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Engineered Human Acidic Fibroblast Growth Factor (FGF1) with an Enhanced Thermal and Proteolytic Stability

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Engineered Human Acidic Fibroblast Growth Factor (FGF1) with an Enhanced Thermal and Proteolytic Stability

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by

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Abstract:

Fibroblast growth factor receptor (FGFR) is made up of three significant domains. The most important domain is the intracellular domain where the dimerization and autophosphorylation occur. Fibroblast growth factor (FGF) interacts with specific FGFR to regulate many cellular processes during the embryonic stage. Furthermore, FGF is significant for adults because FGF plays an important role in regulating cellular differentiation as well as wound healing. The cellular regulating processes are initiated through binding FGF to heparin followed by binding FGF/heparin to FGFR to form FGF/heparin/FGFR complex. Thus, FGFR is dimerized and autophosphorylated. The phosphorylation of FGFR triggers downstream signaling pathways, which enhance cellular processes. Any destruction of downstream signaling results in several diseases including different types of cancer. FGF1, which consists of 140 or 154 amino acids, is made of 12 β-antiparallel strands. Over the past twenty years, several mutations were engineered in various sites to enhance protein stability and function. In our work, penta mutations Q40P, S47L, H93S, K112N, and R122E were introduced in FGF1 to increase protein stability and enhance its biological activity. Because pFGF1 lost heparin binding ability, pFGF1 was not purified by heparin as WFGF1. The pFGF1 was purified by nickel-sepharose column because of inserting poly His tag in its N-terminal. Based on NMR and fluorescence results, the tertiary structure of pFGF1 is different from WFGF1. The pFGF1 exhibits more stability than WFGF1 because thermodynamic study shows that pFGF1 rises the denaturation temperature by ~20 ºC. Also, urea denaturation study illustrates that pFGF1 is partially unfolded after being exposed to 4M urea. Moreover, pFGF1 is not affected by trypsin because it shows high resistance to proteolytic enzymes even if the time of incubation is increased. Despite the high stability, pFGF1 is more potent than WFGF1.

Acknowledgments:

A lot of thanks for Dr. T.K.S. Kumar who opened his lab, advised me and followed me to make successful results in my research. I would like to thank Dr. Srinivas Jayanthi and Dr. Kumar Ravie for training me and helping me in doing biochemical and biophysical characterizations for my proteins. I am very thankful for Dr. Rory Henderson for helping me in doing nanoscale molecular dynamic simulations for my proteins. A lot of thanks to Julie Eberle and Dr.Kumar's group who provided various helps in the lab. This work is supported by Arkansas Bioscience Institute. Finally, I would like to thank King Abdullah Scholarship Program who gave me this opportunity to study abroad.

Dedication:

This thesis is dedicated to my mother who had an acute stoke since I came to the United States.

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I. The structure of fibroblast growth factor receptors (FGFR):

The fibroblast growth factor receptor (FGFRs) signals are significant in many biological processes involving cellular proliferation, migration, differentiation, wound healing, angiogenesis, and fetal development (Ohkubo et al., 2004; Zhao et al., 2007; Gill& Tsai, 2006; Gill, Moenter, & Tsai, 2004). The FGFR family has five members (FGFR-1 to FGFR-5) and four of them (FGFR-1 to FGFR4) belong to the tyrosine kinase family (Bocharov et al., 2013). The structure of tyrosine kinase FGFR is composed of three major domains which are: extracellular domain, transmembrane domain, and intercellular domain (Figure 1). The extracellular domain is composed of domains one to three (D1, D2, and D3) and is essential important in specifying ligand binding to its receptor in order to promote receptor dimerization (Hung et al., 2005; Vladislav et al., 2006; David et al, 1996). In addition, the D1 and D2 are connected by a short disorder polypeptides called the acid box (AB). The transmembrane domain is mainly made up of a signal alpha helix segment that spans from the extracellular domain into the intercellular domain. On the other hand, the intracellular cytoplasmic domain is a domain whereby the tyrosine kinase activity takes place (Hung et al, 2005). This domain folds into three-dimensional structure, and is made up of two lobes (N-lobe and C-lobe). The N-lobe functions as an enzyme, while the C-lobe functions as a substrate (Bocharov et al., 2013).

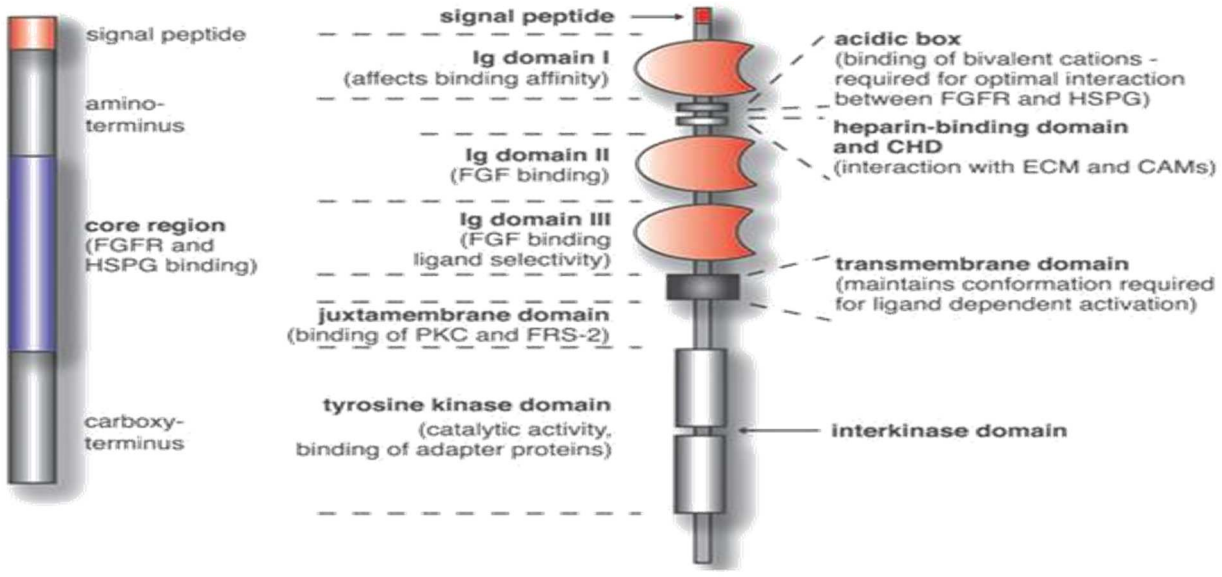

Figure 1: *A cartoon showing the main structure of fibroblast growth factor receptor (FGFR) which mainly consists of three extracellular domains, a transmembrane domain, and the cytoplasmic domain (Kalash, n.d).*

Ig1 domain (D1) primarily has a β-barrel shape, which consists of 10 β-strands forming two β-sheets. Furthermore, Ig1 has a molecular weight of approximately 10.4 kDa (Vladislav et al., 2006). The AB linker placed between D1 and D2 is made up of 20 to 30 amino acids including 4 to 8 acidic residues (Kalinina et al., 2012).

The structure and molecular weight of Ig2 domain (D2) is larger than Ig1 domain (D1). Similar to Ig1, Ig 2 domain is arranged in an antiparallel β manner which consists of 11 β strands forming two β-sheets. Its molecular weight is about 13.7 kDa. The two β-sheets are connected through two critical cysteine residues by a disulfide bond. In terms of triggering physiological processes, Ig2 domain is more significant than Ig1 because it involves the heparin binding site, which consists of five lysine residues (K177, K175, K172, K163, and K160). These lysines interact with sulfate groups in heparin through 9 hydrogen bonds. (Schlessinger et al., 2000; Hung et al., 2005). (Figure 2&3)

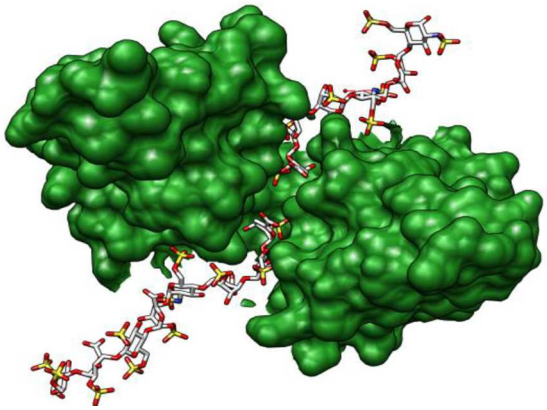

Figure 2: *A cartoon (X-ray structure) of Immunoglobulin like domain 1 (D1) binding to heparin (Schlesinger et al., 2000).*

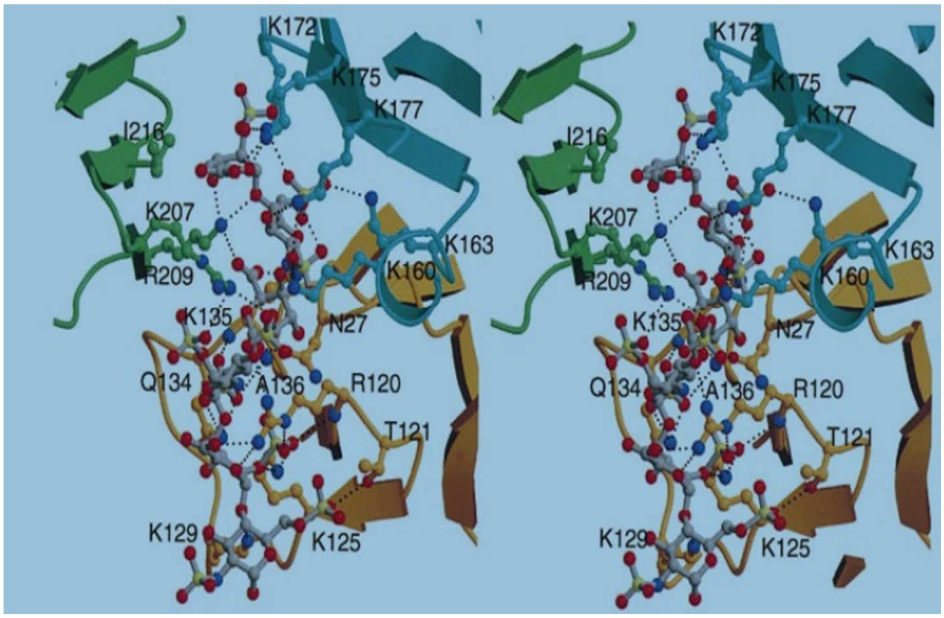

Figure 3*: A PyMOL image which shows five amino acids (K177, K175, K172, K163, and K160) of Immunoglobulin like domain 2 (D2) that interact with heparin sulfate groups (Schlesinger et al., 2000).*

Ig3 domain (D3) is the last extracellular domain. Its structure is similar to both D1 and D2, however, it consists of 9 β-strands which are formed by 97 amino acid residues. Both Ig 2 and 3 domains are extremely important because they are the sites where the fibroblast growth

factors (FGFs) bind. FGFs bind to Ig2 domain at the Aβ stand and C-terminal end of Gβ strand, while on Ig 3 domains, they bind to their loops (Figure 4) (Stauber, Digabriele, & Hendrickson, 2000). Furthermore, FGFs bind to the FGFRs through different affinity binding sites. First, high affinity site contains a group of amino acid residues "Y15, R35, N92, Y94, L133, L135". The other binding sites are the low binding site which located in FGF1's loop and includes the amino acid residues "L101, H102, and W107" (Taylor, n.d).

Over all, FGFR1 to FGFR5 share similar structure over 70%, but FGFR5 does not have the intracellular domain (tyrosine kinase domain) (Zakrzewska et al., n.d).

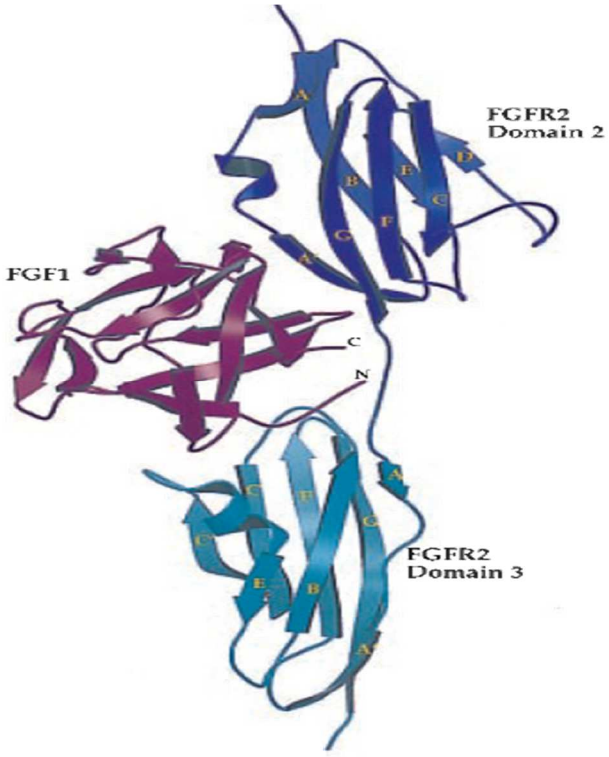

Figure 4: *A cartoon of FGF1-FGFR2 complex where the FGF1 binds to FGFR 2 specifically at domains 2&3 (Stauber, Digabriele, & Hendrickson, 2000).*

There are several splicing methods that occur in mRNA which result in forming various isoforms of FGFRs. These new forms can increase or decrease the binding affinities of FGFRs to

their ligands. One splicing method can cause truncation of D1 while connecting D1 and D3 to produce a shorter polypeptide. Another splicing method is that the D3 of FGFR1,2, and 3 are connected to result in three types of domian3 (IIIa,IIIb,and IIIc) in all three receptors. In contrast to FGFR1-3, a splicing method does not occur on domain 3 of FGFR4 (David et al., 1996). One common feature of FGF1 is that can bind to all four receptors (Yamashita et al., 2002). Table (1) summarizes various FGFs that bind to FGFR isoforms.

FGFRs Isoforms	FGF ligand capacity	The regulator expressions of the receptor
FGFR1b	FGF1, FGF2, FGF3, FGF10	Epithelial cells
FGFR1c	FGF1, FGF2, FGF4, FGF5, FGF6	Mesenchymal cells
FGFR2b	FGF1, FGF3, FGF7, FGF10, FGF22	Epithelial cells
FGFR2c	FGF1, FGF2, FGF4, FGF6, FGF9, FGF17, FGF18	Mesenchymal cells
FGFR3b	FGF1, FGF9	Epithelial cells
FGFR3c	FGF1, FGF2, FGF4, FGF8, FGF9, FGF17, FGF18, FGF23	Mesenchymal cells
FGFR4	FGF1, FGF2, FGF4, FGF6, FGF8, FGF9, FGF16, FGF17, FGF18, FGF19	

Table 4: *Different FGFs can bind to FGFR isoforms. FGF1 has the ability of binding to four receptors (FGR1-FGFR4) (Thurman, 2013, pp.5) & (David et al., 1996).*

II. Classification of Fibroblast Growth Factor:

FGFs are mitogens that play a critical role in fetal and embryonic developments, pathological conditions, and some biological processes in adult organisms. Polypeptide factor signaling is expressed in the evolutionary of not only vertebrates but also invertebrates. The FGF family consists of 23 genes that encode specific polypeptide, with 22 of them found in mice and humans (Zakrzewska et al., n.d). The growth factors that exhibit a high affinity to heparin consist of three

major exons which are formed with amino acids sequence ranging between 150-250 with molecular weight rate between17 to 34kDa (Ornitz, 2000). In general, all FGFs structures are β-trefoil fold which are primarily made up of 12 β-antiparallel strands (Kalinina et al., 2012). All FGFs have 120 amino acid residues in the core region, 28 of them are conserved and six of them are identical (Zakrzewska et al., n.d).

