherently unstable protein and enhancing the bioavailability of the protein in reservoirs near the cell surface. hFGF1 is defined as an unstable protein at temperatures close to physiological due to its poor thermal stability and short half-life in vivo [42, 60-62]. The poor stability of hFGF1 can be known as a regulatory mechanism that protects against overactive or unregulated FGF1 signaling, which leads to tumorigenesis [63]. The studies, that claimed heparin is not essential for hFGF1 biological activity, have reported that function of hFGF1 can be heparin independent if the thermal stability of the protein could be increased [62].

At another study, they reported the hFGF1 mutant K132E with reduced heparin-binding and completed FGF receptor binding and activation, and further was shown to induce early intermediate gene transcription [52]. Further study indicates however substitution of serine for cysteine residues in three positions (30, 97, and 131) increases hFGF1's half-life from 0.26 hours up to 73 hours, it thermally destabilizes the protein [61]. Further studies attempted to design mutations with increased stability and increased the core-packing arrangement of hFGF; however, one mutation, L58F, showed an increasing the thermal stability of hFGF1 [42].

In the recent study, a sequence homology approach was applied to introduce the three mutations: H35Y, H116Y, F122Y [62]. However, each mutation enhanced the thermal stability of hFGF1 by a couple of degrees, they were all finally combined with L58F to introduce the quadruple mutant H35Y/L58F/H116Y/F122Y with the increased thermal stability of hFGF1 by 7.8°C [62].

In addition, the quadruple mutant showed similar biological activity to the wildtype protein. Another study utilized a sequence homology approach to introduce two additional stabilizing

mutants Q54P and S61I [64]. The two mutants were then combined with the previously described quadruple mutant as well as an additional stabilizing mutation, H107G, to introduce septuplet mutant:H35Y/Q54P/S61I/L58F/H107G/H116Y/F122Y. The seven mutations showed increasing thermal stability of hFGF1 by 27°C [64]. The septuplet and triple mutants (Q54P/S61I/H107G) in the absence of heparin are six and ten times higher than the wildtype hFGF1.

In 2009, Zakrzewska and coworkers redesigned the K132E mutation (that was previously introduced by Wong et al.) to examine the role of heparin within hFGF1 activity. Introduction of charge reversal K132E mutation is predicted to reduce hFGF1 heparin binding affinity due to its position within the heparin-binding pocket. In addition, the study has been reported that the hFGF1 K132E mutant is inactive toward DNA synthesis [65].

The combination of the K132E mutant with the stabilizing triple mutant (Q54P/S61I/H107G) implied that in the absence of heparin, the mitogenic activity of this quadruple mutant was equivalent to the level of heparin-bound wildtype hFGF1 [65]. In conclusion, the heparin has no essential role in hFGF1 binding and activation of its FGFR.

Another study indicated that the hydrogen bonds have important roles in stabilization of N and C terminal flexible strands, hypothesizes that the stability of hFGF1 could be enhanced if disulfide bonds were inserted into the N and C terminal tails. Although K26C and P148C mutants were introduced to generate disulfide bonds, increasing thermal stability and mitogenic activity of hFGF1are reported under reducing conditions, with no disulfide bond formation.

Despite disagreement on the role of heparin for hFGF1 activity with FGFRs, there have been several studies indicating the ability of hFGF1 to facilitate cell signaling in a heparin independent manner [37, 28, 66, 67].

FGFR Gene Evolution

FGFR genes were found in both humans and mice with approximately 810 amino acids with 56-71% sequence homology and identical exon-intron sequencing [3, 1]. The FGFRs variation is a result of alternative splicing of the extracellular immunoglobin-like (Ig) domains giving rise to FGFRs with either two or three Ig domains [3]. The FGFR splicing regulation has conserved mechanism throughout evolutionary history. Alternative splicing of FGFRs 1, 2, and 3 gives rise to two different Ig domain III (IIIb and IIIc) forms. Ig domains II, III and the linker region between the two domains are involved in the ligand-binding specificity of the receptor [68]. Furthermore, there are seven various FGFR isoforms with unique ligand-binding specificity from the four known FGFR genes (1-IIIb, 1-IIIc, 2-IIIb, 2-IIIc, 3-IIIb, 3-IIIc, and 4) [1].

FGFR Structure and FGFR -FGF Binding Interface

FGFRs are composed of an extracellular domain included three Ig-like domains that facilitate ligand-binding, a single pass α helical transmembrane domain, and an intracellular tyrosine-kinase domain [1]. All receptor isoforms contain around 810 amino acids with molecular weights of approximately 90 kDa [37, 69].

Protein-receptor binding interface study reveals that hFGF1 contacts Ig domains II (D2) and D3 of the receptor at the junction between the two domains [37]. Ig domain I (D1) and the acidic sequence of residues between D1 and D2 inhibit ligand binding. In addition, recombinant receptor proteins lacking D1 have a higher binding affinity for FGFs [37]. While there are many bounds between the FGF1 ligand and D3, making contacts with 23 residues, only 13 contacts are reported for FGF1-D2 [70]. In FGF1, 39 of 140 residues are involved in receptor binding that none of them include heparin-binding residues. According to the crystal structure of a heparin bound FGF1 dimer complexed with an FGFR2 dimer, the receptor's contacts between FGF1 and

D2 are prominently hydrophobic [56]. In addition, the 1:1 FGF1:FGFR2 structure complex also indicates contact between the N and C terminal ends extending from the core of the FGF1 ligand and the D2-D3 linker region (Fig. 5).

In the presence of heparin, it is bonded to the FGF1: FGFR complex through the heparin-binding region of FGF1. There are symmetrical complexes between FGF-receptor dimer and heparin with contacts of heparin with both FGFs and both receptor chains, or asymmetrical with contacts of heparin with both FGF ligands and only one receptor chain. A study of the FGF1-FGFR2 crystal structure in the presence (PDB 1E0O) and absence (PDB 1DJS) of heparin implies two conformations for the receptor D3. In the presence of heparin D3 of the receptor is rotated around the D2-D3 linker region by an additional 170 Å.

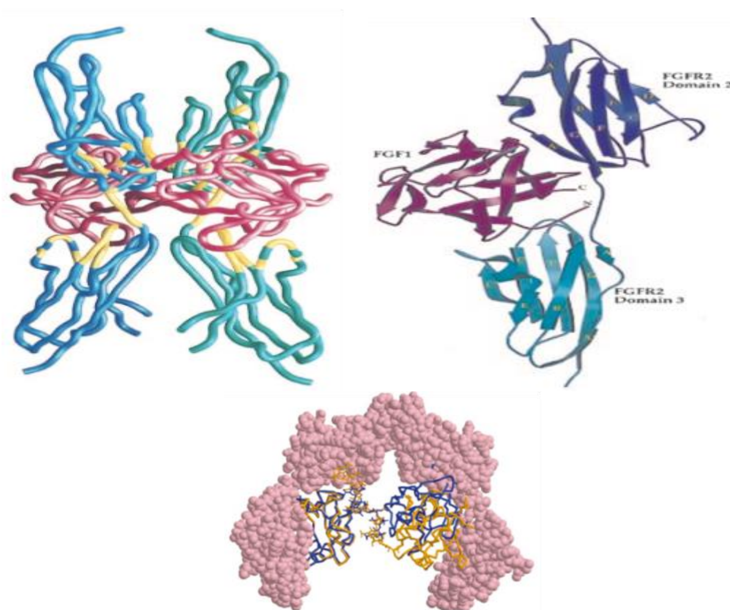


Figure 5. Top left, 1:1 FGF1:FGFR2 complex in the absence of heparin (PDB 1DJS). Top right, 2:2 FGF1:FGFR2 complex in the absence of heparin (D2 and D3 domains are colored in blue and green, FGF1 is colored in red, and receptor contacts with the FGF1 ligand are colored in yellow). Bottom left, 2:2 FGF1:FGFR2 complex in the presence of a heparin disaccharide (FGFR2 is colored in pink, FGF1 ligand is colored in yellow and blue, and heparin is in yellow stick formation) (PDB 1E0O).

Signaling Cascades

The extracellular domain of FGFRs promote receptor-ligand complex dimerization upon ligand binding. The FGF-FGFR dimerization brings the intracellular tyrosine kinase domains of both receptors in proximity to each other [71, 28]. The receptor is activated by phosphorylation of a maximum of six tyrosine residues, and different signaling pathways are activated based on specificity of the FGF ligand. The final phosphorylation of Y677 and Y766 for FGFR1 induces substrate binding of STAT3, phospholipase Cγ (PCγ), and adaptor proteins such as FGFR substrate 2α. Once activation of the receptor happened, adaptor proteins are phosphorylated by the receptor for various intracellular signaling pathways. One pathway known as PCγ pathway, induces the cleavage of phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) into diacyl glycerol (DAG) and inositol 1,4,5-triphosphate (IP3).

Mitogen-activated protein kinase (MAPK) pathway, as another pathway activated by FGFRs, mediates cell proliferation, survival, and mitosis through the activation of specific transcription factors that regulate target gene expression [37]. The third pathway included the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway to mediate cell growth and differentiation and regulate the immune response. The last signaling pathway related to FGFRs involves activating of phosphoinositide 3-kinase and protein kinase B (PI3K-AKT). The PI3K- AKT pathway stimulates cell growth and proliferation through phosphorylation.

FGF and FGFR Regulation

Aberrant FGF/FGFR signaling plays a role in tumorigenesis and many other diseases due to irregulating in signaling process [71]. One mechanism for signaling regulation is found in interaction of ubiquitin ligase Cbl to FGFRs, which results in internalization and degradation of the receptors [37]. Another type of regulation includes phosphatases involved in controlling the

receptor kinase activity and downstream signaling. Growth factor receptor bound 2 (GRB2), as an adaptor protein in dimeric form, bind the C-terminal of FGFR2 to inhibit complete phosphorylation. The binding of GRB2 also sterically hinders the downstream binding of other adaptor proteins necessary for MAPK and PI3K-AKT signaling pathways [37]. In addition, signaling pathways such as RAS-MAPK can inhibit the the receptor's kinase activity upon phosphorylation of specific C-terminus residues by ERK1 and ERK2 (downstream kinases activated by the pathway). Another type of FGF-FGFR regulation known as microRNAs directly affects FGF and FGFR expression at the post-translational stage [37]. Several studies have found that overexpression of FGFs and/or corresponding FGFRs is caused the suppression of microRNAs. MicroRNA-152 and microRNA-198 are found to regulate FGF2 down, and FGFR1 expression respectively. Suppression of microRNAs 152 and 198 in non-small-cell lung cancer causes the overexpression of FGF2 and FGFR1, which leads to hyper proliferation and reduces apoptosis [37].

FGF Secretion

As FGFs are released from the cell into the extracellular matrix since they affect their biological properties through binding to cell surface receptors. All FGF family members except the prototypical ones (FGF1 and FGF2) contain some signal peptide that directs their secretion out of the cell. FGFs 3-8, 10, 17-19, 21, and 23 contain N- terminus peptides that signal their secretion through the classical endoplasmic reticulum-Golgi pathway [36]. FGFs 9, 16, and 20 have N-terminal hydrophobic sequences that regulate their secretion from the cell [3, 36]. FGF1 and FGF2 do not contain signal peptides since they are secreted non-classically. The stress response to different stimuli such as heat shock and hypoxia induces the secretion of FGF1 and FGF2 [67]. Several studies have been reported regarding the non-classical release of FGF1 to

show the order of prerequisites that lead to protein secretion. At the first event, the oxidation of C30 on FGF1 leads to the formation of an FGF1 homodimer (causing the formation of intermolecular disulfide bond) by intracellular Cu^{2+}. FGF1 is associated with S100A13, a calcium-binding protein that anchors the complex to the cell membrane. A bond between FGF1/S100A13 complex and the C2A portion of the integral transmembrane protein (p40Syt1) is created. Lastly, the ternary complex FGF1/S100A13/p40Syt1 is transported across the membrane.

FGF/FGFR Diseases

As FGFs/FGFRs are involved in several diseases due to their role in mammalian development, abnormal signaling is by ligand and/or receptor mutations. In this context, Pfeiffer syndrome was reported due to D321A mutation occurring in FGFR2c. This mutation reduces binding of FGFR2c with FGF2 and increases the aberrant autocrine signaling with FGF10 [28]. Several diseases have been studied for the mutations, such as S252W in FGFR2, N546K in FGFR1, N540K in FGFR3, and G380R in FGFR3 [71, 36, 72]. Kallmann syndrome, a form of hypogonadotropic hypogonadism, is a disease that has been related several different frameshift, nonsense, or donor splice mutations within various exons in FGFR1 coding genes [71]. Kallmann syndrome due to loss of function mutations is marked by a lack of hormones needed for sexual development as well as impairment in the sense of smell [73]. Kallmann syndrome without treatment causes infertility. FGFs and their corresponding FGFRs have a critical role in tumorigenesis in many types of cancers due to their role in cell survival, growth, and migration. Several drugs with similar function to FGFR selective tyrosine kinase inhibitors are designed in phase II and III clinical trials for the treatment of FGFR mutations that lead to cancer. In addition, there are many therapeutics targeting FGFR related cancers, such as FGFR antibodies

as isoform specific to avoid unwanted side effects, FGF-ligand traps, which utilize the extracellular portion of an FGFR and target mitogenic FGFs, and allosteric molecules capable of limiting FGFR signaling with reducing pancreatic cancer progression in mice [71].

Therapeutic Applications of FGFs:

There is a wide range of clinical applications for many different FGFs; However, only those related to hFGF1 will be described due to stay relevant to the aims of this project. Due to FGF1 ability to induce cell proliferation, migration, differentiation, and angiogenesis, it plays an essential role in regenerating tissues such as skin, blood vessels, muscle, adipose, bone, and nerve.