II.1. The role of FGFs during fetal and embryonic developments:

FGFs, which are fundamental regulators in fetal and embryonic development, show their function even before implantations. Moreover, they act in the formation of gastrulation as well as mesoderm (Zakrzewska et al., n.d). These growth factors can participate in embryonic differentiation and organ development. They are also effective in the functioning of lung, heart, circulatory, limbs, and nervous systems formation. In addition, FGFs are essential for inner ear and midbrain development as well as development of prostate gland (Wesch, Haglund, & Haugsten, 2011).

II.2. The role of FGFs in biological process in adult organisms:

FGFs express some biological functions including cellular migration and differentiation, along with cell survival. In adult organisms, these growth factors involve to angiogenesis, tissue repair, and wound healing. FGFs contribute to atherosclerosis, diabetic retinopathy, and a number of pathological conditions such as neoplastic. FGF2 is an example of a growth factor that can act as an oncoprotein. It can control tumor and cancer cell growth (Zakrzewska et al., n.d).

II.3. FGFs subfamilies:

As mentioned previously, FGFs genes were recognized in multicellular organisms such as humans, mice, and nematodes, but not found in unicellular organisms including *E. coli*. FGF1

- FGF23 were identified in both mice and humans. FGFs have been classified into 7 subfamilies depending on their structural similarities, biochemical characteristics, and developmental features. FGF15 in mice orthologous to FGF19 in the humans. Also, some FGFs do not activate FGFR such as FGF11-14. Therefore, by removing FGF11-14 and consideration of FGF15 as orthologous to FGF19, the number of FGF is 18 members in 6 groups (Figure 5). The first subfamily includes FGF1/2, while the second subfamily contains FGF4,5, and 6. The third one has FGF3,7,10, and 22. The fourth subfamily involves FGF9,16, and 20, while FGF8,7, and18 were categorized under the fifth subfamily. The last one has FGF15/19, 21, and 23.

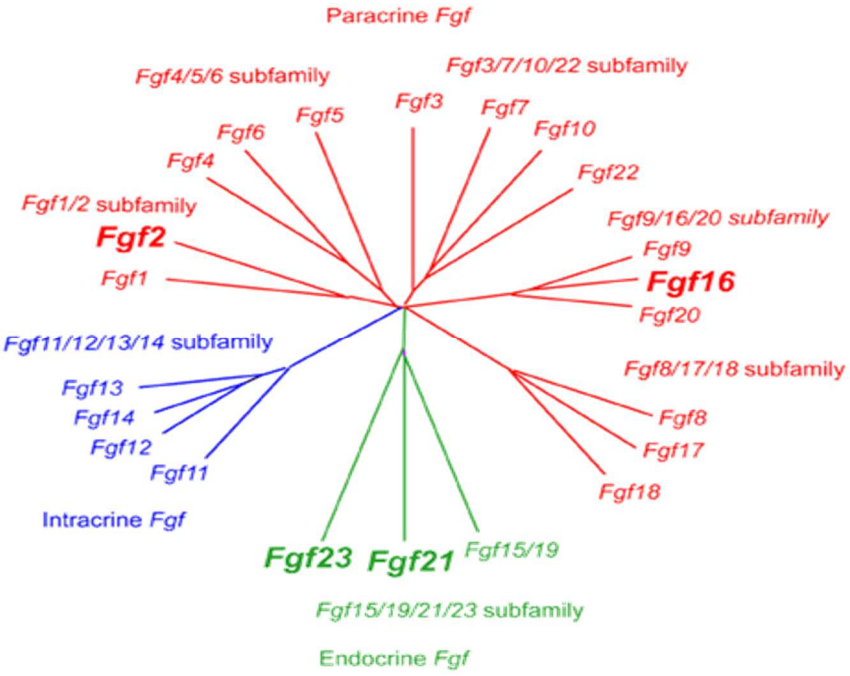

Figure 5*: A cartoon shows various FGF subfamilies (FGF1-FGF6 subfamilies) which undergo one family (fibroblast growth factor family) (Itoh & Ohta, 2013).*

As shown in the Figure 5, the first five subfamilies are paracrine factors, which secret their signals to nearby cells. This is significant in cellular communications. However, the sixth

subfamily is an endocrine. Endocrine is defined as factors that send their signals directly to the target organs.

There are various secretion pathways for FGFs. A common pathway for the majority of FGFs, including FGF3,4,5,6,7,8,10,17,18,19,21, and 23, is the cleavage to the signaling N-terminal peptides followed by their secretions outside the cell via the Golgi apparatus (Zakrzewska et al., n.d). Nevertheless, in the second way, some FGFs, such as FGF20,16, and 9, do not have cleavable signal peptides, but they are still secreted from the cells because of the presence of uncleavable hydrophobic N-terminal sequence. A growth factor, FGF22, prefers the attachment to cellular surfaces instead of being secreted (Itoh & David, 2004). By contrast, FGF1 and FGF2, which are synthesized in endothelia cells, are secreted by non-classical pathways because of the absence of a classical signal peptide. They are released through independent Endoplasmic Reticulum-Golgi mechanisms under abnormal conditions including cell damage, heat shock, hypoxia, cell injury, and cell death. A study has revealed that FGF1 cannot translocate across the cell membrane by itself. However, it interacts with the calcium binding protein "S100A13" to facilitate its secretion throughout the cell membrane (Kathir et al., 2007; Paul et al., 1989).

II.4. The binding affinity of FGFs to heparin:

As mentioned before, FGFs have a high ability of binding to either heparin or heparan sulfate proteoglycans (HSPGs). Heparin and HSPGs protect the growth factors from proteolytic cleavages, thermal and chemical denaturation, mild oxidation, as well as activation of FGFRs (Zakrzewska et al., n.d; Taylor et al., n.d).

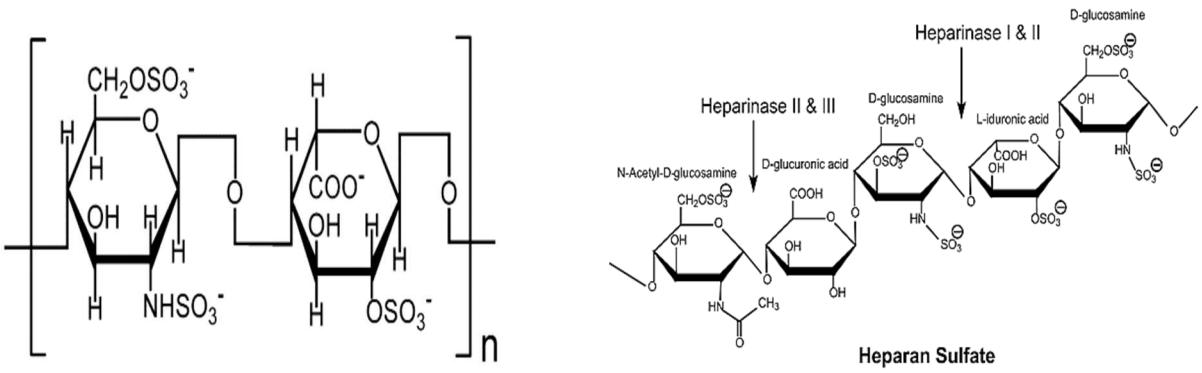

Figure 6: *The general structure of heparin and heparin sulfate (ou et al., 2015).*

HSPGs and heparin have similar structure which consists of repeating alpha 1, 4 disaccharides (Figure 6). Every disaccharide unit is made of amino sugar and uranic acid. The biosynthesis of both HSPGs and heparin is initiated in the endoplasmic reticulum and continued in the Golgi apparatus. Heparin is more sulfated than HSPGs, so it has more negative charges than HSPGs (Maria et al., 2015).

The name of molecules	HSPGs	Heparin
Cell types production	Produced through different types of cells	Produced by only mast cells
Location	Can be found in cellular matrix or attached to the plasma membrane	Can be found in the secretory granules of mast cells
Amino sugar type	N-Acetyl-D-galactosamine	N-Acetyl-D-glucosamine
Uranic acid type	D-glucuronic acid	L-iduronic acid
Molecular weight	50kDa	20kDa

Table 5: *Shows the major differences between HSPGs and heparin (Maria et al., 2015; Rapraeger, Krufka, & Olwin, 1991).*

To activate FGFRs through heparin, FGFs, such as FGF1 and FGF2, bind to heparin through the heparin binding pocket. This pocket which is located in β 10 -12 loops (C-terminal) is rich with positively charge amino acids (K112, K113, K118, R119, R122, K128). The positive

residues interact with heparin by electrostatic interactions. Additionally, beside electrostatic interaction, all FGFs can make 16 hydrogen bonds with heparin, 10 of them occur by sulfate and the remaining six are by oxygen atoms of the heparin (Deborah et al.; Schlessinger et al., 2000). Next, FGFs are able to bind to one of either four receptors (FGFR1, FGFR2, FGFR3, FGFR4). As mentioned earlier, the conserved binding site of the FGFs on FGFRs are D2 and D3. Interaction of FGFs with FGFRs is placed in β1, β2, β9, and β12 strands, β1-2,8-9 turns, and β2-4 loops. Most of the interaction of FGFs with FGFRs are hydrophobic even though there are some hydrogen bonds (Deborah et al., 2000). A dimerization of two tyrosine kinase receptors is the result after binding FGF-heparin complex to Domains 2, and 3 of the receptor (Furdui, Schlessinger, & Anderson, 2006; Mohammadi et al., 1996). The dimerization triggers FGFRs activations through bringing two intercellular kinase domains in close proximity to each other. That enables the kinase protein to auto phosphorylate the activating loop in several tyrosines (Tyr 436, Tyr583, Tyr 585, Tyr653, Tyr654, Tyr730, Tyr766) (Wesche, 2011).

III. FGF-FGFR complex signaling pathways:

Some of the autophosphorylated tyrosines increase FGFRs activity, while others act as recruitment for protein binding sites (docking) and downstream signaling activation of molecules having Src homolog 2 domain (SH2). The active FGFRs elicit several singling pathways such as: Ras/mitogen-activated protein kinase (MAPK), Phospholipase C$^{\gamma}$ (PLC$^{\gamma}$), and phosphoinositide 3-kinase (P13K)/Akt" to send all the signals into the nucleus (Zakrzewska et al., n.d).

III.1. Ras/mitogen-activated protein kinase (MAPK):

Growth factor receptor-bound protein 2 (Grb2) and son of seven less (Sos) are two main proteins for activation this pathway (Zakrzewska et al., n.d). First, Grb2 binds to SH3 domain of Sos followed by a binding of Grb2 SH2 domain to the phosphorylated docking site of tyrosine (Schlessinger, 2000). Grb2 and Sos complex bring inactive Ras, which is bound to guanosine triphosphate (GTP), from the cytoplasm to the membrane. The complete complex is activated through hydrolyzing of the GTP to GDP (guanosine diphosphate) by Sos. Activated Ras is able to activate a serine /threonine kinase (Raf) through binding to its N-terminal domain. MEK1 and MEK2, which have the ability of phosphorylation f not only for tyrosine but also serine residues, are phosphorylated and activated by active Raf. A study has shown that the activation process of MEK happens through binding to Raf's catalytic domain. Finally, further protein phosphorylation, involving transcription factors, has occurred by Ras/MAPK cascade pathway to promote cellular responses (Lodish et al., 2000). See Figure (7).

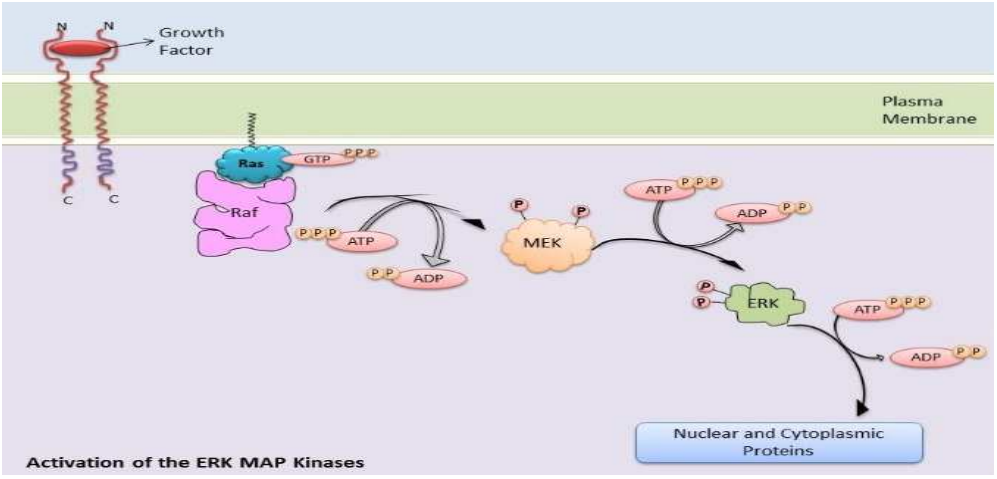

Figure 7: *Shows MAPK kinase pathways where both Grb2 and Sos are essential in activating Ras. Ras is important in Raf activation followed by activating MEK and ERK which is significant in eliciting cellular division (Heda, 2013).*

III.2. Phospholipase C γ (PLCγ) pathway:

The activation of FGFRs stimulates the metabolism of phosphoinositol. The SH2 domain of PLCγ binds to phosphorylated tyrosine (Tyr-766) of FGFRs. PLCγ can generate Inositol triphosphate (IP3) and diacylglycerol (DAG) by phosphatidyl -inositol-4, 5-biophosphate hydrolysis. While IP3 helps to release calcium ions from endoplasmic reticulum, protein kinase C (PKC) is activated by these calcium and DAG components. A mutation on phosphorylated Tyr 766 of FGFRs to phenylalanine was made to test the importance of this residue. The result proves that this tyrosine plays a key role in phosphatidylinositol hydrolysis. There is no obvious function of this pathway even though its mutation and distribution do not effect cellular proliferation or differentiation. Nevertheless, it was indicated that this pathway might participate in some cellular adhesion (Zakrzewska, n.d; Powers McLeskey, & Wellstein s; Schlessinger, 2000).

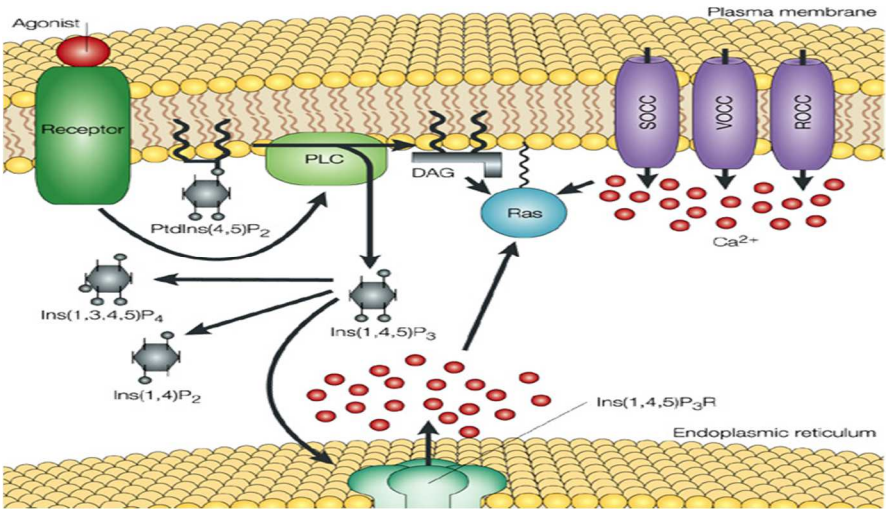

Figure 8: *A cartoon shows PLCγ pathway which participates in the hydrolysis of phosphoinositol (Cullen&Lockyer).*

III.3. Phosphoinositide 3-kinase pathway (PI3):

All tyrosine kinase receptors (TKRs) can activate PI3. The major compositions of PI3 are regulatory subunit (p85) and a catalytic subunit (p110). There are three main mechanisms by which PI3 are activated. First, after FFRs are dimerized and phosphorylated, the phosphorylated tyrosine of the receptor binds to p85 via SH3 domain. That leads to the recruitment of p110 to activate PI3.The second way to activate PI3 is through Grb2 which activates Sos to trigger PI3 activation. Finally, PI3 is activated by Insulin receptor substrate (IRS) that binds to the phosphorylated tyrosine receptor. Activated PI3 generates the second messenger phosphatidylinositol 3, 4, 5 triphosphates "PI (3, 4, 5) P3" through phosphorylation of lipid membrane phosphatidylinositol 4 monophosphate "PI (4) P" and phosphatidylinositol 4, 5 diphosphates "PI (4, 5) P2". PI (3, 4, and 5) P3 mediate signaling proteins such as: serine/threonine kinases involving protein kinase B "PKB" or called "Akt". Activated Akt is important in activating several of downstream pathways which function in preventing cellular apoptosis and enhancing protein translation and synthesis (Zakrzewska et al., n.d; Schlessinger, 2000). See Figure (9)

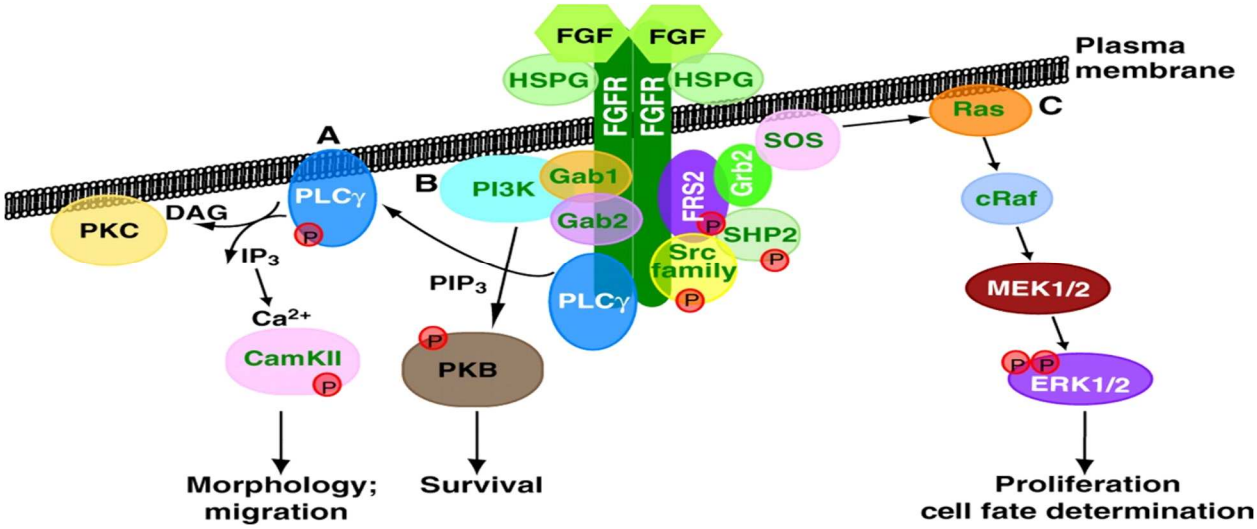

Figure 9: *Shows different signaling pathways which occurred through FGFR-FGF complex. Phosphoinositide 3-kinase pathway (PI3) activates cAkt/PKB which is considerably important in cell survival (Dorey& Amaya, 2010).*

IV. Diseases related to FGF and FGFR:

FGF/FGFRs complex signaling is important in regulating and developing several cellular processes, and this signaling should be highly regulated (Wesche et al., 2011). Nevertheless, several studies have shown that FGF signaling destruction can lead to some diseases including various types of cancer and large numbers of congenital disorders. Moreover, it can cause other pathological conditions such as: skeletal dysplasia, deafness, and cranial abnormalities (Sarabipour & Hristove, 2016). Mutations, single-nucleotide polymorphisms, and overexpression are three main causes of diseases.

IV.1. Mutations:

Deregulation and diseases production mostly occur through either the increase or decrease of functional mutations in the receptors or ligands (Teven et al., 2014). For instance, changing the glycine to arginine at 380 position in the transmembrane segment of FGFR3 can

result in achondroplasia. However, other changes in the extracellular domain of FGFR3 generate a new cysteine (S249C or Y373C). This results in connection between two receptors via a new disulfide bond and leads to lethal skeletal dysplasia. Therefore, a dimerization of the receptors through bond forces cause ligand independent signaling. Moreover, the previous two mutations in the FGFR3 can also be seen in bladder cancer. In addition, some mutations in the cytoplasmic domain of FGFR4 can cause a childhood sarcoma by triggering receptor's auto phosphorylation. Moreover, the receptor auto phosphorylation occurs in the absence of growth factors. Some mutations in growth factors, including FGF3, can cause deafness while Kallmann syndromes can be a consequence of mutated FGF8 (Wesche et al., 2011).

IV.2. Single-nucleotide polymorphisms (SNPs):

SNPs were expressed as changing of a single nucleotide which takes place at a specific site on the genome. SNPs were found in FGFRs to modify the malignant rates in certain cancer types. In FGFR4, metastatic possibility in different types of cancers, involving breast, skin, lung cancer, have become more aggressive and increased when the single nucleotide glycine at 388 was changed to arginine. The mechanisms of developing cancer by FGFR4 G388R are not completely known. However, it was proposed that FGFR4 G388R induced non-stop signaling because of lysosomal disorders (Wesche et al., 2011).

IV.3. Over expression:

FGFRs over expression can lead to ligand independent signaling. In FGFRs over expression, the interaction followed by phosphorylation between single receptors have occurred (Wesche et al., 2011). According to Wesche et al, the main cause of FGFRs over expression is having several copies of the gene among the chromosome because of gene amplification. For

example, breast cancer develops from 8p11-12 FGFRs amplification (2011). Moreover, either FGF1 or FGFRs over expression can cause paracrine or autocrine secretion which initiate cancer cell growth (Power, 2000).

IV.4. FGF3 and Labyrinthine or Michel aplasia:

Labyrinthine or Michel aplasia was defined as hearing loss and balance disorder because of missing a complete inner ear structure involving vestibule, semicircular canals, and cochlea. It affects more than 1,000 births. According to a study composed of 10 children, R95W, R132G, L6P, S156P, R104X, V2016S, and G66C are mutations related to this disease (Riazuddin et al., 2011). In mice, the FGF3 deficiency is not a cause of this syndrome, even though they do not have a normal inner ear. The main expression place of FGF3 in mice is the otic vehicles. One study proved that having abnormal inner ear can result from the absence of otic genes (Hatch et al., 2007).

IV.5. FGFR2, FGFR3, FGF10 and lacrimo-auriculo-dento-digetal syndroms (LADD):

LADD is also called Levy-Hollister syndrome. There are multiple symptoms associated with LADD including several congenital anomalies in lacrimal and salivary ducts and glands, respiratory system and kidney malformations, teeth, ear, and genitals abnormalities. A study has shown that several mutations in FGFR2 affects tyrosine kinase activity which lead to LADD. The FGFR2 mutations are A648T, A628T, R649S, G1882A, and G1942A. Moreover, not only mutations but also deletions of the amino acid (sp650) lead to LADD. According to Rohmann et al., mutations (G1537A, D513N) were found in FGFR3 and lead to LADD. Moreover, it was obvious that G137T and C106F are two main mutations found in FGF10 and caused LADD (2006).

IV.6. Kallmann Syndrome and FGF8:

Kallmann Syndrome, a known genetic disorder exhibit the symptoms of hypogonadotropic and anosmia. It results from a gonadotropin releasing hormone deficiency. Kallmann Syndrome was first clinically identified in 1944. Multiple mutations (H14N, K71E, R98G, K100E, R127G, T200M, and T229M) in FGF8 are leading causes of this syndrome. Structural studies on FGF8 shows that theses mutations impair FGF8 interactions with heparin and result in decreasing FGF8 signaling pathways (Hardelin & Dode; Falardeau et al., 2008).

IV.7. Apert syndrome and FGFR2:

Apert syndrome is characterized by early fusion of skull bones. This premature fusion inhabits normal skull growth, which cause midface hypoplasia, mental retardation, developmental delay, and syndactyly of toes and fingers. Two mutations (S252W, P253R) are responsible for brain dysmorphologies. These mutations affect FGFR2 signaling pathway because they increase ligand or receptor binding affinity. Therefore, an excessive activation was stimulated (Teven et al., 2014; Yu et al, 2000).

V. The structure of FGF1:

Our lab focuses on studying the properties of FGF1 as wound healing agents for medical application. FGF1 is called acidic fibroblast growth factor due to its acidic isoelectric point (~6.52).

FGF1is a single polypeptide that contains approximately 140 or 154 amino acids. The FGF1 which consists of 140 amino acid residues is considered to be a truncated form. The first 14 amino acids in the complete form is a flexible loop which prone to proteolytic cleavage.

Removing this flexible loop participates in increasing the rigidity of the protein and show similar biological activity as the complete form. FGF1 can be either monomer or homodimer with molecular weight ranges between16-17kDa. Similar to fibroblast growth factor family, the structure of FGF1 is β-trefoil fold, which mainly consists of 12 β-antiparallel strands. Six β-strands, β1, β4, β5, β8, β9, and β12 (numbering from FGF1-N terminal domains), are arranged in antiparallel barrel form. This barrel shape is made via β-sheet interactions, which has the remaining β-strands. There are several loops that connect between β-strands (3 and 4, 7 and 8, and 11 and 12). Both loops and β turns are essential in receptor recognition following receptor binding (Seddon et al., 1994). One feature of β-trefoil architecture that it contains a 50 cubic angstrom central cavity. This cavity has several amino acids such as L14, L23, L-44, I56, F85, Y97, V109, F132. As mentioned previously, FGF1 has a high heparin binding affinity, so heparin is used to purify FGF1in Dr.Kumar's lab. Even though FGF1 has three cysteines (Cys16, Cys83, Cys117), it does not contain disulfide bonds (Zhu et al, 1991) & (Zakrzewska et l., n.d).

VI. Mutational studies reported on FGF1:

FGF1 has been studied since it plays a significant role in endothelia cell proliferation, migration, and angiogenetic function. Several mutations were engineered in FGF1 to see if the mutation affects the protein stability, cellular proliferation, and binding affinity. In early FGF1 mutational study, it was proven that the replacement of lysine K118 with glutamic acid (E) could reduce FGF's heparin binding, although FGF was capable of binding to FGFR (Conrad, 1998). In 1990, Burgess et al. studied the effects of altering K132 to E. This mutation displayed heparin binding and mitogenic activity reduction although it stimulated receptor binding affinity and tyrosine kinase activity. Furthermore, the results demonstrated that K132E was able to trigger proto-oncogene expression. In 1991, a substitution of two of the three cysteine residues with

serine (S16, S83, S117) or two of the three cysteines (S16 S83, S16 S117, S83 S117) were made. Mitogenic assay showed different biological activities for theses mutations. In the presence of heparin, S83 S117 was active as wild types FGF1 (WFGF1) while S16 exhibited less activity. Additionally, S16 revealed minimum activities in the absence of heparin though S83 and S117 had similar activity. These mutations were more active than WFGF1 only if they contained one or no cysteines. Stability studies revealed various results. To illustrate, S16 was less stable than WFGF1 in the absence of heparin. Conversely, S16 S83 S117 had longer life and larger stability than WFGF1 (Ortega et al., 1991). In 1995, eight mutations (K23G, K24G, K26G, K114G, K115G, K126G, K127G, K132G) were designed in FGF1 heparin binding domain to determine the level of their binding to heparin. This study was based on computer modeling. Results verified that K23G, K24G, and K26G did not influence heparin binding activity whereas K114G, K115G, and K126G slightly reduced heparin binding affinity. K127G and K132G were only two mutations that lost their binding affinity to heparin and they could bind to FGFR as well as WFGF1 (Wong et al.). In 1997, Klingenberg found out the reason behind mitogenic activity reduction of K132E. He reported that K132E prevented FGF1 transmission to the cell nucleus. Thus, K132E FGF1 was not capable for triggering DNA synthesis. Moreover, WFGF1 is usually phosphorylated by protein kinase C, but this phosphorylated feature does not apply to K132E. In 1998, since protein kinase C (PKC) could not phosphorylate K132E, Klingenberg carried out multiple mutations in the phosphorylated region of FGF1. The amino acid residue S130, which is in the phosphorylation region, was substituted to either glutamic acid (S130E) or alanine (S130A). A similar mutation on Lys132 was produced, but K132 was mutated to Ala (K132A) and Arg (K132R) instead of glutamic acid. Also, a double mutant of S130E, K132A was made and it was a phosphorylated control. Another double mutation of Cys131 to Ser and Lys132 to

Glu was produced (C131S/K132E). After phosphorylated analysis, results confirmed that S130E or S130A were not phosphorylated, although S130 is the main phosphorylated position in FGF1. Moreover, this mutant exhibited high affinity for heparin as well as WFGF1. Similarly, the double mutation C131S/K132E, which can exhibit a potential phosphorylated site, was not phosphorylated. However, K132E or K132R showed a faint phosphorylated band. In fact, either K132E or K132R is PKC's substrate, and they reduced heparin binding affinity. Mitogenic and cellular proliferation tests indicated that S130E was more potent and trigger more cellular proliferation than WFGF1, but S130A had similar mitogenic activity as wild type and less proliferation activity. Nevertheless, both K132A and S130E/K132A were less potent and exhibited less cellular proliferation. Overall, FGF1 phosphorylation is not essential for its mitogenic effect, and K132 is significant for not only heparin binding but also mitogenic activity. In 1999, double mutants were designed in FGF1 by the replacement of tyrosine at 139 (Y) to phenylalanine(F) and cysteine 131 (C) to serine (S) in order to see the mutational effects on heparin binding. It was reported that this double mutant growth factor lost its heparin binding activity. Losing heparin properties was affected by C131S because C131 is essential in heparin binding while Y139F stimulated heparin binding affinity as WFGF1 (Patrie, Botelho, Franklin, & Chiu). In 2000, four point mutations (C16S, C83S, C117S, H93G), two double mutations (C83S/C117S) and (C16S/H93G), A triple mutation (C16S/C83S/C117S), and quadruple mutation (C16S/C83S/C117S/H93G) were made to test protein stability. It was found that the two single mutations C117S, H93G, the double, triple, and quadruple mutations were more stable than WFGF1 due to the replacement of cysteine 117 to serine and histidine 93 to glycine (Culajay, Blaber, Khurana, & Blaber). In 2004, five point mutations (H21Y, L44F, H102Y, F108Y, V109I), double mutant (H21Y/L44F), triple mutant (H21Y/L44F/H102Y), and

quadruple mutant (H121Y/L44F/H102Y/F108Y) were engineered in FGF1 to see the effect of all these mutations in protein stability and cellular proliferation activity. The results proved that all these mutations improved FGF1 stability as well as mitogenic activity (Zakrzewska, Krowarsch, Wiedlocha, & Otlewski). In 2005, Zakrzewska and his coauthors showed several mutations on FGF1 led to increase FGF stability more than WFGF1. These mutations include a double substitution (Q40P/S47I), a triple mutation (Q40P/ S47I/H93G), and sextuple mutations (H21Y/Q40P/S47I/L44F/H93G/H102Y/F108Y). Further mitogenic, binding, and proteolytic studies were carried out on these mutations. These mutations stimulated mitogenic activity and DNA synthesis as WFGF1in the presence of heparin, while only triple mutant type showed more mitogenic activity and DNA synthesis than WFGF1 in the absence of heparin. All these mutations were strongly bound to FGFR as WFGF1. In proteolytic assay, it was proved that sextuple mutations were easily degraded by trypsin while double and triple mutations were highly resistance to trypsin (Zakrzewska, Krowarsch, Wiedlocha, Olsnes, & Otlewski). In 2006, a number of mutations in FGF1 (Y15A, E87A, Y94A, N95A, Y94A/N95A) caused weak binding to FGFR. The reason behind the diminishing in receptor binding is that these mutations were engineered in the receptor binding position where several of intrinsic amino acids play a significant role in FGF1binding to the receptor. For instance, both tyrosine (Y15, Y94) are important in forming a hydrophobic interaction with A167 and P169 in FGFR. In addition, Y15 is significant in making two hydrogen bonds with L165 and A167. N95 plays an essential role in connecting the growth factor to the receptor through binding to the D2-D3 linker (Zakrzewska, Krowarsch, Wiedlocha, Olsnes, Otlewski). In 2009, multiple mutations were designed in the heparin binding pocket of FGF1 in order to increase its stability and reduce heparin binding affinity. These mutations include six point mutations (K112A, K112E, K118N, K118A, K118E)

and one double mutation (K112N/K118E). Thermodynamic stability and heparin binding assay revealed that all the single point mutations except K112N showed less thermal stability but all of them showed less heparin binding than WFGF1. However, the double mutant was more stable than WFGF1and could not bind to heparin. In mitogenic activity assay with heparin present, K112A, K112E, K118A, K118E and K112N/K118E were less potent than WFGF1 whereas both K112N and K118N stimulated cellular proliferation activity as WFGF1. Proteolytic test (without heparin) proved that all the mutations were susceptible and easily degraded by trypsin, with the exception of K112N and the double mutant K112N/K118E which were considerably resistance to trypsin (Zakrzewska et al.). In 2012, single mutation (A66C), triple mutation (C83T/C117V/K12V) and quadruple mutation (C83T/C117V/L44F/F132W) demonstrated heparin binding affinity, higher stability, and higher mitogenic activity than WFGF1. Because of high biophysical properties, these mutations can be significantly used for therapeutic applications in the future (Alsenaidy et al.). In 2014, substitution of the three-buried cysteines (Cys16, Cys83, Cys117) to alanine, serine, threonine, and valine were designed to test the stability effect of the mutations. The result confirmed that one of the previous mutations, C117, contributed to protein stability only if it was mutated to alanine (Xia, Longo, & Blaber).