As FGF1 is involved in all phases of the wound healing process, such as inflammation, tissue regeneration, and re-modeling, it is a critical in the accelerate the novel wound healing therapies. Based on the United States health care system, acute and chronic wounds such as diabetic foot ulcers, pressure ulcers, and chronic venous leg ulcers causes serious problems. However, 71,000 diabetic foot ulcers receive amputations each year; a 68% mortality rate exists for patients with stage II or IV pressure ulcers [74]. Studies have displayed a vital role of FGF1 in improvement and acceleration of wound healing in diabetic mice and patients with burn wounds [75]. Diffusion and inherent limitations of FGF1 cause significant thermal unfolding and protease degradation during directly injection to the wound site [76].

In addition, when FGF1 is continuously applied to the defective area, it acts more efficiently. To address these issues, there have been several studies regarding the application of FGF1 using delivery methods such as porous scaffolds, hydrogels, and nano-particulates. Porous scaffolds are composed of natural polymers such as collagen, fibrin, and glycosaminoglycans, and binding FGF1 to the polymers is through electrostatic interactions that prolong the delivery of the protein

by a slower release of the protein from the scaffold [77]. The application of self-hardening bioceramics is also a progressive field for the delivering of FGFs to hard tissues such as bone and teeth [77]. There is another delivery method known as hydrogels that resemble in vivo extracellular matrices better than other delivery methods. FGFs fabricated into hydrogels are very secure and are released by enzymatic reactions or hydrolytic cleavages [76]. The release rate of FGFs from hydrogels is dependent on the charge characteristics and the biodegradability of the hydrogel material

Scope of the Present Work:

hFGF1 as a universal ligand binds to all the four types of FGFRs. hFGF1 plays a vital role in regulating wound healing and angiogenesis due to increasing proliferation of fibroblasts and embryonic cells. However, hFGF1 as a therapeutic agent has low thermodynamic stability and a short half-life *in vivo*.

One of the research interests of Dr. Kumar's group is to investigate the structural and functional aspects of the FGF-1 protein as a vital protein involved in the various vital cellular processes, especially in wound healing. Based on the importance of the subject, several studies have been reported regarding the development of a potential therapeutic agent to accelerate wound healing. To achieve this aim, the engineering of growth factors and cytokines has lately attracted lots of attention in medicine. However, there are numerous commercial growth factors to improve wound healing in today's market, such as PDGF, TGF, EGF, FGF, the development of a novel cure is still a clinical challenge.

The previous work done in our group (R136E FGF1 mutant) showed an increase in cell proliferation activity, stability of FGF1 and decreased heparin binding affinity significantly compared to wildtype hFGF1. In the first study, we focus on the introduction of another single

mutation in FGF1(R136sY) to increase thermal stability, resistance to proteases, half-life, and biological activity of FGF1 using site directed mutagenesis compared to wtFGF1. Tyrosine sulfation is a common post-translational modification in secreted and membrane-bound proteins [77]. Sulfotyrosine is involved in several types of protein-protein interactions due to its biological function. For example, sulfotyrosine has been identified in binding of chemokine to the chemokine receptors [78]. Tyrosine sulfation also plays a determined role in the coagulation cascade; it has been involved in several clotting factors as well as in natural thrombin inhibitors [79-80]. To obtain the aim, we designed a single mutation on wtFGF1 by substituting one positive charge residue at position 136 with a negatively charged residue. The introduced mutation (R136sY) was placed in heparin binding pocket. It was predicted that the designed variant would reduce the electrostatic interaction between the positively charged residues to develop a clear vision of the role of heparin in hFGF1 activation and cell signaling.

The second study aims to characterize the variant using biophysical methods to monitor the overall structure, conformational stability, and backbone flexibility. Therefore, the engineered variant gives us a better understanding to overcome the burdens and challenges of wild-type FGF, such as unfolding at physiological temperature, susceptibility to proteolytic degradation, and severely limiting its bioavailability. Our results lead us to future development in compatible biomaterials for topical and internal applications such as treating a variety of biomedical conditions, including wound healing.

REFERENCES

[1] Itoh N, Ornitz DM. Fibroblast growth factors: from molecular evolution to roles in development, metabolism, and disease. The Journal of Biochemistry. 2011 Feb 1;149(2):121-30.

[2] Hui Q, Jin Z, Li X, Liu C, Wang X. FGF family: from drug development to clinical application. International journal of molecular sciences. 2018 Jul;19(7):1875.

[3] Itoh N, Ornitz DM. Evolution of the FGFand FGFr gene families. TRENDS in Genetics. 2004 Nov 1;20(11):563-9.

[4] Kerr R, Agrawal S, Maity S, Koppolu B, *et al*. Design of a thrombin resistant human acidic fibroblast growth factor (hFGF1) variant that exhibits enhanced cell proliferation activity. Biochemical and biophysical research communications. 2019 Oct 15;518(2):191-6.

[5] The Universal Protein Resource. The Universal Protein Resource for fibroblast growth factor-15 in mouse. Available at: https://www.uniprot.org/uniprot/O35622.

[6] Ornitz DM, Itoh N. Fibroblast growth factors. Genome biology. 2001 Mar;2(3): eviews3005-1.

[7] Wang YC, Peterson SE, Loring JF. Protein post-translational modifications and regulation of pluripotency in human stem cells. Cell research. 2014 Feb;24(2):143-60.

[8] Miller, D., Ortega, S., Barhayan, O., Basilico, C., Compensation by Fibroblast Growth Factor 1 (FGF1) Does Not Account for the Mild Phenotypic Defects Observed in FGF2 Null Mice. American Society for microbiology, 2000. 20: p. 2260-2268.

[9] Zechel, S., Werner, S., Expression and Functions of Fibroblast Growth Factor 2 (FGF-2) in Hippocampal Formation. The Neuroscientist, 2010. 16(4): p. 357-373.

[10] Liao, S., Bodmer, J., Pietras, D., Azhar, M., Doetschman, T., Schultz, J.J., Biological Functions of the Low and High Molecular Weight Protein Isoforms of Fibroblast Growth Factor-2 in Cardiovascular Development and Disease. Developmental Dynamics, 2009. 238: p. 249-264.

[11] Giakoumopoulos, M., Golos, T., Embryonic stem cell-derived trophoblast differentiation: a comparative review of the biology, function, and signaling mechanisms. Journal of Endocrinology, 2013. 216: p. 33-45.

[12] Naiche, L., Holder, N., Lewandoski, M., FGF4 and FGF8 comprise the wavefront activity that controls somitogenesis. PNAS, 2011. 108(10): p. 4018-4023.

[13] Allerstorfer, S., et al., FGF5 as an oncogenic factor in human glioblastoma multiforme: autocrine and paracrine activities. Oncogene., 2008. 27(30): p. 4180-4190.

[14] Drogemuller, C., Rufenacht, S., Wichert, B., Leeb, T., Mutations within the FGF5 gene

are associated with hair length in cats. Animal Genetics, 2007 38(3): p. 218-221.

[15] Bing M, Da-Sheng C, Zhao-Fan X, *et al*. Randomized, multicenter, double-blind, and placebo-controlled trial using topical recombinant human acidic fibroblast growth factor for deep partial-thickness burns and skin graft donor site. Wound repair and regeneration. 2007 Nov;15(6):795-9.

[16] Sweet RM, Wright HT, Janin J, Chothia CH, Blow DA. Crystal structure of the complex of porcine trypsin with soybean trypsin inhibitor (Kunitz) at 2.6 Å resolution. Biochemistry. 1974 Sep 1;13(20):4212-28.

[17] Powers, C., McLeskey, S., Wellstein, A., Fibroblast growth factors, their receptors and signaling. Endocrine-Related Cancer, 2000. 7: p. 165-197.

[18] Li Y, Sun C, Yates EA, Jiang C, Wilkinson MC, Fernig DG. Heparin binding preference and structures in the fibroblast growth factor family parallel their evolutionary diversification. Open biology. 2016 Mar 1;6(3):150275.

[19] Higgins CA, Petukhova L, Harel S, Ho YY, Drill E, Shapiro L, Wajid M, Christiano AM. FGF5 is a crucial regulator of hair length in humans. Proceedings of the National Academy of Sciences. 2014 Jul 22;111(29):10648-53.

[20] Jarosz, M., Robbez-Masson, L., Chioni, A.M., Cross, B., Rosewell, I., Grose, R., Fibroblast Growth Factor 22 Is Not Essential for Skin Development and Repair but Plays a Role in Tumorigenesis. PLos-One, 2012. 7(6).

[21] Rohmann E, Brunner HG, Kayserili H, Uyguner O, Nürnberg G, Lew ED, Dobbie A, Eswarakumar VP, Uzumcu A, Ulubil-Emeroglu M, Leroy JG. Mutations in different components of FGF signaling in LADD syndrome. Nature genetics. 2006 Apr;38(4):414-7.

[22] Itoh N, Ornitz DM. Functional evolutionary history of the mouse Fgf gene family. Developmental dynamics: an official publication of the American Association of Anatomists. 2008 Jan;237(1):18-27.

[23] Tsai, S.M., Wang, W.P., Expression and Function of Fibroblast Growth Factor (FGF) 7 during Liver Regeneration. Cellular Physiology and Biochemistry, 2011. 27: p. 641-652.

[24] Sekine, K., et al., FGF10 is essential for limb and lung formation. Nature Genetics, 1999. 21: p. 138-141.

[25] Umemori, H., Linhoff, M.W., Ornitz, D.M., Sanes, J.R., FGF22 and Its Close Relatives Are Presynaptic Organizing Molecules in the Mammalian Brain. Cell, 2004. 118: p. 257-270.

[26] Terauchi A, Johnson-Venkatesh EM, Bullock B, Lehtinen MK, Umemori H. Retrograde fibroblast growth factor 22 (FGF22) signaling regulates insulin-like growth factor 2 (IGF2) expression for activity-dependent synapse stabilization in the mammalian brain. Elife. 2016 Apr 15;5: e12151.

[27] Zinkle A, Mohammadi M. Structural biology of the FGF7 subfamily. Frontiers in Genetics. 2019 Feb 12; 10:102.

[28] Goetz, R., Mohammadi, M., Exploring mechanisms of FGF signalling through the lens of structural biology. Nat Rev Mol Cell Biol 2013. 14(3): p. 166–180.

[29] Crossley, P.H., Martin, G.R., The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. Development, 1995. 121: p. 439-451.

[30] Olsen, S.K., Li, J.Y., Bromleigh, C., Eliseenkova, A.V., Ibrahimi, O.A., Lao, Z., Zhang, F., Linhardt, R.J., Joyner, A.L., Mohammadi, M., Structural basis by which alternative splicing modulates the organizer activity of FGF8 in the brain. Genes Dev., 2006. 20: p. 185-198.

[31] Haque, T., Nakada, S., Hamdy, R.C., A review of FGF18: Its expression, signaling pathways and possible functions during embryogenesis and post-natal development. Histol Histopathol 2007. 22: p. 97-105.

[32] Fon Tacer K, Bookout AL, Ding X, Kurosu H, John GB, Wang L, Goetz R, Mohammadi M, Kuro-o M, Mangelsdorf DJ, Kliewer SA. Research resource: comprehensive expression atlas of the fibroblast growth factor system in adult mouse. Molecular endocrinology. 2010 Oct 1;24(10):2050-64.

[33] Duarte, M., et al., Thrombin induces rapid PAR1-mediated non-classical FGF1 release. Biochem Biophys Research Communications. 2006. 350: p. 604-609.

[34] Thallapuranam, S.K., Engineered Compositions of FGF and Methods of Use Thereof. Patent Application No. 15/474,268, filed March 30, 2017.

[35] DiGabriele, A., Structure of a heparin-linked biologically active dimer of fibroblast growth factor. Nature, 1998. 393: p. 812-817.

[36] Beenken A, Mohammadi M. The FGF family: biology, pathophysiology and therapy. Nature reviews Drug discovery. 2009 Mar;8(3):235-53.

[37] Ornitz, D.M., Itoh, N., The Fibroblast Growth Factor Signaling Pathway. WIREs Dev Biol, 2015. 4: p. 215-266.

[38] Inagaki, T., et al., Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis. Cell Metabolism, 2005. 2: p. 217-225.

[39] Kliewer, S.A., Mangelsdorf, D.J., Fibroblast growth factor 21: from pharmacology to physiology. American J Clinical Nutrition, 2010. 91: p. 254-257.

[40] Fukumoto, S., Physiological Regulation and Disorders of Phosphate Metabolism; Pivotal Role of Fibroblast Growth Factor 23. International Medicine, 2007. 47: p. 337-343.

[41] Bernett, M.J., Somasundaram, T., Blaber, M., An atomic resolution structure for human fibroblast growth factor 1. Proteins-Structure Function and Bioinformatics, 2004. 57(3): p. 626-634.

[42] Brych, S.R., Blaber, S.I., Logan, T.M., Blaber, M., Structure and stability effects of mutations designed to increase the primary sequence symmetry within the core region of a β-trefoil. Protein Science 2001. 10: p. 2587–2599.

[43] Zhu, X., Komiya, H., Chirino, A., Three-dimensional structures of acidic and basic fibroblast growth factors. Science, 1991. 251: p. 90-93.

[44] Sweet, R., et al., Crystal structure of the complex of porcine trypsin with soybean trypsin inhibitor (Kunitz) at 2.6 angstrom resolution. Biochemistry, 1974. 13: p. 4212– 4228.

[45] DePaz, J.L., et al., The Activation of Fibroblast Growth Factors by Heparin: Synthesis, Structure, and Biological Activity of Heparin-Like Oligosaccharides. ChemBiochem, 2001 2: p. 673–685.

[46] Casu, B., Naggi, A., Torri, G., Re-visiting the structure of heparin. Carbohydrate Research, 2015. 403: p. 60-68.

[47] Carlsson, P., Kjellen, L., Heparin Biosynthesis. Handbook of Experimental Pharamacology, 2012(207): p. 23-41.

[48] Jacobsson, K.G., Riesenfeld, J., Lindahl, U., Biosynthesis of Heparin, Effects of NButyrate on Cultured Mast Cells. J Biol Chem, 1985. 260(22): p. 12154-12159.

[49] Carlsson, P., Presto, J., Spillmann, D., Lindahl, U., Kjellen, L., Heparin/Heparan Sulfate Biosynthesis: Processive formation of N-Sulfated Domains. J Biol Chem, 2008. 283(29): p. 20008-20014.