VII. The different classification of the FGF and their individual functions

FGF ligand	Location	Molecular weight	Function	References
FGF1	[4] 5q31	[5] 16	Physiological function: there is no specific function for FGF1, but it functions in angiogenesis as all FGF and wound healing process (1). FGF1 knockout: can cause resistance against insulin in mice when they are under high diet (2). Medical applications: FGF1 can be a treatment for diabetic hyperglycemia in rodent without increasing any risk of hypoglycemia (3).	[1]Xue at al., 2002. [2] suh et al., 2014. [3] Scarlett et al., 2016. [4]Ornitz&Itoh, 2001. [5] Burroughs, 2016.
FGF2	[4] 4q26-27	[1] 18 22.5 23.1 24.2	Physiological function: similar to FGF1, there is no certain function for FGF2, but it plays an important role in vascular tone such as cardiovascular development (1). FGF2 knockout: causes an increasing in mineral density in mice bones including femurs and lumbar vertebrate (2). Medical application: FGF2 is a novel therapeutic protection and treatment for coronary artery disease, and it is a stress reduction factor from ischemia. Moreover, FGF2 is very important for prostate cancer treatment (1,3).	[1] Liao et al., 2009.[2] Bouthiette et al., 2014. [3] Dorey & Amaya, 2010. [4]Ornitz&Itoh, 2001.
FGF3	[2] 11q13	[3] 28-32	Physiological function: FGF3 is necessary for inner ear improvement. FGF3 Knockout: causes Labyrinthine or Michel aplasia (1). Medical application: so far, there is no therapeutic implementation for FGF3.	[1] Vendrell et al., 2000. [2] Ornitz&Itoh, 2001. [3] Burroughs, 2016.

Table 6: *Shows different types of FGFs family, their chromosomal locations, molecular weights, and functions.*

FGF ligand	location	Molecular weight	Function	References
FGF4	[2] 11q13.3	[3] 22	Physiological function: FGF4 plays an essential role in vertebrate embryogenesis. FGF4 knockout: causes lethal effects on embryonic mice after implantation. Medical applications: FGF4 can be used as inhibitor for testicular tumors (1).	[1] Kosaka et al., 2009. [2]Ornitz&Itoh, 2001.[3] Burroughs, 2016.
FGF5	[2] 4q21	[3] 32-38	Physiological role: FGF5 is anti- hair growth factor. FGF5 knockout: causes trichomegaly or long hair growth in human. Medical application: there is a possibility of creating FGF5 inhibitor in order to promote hair growth (1).	[1] Higgins et al., 2014. [2]Ornitz&Itoh, 2001. [3] Burroughs, 2016.
FGF6	[3] 12p13	[4] 25	Physiological role: FGF6 plays a critical role in myogenesis (muscle regeneration). FGF5 knockout: causes abnormal muscle regeneration with fibrosis and muscle degeneration with freeze crush injury (1). Medical application: FGF6 injection is able to increase soleus muscle regeneration in mice (2).	[1] Armand et al., 2006. [2] Armand et al., 2003. [3]Ornitz&Itoh, 2001. [4] Burroughs, 2016.

Table 4: *Shows different types of FGFs family, their chromosomal locations, molecular weights, and functions.*

FGF ligand	Location	Molecular weight	Function	References
FGF7	[4] 15q15-21.1	[5] 28	Physiological function: FGF7 is beneficial for liver regeneration and liver injury repair (1). FGF7 knockout: causes epileptic seizures in mice (2). Medical application: FGF7 is a treatment for burned skin. It promotes bioengineered skin growth (four times faster than normal growth) (3).	[1] Steiling et al., 2003.[2] Lee et al., 2012. [3] Erdag et al., 2004. [4] Ornitz&Itoh, 2001. [5] Burroughs, 2016.
FGF8	[3] 10q24	[4] 28-32	Physiological function: FGF8 is a regulator for ventral telencephalon. FGF8 knock out: causes abnormal and small telencephalons in mice (1). Also, it triggers Kallman syndrome in humans where the palate is cleaved (2). Medical application: there is recent medical application of FGF8.	[1] Storm et al., 2006. [2] Dorey& Amaya, 2010. [3] Ornitz&Itoh, 2001. [4] Burroughs, 2016.

Table 5: *Shows different types of FGFs family, their chromosomal locations, molecular weights, and functions.*

FGF ligand	Location	Molecular weight	Function	References
FGF9	[5] 13q11-q12 of exon 4	[6] 30	Physiological function: FGF9 is important for lung development because it is necessary for epithelial branching and mesenchymal growth. FGF9 knockout: causes perinatal death in mice because of uncompleted respiratory system (1). In humans, knockout this factor can lead to lung cancer, colorectal and ovarian carcinoma (2,3). Medical application: there is no present therapeutic application of FGF9. However, FGF9 over expression can lead to anxiety disorders. Therefore, there is a potential drug will be designed in the future to inhibit the excess FGF9 and modulate the mood and behavioral disorders (4).	[1] Yin et al., 2015. [2] Ohgino et al., 2013. [3] Dorey& Amaya, 2010. [4] Aurbach, 2015. [5]Ornitz&Itoh, 2001. [6] Burroughs, 2016.
FGF10	[3] 5p12-p13	[4] 30	Physiological function: FGF10 play a significant role in embryonic morphogenesis involving lung, limb, and brain development. Knockout FGF10: causes perinatal death in mice because their lungs are completely absent (1). Medical application: exogenous FGF10 is able to trigger eyelid closure in eye-opened mice at birth (2). Medical application: there is no specific recent medical application published about FGF10	[1] Min et al., 1998. [2] Tao et al., 2006. [3] Ornitz&Itoh, 2001. [4] Burroughs, 2016.
FGF13	[4] Xq26	[5] 22	Physiological function: FGF13 is expressed in the nervous system. Therefore, FGF13 involved in regulating the function of sodium channel as well as peripheral and central nervous system development. FGF13 knockout: causes inhibiting then increasing in synaptic excitatory (1,2). Because FGF13 participates in cerebral cortex development, its deficiency can result in mental retardation (3). Medical application: none	[1] Puranam et al., 2015.[2] Hartung et al., 1997. [3] Xu et la., 2012. [4] Ornitz&Itoh, 2001.[5] Burroughs, 2016.

Table 6: *Shows different types of FGFs family, their chromosomal locations, molecular weights, and functions.*

FGF ligand	Location	Molecular weight	Function	References
FGF14	[3] 13q34	[4] 27	Physiological function: like FGF13, FGF14 contributes in cerebral cortex development (1). FGF14 Knockout: causes paroxysmal dystonia, cognitive disorders, and ataxia in mice (2). Medical application: none	[1]Xu et la., 2012. [2] Laezza et al., 2007. [3] Ornitz&Itoh, 2001. [4] Burroughs, 2016.
FGF16	---	[2] 26	Physiological role: FGF16 plays a fundamental role in cardiomyocyte proliferation and heart development. FGF16 knockout: in embryonic mice, FGF16 knockout results in decreasing in the number of cardiac cells as well as hart weight even though the blood pressure and heart performance in effected embryonic mice remains the same as normal mice (1). Medical application: none	[1] Hotta et la., 2008. [2] Burroughs, 2016.
FGF17	[2] 8p21	[3] 25	Physiological role: FGF17 assists to proliferate and differentiate the cerebellar structure. FGF17 knockout: causes severe effects on mice involving losing their cerebellar primordium at early fatal age (1). Medical application: none	[1] Xu et al., 2000. [2] Ornitz&Itoh, 2001.[3] Burroughs, 2016.

Table 7: *Shows different types of FGFs family, their chromosomal locations, molecular weights, and functions.*

FGF ligand	location	Molecular weight	Function	References
FGF18	[3] 8p21.3	[4] 31	Physiological function: FGF18 is important in cellular proliferation including: chondrocytes and osteoblast (1). FGF18 knockout: causes skeletal abnormalities and uncompleted respiratory system in mice. Medical application: FGF18 knockout can result in rising chondrocyte proliferation in mice. When these mice were treated with exogenous FGF18, FGF18 was able to drop this negative impact (2).	[1]Yun et al., 2010. [2] Haque& Hamdy, 2007. [3] Ornitz&Itoh, 2001. [4] Burroughs, 2016.
FGF19	[4] 5q35.1		Physiological function: FGF19 regulates the metabolism of lipid and glucose (1). FGF19 knockout: leads to increase in the bile acid rate (2). Medical application: It is possible to use FGF19 in diabetic treatment (3).	[1] Wu&Li, 2012. [2] Luo et al., 2014.[3] Perry1et al., 2015. [4] Ornitz&Itoh, 2001.
FGF20	[3] 11q13.3	[4] 23	Physiological function: FGF20 is important in maintaining fatal nephron progenitors. FGF20 knockout: causes absence of kidneys (1). Medical application: there is a potential usage of FGF20 in Parkinson diseases treatment (2).	[1] Bakar et al., 2012. [2] Yun et al., 2010. [3] Ornitz&Itoh, 2001.[4] Burroughs, 2016.

Table 8: *Shows different types of FGFs family, their chromosomal locations, molecular weights, and functions.*

FGF ligand	Location	Molecular weight	Function	References
FGF21	[3] 19q13.32	[4] 19.5	Physiological function: FGF21 serves as a metabolic agent to control the metabolism of lipid and glucose in several targets involving liver, pancreas, brain, and adipose tissue (1). FGF21 knockout: mice knockout of FGF21 have low lipolysis rate in adipocytes. That results in increasing the level of fatty acids (2). Medical application: FGF21 can be used as a therapeutic factor for obesity and diabetes (1).	[1] Ge et al., 2012. [2] Itoh& Ornitz, 2010. [3] Ornitz&Itoh, 2001.[4] Burroughs, 2016.
FGF22	[4] 19p13.3	----------	Physiological function: FGF22, which is an endocrine, participates in hair progression because FGF22 is secreted in the hair follicles (1). FGF22 knockout: in mice, it was proved that inhibition or knocking out of FGF22 contributes in relieving epileptic seizures (2). Medical application: FGF22 is a therapeutic agent for weak wound healing (3).	[1] Nakatake et al., 2001. [2] Lee&Hisashi, 2013. [3] Beyer et al., 2003. [4] Ornitz&Itoh, 2001.
FGF23	[2] 12p13.32	----------	Physiological function: FGF23, which preforms as hormones, contributes in regulating a phosphate and vitamin D level in the serum. FGF23 knockout: knocking out the FGF23 in mice shows a high rate of phosphate and calcium in the serum. Medical application: FGF23 is a potential treatment for hypophosphatemic diseases (1).	[1] Shimada at al., 2016. [2] Ornitz&Itoh, 2001.

Table 9: *Shows different types of FGFs family, their chromosomal locations, molecular weights, and functions.*

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Abstract:

Fibroblast growth factor1 (FGF1) has played a significant role in medical applications including repairing injuries. Because FGF1 is partially denatured at physiological conditions, penta mutations Q40P, S47L, H93S, K112N, and R122E were engineered in different sites of the protein to increase protein stability and mitogenic activity. Since pFGF1 lost heparin binding ability, pFGF1 was not purified by heparin as WFGF1. The pFGF1 was purified by nickel because of inserting poly His tag in its N-terminal. Based on NMR and fluorescence results, the tertiary structure of pFGF1 is different from WFGF1. The pFGF1 exhibits more stability than WFGF1 because thermodynamic study shows that pFGF1 rises the denatured temperature by ~20 ºC. Also, urea denaturation study illustrates that pFGF1 is partially unfolded after being exposed to 4M urea. Moreover, pFGF1 is not effected by trypsin because it shows high resistance to proteolytic enzymes even if the time of incubation is increased. Despite the high stability, pFGF1 is more potent than WFGF1.

Chapter2: Engineered Human Acidic Fibroblast Growth Factor (FGF1) with an Enhanced Thermal and Proteolytic Stability

Introduction:

Acidic fibroblast growth factor (FGF1), which is mostly made up of antiparallel β-sheet forming a β trefoil structure, belongs to fibroblast growth factor family (Zakrzewska et al., 2009). Several cellular types involving, fibroblasts, cardiomyocytes, macrophages, and endothelia cells, are responsible for FGF1 secretions (Palmen et al., 2004). Growth factors in general play a significant role in cellular proliferation, differentiation, migration as well as cellular survival. In addition, several studies indicate that FGFs can be used as therapeutic agents for multiple diseases such as Ischemia diseases (Zakrzewska, n.d). FGF1 shows its activity only if it binds to one of four extracellular receptors (FGFR1-FGFR4). Because of binding FGF1 to FGFRs, FGFR is dimerized and autophosphorylated (Spivak-Kroizman, 1994). FGFR phosphorylation promotes several downstream signaling pathways, one of which is a mitogen activated protein kinase pathway (MAPK). As stated, FGF1can cross the cell and perform intracellular functions (Mohammadi, Olsen, & Ibrahimi,2005).

FGF1 consists of either 154 or 140 amino acids residues after the starting amino acid (methionine) is cut off (Ornitz, & Itoh, 2001). Two truncated FGF1, which built of 21-154 and 15-154 amino acids, were biologically characterized and show equivalent activity as in intact FGF1 (Zakrzewska, n.d).

Even though FGF1 shows weak thermal stability, FGF1 tightly binds to heparin. Heparin consists of repeating alpha 1,4 disaccharides. Every disaccharide unit is made of amino sugar (N-Acetyl-D-glucosamine) and uranic acid (L-iduronic acid) (Waksman, & Herr, 1989). Heparin is

very effective in protecting FGF1 from denaturation by several thermal, chemical, and proteolytical factors (Culajay et al., 2000).

Until now, heparin's role on FGF1 is still a contentious issue. Many studies demonstrate that heparin is necessary for triggering FGF1 signals, dimerizing FGFRs, and stimulating DNA synthesis as well as cellular proliferation activity (Wu et al., 2003; Waksman, & Herr,1998). However, other studies prove that heparin only helps FGF1 in binding to its receptors even though others show that FGF1 is able to interact with FGFRs and activate MAPK pathways in the absence of heparin (de Paz et al., 2001; Delehedde et al., 2002).

FGF1/ heparin binding is controlled by several positive amino acids residues located in the heparin binding pocket. These amino acids include K112, K113, K118, R119, R122, K128. In addition, not only these positive amino acids but also some polar amino acids including N114 and Q127 participate in heparin binding (Zakrzewska et al., 2009). Beside heparin binding function, it was found that K118 is important in FGF1 biological function. The mutation of K118 to E inhabits cellular proliferation activity, even though K118E did not reduce FGFRs binding affinity as well as promote signal cascade pathways (Klingenberg et al., 1989).

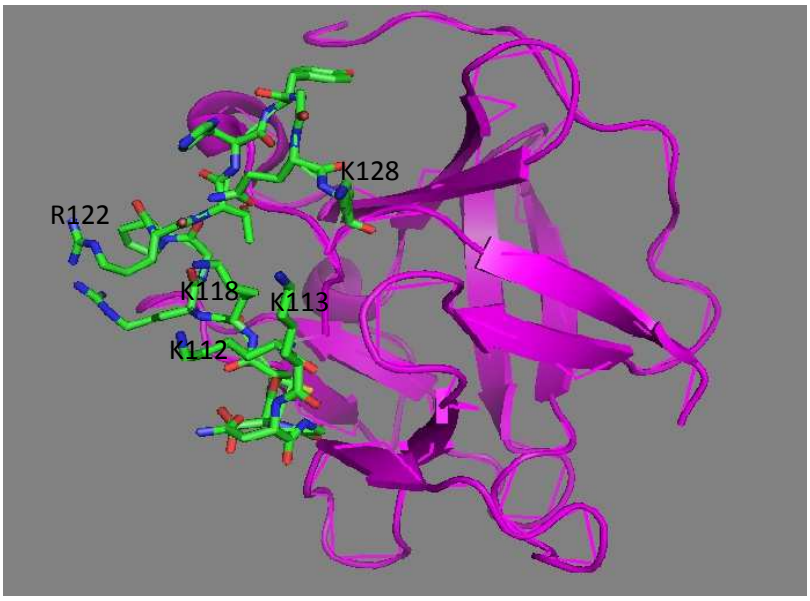


Figure 1: *A PyMOL image which shows the heparin binding pocket (green color). Some amino acids residues, which interact with heparin, are labeled in this figure (PDB1RG8).*

In previous work, a triple mutation (Q40P, S47I, H93G) was introduced in FGF1 to increase protein stability. This mutation strongly binds to FGFR, showing more mitogenic activity and DNA synthesis than WFGF1 in the absence of heparin, increases protein stability, and exhibits resistance to proteolytic and thermal factors (Zakrzewska et al., 2005). In the heparin binding pocket, a single point mutation K112N was inserted to have more understanding about the impacts of this mutation on FGF1. The results prove that K112N increase FGF1 stability, reduce heparin binding activity, and show mitogenic activity as WFGF1 (Zakrzewska et al., 2009).

In this paper, five different mutations (Q40P, S47L, H93S, K112N, and R122E) were introduced in FGF1 to also increase protein stability and enhance protein biological activity. Two of these mutations (K112N and R122E) were inserted into heparin binding pocket. Our results are similar to previous work results. However, Zakrzewska et al did not determine the structural changes of the FGF1 after mutations. Also, he did not study the chemical effects, urea, on this

mutation. In our lab, structural changes on penta FGF1 (pFGF1) was studies by using NMR. It was found that pFGF1 has a different structure than WFGF1. Because pFGF1 is resistance to proteolytic enzyme, such as trypsin, we proved that truncated pFGF1can show biological activity even though it lost part of its structure.

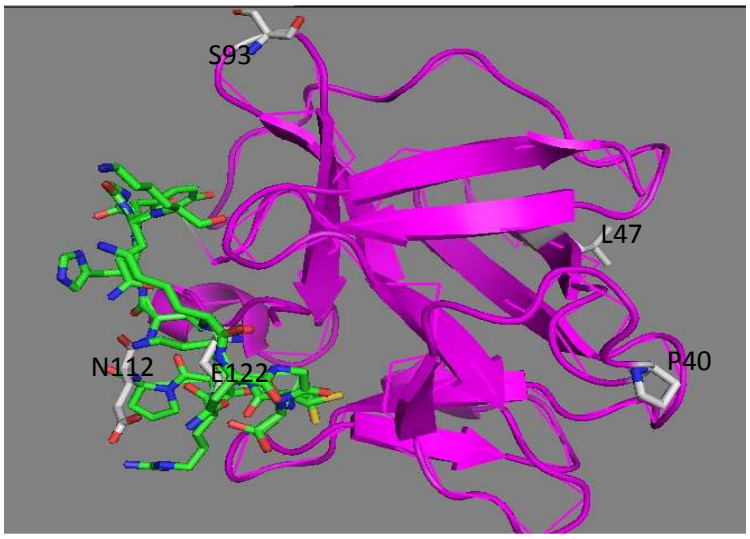


Figure 2: *A PyMOL image which shows different mutations (P40, L47, S93, N112, and E122) among FGF1 (PDB1RG8).*

Materials and methods:

All heparin and nickel spherose resins were obtained from GE Healthcare bioscience. All the low molecular weight of heparin (~3000kD), lysozyme, and trypsin (purified from pancreatic bovine) were obtained from Sigma. The antibiotic (ampicillin) was purchased from VWR. All of the chemical reagents and buffers were obtained by VWR, J.T.Baker,and Omnipur. The Isopropyl β-D-1-thiogalactopyranoside (IPTG) was purchased from Omnipur. All the labeled ammonium chloride (N^{15}) was purchased from ISOTEC. All the components of cell culture media were purchased from EMD Millipore Corporation. Guanidine hydrochloride was purchased from Amresco. A mixture phosphate buffered saline with 100mM NaCl (PBS) with ranging pH (from 6.9 to 7.2) was used in this study. All the proteins were biophysically and

biochemically characterized in a buffer having 50mM of $(NH_4)_2SO_4$, 100mM NaCl, and 10mM of PBS. In the all heparin experiments, a molar ratio 10:1 of the heparin was used.

1) Site Directed Mutagenesis and bacterial transformation:

All the five mutations were engineered on FGF1 plasmid by using the Quick Changes site directed mutagenesis kit (Agilent Inc. USA). DNA sequencing was verified to establish the correctness of the designed mutations. Two of the mutations were made in the heparin binding pocket of FGF1, while the remaining three mutations were applied at different sites.

1µL of the vector pET20b-pentaFGF1 plasmid was added to 100µL of either BL-21 (DE3) or BL-21 (DE3) pLysS competent *E. coli* cells. Well-standardized heat shock based transformation, procedure was employed to transform the cells. The following steps were used a mixture of the competent *E.coli* cells and the plasmid of pFGF1 was placed in a water path for 45seconds. Next, 800µL of the sterile lysogeny broth (LB) was added to the cells in order to nurture them while they were incubated in a shaker at 250rpm for 45 minutes at 37ºC. A 100µL of competent *E.coli* and the plasmid of pFGF1 was spread on an Agar plate which consisted of the desired concentration antibiotic (25µg/µL). The Ampicillin (Amp) was used since FGF1 plasmid has genes resistant to Amp. Therefore, the only cells that can survive are BL-21 (DE3) and BL-21 (DE3) pLysS competent E. coli cells which have the FGF1 plasmid. Finally, the Agar plate that has the bacterial cells and the Amp was stored upside down in an incubator for 14-15hours at 37ºC.

2) **Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE):**

All the protein samples were tested by using this technique via 15% polyacrylamide gel. The gel was running in 200 V, 100 mA. Then, it was stained by coomassie Brilliant Blue dye and destained by a destainer (composition of methanol and acetic acid).

3) **Precipitation of protein by trichloroacetic acid (TCA):**

TCA is an acidic compound that consists of ester (COO+ OH) and salt (CL-). Before running the protein samples, a specific amount of TCA (10% of the protein volume) was added. When additional of TCA is added, more protein precipitation occurs. The samples were centrifuged, discarded, washed with 100% acetone, and centrifuged again. The pellets were dried by heating block, and dissolved in 8M urea and blue loading dye (made up from 95% of blue dye plus 5% of 2-mercaptoethanol). The purpose of doing TCA preparation is to have complete denatured protein through disrupting non-covalent bonds and the addition of a reducing agent (2-mercaptoethanol) to reduce any disulfide bonds or disulphide bridges.

4) **Recombinant protein expression:**

The small-scale expression was done through inoculation of one or more colonies (from the Agar plate) into 10mL of LB broth containing 10µL Amp for 14-15 hours. Next morning, 5% of the overnight culture was inoculated into 10mL of LB having 10µL of Amp and incubated at 37ºC for 2.15 hours at 250rpm. After 2.15 hours, the culture was checked by UV visible spectrometer at wavelength 600nm to measure the optical density (OD). When the culture met the required OD (between 0.6 to 0.8), it was induced by 9µL of the isopropyl β-D-1-thiogalactopyranoside (IPTG). 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 pFGF1 in BL-21 (DE3) and BL-21 (DE3) pLysS competent E. coli cells. After 3.5 hours, the bacterial cells were collected, sonicated, then centrifuged in order to obtain the protein of interest. Four samples were run in the SDS-PAGE preinduce, postinduce, pellet, and supernatant. The purpose of doing small scale expression before large scale expression is to check the presence of protein and not waste time and money. Similar procedures were used for expressing the larger volumes of the culture.

5) Protein Purification:

5.1. Purification by affinity chromatography*:*

To purify the pFGF1, an affinity chromatographic nickel- Sepharose column (approximately 25ml bed or resin capacity with diameter 1cm) was used. The nickel- Sepharose column was used due to the presence of His tag in the N terminal of pFGF1. Poly His tag was engineered in the N terminal to simplify the purification process in case if the protein shows reducing bindng affinity to heparin. The bacterial pellets, which were stored at -20ºC and containing the pFGF1 protein, were thawed and resuspended in 30ml of a 1XPBS buffer at a pH range from 6.9 to 7.2. The bacterial cells lysis through ultrasonication with a power 13 output for 30 cycles by operating the ultrasonication 10 seconds on plus 10-seconds off with five minutes resting time every 10 cycles. The cell lysate was centrifuged for 30minutes at 19000rpm. Before loading the supernatant, which has the pFGF1, the nickel column must be pre-equilibrated with 1XPBS. After running the supernatant at speed of 1ml/minute, the nickel column should be wash with 1XPBS. Next, wash the column with 10mM of PBS containing different imidazole gradient 20mM, 100mM, 250mM, and 500mM at a pH6.9 to7.2. All the fractions from the purification

were collected and prepared them for SDS-PAGE analysis by doing TCA precipitation. The pure protein was determined in the SDS-PAGE by a thick band that matches FGF1's molecular weight (~16kDa). The pure pFGF1 was dialyzed for 3hours by using a dialysis bag with a cut off of 3500Da and 4000Da in a 50mM $(NH_4)_2SO_4$, 100mM NaCl, and 10mM PBS. To shrink down the volume for the pFGF1, ultra-Millipore centrifugal concentrator with a 30kDa cut off was used. After obtaining a high concentration of the pFGF1, the pFGF1 was kept in a -80 freezer for biophysical and biochemical characterizations.

However, the WFGF1 was purified by using a different column and different salt gradients. WFGF1 was purified by a chromatographic heparin-Sepharose column with an approximate capacity of 20ml of beads and 1cm in diameter. After loading the supernatant through the heparin-Sepharose column, a 10mM phosphate buffer with various NaCl concentration 250mM, 800mM, and 1500mM were passed over the column.

5.2. Size exclusion chromatography connected via Fast protein liquid chromatography (FPLC):

Fast protein liquid chromatography(FPLC) was used to get a pure pFGF1 through gel filtration (size exclusion) at 25ºC by using a Superdex -75 column. This experiment was carried out at different pHs. Before loading the protein (at 0.5mg/ml), the column must be pre-equilibrated a buffer containing 50mM of $(NH_4)_2SO_4$, 100mM NaCl, and 10mM of PBS. The flow through elution was adjusted to 1ml/min. This machine can detect the protein at 280nm. All the peaks fractions were collected, and run using SDS-PAGE.

6) Ultraviolet visible absorbance spectroscopy (UV):

UV spectroscopy is used in our lab to measure bacterial growth during small and large scale expression.

7) Fluorescence Spectroscopy:

Intrinsic fluorescence spectroscopy through F-2500 fluorometer (at 25ºC and 2.5nm resolution) was used to examine any alternation in the three-dimensional structure and folding manner of both pFGF1 and WFGF1 in the presence and absence of heparin. A quartz cell with a 1.0cm path length was used. The protein concentration of 0.1mg/ml was prepared for fluorescence measurements. Our samples usually are in a 50mM $(NH_4)_2SO_4$, 100mM NaCl, and 10mM PBS. A 280nm was used as an excited wavelength, but all the data for WFGF1 and pFGF1 was acquired at an intrinsic wavelength range between 300-450nm. Intrinsic fluorescence was checked at these wavelengths to study if there were any changes among tyrosine (308nm) and tryptophan (350nm) in the pFGF1 compared to WFGF1.

8) Circular Dichroism (CD) Spectroscopy:

Jasco 1500 spectropolarimeter was used to study the secondary structural changes among FGF1 after mutations. A data of pFGF1 and WFGF1 in the presence and absence of heparin was collected from protein with a concentration of 0.5mg/ml. The samples were in a 50mM $(NH_4)_2SO_4$, 100mM NaCl, and 10mM PBS, and were loaded in a quartz cell whose path length is 2cm. The wavelength of a Jasco 1500 spectropolarimeter was set in a range of 190- 250nm at 25 ºC, and the scanning speed was 20nm/min. The data of CD was collected as an average of three scans. Molar ellipticity was calculated and plotted against a wavelength.

9) Isothermal Titration Calorimetry (ITC):

ITC was performed to see if the mutations affect the binding affinity of the pFGF1 to heparin comparing that with WFGF1. All the measurements were done by a MicroCal ITC_{200} at 25ºC. For protein samples, two different concentrations were prepared 50mM and 100mM pFGF1 in a buffer solution consisting of 50mM $(NH_4)_2SO_4$, 100mM NaCl, and 10mM PBS. 1000mM and 5000mM heparin, with a molar ratio of 10:1 of heparin protein sample, were used. The samples were centrifuged before loading to remove any precipitation or aggregation. Moreover, before titration, the samples should be degassed. A titration correction was made to avoid any background heat changes. Then, the data was analyzed through one program available on origin software "a set of sites binding model". Moreover, the binding affinity of the protein was identified after subtracting the additional heparin by dissociation constant factor ($K_{d)}$.

10) Proteolytic digestion:

Trypsin digestion was done to confirm if both WFGF1 and pFGF1with and without heparin were resistant to proteolytic degradation or not. A water path at 37ºC was used for protein- trypsin incubation and TCA was used for stopping the enzymatic activity of the trypsin. Both WFGF1 and pFGF1 were in 50mM $(NH_4)_2SO_4$, 100mM NaCl, and 10mM PBS. To determine the optimal trypsin concentration, 10µL of 50µg pFGF1 and WFGF1 was added to 8 tubes. Seven different concentrations of the trypsin (0.001, 0.005,0.01,0.05,0.1,0.5, 1 mg/ml) were added to both proteins. After incubating the mixture of protein with trypsin in a water bath for 10 minutes, we usually stopped the proteolytic reactions by adding 10µL of TCA. After completing the TCA prep, all the samples were loaded in SDS-PAGE. The best trypsin concentration is 1mg/ml.

To start the experiment, lml of two different samples for pFGF1 and WFGF1 were made containing 1mg/ml of trypsin solution and 0.250mg/ml of protein. Two other samples were created (total of 4 samples), and they were similar to the previous ones, but they contained heparin (10X of the protein concentration). 100µL of every protein (with and without heparin) were taken before adding the trypsin to consider as a time zero sample. After adding the trypsin, we incubated the protein samples in a water bath for 40minutes. 100µL was taken every 5 minutes from four solutions and immediately the reaction was stopped by TCA. Over time, every sample (a total of 36 samples including zero-time sample) has a different level of trypsin digestion. All the samples were analyzed by SDS PAGE and a scanning analysis program.