[50] Gray, E., Hogwood, J., Mulloy, B., The Anticoagulant and Antithrombotic Mechanisms of Heparin. Handbook of Experimental Pharmacology, 2012(207): p. 43-61.

[51] Nugent, M.A., Heparin sequencing brings structure to the function of complex oligosaccharides. PNAS, 2000. 97(19): p. 10301-10303.

[52] Wong, P., Hampton, B., Szylobryt, E., Gallagher, A.M., Jaye, M., Analysis of Putative Heparin-binding Domains of Fibroblast Growth Factor-1. J. Biol. Chem., 1995. 270(43): p. 25805-25811.

[53] Pellegrini, L., Role of heparan sulfate in fibroblast growth factor signalling: structural view. Current Opinion in Structural Biology 2001. 11: p. 629–634.

[54] Raman, R., Venkataraman, G., Ernst, S., Sasisekharan, V., Sasisekharan, R., Structural specificity of heparin binding in the fibroblast growth factor family of proteins. PNAS, 2003 100: p. 2357–2362.

[55] Harmer, N.J., Insights into the role of heparan sulfate in fibroblast growth factor signaling. Biochemical Society Transactions, 2006. 34(3): p. 442-445.

[56] Pellegrini, L., et al., Crystal structure of fibroblast growth factor receptor ectodomain bound to ligand and heparin. Nature, 2000. 407(6807): p. 1029-1034.

[57] Arunkumar, A.I., et al., Oligomerization of acidic fibroblast growth factor is not a prerequisite for its cell proliferation activity. Protein Science, 2002. 11: p. 1050-1061.

[58] Angulo, J., et al., Dynamic properties of biologically activity synthetic heparin-like hexasaccharides. Glycobiology, 2005. 15(10): p. 1008-1015.

[59] Canales, A., et al., Solution NMR structure of a human FGF-1 monomer, activated by a hexasaccharide heparin-analogue. Febs Journal, 2006. 273(20): p. 4716-4727.

[60] Chi, Y., Kumar, T.K., Wang, H.M., Ho, M.C., Chiu, I.M., Yu, C., Thermodynamic Characterization of the Human Acidic Fibroblast Growth Factor: Evidence for Cold Denaturation. Biochemistry, 2001. 40: p. 7746-7753.

[61] Culajay, J.F., et al., Thermodynamic characterization of mutants of human fibroblast growth factor 1 with an increased physiological half-life. Biochemistry, 2000. 39(24): p. 7153-7158.

[62] Zakrzewska, M., Krowarsch, D., Wiedlocha, A., Otlewski, J., Design of fully active FGF-1 variant with increased stability. Protein Engineering, Design & Selection, 2004. 17(8): p. 603-611.

[63] Blaber, S.I., Culajay, J.F., Khurana, A., Blaber, M., Reversible Thermal Denaturation of Human FGF-1 Induced by Low Concentrations of Guanidine Hydrochloride. Biophysical Journal, 1999. 77(1): p. 470-477.

[64] Zakrzewska, M., et al., Highly stable mutants of human fibroblast growth factor-1 exhibit prolonged biological action. J Mol Biol, 2005. 352(4): p. 860-75.

[65] Zakrzewska, M., et al., Increased Protein Stability of FGF1 Can Compensate for Its Reduced Affinity for Heparin. Journal of Biological Chemistry, 2009. 284(37): p. 25388-25403.

[66] Kanodia, J., Greg, F., Role of HEPARINGAGs in Regulation of FGF Signaling Pathway:

Insights from Mathematical Modeling. Glycobiology, 2014. 4(1): p. 1000113.

[67] Kimura, H., Okubo, N., Chosa, N., Kyakumoto, S., Kamo, M., Miura, H., Ishisaki, A., EGF Positively Regulates the Proliferation and Migration, and Negatively Regulates the Myofibroblast Differentiation of Periodontal Ligament-Derived Endothelial Progenitor Cells through MEK/ERK- and JNK Dependent Signals. Cellular Physiology and Biochemistry 2013. 32: p. 899-914.

[68] Givol, D., Yayon, A., Complexity of FGF receptors: genetic basis for structural diversity and functional specificity. FASEB J., 1992. 6: p. 3362-3369.

[69] FGFR. Available from: https://www.sigmaaldrich.com/technicaldocuments/ articles/biology/rbi-handbook/protein-serine-threonine-tyrosinekinases/ fgfr.html.

[70] Stauber, D.J., DiGabriele, A.D., Hendrickson, W.A., Structural interactions of fibroblast growth factor receptor with its ligands. PNAS, 2000. 97(1): p. 49-54.

[71] Carter, E.P., Fearon, A.E., Grose, R.P., Careless talk costs lives: fibroblast growth factor receptor signalling and the consequences of pathway malfunction. Trends Cell Biol., 2015. 25(4): p. 221-233.

[72] Rand, V., et al., Sequence survey of receptor tyrosine kinases reveals mutations in glioblastomas. PNAS, 2005. 102(40): p. 14344-14349.

[73] Dode, C. et al., Loss-of-function mutations in FGFR1 cause autosomal dominant syndrome. Nature Genetics, 2003. 33: p. 1-3.

[74] Barrientos, S. et al., Clinical Application of Growth Factors and Cytokines in Wound Healing. Wound Repair Regen., 2014. 22(5): p. 569-578.

[75] Borena, B.M., et al., Regenerative Skin Wound Healing in Mammals: State-of-the-Art on Growth Factor and Stem Cell Based Treatments. Cell Physiol Biochem, 2015. 36: p. 1-23.

[76] Yun, Y.R., Won, J.E., Jeon, E., Lee, S., Kang, W., Jo, H., Jang, J.H., Shin, U.S., Kim, H.W., Fibroblast Growth Factors: Biology, Function, and Application for Tissue Regeneration. Journal of Tissue Engineering, 2010. 2010: p 218142.

[77] Kehoe, J.W. & Bertozzi, C.R. Tyrosine sulfation: a modulator of extracellular proteinprotein interactions. Chem. Biol. 7, R57–R61 (2000).

[78] Preobrazhensky, A.A. et al. Monocyte chemotactic protein-1 receptor CCR2B is a glycoprotein that has tyrosine sulfation in a conserved extracellular N-terminal region. J. Immunol. 165, 5295–5303 (2000).

[79] Dong, J.F., Li, C.Q. & Lopez, J.A. Tyrosine sulfation of the glycoprotein Ib-IX complex:

identification of sulfated residues and effect on ligand binding. Biochemistry 33, 13946–13953 (1994).

[80] Bagdy, D., Barabas, E., Graf, L., Petersen, T.E. & Magnusson, S. Hirudin. Methods Enzymol. 45, 669–678 (1976).

CHAPTER 2

Overexpression, Purification, and Characterization of the Human Acidic Fibroblast Growth Factor Variants and Evaluation of its effects on the Structure, Stability, and Cell Proliferation Activity of the hFGF1.

Abstract

Human acidic fibroblast growth factor (hFGF1) plays an important role in cellular processes such as cell proliferation, cell differentiation, angiogenesis, and tumor growth. hFGF1 is found to have a low half-life *in vivo* and low melting temperature in the physiological range (Tm =42˚C). It's also believed that heparin's interaction with FGFRs is mandatory to stabilizes the FGF1-FGFR binary complex and signaling.

In this context, engineering via charge reversal mutation (R136 sulfoY or R136sY) at heparin binding pocket is performed to study the effects of decreasing the positive charge at the heparin binding pocket on the protein stability and cell proliferation as compared to R136E.

After obtaining the pure protein, characterization of the single (R136sY) variant was performed to monitor the overall structure, conformational stability, and backbone flexibility. A cell proliferation assay is used to assess the bioactivity of the designed variant. The results of this study clearly indicate the introduced mutant doesn't change the secondary and tertiary structure of hFGF1. Based on the limited enzymatic digestion and 8-Anilinonaphthalene-1-sulfonic acid (ANS) binding experiments, the designed mutation renders the growth factor more resistant to proteolytic enzymes and more flexible compared to wtFGF1 molecule. Moreover, thermal denaturation data indicates R136sY mutant shows higher T_m than wtFGF1. In conclusion, the introduction of the R136sY mutant makes hFGF1 more resistant to thermal, chemical, and proteolytic digestion and increases mitogenic activity.

Introduction

As a large class of biomolecules, growth factors play a vital role in cell growth, development, and differentiation [4]. Fibroblast growth factors (FGFs) consist of a family with twenty-two related proteins that have an important role in mitogenic and cell-survival activities. All the FGF family members show strong mitogenic properties needed for proper growth and development [1-3]. One family member is known as the acidic fibroblast growth factor (FGF1). The human FGF1 (hFGF1) is found as a positively charged protein with a molecular weight around 16kDa. The hFGF1 has a relatively short half-life of 5 minutes and low thermal stability in the body, with a melting temperature ($T_m = 42^{\circ}C$) close to the physiological range [5].

Binding of hFGF1 to heparin not only increases the stability but also protects the protein against proteolytic digestion and denaturation via heat. Heparin is a polydispersed sulfated molecule known as glycosaminoglycans by repeating disaccharide chains of L-iduronic acid, and D-glucosamine found in the extracellular matrix and cell surfaces. hFGF1 binds to the heparin to eliminate the charge-charge repulsion via electrostatic interactions with the positively charged residues near the c-terminus in the region known as the heparin-binding pocket, and it renders the protein less susceptible to proteolytic cleavage and degradation [6].

Despite several studies, the role of heparin in the activation of fibroblast growth factor proteins and the protein receptor interactions (FGFRs) is still controversial. Tyrosine kinases (RTKs) as a member of the FGFRs family, on the surface of cell membranes, stimulating intracellular signaling pathways which are responsible for processes such as cell proliferation, cell differentiation, and cell migration. Based on the multidimensional nuclear magnetic resonance (NMR) technique, 3D solution structure of protein shows that hFGF1 consists of twelve β-strands arranged in an antiparallel fashion into a β-barrel structure [7-10].

The introduction of R136E variant as a point mutation into the receptor binding site and heparin binding pocket of hGFGF1 was constructed by previous research in our lab. The variant increased the inherent stability and enhance bioactivity of the hFGF1, which can be applied for potential pharmaceutical use in wound healing medications [11-15]. Most of these hFGF1 variants have been reported based on the introduction of point mutations into the receptor binding site and heparin binding pocket of hFGF1 and then increases its stability.

The Prior work performed in our lab demonstrated that mutations in the heparin binding pocket could modulate the stability and mitogenic activity, which is the purpose of this project [16-19]. This chapter is focused on introduction of R136sY as a variant of hFGF1 using site directed mutagenesis and characterization of R136sY mutant compared to R136E and wtFGF1(Fig. 1).

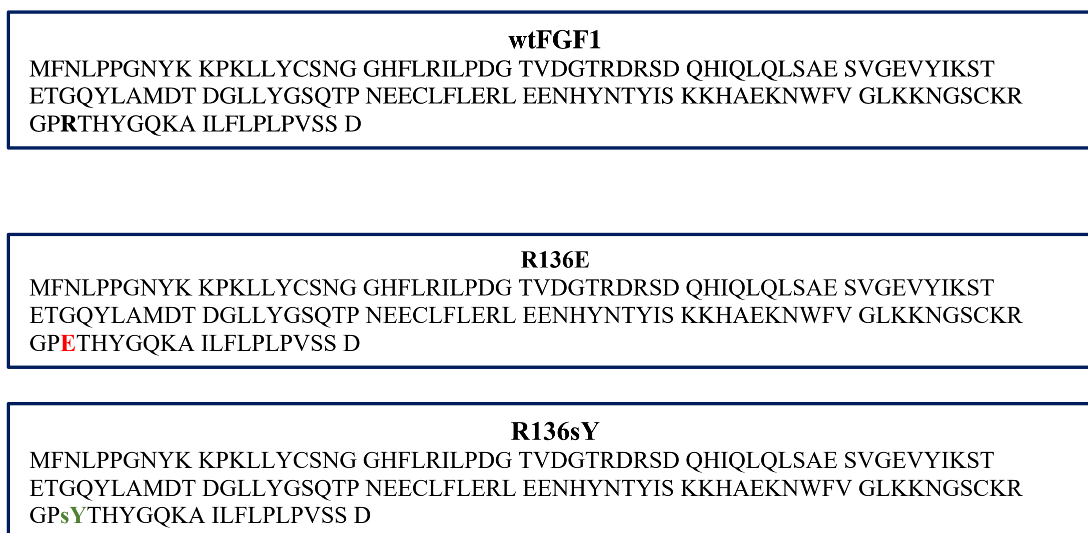


Fig. 1: Amino acid sequence of wtFGF1. The residues highlighted in red, and green represent the mutated residues in wtFGF1 to form R136E and R136sY, respectively.

Both R136E and R136sY are located in hFGF1's heparin-binding region (Fig. 2). At both mutations, a polar positively charged amino acid is replaced with a polar negatively charged one [20]. Replacement of the arginine residue with negatively charged residue decreases the

repulsion between the positively charged amino acid residues in the heparin-binding region, which can plausibly lead to increase the inherent stability of the hFGF1. Moreover, the substitution of Arg by Glu or sulfo-Tyr will increase the variant hFGF1's resistance to trypsin digestion since trypsin cleaves at the C-terminal of arginine and lysine residues.

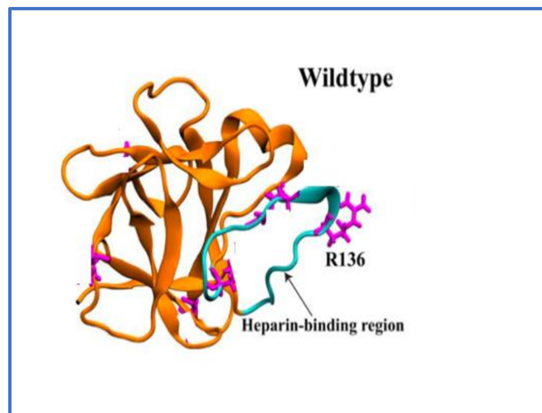


Fig. 2: Ribbon representation of mutated residue location in wtFGF1 structure.