11) 8-Anilinonaphthalene-1-sulfonic acid (ANS):

8-Anilinonaphthalene-1-sulfonic acid (ANS), which is known as extrinsic fluorophore, is used to preform structural and binding studies. ANS is an organic compound consisting of an amine group and sulfonic acid group.

ANS binding assay is used to illustrate if there are any alterations in the three-dimensional structure of the pFGF1 or folding properties by binding the ANS to hydrophobic pocket. A Hitachi F-2500 spectrofluormeter with an excitation of 380nm and emission of 450-600nm was used. Both pFGF1 and WFGF1 in the presence and absence of heparin were tested. 0.5mg/ml (31.25µM) of pFGF1 and WFGF1 and 5mM ANS stock were prepared. The heparin concentration was 10X of the protein. 1µL of ANS stock was added to the protein, and a measurement was taken. After adding 1µL of ANS at a time, the ANS concentration increased by 10µM.This process was repeated 25 times until the final ANS concentration reached 250µM.

Finally, after the data was overlaid and smoothed, the reading of relative fluorescence intensity (RFI) was taken at 520nm.

12) Thermal denaturation:

A Jasco 1500 spectropolarimeter was used to study thermodynamic stability changes in both WFGF1 and pFGF1(in the presence and absence of heparin). 1mg/ml of proteins were prepared in a buffer containing low salt concentration 5mM$(NH_4)_2SO_4$, 1mM PBs, and 10mM NaCl. The setting temperature was ranged from 20ºC to 90ºC. The data from a different wavelength at 228nm for CD and 308 and 350 for fluorescence was collected, overlaid, smoothed, and plotted. The graphing criteria for thermal denaturation was the reading of different spectra at 228nm against temperature as well as the average of 308/350 against temperature.

13) Urea denaturation:

Urea, which is an organic compound, is useful in protein denaturation due to non-covalent bonds distraction. Urea denaturation study was carried out by using a Jasco 1500 spectropolarimeter. 8M pure urea and 1mg/ml of WFGF1 and pFGF1 were used. Both Proteins were in a buffer containing low salt concentration. Urea denaturation parameters and collections are the same as thermal denaturation study.

14) Bioactivity assay:

The bioactivity of the pFGF1 was studied to check if the mutations influence the cellular proliferation function of pFGF1. 1mg/ml WFGF1 and pFGF1 were prepared in a buffer containing 50mM ammonium sulfate, 10mM PB, and 100mM NaCl. Different concentrations of

the protein starting from (0.4 to 50ng/ml) were tested on NIH/3T3 fibroblast cell lines. The desired proteins were usually inoculated to NIH/3T3 fibroblast cell lines and incubated for 24 hours then counted.

15) Nuclear magnetic resonance (NMR spectroscopy):

All the NMR studies were performed to check the changes of the three-dimensional structure of the protein after mutations, and it was done by Bruker Avance 500mHz. The protein sample is required to have N15 isotope during its expression and deuterium D2O after its purification in order to run heteronuclear single quantum coherence spectroscopy (1H-15N HSQC) at 25ºC. The concentration of the protein sample should be between 150µM to 500µM in a buffer having 50mM $(NH_4)_2SO_4$, 10mM PBs, and 100mM NaCl. The spectra of 1H-15NHSQC was scanned 64 times at various pHs. The spectra were analyzed through sparky software.

16) Molecular dynamic simulation:

pFGF1 were designed by using 1RG8 file from the protein data bank. Then, molecular dynamic simulations of pFGF1 were run through using pFGF1/1RG8 file. Before running the simulation, the protein structure should be in a relaxed form through subjecting the simulation system to several refinement steps. This can relax protein's backbones, side chains, Cl^- and Na^+ ions, and surrounding water molecules. To minimize and equilibrate the system, isothermal isobaric (NTP) ensemble was carried out along with CHARMM36 or CHARMM27 force field and NAMD 2.9. The simulation was performed at 50/100ns through using 2-fs time steps to determine the protein stability and evaluate different changes in noncovalent interactions. Finally, visual molecular dynamics (VMD) was used for data analysis.

Results:

Significance of the project:

The purpose of this research work is to enhance the stability of FGF1 protein by introducing mutations using a knowledge based approach. From the literature, it was shown that mutations of residues (Q40P, S47I & H93G) contributed to an increased thermal stability. Here in this research study, we have introduced an additional two sites along with those published. These sites are located in the vicinity of the heparin binding pocket. Mutations of K112N and R122E have been added to the list to generate a penta mutant version of FGF1. Our recent study on R122E mutation of FGF1 showed that this one site is very critical for conferring proteolytic resistance to FGF1. This single point charge reversal mutant of FGF1 exhibited an enhanced bioactivity with a robust stability was filed for a provisional patent. We have continued our research to develop a more stable variant of FGF1 that can be used as an ideal therapeutic agent.

Site-directed Mutagenesis bacterial, transformation, and expression:

```
ATGTTTAATCTGCCTCCAGGGAATTACAAGAAGCCCAAACTCCTCTACTGTAGCAACGGGGGCCACTTCCTGAG
GATCCTTCCGGATGGCACAGTGGATGGGACAAGGGACAGGAGCGACCAGCACATTCAGCTGCAGCTCAGTGCGG
AAAGCGTGGGGGAGGTGTATATAAAGAGTACCGAGACTGGCCAGTACTTGGCCATGGACACCGACGGGCTTTTA
TACGGCTCACAGACACCAAATGAGGAATGTTTGTTCCTGGAAAGGCTGGAGGAGAACCATTACAACACCTATAT
ATCCAAGAAGCATGCAGAGAAGAATTGGTTTGTTGGCCTCAAGAAGAATGGGAGCTGCAAACGCGGTCCTCGGA
CTCACTATGGCCAGAAAGCAATCTTGTTTCTCCCCCTGCCAGTCTCTTCTGATTAA
```

```
        10         20         30         40         50         60
MFNLPPGNYK KPKLLYCSNG GHFLRILPDG TVDGTRDRSD QHIQLQLSAE SVGEVYIKST

        70         80         90        100        110        120
ETGQYLAMDT DGLLYGSQTP NEECLFLERL EENHYNTYIS KKHAEKNWFV GLKKNGSCKR

       130        140
GPRTHYGQKA ILFLPLPVSS D
```

```
        10         20         30         40         50         60
MFNLPPGNYK KPKLLYCSNG GHFLRILPDG TVDGTRDRSD PHIQLQLLAE SVGEVYIKST

        70         80         90          100        110        120
```

```
ETGQYLAMDT DGLLYGSQTP NEECLFLERL EENSYNTYIS KKHAEKNWFV GLNKNGSCKR

       130        140
GPETHYGQKA ILFLPLPVSS D
```

Each single point mutation at various sites of FGF1 were introduced sequentially to successfully generate the penta mutant with modified sites at five different locations After determining the DNA sequencing, it was obvious that all of the five desired mutations were introduced with no sequence errors. The acidic polar amino acid glutamine in position 40 was mutated to cyclic non-polar amino acid proline (Q40P). At 47th position, the polar amino acid serine was converted to aliphatic nonpolar amino acid leucine (S47L), and the positive amino acid histidine at 93 positions was mutated to polar amino acid serine (H93S). In position 112, the positively charged lysine was mutated to the acidic polar asparagine (K112N). The last mutation occurred in position 122 where the positively charged amino acid residue arginine was mutated to negatively charged glutamic acid (R122E). Compared to the published literature, in this version of the mutant the residues Q40 and K112 are located on the loop regions and other three residues are present on the secondary structural regions of the FGF1 molecule.

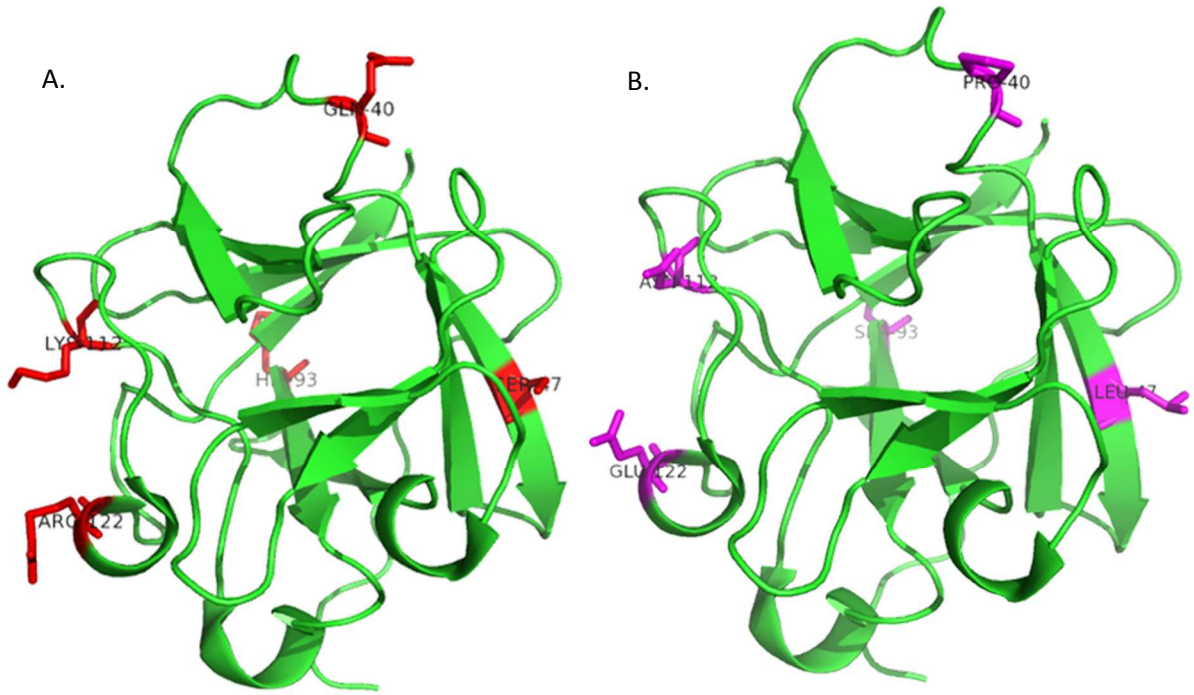


Figure 3: *A) PyMOL image indicating the general structure of WFGF1. Five different amino acids where the mutations occurred and were specify in red color. B. PyMOL image showing the five desire mutations (Q40P, S47L, H93S, K112N, R122E) (PDB1RG8).*

Figure 4: *Shows the transformation of WFGF1 and pFGF1 by using both strains of competent E. coli cells (BL-21 (DE3) or BL-21 (DE3) pLysS competent E. coli cells).*

Transformation of BL21 cells was successfully completed which was shown by the presence of bacterial colonies on ampicillin plate (Figure 4). Further, the cells were subjected to overexpression by inducing with IPTG for expressing the mutant form of FGF1 protein which

was accomplished successfully, and is shown by the presence of a band matching the size of FGF1 on SDS-PAGE (Figure 5&6).

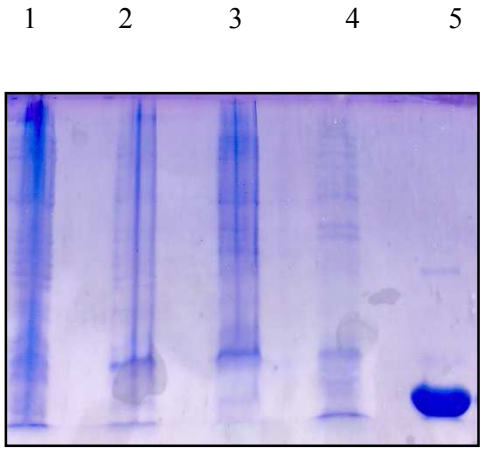


Figure 5: *Shows the overexpression of WFGF1.Lanes (1) uninduced sample (2) induced sample (3) pellet (4) clear cell lysate (5) protein marker (lysozyme).*

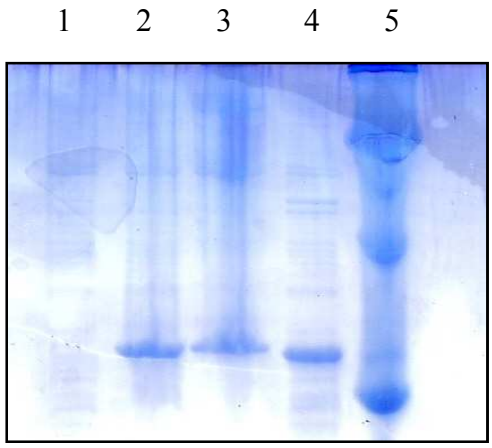


Figure 6: *Shows the over expression of pFGF1. Lane (1) is uninduce sample. Lane (2) is induce sample. Lane (3) pellet and lane (4) is supernatant. The last lane (5) is protein marker (lysozyme).*

Isolation of pFGF1 on nickel- Sepharose column affinity chromatography:

Introduction of mutations in these five sites resulted in a complete loss of heparin binding. Therefore, 6xHis tags version of the clone was engineered in the N-terminal end of pFGF1 to simplify the process of protein purification. The penta-FGF1 with N-terminal 6x his tag (NHFP) was overexpressed and purified using an immobilized metal affinity chromatography. NHFP was eluted from nickel-Sepharose column at two distinct step concentrations of 100mM and 250mM imidazole.

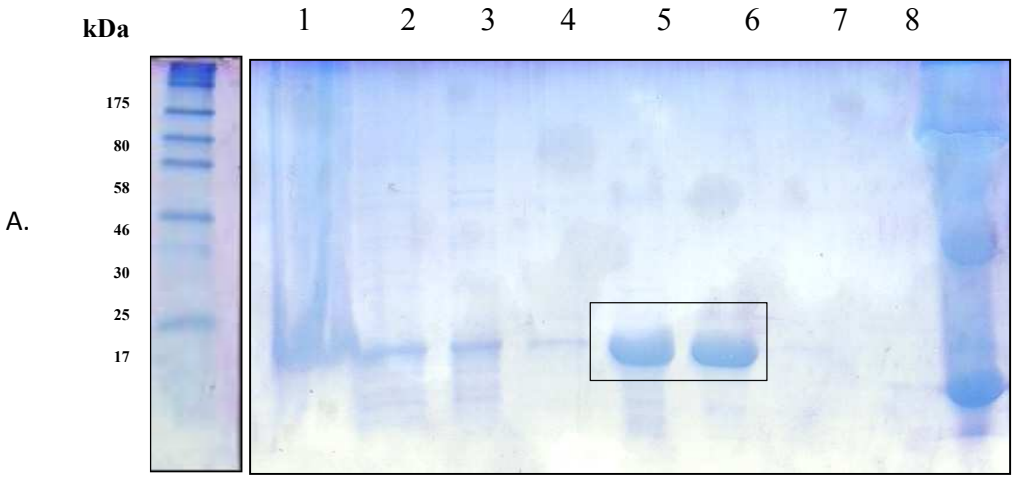


Figure 7: *SDS-analysis of pFGF1purification on nickel- Sepharose column. Lane (1): pellet, Lane (2): superannuant, Lane (3): flow through, Lane (4): 20mM of Imidazole, Lane (5): 100mM of Imidazole, Lane (6): 250 mM of Imidazole, Lane (7): 500 mM of Imidazole, Lane (8): 8M of urea wash, Lane (9): protein marker (lysozyme). The black box bands indicate pure pFGF1 which is known by FGF1's molecular weight (~16kDa).*

Pure WFGF1was isolated on the heparin-Sepharose column through running different gradients of phosphate/NaCl buffers: 250mM, 800mM, and 1500mM of NaCl. Pure WFGF1 is usually eluted at 1500mMof NaCl which is the required salt strength to break down the electrostatic interactions between heparin and WFGF1 protein. SDS analysis was completed to check the purity of the protein.