The purpose of this chapter is to focus on the structural forces that contribute to high stability of the mutation. Altering the primary amino acid sequence can potentially affect the secondary and tertiary structure of the protein; then, analyses are performed to ensure that the three-dimensional structure of the variant is not significantly altered. Structural analyses are utilized to assess the role of the amino acid mutations on hFGF1 variants contributing to its higher stability and increased biological function. In conclusion, the R136sY variant have been demonstrated to have a higher inherent stability as compared to wtFGF1 but similar inherent stability as compared to R136E mutant.

Results and Discussion

Human acidic fibroblast growth factor (hFGF1) is a 155-amino acid, an all β-sheet protein. The region on hFGF1 between residues 124 and 140 facilitates heparin binding (Fig. 2). Net positive

charge related to the six polar, basic amino acids at physiological pH located in the heparin binding pocket [21]. Previous studies have demonstrated that the repulsion interaction between the positive residues in heparin bonding pocket on hFGF1 makes it unstable and unlikely to complex with the FGF receptor. Consequently, binding of hFGF1 to the heparin contributes to the stability of the growth factor [22].

1) Site Directed Mutagenesis and Bacterial Transformation:

The mutations are designed on FGF1 plasmid by using the Quick Changes site directed mutagenesis kit (Agilent Inc. USA). The single mutant (R136sY) is engineered in the heparin binding pocket of FGF1.

1μL of the vector pET20b-R136Y FGF1 plasmid is added to 100μL of BL-21 (DE3) pLysS competent E. coli cells. Well-standardized heat shock-based transformation procedure is applied to transform the cells. In the following steps, a mixture of the competent E.coli cells and the plasmid of R136Y is placed in a water path for 45seconds. At the next step, 800μL of the sterile lysogeny broth (LB) is added to the cells to nurture them while they are incubated in a shaker at 250 rpm for 45 minutes at 37°C. A 100μL of competent E.coli and the R136Y plasmid are spread on an Agar plate included the desired concentration antibiotics. The Ampicillin (Amp) and Chloramphenicol (Cap) are utilized since the gene in FGF1 plasmid is resistant to Amp and Cap. Therefore, only the competent E. coli cells containing FGF1 plasmid can survive. At the end, the Agar plate included bacterial cells and the antibiotics are stored upside down in an incubator for 14-15hours at 37°C. Therefore, transformation of R136Y plasmid is successfully completed which is depiced by the presence of bacterial colonies on the plate (Fig. 3)

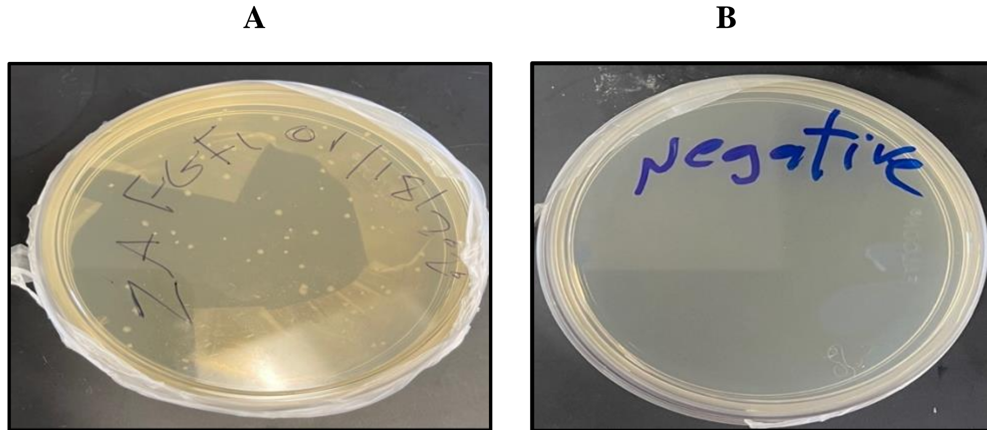


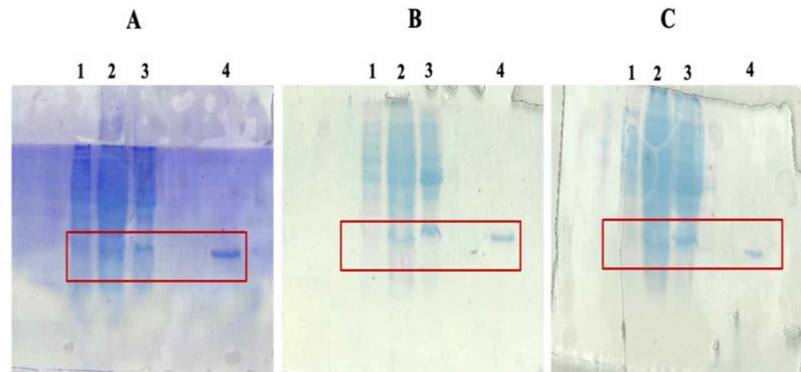
Figure 3: Transformation of R136Y plasmid by using strains of competent E. coli cells (BL-21 (DE3) pLysS). Positive control (Panel- A) and Negative control (Panel- B).

2) Recombinant Protein Expression:

Small scale expression is accomplished before starting large scale expression to check the presence of protein. That is started by inoculation of one or more colonies from Agar plate into LB broth included antibiotics (Amp and Cap). The overnight culture is inoculated into LB included Amp and Cap; then, incubated at 37°C for 2.15 hours at 250 rpm. After 2.15 hours, the culture is checked by UV visible spectrometer at wavelength 600nm to measure the optical density (OD). When the culture reached the required OD (between 0.4 to 0.6), O-Sulfonate-L-tyrosine sodium salt (2 mM) is added to the culture to synthesis sulfated peptide. When the cells meet an OD_{600} between 06-0.8, the isopropyl β-D-1- thiogalactopyranoside (IPTG) is added to induce expression. IPTG is a bimolecular reagent whose chemical structure is analogous to allolactose. IPTG is significant because it stimulates lac operon which can induce the expression of protein in BL-21 (DE3) pLysS competent E. coli cells. Cells were grown for an additional 7 h at 37°C. After 7 hours, cells are harvested by centrifugation and resuspended in buffer containing 10 mM phosphate, 25 mM ammonium sulfate, 100 mM sodium chloride, 5% glycerol, 1mM EDTA, 1% BME and 1mM PMSF at a pH of 7.2. The bacterial cells are sonicated, then centrifuged to obtain the protein of interest. Expression of the protein is checked by running

SDS-PAGE. Optimization of IPTG concentration is then performed. Based on the result, we consider 2 mM as a desired concentration for induced reagent in the expression of R136sY FGF1 protein (Fig. 4).

Fig. 4: SDS-PAGE analysis of R136sY FGF1 protein overexpression by 5 mM IPTG (Panel- A),



2 mM (Panel- B) and 0.8 mM (Panel- C). Induced sample (Lane-1); Supernatant (Lane-2); Pellet (Lane-3); Marker-pure wtFGF1 (Lane-4).

3) Protein Purification:

Purification of the hFGF1 and its mutants is achieved by using heparin Sepharose affinity column chromatography. hFGF1 and its variants are purified using a stepwise sodium chloride gradient in 10 mM sodium phosphate buffer (PB) containing 25 mM $(NH_4)_2SO_4$ at pH 7.2. While pure wtFGF1 is typically obtained in the 1.5 M sodium chloride buffer fraction, R136E and R136sY are eluted in the 0.8 M sodium chloride buffer fraction. Since the binding affinity of the R136E and R136sY variants is weaker than pure wtFGF1, they are eluted in less sodium chloride buffer fraction (Fig.5).

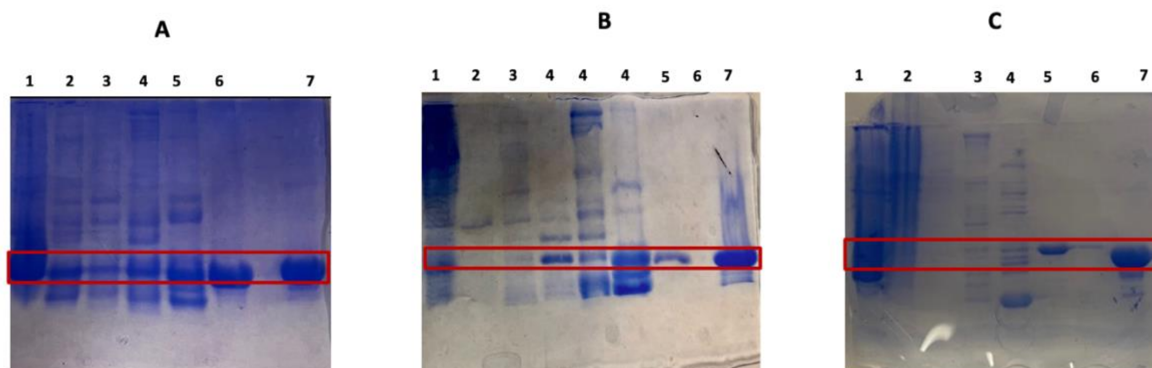


Fig. 5: SDS-PAGE analysis of proteins wtFGF1 (Panel- A), R136E (Panel- B) and R136sY (Panel- C) purified by heparin Sepharose affinity chromatography at different concentrations of NaCl. Supernatant (Lane-1); 10 mM PB + 100mM NaCl (Lane-2); 10 mM PB + 300 mM NaCl (Lane-3); 10 mM PB + 500 mM NaCl (Lane-4); 10 mM PB + 800 mM NaCl (Lane-5); 10 mM PB + 1500 mM NaCl (Lane-6); Marker-pure wtFGF1 (Lane-7); All buffer fractions included 25 mM $(NH_4)_2SO_4$ at pH 7.2

The yields of the purified wtFGF1 and the single mutant proteins are in the range of 10–20 mg per liter of bacterial culture.

4) Fluorescence Spectroscopy:

Fluorescence spectroscopy is utilized to examine the tertiary structure of proteins and monitor the structural differences between the wtFGF1 and its variants. wtFGF1 displays intrinsic fluorescence due to the presence of one tryptophan (W121) and eight tyrosine residues. The fluorescence spectrum is analyzed by examining the presence or absence of peaks at 308 nm and 350 nm. The intrinsic fluorescence spectrum of native wtFGF1 shows an emission maximum at 308 nm, representative of the eight tyrosine residues in the native protein. In the natured state(s) of the protein, the fluorescence of the single tryptophan residue (W121) is mostly quenched by lysine and proline residues. Fig.6 exhibits the overlay of intrinsic fluorescence spectra of wtFGF1 and its variants. The result indicates that the intrinsic fluorescence spectra of all the designed variants shows an emission maximum at 308 nm the same as wtFGF1. Furthermore, the

presence of tyrosine peak at 308 nm and absence of tryptophan peak at 350 nm implies that the tertiary structure of the FGF1 is not significantly changed due to the introduced mutations.

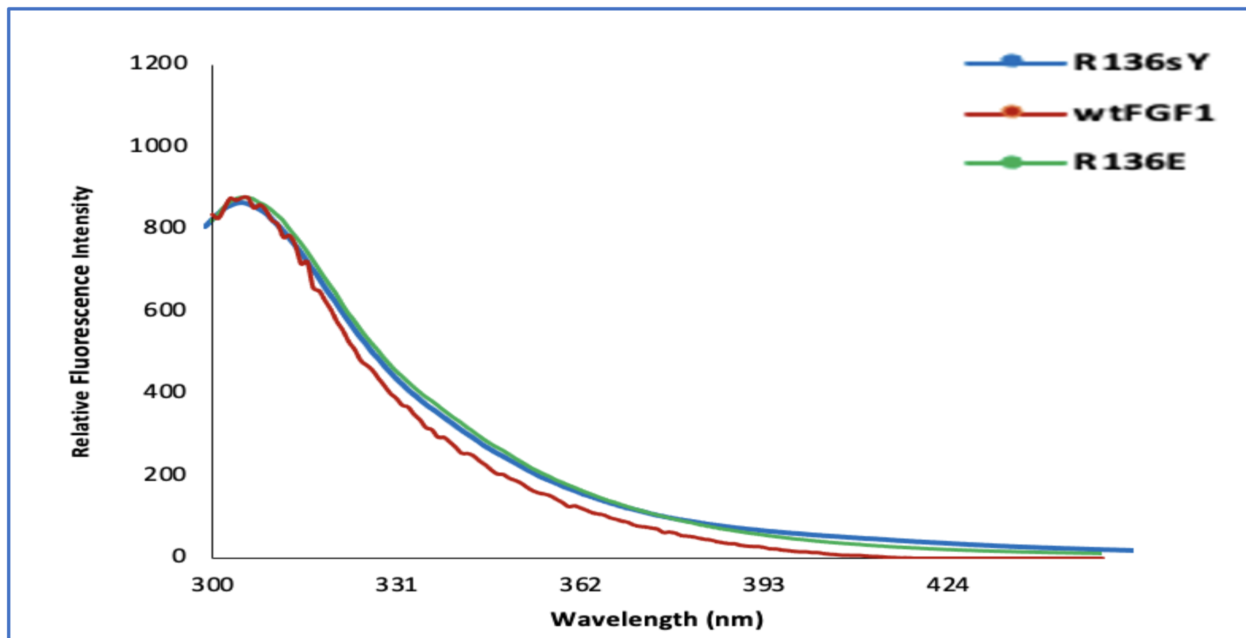


Fig. 6: Intrinsic fluorescence spectra of wtFGF1 and the designed variants. wtFGF1 (red), R136E (green), R136sY (blue).

5) Circular Dichroism (CD) Spectroscopy:

Far-UV CD spectral data is used to examine the secondary structure of proteins and monitor the structural differences between the wtFGF1 and its variants.

The overlay of far-UV CD spectra of wtFGF1 and the variants shows there are no changes in the secondary structures of FGF1 after mutations (Fig. 7). The negative peak at 208 nm (class II β-protein structure) and the positive peak at 228 nm (β-turns, loops and aromatic side chains) show no significant difference in terms of the global secondary structure (β-trefoil).