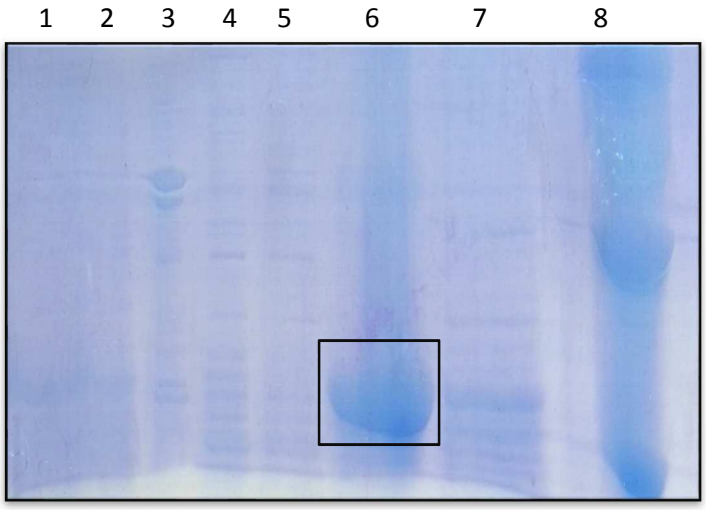


Figure 8: *Shows an SDS- PAGE analysis for WFGF1 purification on heparin-Sepharose column: Lane (1): pellet, Lane (2): supernatant, Lane (3): flow through, Lane (4): 250mM of NaCl, Lane (5): 800mM of NaCl, Lane (6): 1500mM of NaCl, Lane (7): 8M of urea wash, lane (8): protein marker (lysozyme). The black box bands indicate pure WFGF1 which is known by FGF1's molecular weight (~16kDa).*

Biophysical and biochemical characterizations of pFGF1 and tr-pFGF1:

The effects of mutations on the on the secondary structure FGF1:

To understand the molecular details pertaining to the structural differences between the mutant and wild type version of the FGF1 gives an insight into its functional differences. Far-UV CD spectral data was used as a probe to monitor these differences. Gross secondary structural information can be derived from far-UV CD analyses. Figure (9) shows the far UV spectra of pFGF1compared with WFGF1. Normal beta sheet spectra range from 192 nm to 194 nm in the positive CD side and 215 nm to 218nm in the negative CD side. Nevertheless, due to FGF1 β-barrel structure, the dipoles shape of β-sheet is cancelled. Therefore, there are some differences in CD spectra between β-sheet and β-barrel. B-barrel in the WFGF1 is shown in far UV as positive spectra ranging from 220 to 240nm with maximum wavelength at 228nm. Moreover, not

only β- barrel but also aromatic amino acid residues and FGF1 loops contribute to this characteristic spectrum. Comparison of CD spectra of pFGF1 with WFGF1 in the presence and absence of heparin, shows no significant difference in terms of the global secondary structure was observed. In conclusion, the penta mutation does not disrupt the β- barrel structure of FGF1.

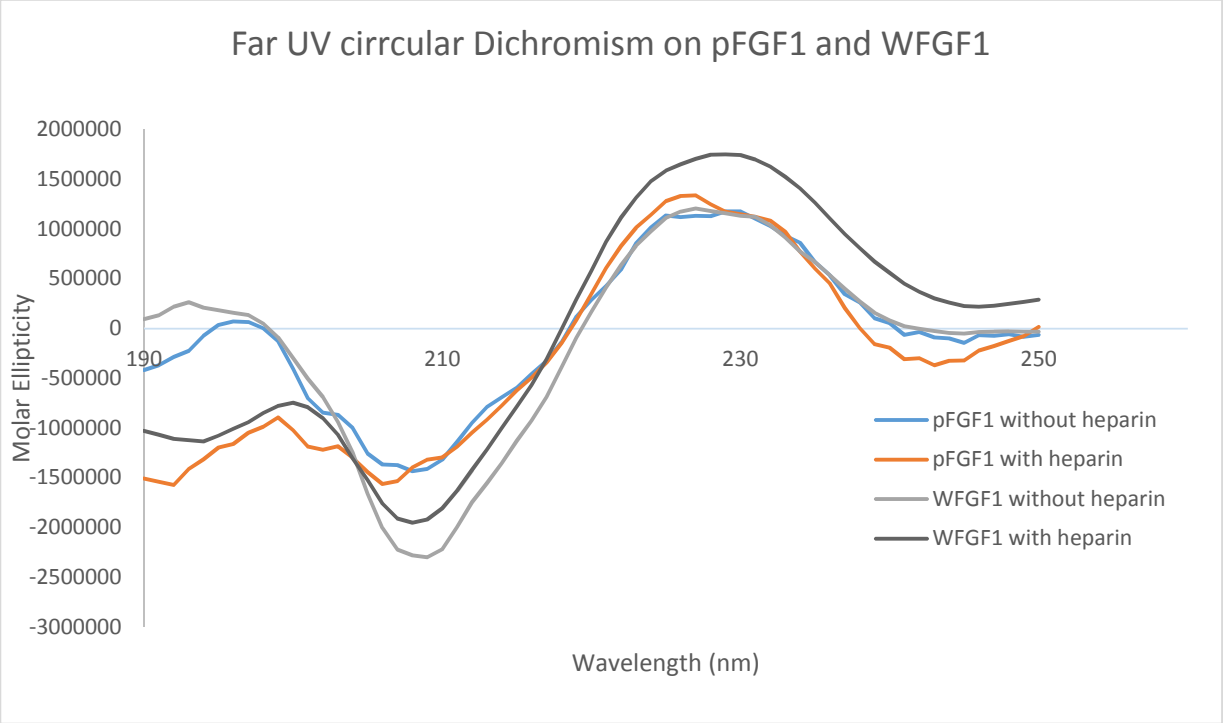


Figure 9: *Determining the secondary structure of pFGF1 and WFGF1 in the presence and absence of heparin by suing CD. The CD result shows that there are no significant changes in the secondary structures of FGF1 after mutations.*

Tertiary Structural changes in pFGF1:

Intrinsic tryptophan fluorescence provides valuable information about the three-dimensional structural alternations which might could occur after the introduction of mutations. The primary structure of FGF1 contains 8 tyrosines (Tyr8, Tyr15, Tyr55, Tyr64, Tyr74, Tyr94, Tyr97, and Tyr125) and 1 tryptophan (Trp107) (Fan, Li, Zhang, & Middaugh, 2006; Copland et al., 1991). To determine any modification in the tertiary structure of pFGF1 in the presence and

absence of heparin with comparison to the WFGF1, intrinsic fluorescence of tyrosine and tryptophan was checked. After excitation at 280nm, the peak for pFGF1 sets at ~ 314nm while the WFGF1 exhibit a maximum emission at ~305nm. The spectra of intrinsic fluorescence of folding WFGF1 is dominated through several tyrosine residues at ~305nm. The fluorescence of tryptophan residue in native FGF1 is quenched because tryptophan is surrounded by several imidazole and proximal pyrrole groups. However, in unfolded FGF1, the maximum wave length can be reached to ~350nm due to the presence of indole ring in tryptophan (Zakrzewska, n.d). However, pFGF1 which shows an emission maximum at 314nm indicates that pFGF1mutaions causes partially exposure to the W107 in the protein. The microenvironment around residue W107 might have lost interaction with the neighboring charged amino acids which is evident from the intrinsic emission florescence spectra of pFGF1 which shows a slightly red shift (Figure 10).

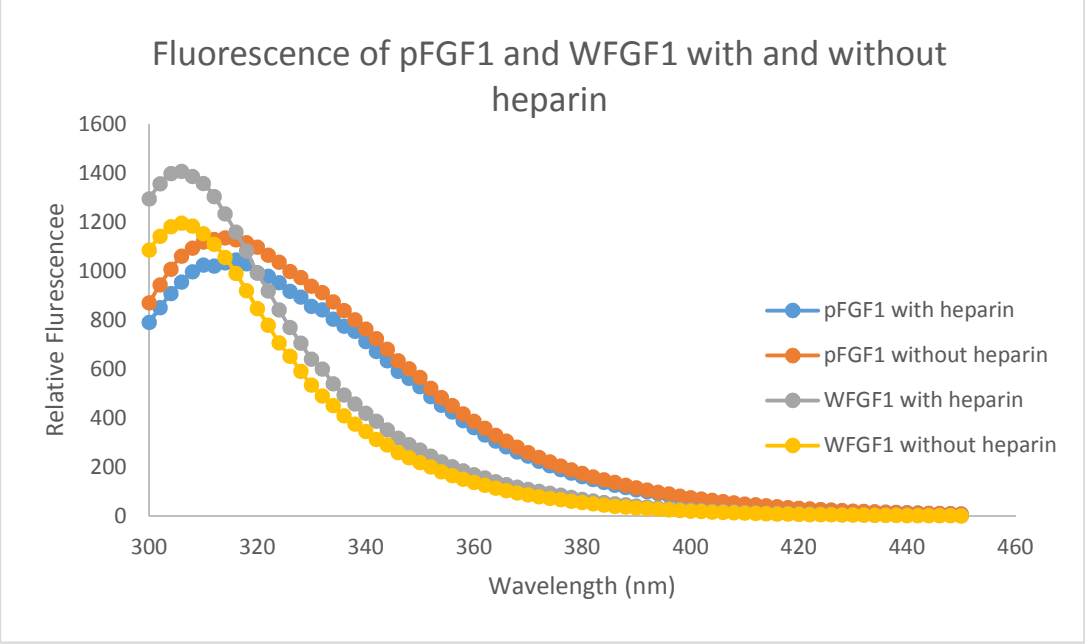


Figure 10: *Intrinsic fluorescence spectra for pFGF1and wild type FGF1in the presence and absence of heparin. pFGF1 exhibits slight changes in the intrinsic fluorescence due to minor exposure of tryptophan.*

Comparison of the non-polar surface between pFGF1 and WFGF1:

8-Anilinonaphthalene-1-sulfonic acid (ANS), which is a hydrophobic dye, shows weak spectrum in aqueous solutions. This spectrum is gradually increased upon binding of ANS to non-polar amino acids of the proteins. Therefore, ANS binding assay is a powerful technique to study the surface hydrophobicity of the protein molecule which to check if the protein in folding state or not (Alsenaidy et al., 2012). ANS binding study was done to see the structural changes and folding among pFGF1 in the presence and absence of heparin. Once the ANS bind to the hydrophobic amino acids of the proteins, it shows a fluorescence spectrum which is different from intrinsic spectra. Thus, the ANS relative fluorescence at 510 nm is directly proportional to the number of the hydrophobic amino acids in the pFGF1. Figure (11) shows a curve of ANS binding study of both pFGF1 and WFGF1 with and without heparin. According to the result, ANS intensity of pFGF1 is similar to WFG1F which prove that there are no major alternations on the surface of the FGF1 because of the mutations. Therefore, pFGF1 exhibits folding and structural manner as WFGF1.

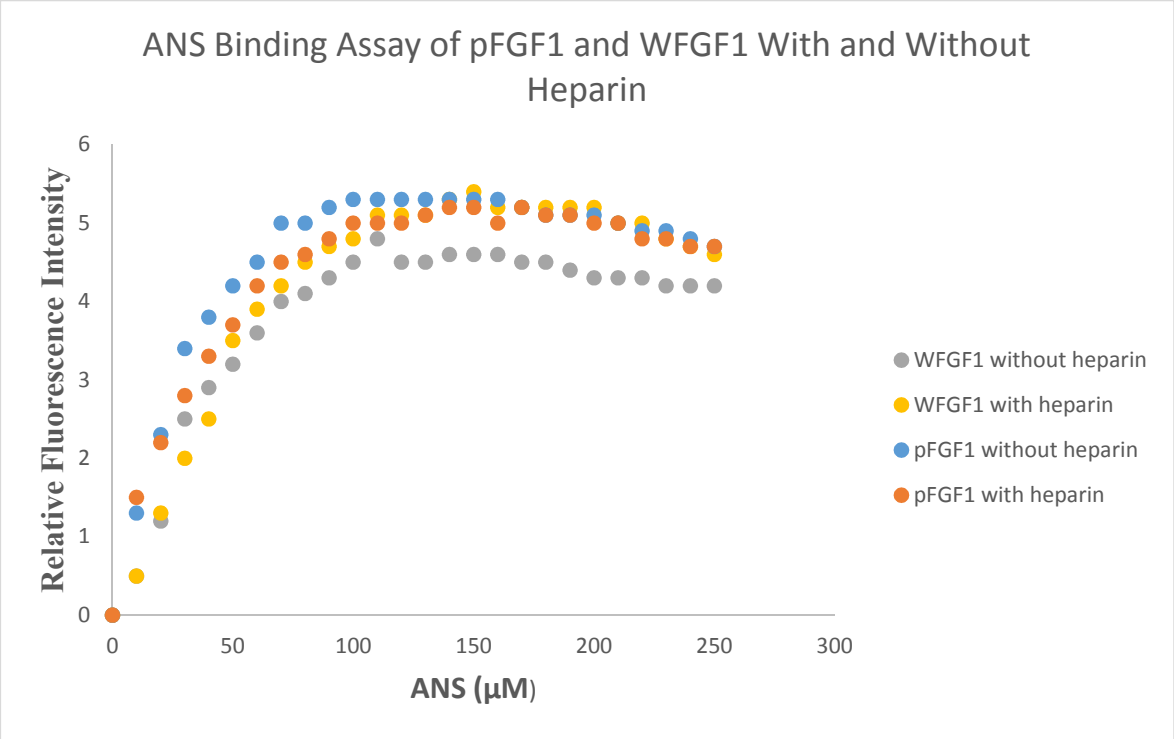


Figure 11: *ANS binding probe for pFGF1and wild type FGF1in the presence and absence of heparin. ANS binding remain the same among both pFGF1 and WFGF1, and that suggests that the five mutations do not produce significant changes in the protein concentration. In fact, these results show that pFGF1 is more stable than WFGF1.*

Measurement of heparin binding affinity for pFGF1 through using isothermal titration calorimetry (ITC):

Heparin is considerably important to activate FGFR (Ohmachi et al., 2000). Heparin binding with FGF through electrostatic interactions is a critical event for the activation of the signaling cascade. Complex of FGF1-heparin is important before binding to FGFR (Schlessinger et al., 2000). Then, the FGF- heparin can bind to FGFR via the positive charge canyon which can be formed after the dimerizing of D2 (Thurman, 2013). In this context, it is significant to study if the penta mutations impact the binding affinity of pFGF1 to heparin.

There are many advantages of using ITC to determining protein- ligand interactions and deriving thermodynamic binding constant (K_d) of specific protein-ligand interactions (Mohan,

Rani, & Yu, 2009). This method usually gives direct measurements of heat which usually absorbs or releases after injection of protein or ligands. In this study, ITC was used to study the binding affinity of the pFGF1and WFGF1 to the heparin. Isothermogram which represent the titration of heparin with WFGF1 is exothermic and sigmodal. From our data, it was proved that the binding affinity of WFGF1 is ~ 5µM , and the binding stoichiometry between heparin and WFGF1 is approximately 1:1 (Figure 13). Nevertheless, pFGF1 lost heparin binding affinity comparing to WFGF1.Therefore, there is no K_d value for pFGF1 (Figure 13). We hypothesize that pFGF1 lost heparin binding affinity because of designing two different mutations N112 and E122 in the heparin binding pocket. In a previous study, N112 reduces heparin binding affinity, but does not completely lose the binding (Zakrzewska et al., 2009). Therefore, we hypothesize that pFGF1 is unable to bind to heparin because of the combining of two mutations N112 and E122.