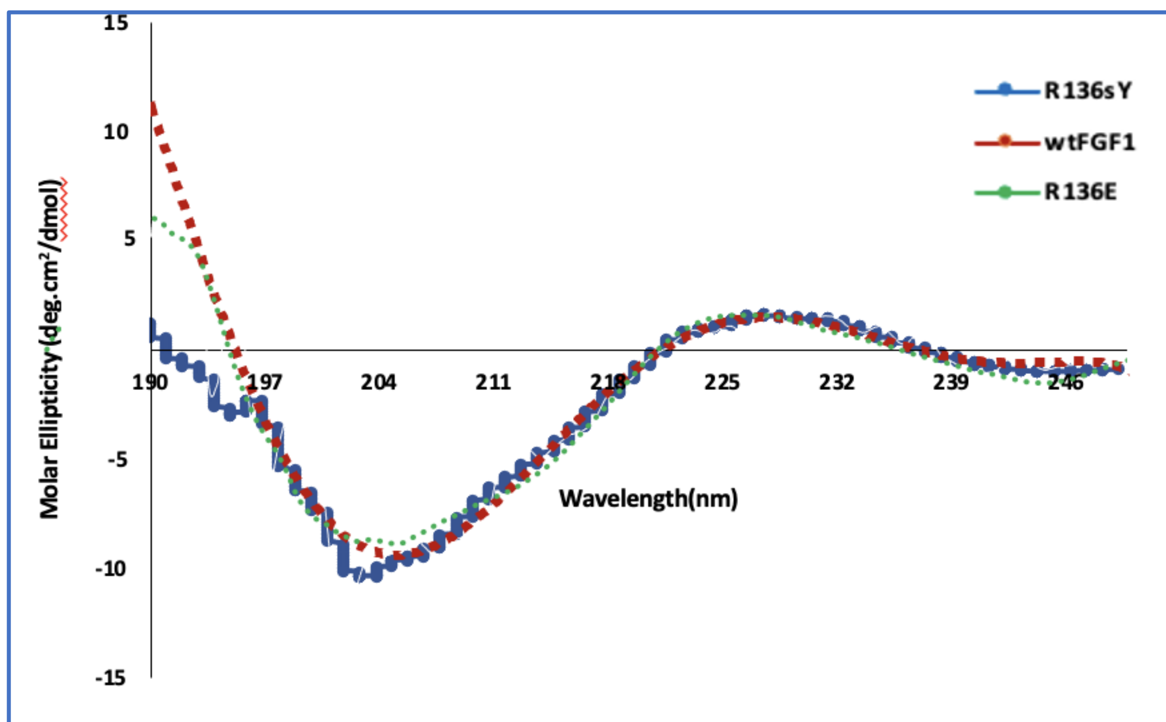


Fig. 7: Circular dichroism (CD) spectra of wtFGF1 and its variants. wtFGF1 (red), R136E (green), R136sY (blue).

6) 8-Anilinonaphthalene-1-sulfonic acid (ANS) Binding Assay:

8-anilino-1-nathalenesulfonate (ANS) is a non-polar fluorescent dye that is generally used to evaluate the presence of solvent-exposed hydrophobic surface(s) in proteins [26]. The relative fluorescent intensity is directly proportional to the number of the hydrophobic amino acids in the protein and the number of ANS molecules bound to the protein. Normally, hydrophobic residues are buried inside the protein core. Therefore, increasing the ANS fluorescence indicates exposure of these hydrophobic residues toward the surface of the protein.

A comparison of the ANS curves reveals that the relative fluorescence intensity for R136E, and R136sY mutants is similar and lower than wt-hFGF1 (Fig. 8). These results show that the charge-reversal at position 136 renders the proteins less flexible, increases compactness and induces changes in the solvent exposed surface.

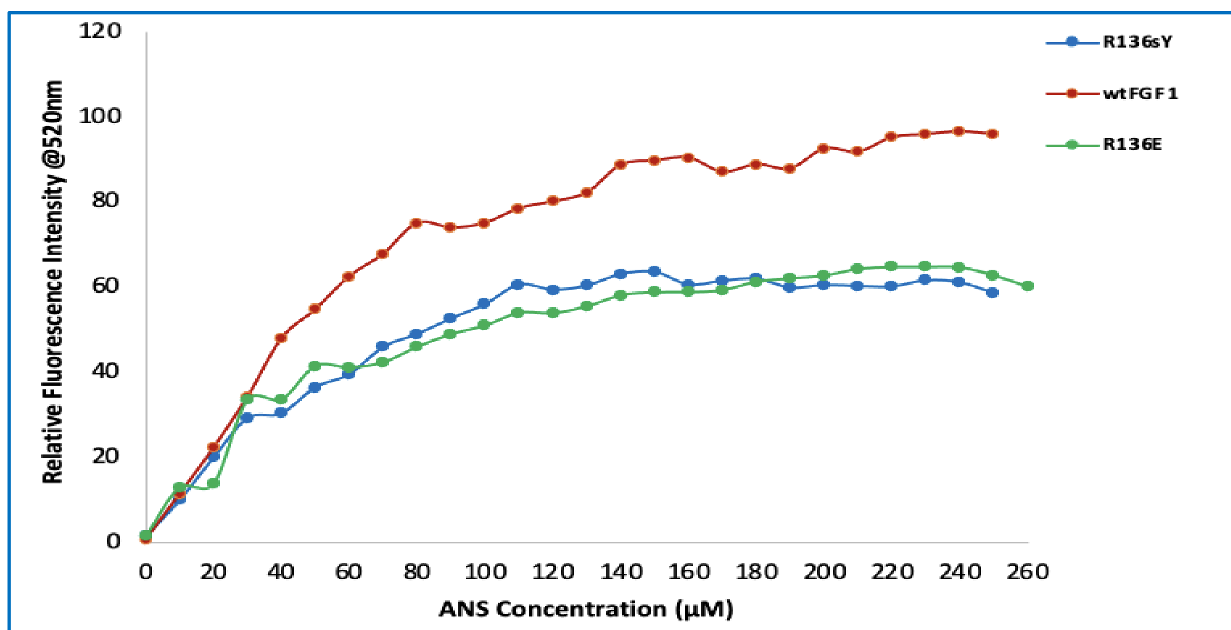


Fig. 8: ANS binding curves of wtFGF1 and the variants in the absence of heparin. wtFGF1 (red), R136E (green), R136sY (blue).

7) Proteolytic Digestion:

Limited trypsin digestion (LTD) assay is a helpful technique to evaluate the subtle changes in flexibility of the backbone caused by engineered mutations in the heparin-binding pocket of hFGF1 [27]. Trypsin as a serine protease cleaves polypeptides at the carboxyl end of positively charged amino acids lysine and arginine residues [28]. Moreover, wtFGF1 contains three arginine and nine lysine residues. Limited trypsin cleavage is then accomplished on wtFGF1 and the designed mutations to reveal the effects of the individual mutations on the conformational flexibility of the arginine and lysine residues in FGF1.

At the first, the appropriate trypsin concentration is determined by preparing different concentration of the enzyme (0.001, 0.005, 0.01, 0.05, 0.1, 0.5 and 1mg/ml). The trypsin with different concentration is incubated with the protein at the same time duration, 30 min (Fig.9).

The desired trypsin concentration is obtained based on 10% undigested protein (Fig.10). Trypsin digestion time dependent is then started with the desired trypsin concentration (0.05 mg/ml). Based on the result, after 40 minutes incubation with the enzyme, wtFGF1, R136E and R136sY mutants are digested by 90%, 75% and 10% respectively (Fig. 12). The densitometric analysis results indicate that R136E and R136sY mutants are resistant against the action of trypsin whereas wtFGF1 is digested by 80% in the first 20 minutes (Fig. 12). Manipulating of wtFGF1 by negative charged residue introduced at position R136 decreases repulsions in the heparin binding pocket, renders the tertiary structure of hFGF1 more compact and increases the resistance of protein to proteolytic digestion. Moreover, substitution of one of the three arginine residues by glutamic acid or sulfotyrosine renders the designed hFGF1 mutations resistant due to eliminate a trypsin cleavage site. The trypsin digestion results are in a good agreement with ANS binding experiment regarding decreasing flexibility of FGF1 due to the designed variants.

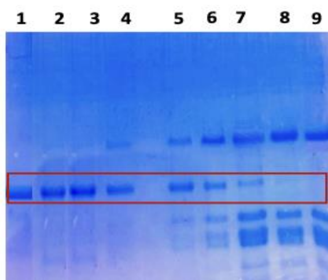


Fig. 9: SDS-PAGE analysis of concentration dependent limited trypsin digestion of R136sY variant. 0.1 mg/mL of proteins only (Lane-1); 1 mg/mL of trypsin only (Lane-9); Every lane represents specific concentration of trypsin at 30 min incubation, 0.001 mg/mL of trypsin (Lane-2); 0.005 mg/mL of trypsin (Lane-3); 0.01 mg/mL of trypsin (Lane-4); 0.05 mg/mL of trypsin (Lane-5); 0.1 mg/mL of trypsin (Lane-6); 0.5 mg/mL of trypsin (Lane-7); 1 mg/mL of trypsin (Lane-8).

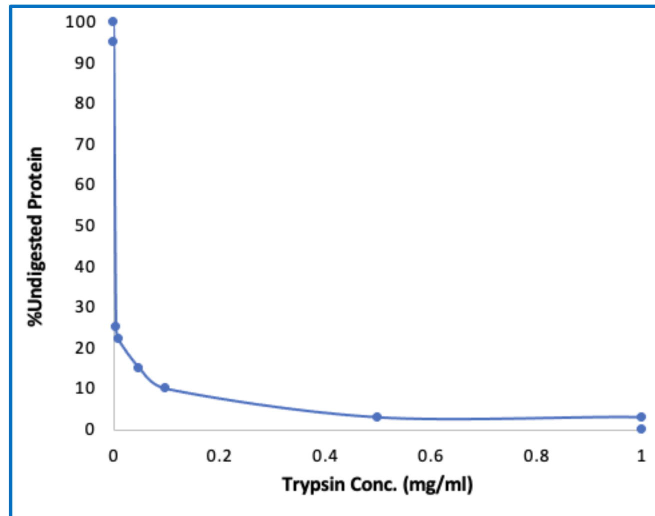


Fig. 10: Densitometric analysis of concentration dependent limited trypsin digestion of R136sY variant in absence of heparin as monitored by SDS-PAGE.

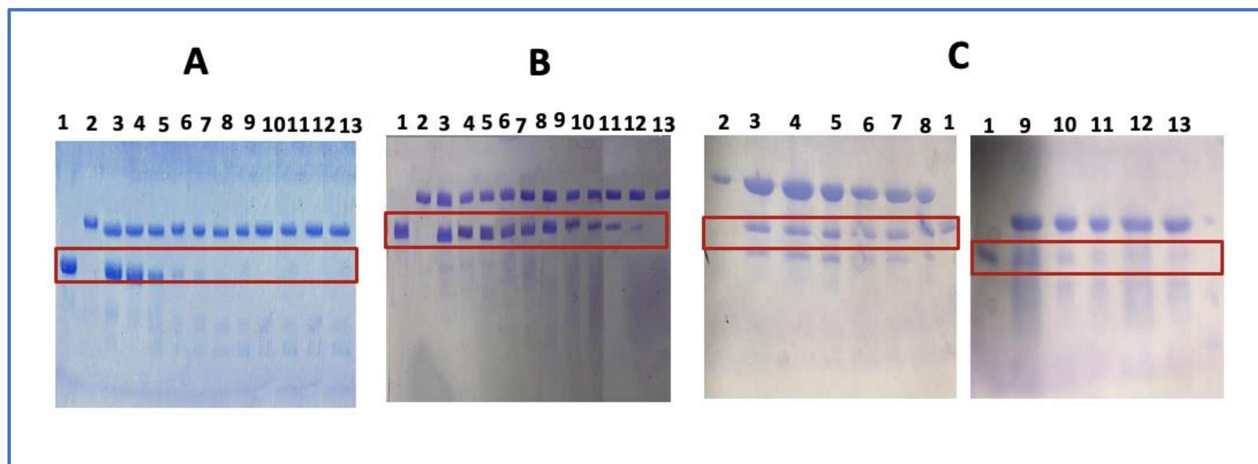


Fig. 11: SDS-PAGE analysis of time dependent limited trypsin digestion of wtFGF1 (Panel –A), R136sY (Panel-B) and R136E (Panel-C) variants. 0.1 mg/mL of proteins only (Lane-1); 0.05 mg/mL of trypsin only (Lane-2); Every lane represents specific time for trypsin incubations, 2 minutes (Lane-3); 4 minutes (Lane-4); 6 minutes (Lane-5); 10 minutes (Lane-6); 15 minutes (Lane-7); 30 minutes (Lane-8); 45 minutes (Lane-9); 60 minutes (Lane-10); 90 minutes (Lane-11); 120 minutes (Lane-12); 150 minutes (Lane-13).

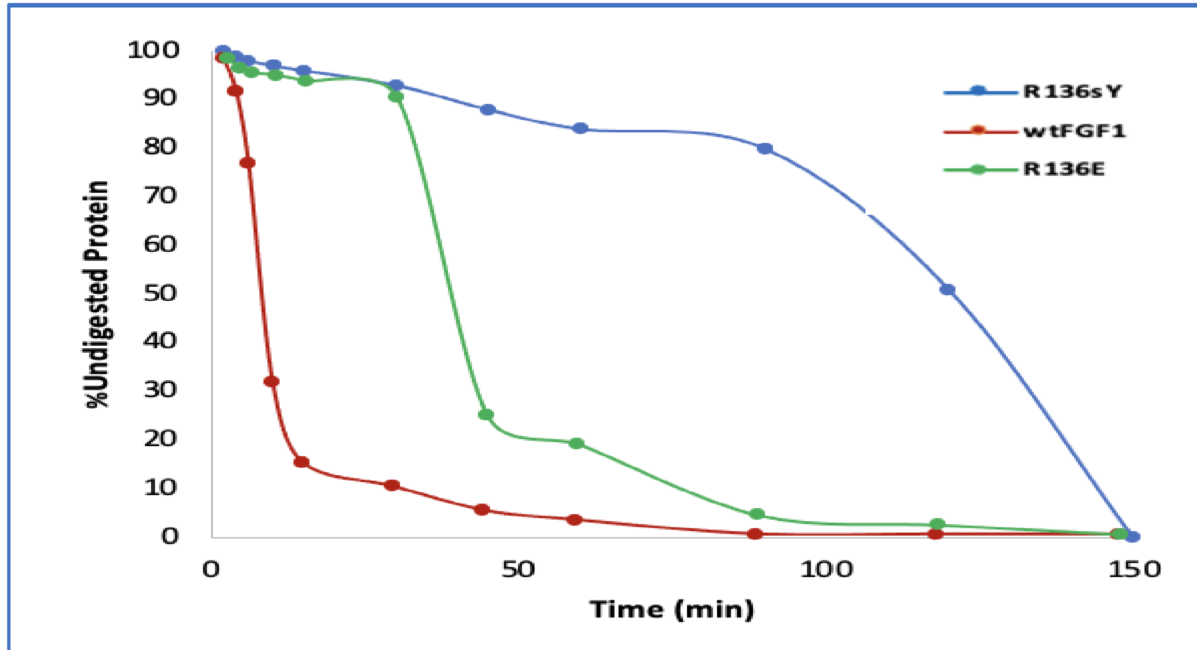


Fig. 12: Densitometric analysis of time dependent limited trypsin digestion of wtFGF1 and the designed variants in absence of heparin as monitored by SDS-PAGE, wtFGF1 (red), R136E (green), R136sY (blue).

8) Thermal Denaturation:

The thermal denaturation technique, based on far-UV CD, is used to measure protein's conformational stability under high temperatures and compare the thermal stability changes of wtFGF1 and its variants. In CD, thermal stability is determined by fractional unfolded calculated by the average molar ellipticity at different temperature (25°C to 90°C). Thermal denaturation plot indicates melting temperature (T_m), which is defined as the temperature at which 50% of the protein denatures [28].

The analysis of the thermal stability data of the wtFGF1 and the FGF1 mutants reveals higher thermal stability level for R136E and R136sY with the T_m being 56°C and 54°C respectively compared to the wild type with the T_m = 42°C (Fig. 13).

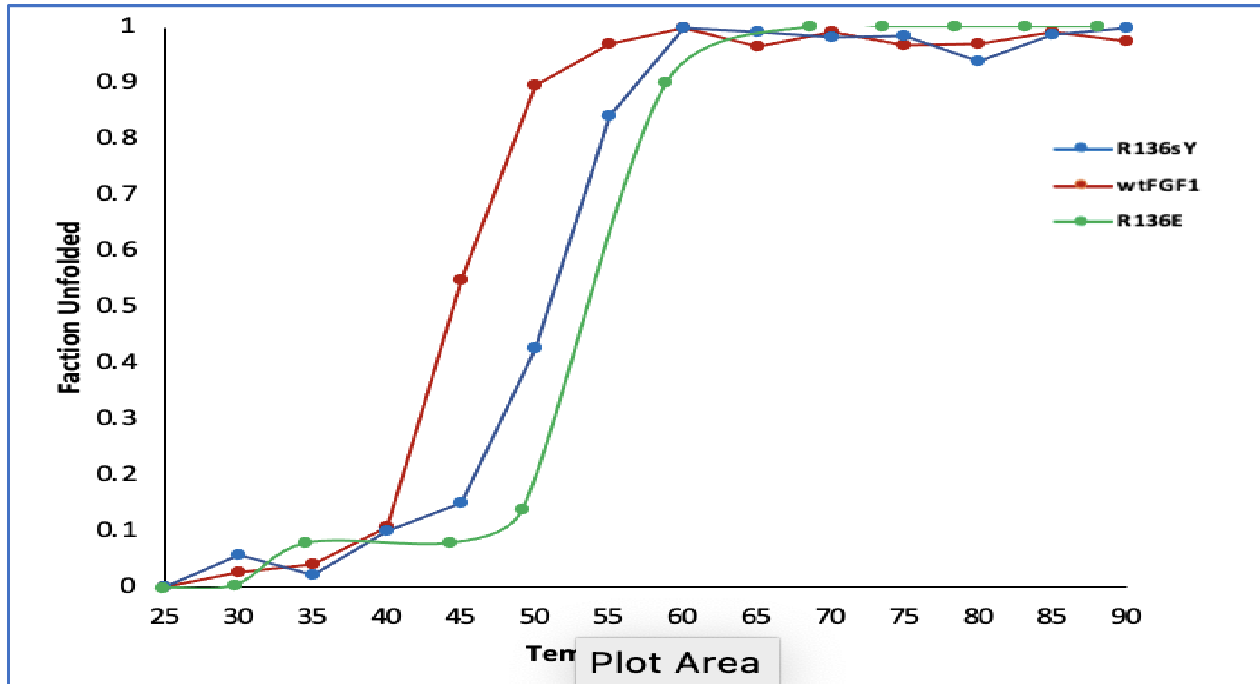


Fig. 13: Thermal stability analysis of the wtFGF1 and its variants in the absence of heparin using Circular Dichroism, wtFGF1 (red), R136E (green) and R136sY (blue).

The thermal equilibrium unfolding data depicts that the introduction of positively charged residue at position 136 increases the stability of the protein variants with 12-14°C in the thermal resistant. It also seems substitution of arginine by sulfotyrosine and replacement of Arginine with Glutamic acid render the protein more compact compared to wtFGF1. This compactness might strengthen the interactions inside the protein core and stabilize the protein against thermal and enzymatic degradation. Improvement the stability level of the variant protein compared with the wtFGF1strongly supports our hypothesis. These reported results also are in a good agreement with the data from limited trypsin digestion.

9) Differential Scanning Calorimetry:

Differential scanning calorimetry is performed to examine the thermal stability and refolding of proteins using DSC (Fig.14). According to the DSC profiles, designing charge-reversal mutation at heparin binding pocket on FGF1(R136sY) increases the thermal stability with 12°C compared

to wtFGF1and reduces the thermal stability 2°C compared to R136E. The results of thermal refolding by DSC show that the T*m* of refolded wtFGF1, R136sY and R136E is ~ 67°C, 69°C and 42°C, respectively. Furthermore, the designed proteins (R136sY and R136E) are more thermal stable than wtFGF1.

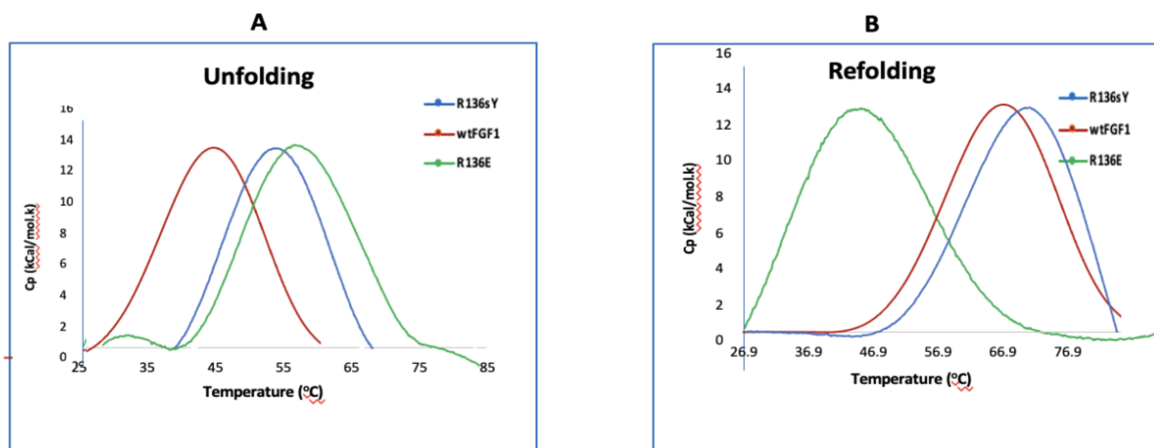


Fig. 14: Thermal unfolding (Panel -A) and refolding (Panel -B) of proteins measured by DSC, wtFGF1 (red), R136E (green) and R136sY (blue).

10) Urea Denaturation

The urea denaturation technique, based on the change in intrinsic fluorescence intensity at 305 nm/350 nm, is accomplished to monitor protein's conformational stability under increasing urea concentration and compare the chemical stability changes of wtFGF1 and its variants. However, the urea denaturation mechanisms are not still clear. Several studies reported the correlation between urea-induced unfolding proteins and urea interactions to protein's peptide bonds, polar amino acids, non-polar amino acids, side chains or both polar and non-polar amino acids. In addition, researchers have challenges regarding the effect of urea on electrotactic interactions or hydrogen bonding [29].

There is a correlation between protein thermal, chemical stability, and protein's half-life. Therefore, higher biological activity at physiological temperature for an extended time duration is expected for proteins with higher T_m and C_m values. The denaturation concentration (C_m) was defined as the urea concentration which 50% of the protein population is in a denatured state [30]. The overall trend of the curves of wtFGF1 and its variants displays that both R136E and R136sY are more stable at higher urea concentrations than in comparison to wtFGF1 without heparin (Fig.15). Substitution of Arginine by glutamic acid or sulfotyrosine at 136 positions reduces positive charge repulsion in the heparin binding pocket and makes the protein more compact and stable against chemicals compared to wtFGF1.

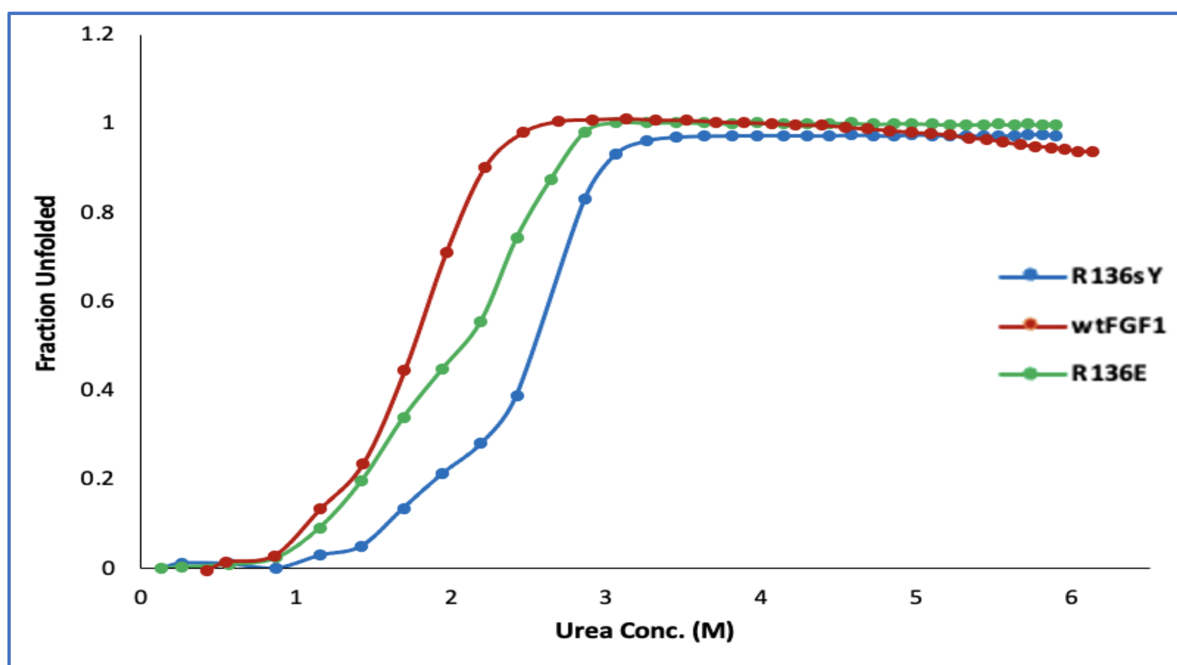


Fig. 15: Urea stability analysis of wtFGF1 and its variants in the absence of heparin, wtFGF1 (red), R136E (green) and R136sY (blue).

The results implies that wtFGF1 can be simply lost its tertiary structure by exposed to 1.5 M of urea while the tertiary structure of the engineered R136E and R136sY mutants would be lost by 1.8 M and 2.8 M of urea respectively (Fig. 15). Therefore, the designed variants are thermally

unfolded at high temperatures and chemically denatured in high urea concentration. In conclusion, the results from the urea denaturation experiments indicate that the introduction of R136E and R136sY mutations make the protein more stable than wtFGF1. These results are consistent with the structural flexibility data.

11) Bioactivity Assay:

This experiment is accomplished to assess how the introducing mutations affect the cell proliferation activity of the protein at different concentrations. hFGF1 as a heparin binding protein plays an important role in stimulating mitogenic and angiogenic activity. Several studies indicated that heparin is critical for FGF receptor activation. In this context, decreased heparin binding affinity of the charged reversed single mutation in FGF1(R136sY) is expected to result in decreased activation of the receptor. However, this cell proliferation study implies that the hFGF1 variants with reduced heparin binding affinity show increased cell proliferation activity. Both R136E and R136sY mutants display same trend in bioactivity assay at protein concentrations below 69 ng/ml. Fig. 16 depicts that there is no significant difference between bioactivity level of wtFGF1 and its variants at the highest protein concentration (69 ng/ml) but that displays higher cell proliferation activity for R136sY and R136E in compared to wtFGF1 at protein concentration range of 2-69 ng/ml.

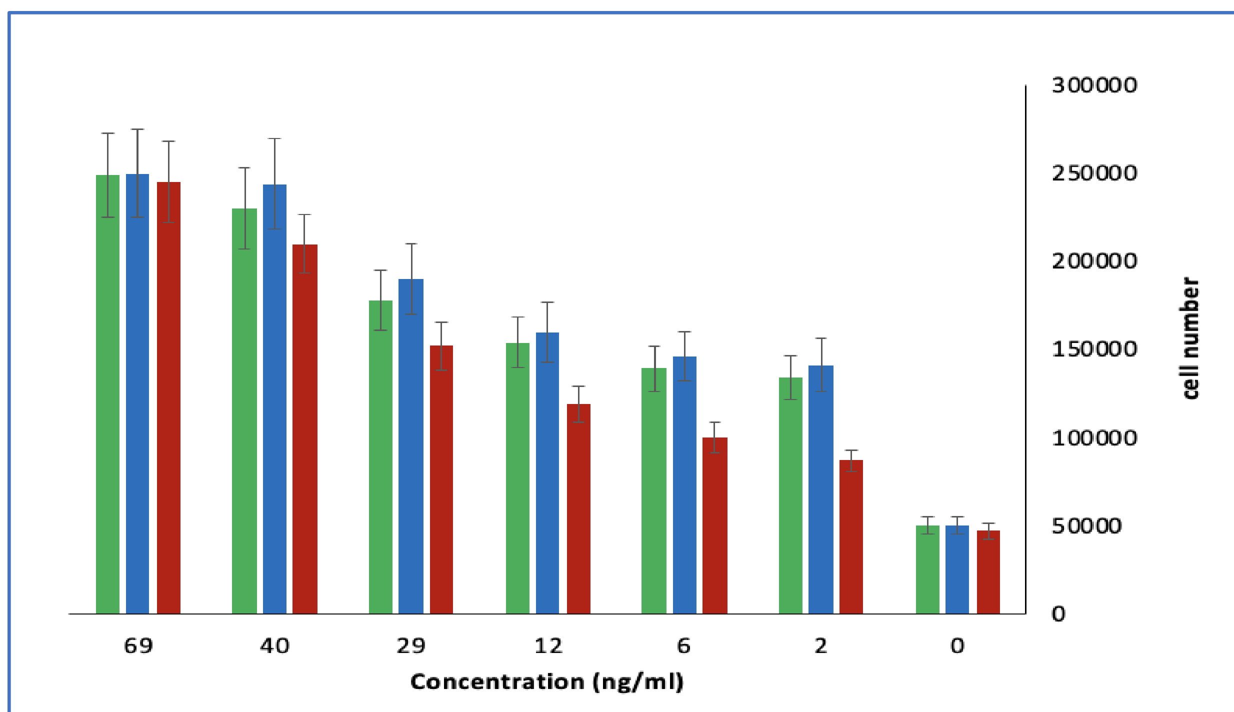


Fig. 16: Cell proliferation activity of NIH 3T3 cells treated with wtFGF1(red), R136E (green) and R136sY(blue) at different concentration (69, 40, 29, 12, 6, 2 and 0 ng/ml).

Based on the results, the reduction in the charge-charge repulsion in the heparin binding region can led to decreasing interaction of fibroblast growth factors with heparin and increasing cell proliferation activity. In the studies, reported by Moscatelli and coworkers, have showed that heparin affinity binding is not mandatory for activation of FGF2-receptor interaction.

In another study, substitution of two or all three cysteine residues in hFGF1 with serine reduces hFGF1 affinity towards heparin despite increasing physiological half-life and exhibition prolonged biological action [31]. Several mutagenesis studies have indicated that interaction of fibroblast growth factors with heparin is not essential for FGFR activation and inducing downstream signaling pathways. Similar observation by other groups showed that there is a correlation between increased stability and enhanced cell proliferation activity irrespective of heparin binding.

In conclusion, R136E and R136sY mutations prolong mitogenic activity in comparison to wtFGF1 [16, 17].

Conclusions

FGF1 protein participates in the various key cellular processes such as wound healing. Based on importance of this subject, several studies have reported in developing a potential therapeutic agent to enhance the wound healing process. For this purpose, engineering of growth factors has currently attracted lots of attention in the field of medicine.

A previous study of the introduced hFGF1 variant in our lab, R136E, has indicated an increase in thermal and proteolytic stability of hFGF1 and a decrease in the protein's affinity for HS [14]. In addition, R136E mutant showed higher (about seven times) cell proliferation activity compared to wt-hFGF1 [14]. Furthermore, it can be inferred that increased structural stability of mutant protein leads to enhanced activity of the protein.

In this research, we have introduced another single charge-reversal mutation by substitution of R136 to sulfotyrosine in the HBR of hFGF1 to monitor the overall structure, conformational stability, backbone flexibility and bioactivities of the protein compared to R136E and wtFGF1. This project's basis is related to understanding the role of individual residues in the heparin binding pocket region of FGF1 and increasing the half-life, and biological stability of the protein. Based on the bioactivity assay outcomes, there are higher mitogenic activity for the introduced of R136sY mutant compared to wtFGF1 (what about compared to R136E in this paragraph). The overall data obtained in the current chapter indicate the designed mutants make hFGF1 reduced heparin binding affinity, increased cell proliferation activity and more stable against chemical, thermal and proteolytic stability compared to wtFGF1. In addition, the results imply that increased affinity for heparin does not necessarily result in increased cell proliferation activity.

Materials and methods

Quikchange II XL mutagenesis kits and the competent cell (BL-21 (DE3) pLysS) were produced by Agilent, and Qiagen, USA supplied the DNA plasmid isolation kit. Lysogeny broth was obtained from IBI Scientific, USA. Cell culture medium was purchased from EMD Millipore, USA. Heparin Sepharose resin, used for protein purification(s), was supplied from GE Healthcare, USA. VWR Scientific., USA produced the buffer components (Na_2HPO_4, NaH_2PO_4, NaCl). American Type Culture Collection (ATCC) was the producer of NIH 3T3 cells, and additional cell culture reagents such as, fetal bovine serum (FBS), DMEM media and penicillin streptomycin was obtained from Thermo Fisher Scientific (Waltham, MA).

All other chemicals and materials were high-quality analytical grade. Unless otherwise stated, samples were made in 10 mM phosphate buffer containing 25 mM ammonium sulfate and 100 mM NaCl (pH 7.2).

Construction and Purification of hFGF1 and the variants

hFGF1 variant is constructed sing the site-directed mutagenesis (SDM) kit (Quik- Change II XL) (Fig. 17). After checking protein presence by small scale expression, large scale expression would be started. For expression of hFGF1, BL-21 (DE3) pLysS cells are grown to an Optical Density of 0.4–0.6 at Abs of 600 and incubated with O-Sulfonate-L-tyrosine sodium salt. At OD_{600} ranging from 06-0.8, the cells are then incubated with 1 mM isopropyl β-D-thiogalactopyranoside for 7 hours at 37°C. IPTG as an allolactose analogous is used to stimulate lac operon which can induce protein expression in competent cells. Cells are harvested by centrifugation at 6000 rpm for 20 minutes, and the resultant pellet is stored in a -20ºC freezer until purification. To purify the R136sY mutant protein, the affinity chromatography separation method is accomplished using a heparin-Sepharose column. The bacterial pellet containing the

protein of interest obtained from the previous step is resuspended in 10 mM phosphate buffer containing 25 mM ammonium sulfate, 100 mM sodium chloride, 5% glycerol, 1mM EDTA and 1% BME at a pH of 7.2. They are then ruptured using ultrasonication for 30 cycles with 20 second on/off pulses. After adding 1mM PMSF, centrifugation at 19,000 rpm for 20 minutes is done to separate the supernatant without cell debris (extra contamination) from the pellet. Subsequently, the resultant clear supernatant containing R136sY-FGF1 is loaded to the heparin Sepharose affinity column, washed with the same buffer used to resuspend the pellet, and then exposed to a stepwise sodium chloride gradient (100, 300, 500, 800, and 1500 mM NaCl). Fractions are collected and 15% Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) analysis is performed for each sample at different salt concentration to verify the purity of proteins followed by staining with Coomasie brilliant blue. The desired fraction (800 mM NaCl) is buffer exchanged to 100 mM NaCl and concentrated down to 5 ml using a 10 kDa MW cutoff Millipore centrifugal concentrator. The concentration of the pure R136sY -FGF1 protein is measured using Nanodrop and Bradford methods at A280 nm. The protein is then aliquoted into 1ml eppendorf tubes and stored in a -80 ºC freezer for further experiments.

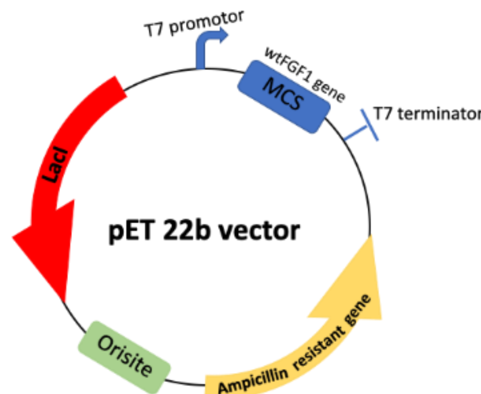


Fig. 17: pET20 expression vector with recombinant wtFGF1 gene and ampicillin resistant gene [24].

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE):

The protein samples are resolved on a 15% polyacrylamide gel. The gel is running in 200 V, 100 mA. The gel is then stained using Coomassie Brilliant Blue dye and destained by a detainer (composition of methanol and acetic acid).

Precipitation of protein by Trichloroacetic Acid (TCA):

TCA preparation is performed to disrupt non-covalent bond and the addition of a reducing agent (2-mercaptoethanol) causes breaking any disulfide bonds or disulfide bridges to have a complete denatured protein. TCA is an acidic compound that consists of ester and salt (Cl^-). At the first, TCA (10% of the protein volume) is added to protein samples to get more protein precipitation. After centrifugation, the supernatant is discarded, and the pellet is washed with 100% acetone and centrifuged again. Heating block is then used to dry the pellets and that is dissolved in 8M urea and blue loading dye (made up from 95% of blue dye plus 5% of 2-mercaptoethanol).

Circular Dichroism and Fluorescence Spectroscopy

CD and fluorescence spectra data are recorded as an average of 3 accumulations at 25 °C using a Jasco J-720 spectropolarimeter. The results of the CD and fluorescence spectra are obtained to determine the secondary and tertiary structural changes of wtFGF1 and FGF1 after mutations. For analysis, 13µM of protein in 10 mM PB + 25 mM $(NH_4)_2SO_4$+100 mM NaCl at pH 7.2 are recorded using a quartz cell of 0.1 mm path length in the standard sensitivity mode with a scan speed of 50 nm per minute. The wavelength of the spectrophotometer is set in a range of 190-250 nm at 25ºC, and the scanning speed is 20 nm/min. The CD data are expressed as molar ellipticity (deg x cm^2 x $dmol^{-1}$). Molar ellipticity is calculated and plotted against a wavelength.

Intrinsic fluorescence spectroscopy is used to determine any alternation in the tertiary structure and folding of protein by combining 13 µM protein with 10 mM PB + 25 mM $(NH_4)_2SO_4$+100

mM NaCl. The excitation wavelength for the fluorescence measurements is 280 nm and the emission is recorded from 300–450 nm. Intrinsic fluorescence is performed at these wavelengths to study if there are any changes between tyrosine and tryptophan residues, which fluoresce at 308 and 350 nm, respectively. A buffer subtraction is made to correct any background noise.

8-Anilino-1-Napthalenesulfonic Acid (ANS) Binding Assay

ANS binding assay measurements are performed using a Fluorescence Spectrophotometer F-2500 (Hitachi) at 25°C using a slit-width of 2.5 nm and a 10mm quartz cuvette. Extrinsic fluorescence experiments are made with protein concentration of 13 μM in 10 mM phosphate buffer containing 100 mM NaCl, and 25 mM (NH4)2SO4 (pH 7.2). Titrations using an ANS stock are prepared by addition of 10 μM increments of ANS followed with mixing and incubation for 2 minutes preceding each reading at 25ºC. Fluorescence intensity is recorded with an excitation at 380 nm and emission intensity is measured at 510nm.

Limited Trypsin

Concentration and time-dependent limited trypsin digestion (LTD) are performed for the proteins dissolved in 10 mM phosphate buffer containing 100 mM NaCl, and 25 mM (NH4)2SO4 (pH 7.2) with a total volume of 100μl. The trypsin concentration at the concentration-dependent LTD experiment is set over the range of 0.001-0.5 mg/ml (30 minutes incubation at 37℃) while the concentration for the time-dependent LTD is 0.05 mg/ml. After addition of trypsin to all protein samples at time-dependent LTD experiment, they are incubated at 37℃ for 2-150 minutes. The reaction is then stopped by the addition of 10 % trichloroacetic acid prior to analysis on 15% SDS-PAGE. Gels are stained using Coomassie Brilliant Blue (Sigma Aldrich) and the band intensity at different time points is calculated by using UN-SCAN IT software densitometric through plotting the average pixel intensity versus time intervals.

Thermal Equilibrium Unfolding

Thermal denaturation experiments are monitored using a Jasco-1500 spectropolarimeter equipped with Circular Dichroism to track the unfolding nature of hFGF1 and related variants at increasing thermal intervals. The folding mechanism of the protein as a function of temperature is evaluated in the absence of heparin. The data is scanned in the wavelength range of 190-250 nm, the scan speed of 50 nm/min and the CD data is collected at a wavelength of 228 nm. For spectra, protein samples are prepared using a protein concentration of 13 μM in 10 mM phosphate buffer containing 100 mM NaCl, and 25 mM $(NH_4)_2SO_4$ (pH 7.2). A temperature probe is inserted into the sample cell to heat the sample, and the spectra are collected in 5 degrees increments from 25 to 90°C. For thermal denaturation, the fraction unfolded is calculated using $(M_x-M_{25})/(M_{90}-M_{25})$ where M is the molar ellipticity value at 228 nm and M_{25} and M_{90} are the molar ellipticity value at 25°C and 90°C respectively. T_m, temperature at which 50% of the protein molecules exist in the denatured state(s), is calculated from the plot of fraction of unfolded protein population versus temperature.

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) is conducted for the proteins dissolved in 10 mM phosphate buffer containing 100 mM NaCl, and 25 mM $(NH_4)_2SO_4$ (pH 7.2) to determine the thermal stability of proteins using changes in heat capacity due to exothermic or endothermic reactions. All samples are subjected to degassing at 25°C for 15 minutes, and the cells then are equilibrated for 10 minutes at the same temperature. Scans are processed from 10 to 90 °C with a 1°C /min ramping temperature. Before running the protein samples, buffer blanks experiments are performed to obtain a stable baseline. CpCalc Version 2.2.0.10 software provided by the manufacturer is utilized to process the data.

Urea Denaturation

Chemical denaturation experiments are monitored using a Jasco-1500 spectropolarimeter equipped with intrinsic fluorescence to track the conformational changes of hFGF1 and related variants at increasing urea concentration intervals.

Urea, an organic compound, is useful in protein denaturation due to non-covalent bonds distraction. This experiment uses 8 M pure urea, 13 μM of wtFGF1 and the FGF1variants. All proteins are eluted in a buffer containing low salt concentration.

For the urea denaturation, urea is steadily added to increase the urea concentration from 0 M to 8 M, and the fluorescence value at 308 nm and 350 nm is recorded at each addition. The ratio of 308 nm to 350 nm is recorded, and the fraction unfolded is calculated using $(F_x-F_0)/(F_0-F_8)$. F is the fluorescence value at 308 nm divided by the fluorescence value at 350 nm, and F_0 and F_8 are the fluorescence fractions at 0 M urea and 8 M urea, respectively.

C_m, urea concentration at which 50% of the protein molecules exist in the denatured state(s), is calculated from the plot of fraction of unfolded protein population versus different concentration of urea.

Bioactivity Assay

3T3 fibroblast cells obtained from ATCC (Manassas, VA) are cultured in media including DMEM supplemented with newborn calf serum (NCS) and L-glutamine, penicillin-streptomycin is purchased from Thermo Fisher Scientific (Waltham, MA). Cells are grown and incubated overnight at 37 °C. The bioactivity of wtFGF1and the related variants are determined by quantifying the cell number increase after the cells are incubated with the proteins. Starved 3T3 fibroblasts is collected and seeded in a well plate with a seeding density of 20,000 cells/well. The

bioactivity assays are repeated three times at the same condition. 3T3 cell proliferation is assessed by the Cell Titer-Glo (Promega, Madison, WI) after 72 hours.

REFERENCES

[1] Brewer JR, Mazot P, Soriano P. Genetic insights into the mechanisms of Fgf signaling. Genes & development. 2016 Apr 1;30(7):751-71.

[2] Hui Q, Jin Z, Li X, Liu C, Wang X. FGF family: from drug development to clinical application. International journal of molecular sciences. 2018 Jul;19(7):1875.

[3] Ornitz DM, Itoh N. Fibroblast growth factors. Genome biology. 2001 Mar;2(3): reviews3005-1.

[4] Itoh N, Ornitz DM. Fibroblast growth factors: from molecular evolution to roles in development, metabolism and disease. The Journal of Biochemistry. 2011 Feb 1;149(2):121-30.

[5] Arunkumar AI, Srisailam S, Kumar TK, Kathir KM, Chi YH, Wang HM, Chang GG, Chiu M, Yu C. Structure and stability of an acidic fibroblast growth factor from Notophthalmus viridescens. Journal of Biological Chemistry. 2002 Nov 29;277(48):46424-32.

[6] Davis JE, Alghanmi A, Gundampati RK, Jayanthi S, Fields E, Armstrong M, Weidling V, Shah V, Agrawal S, prasanth Koppolu B, Zaharoff DA. Probing the role of proline− 135 on the structure, stability, and cell proliferation activity of human acidic fibroblast growth factor. Archives of biochemistry and biophysics. 2018 Sep 15; 654:115-25.

[7] Itoh N, Ornitz DM. Evolution of the Fgf and Fgfr gene families. TRENDS in Genetics. 2004 Nov 1;20(11):563-9.

[8] Häcker U, Nybakken K, Perrimon N. Heparan sulphate proteoglycans: the sweet side of development. Nature reviews Molecular cell biology. 2005 Jul;6(7):530-41.

[9] Copeland RA, Ji H, Halfpenny AJ, Williams RW, Thompson KC, Herber WK, Thomas KA, Bruner MW, Ryan JA, Marquis-Omer D, Sanyal G. The structure of human acidic fibroblast growth factor and its interaction with heparin. Archives of biochemistry and biophysics. 1991 Aug 15;289(1):53-61.

[10] Eswarakumar VP, Lax I, Schlessinger J. Cellular signaling by fibroblast growth factor receptors. Cytokine & growth factor reviews. 2005 Apr 1;16(2):139-49.

[11] Lee J, Blaber M. The interaction between thermodynamic stability and buried free cysteines in regulating the functional half-life of fibroblast growth factor 1. Journal of molecular biology. 2009 Oct 16;393(1):113-27.

[12] Kerr R, Agrawal S, Maity S, Koppolu B, Jayanthi S, Kumar GS, Gundampati RK, McNabb DS, Zaharoff DA, Kumar TK. Design of a thrombin resistant human acidic fibroblast growth factor (hFGF1) variant that exhibits enhanced cell proliferation activity. Biochemical and Biophysical Research Communications. 2019 Oct 15;518(2):191-6.

[13] Szlachcic A, Zakrzewska M, Krowarsch D, Os V, Helland R, Smalås AO, Otlewski J. Structure of a highly stable mutant of human fibroblast growth factor 1. Acta Crystallographica Section D: Biological Crystallography. 2009 Jan 1;65(1):67-73.

[14] Ortega S, Schaeffer MT, Soderman D, DiSalvo J, Linemeyer DL, Gimenez-Gallego G, Thomas KA. Conversion of cysteine to serine residues alters the activity, stability, and heparin dependence of acidic fibroblast growth factor. Journal of Biological Chemistry. 1991 Mar 25;266(9):5842-6.

[15] Brych SR, Blaber SI, Logan TM, Blaber M. Structure and stability effects of mutations designed to increase the primary sequence symmetry within the core region of a β-trefoil. Protein Science. 2001 Dec;10(12):2587-99.

[16] Zakrzewska M, Krowarsch D, Wiedlocha A, Olsnes S, Otlewski J. Highly stable mutants of human fibroblast growth factor-1 exhibit prolonged biological action. Journal of molecular biology. 2005 Sep 30;352(4):860-75.

[17] Zakrzewska M, Krowarsch D, Wiedlocha A, Otlewski J. Design of fully active FGF-1 variants with increased stability. Protein Engineering Design and Selection. 2004 Aug 1;17(8):603-11.

[18] Huang Z, Tan Y, Gu J, Liu Y, Song L, Niu J, Zhao L, Srinivasan L, Lin Q, Deng J, Li Y. Uncoupling the mitogenic and metabolic functions of FGF1 by tuning FGF1-FGF receptor dimer stability. Cell reports. 2017 Aug 15;20(7):1717-28.

[19] Xia X, Kumru OS, Blaber SI, Middaugh CR, Li L, Ornitz DM, Suh JM, Atkins AR, Downes M, Evans RM, Tenorio CA. An S116R phosphorylation site mutation in human fibroblast growth factor-1 differentially affects mitogenic and glucose-lowering activities. Journal of pharmaceutical sciences. 2016 Dec 1;105(12):3507-19.

[20] Thallapuranam SK, Agarwal S, Gindampati RK, Jayanthi S, Wang T, Jones J, Kolenc O, Lam N, Niyonshuti I, Balachandran K, Quinn K, inventors. Engineered fgf1 and fgf2 compositions and methods of use thereof. United States patent application US 16/356,872. 2019 Sep 19.

[21] Dubey VK, Lee J, Somasundaram T, Blaber S, Blaber M. Spackling the crack: stabilizing human fibroblast growth factor-1 by targeting the N and C terminus β-strand interactions. Journal of molecular biology. 2007 Aug 3;371(1):256-68.

[22] Pellegrini L, Burke DF, von Delft F, Mulloy B, Blundell TL. Crystal structure of fibroblast growth factor receptor ectodomain bound to ligand and heparin. Nature. 2000 Oct;407(6807):1029-34.

[23] De Paz JL, Angulo J, Lassaletta JM, Nieto PM, Redondo-Horcajo M, Lozano RM, Giménez-Gallego G, Martín-Lomas M. The activation of fibroblast growth factors by heparin:

Synthesis, structure, and biological activity of heparin-like oligosaccharides. ChemBioChem. 2001 Sep 3;2(9):673-85.

[24] Hung KW, Kumar TK, Kathir KM, Xu P, Ni F, Ji HH, Chen MC, Yang CC, Lin FP, Chiu IM, Yu C. Solution structure of the ligand binding domain of the fibroblast growth factor receptor: role of heparin in the activation of the receptor. Biochemistry. 2005 Dec 6;44(48):15787-98.

[25] Powers CJ, McLeskey SW, Wellstein A. Fibroblast growth factors, their receptors and signaling. Endocrine-related cancer. 2000 Sep 1;7(3):165-97.

[26] Gasymov OK, Glasgow BJ. ANS fluorescence: potential to augment the identification of the external binding sites of proteins. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics. 2007 Mar 1;1774(3):403-11.

[27] Wong, P., Hampton, B., Szylobrgt, E., Gallagher, A.M., Jaye, M., Burgess, W.H., Analysis of Putative Heparin-binding Domains of Fibroblast Growth Factor-1. J Biol Chem, 1995. 270(43): p. 25805-25811.

[28] Ornitz, D.M., Itoh, N., The Fibroblast Growth Factor Signaling Pathway. Wiley Interdiscip Rev Dev Biol, 2015. 4(s): p. 215-266.

[29] Zakrzewska, M., Wiedlocha, A., Szlachcic, A., Krowarsch, D., Otlewski, J., & Olsnes, S. (2009). Increased protein stability of FGF1 can compensate for its reduced affinity for heparin. Journal of Biological Chemistry, 284(37), 25388-25403.

[30] Srimathi, T., Kumar, T. K. S., Kathir, K. M., Chi, Y. H., Srisailam, S., Lin, W. Y., ... & Yu, C. (2003). Structurally homologous all β-barrel proteins adopt different mechanisms of folding. *Biophysical journal*, *85*(1), 459-472.

[31] Chen, G., Gulbranson, D. R., Yu, P., Hou, Z., & Thomson, J. A. (2012). Thermal stability of fibroblast growth factor protein is a determinant factor in regulating self-renewal, differentiation, and reprogramming in human pluripotent stem cells. *Stem cells (Dayton, Ohio)*, *30*(4), 623–630. https://doi.org/10.1002/stem.1021.

CHAPTER 3

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Fibroblast growth factors (FGFs) family consists of 23 genes which play an important role in eliciting several biological processes in embryonic and adult organism. hFGF1 as a mitogenic factor with molecular weight of 16kDa shows high affinity for heparin. However, FGF1 has an essential issue that it is prone to thermal and proteolytic degradation at a biological pH and under physiological conditions. Site directed mutagenesis technique was then applied in this study to introduce the R136sY mutation with increased FGF1 stability and biological activity. The single mutation was introduced in the heparin binding pocket. Heparin- Sepharose column was then used to purify R136sY. After obtaining pure protein, it was biophysically and biochemically evaluated through using different techniques. The designed variant doesn't alter overall secondary and tertiary structure of FGF1. The biological study of R136sY indicates higher mitogenic activity compared to wtFGF1. Thermodynamic and proteolytic studies imply that decreasing repulsion of positive charges in the heparin binding site can lead to improvement in structural stability of hFGF1. Based on the results from limited enzymatic digestion, thermal and urea denaturation, R136sY mutation renders FGF1 to be more resistant to the thermal, chemical, and proteolytic degradation. Therefore, the data from this study strongly supported our hypothesis. According to the characteristic, the engineered variant can be a good candidate for wound healing.

Significance of this Thesis

hFGF1 has an intrinsically low thermal stability and short half-life under physiological temperature. There have been several studies to increase the thermal and proteolytic stability of

hFGF1. A previous mutation studied in Kumar lab was related to substitution of arginine at position 136 with glutamic acid which showed increasing in stability and cell proliferation activity when compared to the wtFGF1. At this study, another hFGF1R136 single mutant was introduced to evaluate its effect on the structure, stability, and cell proliferation activity of the FGF1. This study creates an opportunity for better understanding structure-activity relationships of FGFs allowing systematic generation of novel proteins with modified properties as topical and injectable treatments in pharmaceutical wound healing applications.

Future Directions

Since 1980s, proteins have been played a critical role in pharmaceuticals industry with around 200 marketed products including therapeutics, diagnostics, and vaccines [1].

In addition, combination of protein design techniques and recombinant protein technology lead to produce novel therapeutic agents by engineering different types of proteins. Modification of the desired protein by targeting its limitations and overcome the challenges is obtained through rational and knowledge based molecular evolution.

The studied variant in my dissertation with improving the stability of FGF1 can be a good candidate in the field of diabetic research, wherein the role of hFGF1 variant will be to increase the glucose-lowering activity and enhance the cell proliferation ability. The variant could also be a new class of FGF-based protein therapeutics against Type-1 & Type-2 diabetes.

Based on this research results, further studies are more likely to be accomplished toward improving the properties of the native FGF1. The studies need to be conducted on charge-reversal mutation at heparin binding pocket at position 136 on FGF1 to increase the bioactivity of FGF and enhance the stability. For this purpose, site directed mutagenesis technique is utilized to engineer the desired variant that will be more efficient in terms of both activity and stability.

While our single variant (R136sY) has indicated enhanced mitogenic activity, there have been several hFGF1 variants demonstrating increased metabolic activity and decreased potential to promote cell proliferation. In regard the future work, more mutations on FGF1(R136sY) in the heparin binding pocket can be designed to understand the role of individual residue in the overall stability of the molecule.

In addition, future studies are needed to be performed regarding heparin binding affinity of wtFGF1 and its variants. In this regard, isothermal titration calorimetry (ITC) is used to determine thermodynamic binding parameters of interactions in solution.

REFERENCES

[1] Walsh G. (2010). Biopharmaceutical benchmarks. Nature Biotechnology; 28(9):917-24. doi: 10.1038/nbt0910-917.