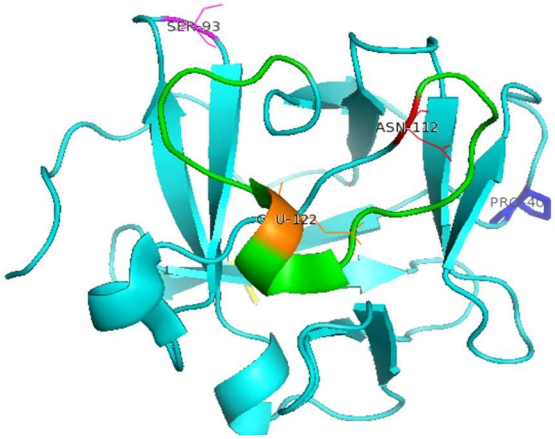


Figure 12: *PyMOL image showing the location of five mutations, where K112N and R122E are in heparin binding pocket (green color) (PDB1RG8).*

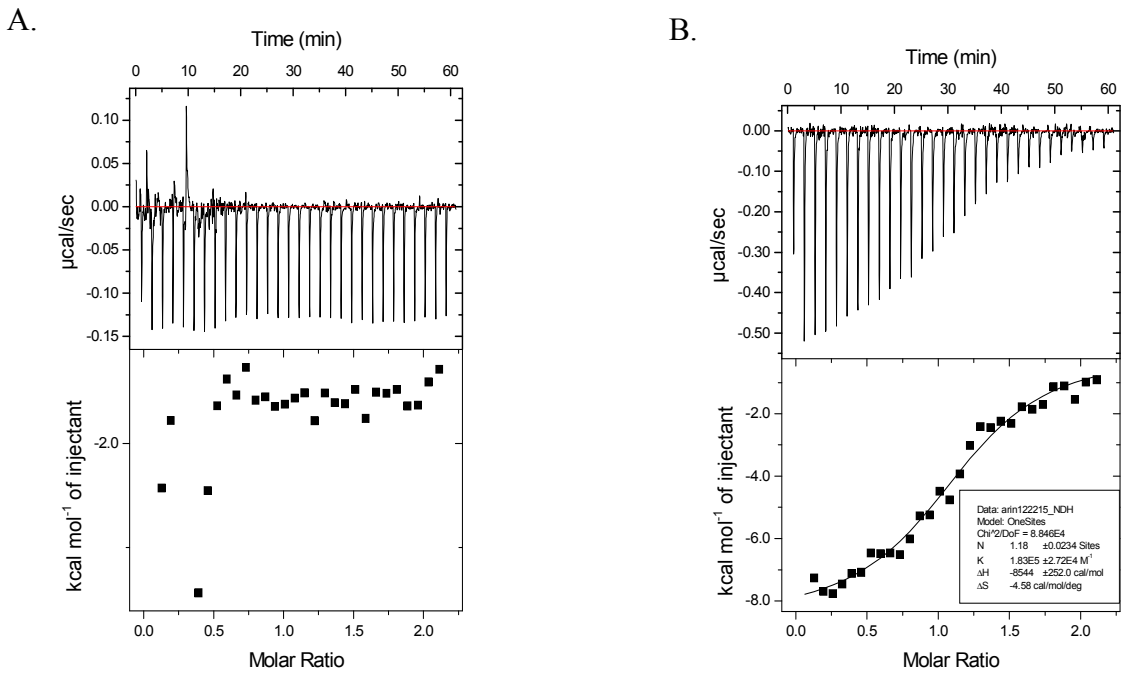


Figure 13: *Isothermogram of pFGF1 titration with heparin. A. 1mM of LMWH was titrated into 100µM pFGF1. In general, pFGF1 does not bind to heparin. B. Shows Isothermogram of WFGF1 titration with heparin, and the power bottom plot displays that the interaction between WFGF1 and heparin is exothermic.*

Determining the stability of pFGF1:

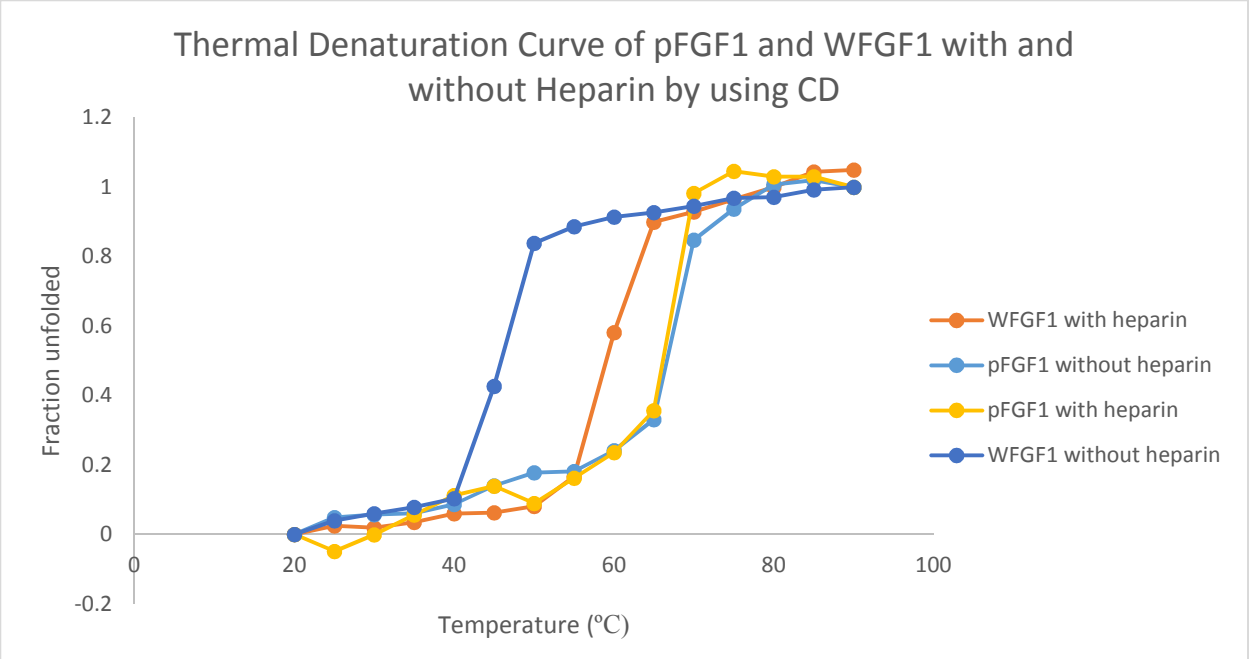
Thermal stability of FGF1 was ascertained by using the fluorescence and CD experiments. In folded WFGF1, the fluorescence spectra show a peak maximum at ~305nm which characterize several tyrosine residues while the single tryptophan (W107) is quenched because it is surrounded by imidazole and pyrrole groups. In unfolded WFGF1, the quenched effects of both imidazole and pyrrole groups on tryptophan becomes weaker and the three-dimensional structure of protein returns to its primary structural state. Therefore, fluorescence spectra characterize tryptophan residue and show a maximum wavelength at ~350nm. In addition, the tryptophan residue is usually used to check the FGF1 denaturation. In CD technique, the beta barrel structural of FGF1 show a maximum emission at ~228nm, which can be switched to -228 because of damaging of the secondary structure.

Both fluorescence and CD were used to measure the thermal stability of pFGF1 and WFGF1 with and without heparin. In fluorescence, thermal denaturation of pFGF1and WFGF1 were determined through calculation of fractional unfolded from wavelength emission 308nm (tyrosine) and 350nm (tryptophan). In CD, thermal stability was determined by finding also fractional unfolded, but fractional unfolded was calculated by having the average of molar ellipticity. The fractional unfolded corresponded to different temperatures (20 ºC to 90 ºC). Table (1) shows thermal denaturation temperature for pFGF1 and WFGF1 with and without heparin by using CD and fluorescence. CD and fluorescence show different melting temperature (Tm), which defines "the temperature at which 50% of the protein molecules exist in unfolded state" for WFGF1 (Srimathi et al., 2002, pp47510). In CD, WFGF1 is partially denatured at 45ºC while fluorescence indicates that WFGF1 can be in an unfolded state at 35 ºC. To illustrate, the data proves that WFGF1 loses a part of its secondary structure at 45 ºC and its tertiary structure at 35ºC. Copeland et al shows that the T_m for WFGF1 is ranges from 35 ºC to 42 ºC (1991). Addition of heparin promotes thermal stability for WFGF1by increasing the denaturation temperature from 14-20 ºC (Zakrzewska, n.d). In our result, WFGF1 with heparin can be thermally denatured after exposure to 60 ºC. However, pFGF1, Q40P, S47L, H93S, K112N, R122E, is denatured at a higher temperature ranging from 60ºC to 65 ºC. To demonstrate, pFGF1's secondary structure is unfolded at 65 ºC while the melting point for tertiary structure is around 60 ºC. The triple mutations (Q40P/S47I/H93G) exhibits similar melting temperature points. They are denatured at 60.7 ºC (fluorescence) and 61.8 ºC (CD) as well as K112N which shows a higher thermal stability (more than 3 ºC) in comparison to WFGF1 (Zakrzewska et al., 2005&2009).

Method	Circular Dichroism	Fluorescence
FGF types	T_m (ºC)	T_m(ºC)
WFGF1 without heparin	45 ºC	35 ºC
WFGF1 with heparin	60 ºC	60 ºC
pFGF1without heparin	65 ºC	60 ºC
pFGF1 with heparin	65 ºC	60 ºC

Table 1*: This table summarizes the melting temperature point (Tm) for pFGF1 and WFGF1 with and without heparin by suing two techniques: CD and fluorescence.*

A.



B.

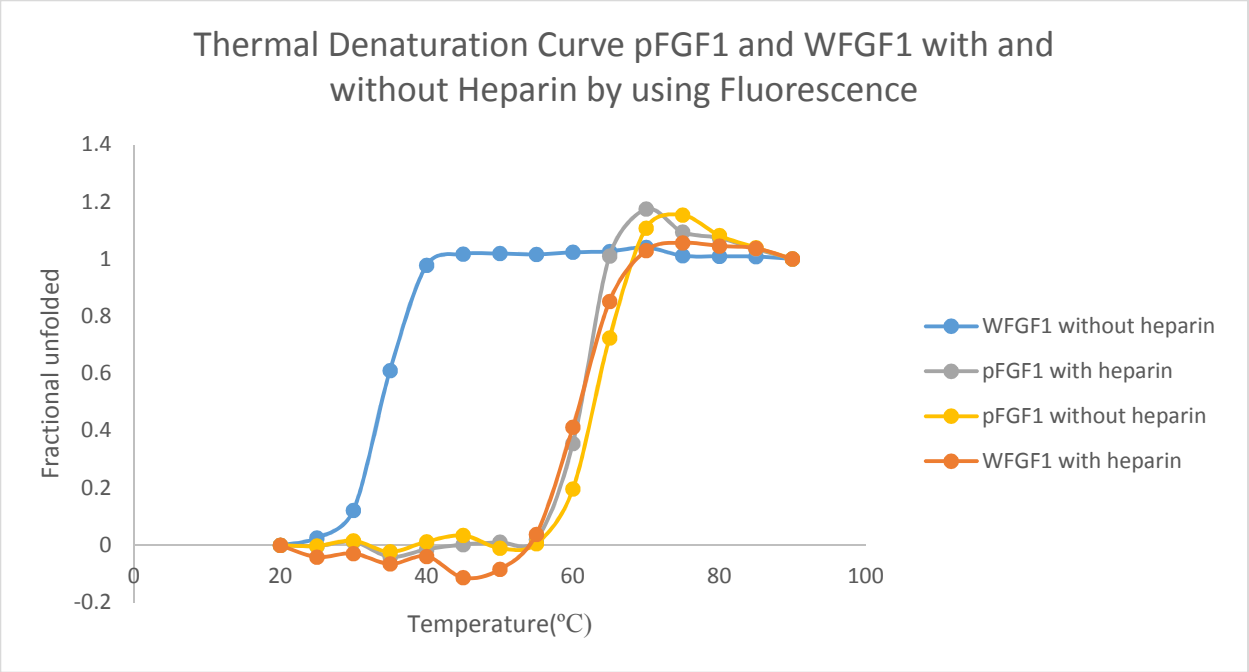


Figure 14: *A/B show thermal denaturation of pFGF1 and WFGF1 in the presence and absence of heparin by using CD and fluorescence. pFGF1 increase thermal stability for FGF1 where WFGF1 is thermally degraded at 35- 45 ºC.*

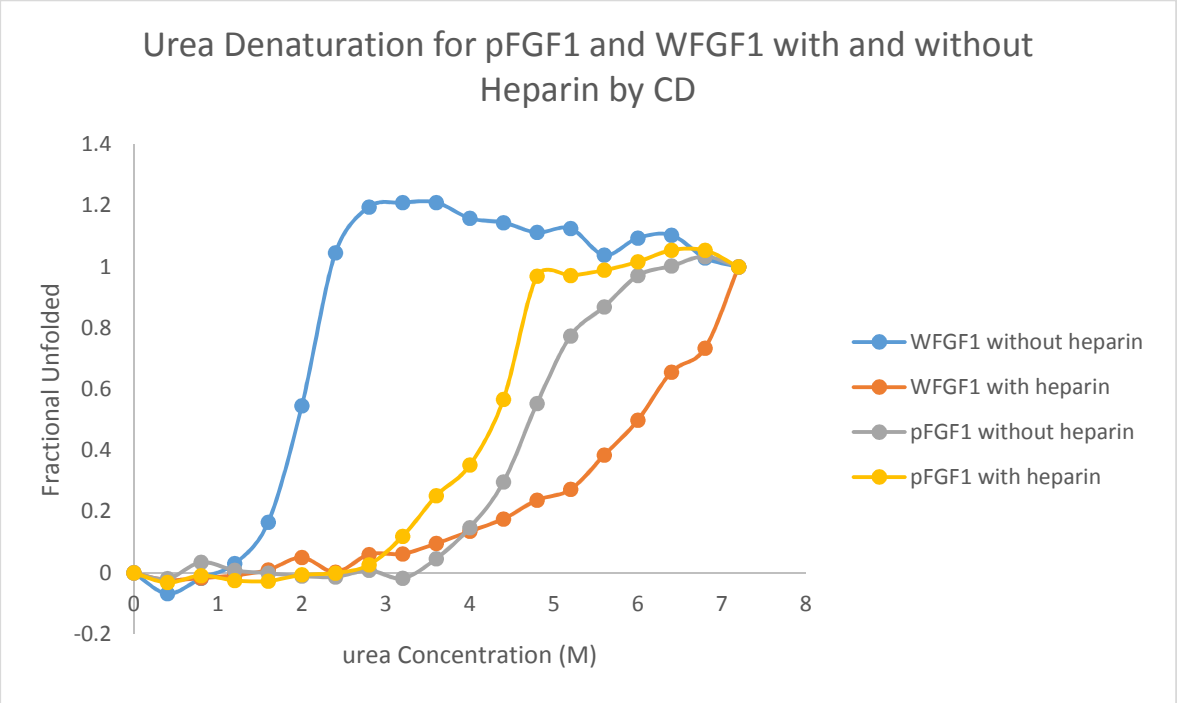
Urea, which is a chemical denaturant, is used in many chemical denaturation studies. Until now, urea denaturation mechanisms are still ambiguous. Several authors suggest that urea can unfold proteins through direct interactions to protein's peptide bonds, side chains, non-polar amino acids, polar amino acids, or both polar and non-polar amino acids. Moreover, scientists have difficulties in specifying the types of interactions whether electrotactic interactions or hydrogen bonding (Candotti et al., 2011). Urea usually promotes equilibrium unfolding (unfolded◂▸refolded state) in FGF1. FGF1 which is denatured by urea at 25 ºC is totally corporative and reversible. The reversibility effects of urea on unfolded FGF1 depends on incubation times of the unfoldedFGF1 on 8M urea. For instance, when FGF1 is incubated in 8M urea for 60 minutes, FGF1is able to return to ~85% of its native state. However, if the FGF1 was incubated in the same urea, but longer time (100 hours), FGF1 stay in unfolded state (Kathir et al., 2007). Urea denaturation of FGF1 was monitored by using both CD and fluorescence. Table

(2) summarizes the urea concentration melting point (C_m) "at which half of the protein (50%) is in unfolded state" (Srimathi et al., 2002, pp47510). The C_m of the WFGF1 was measured to be ranging from 1.2 to 2 M while the C_m of the pFGF1 (Q40P, S47L, H93S, K112N, R122E) concentration was from 3.2 to 4.8 M. To demonstrate, WFGF1 can be simply lost its secondary structure after having exposed to 2M of urea, and easily lost its tertiary structure by 1.2M of urea. The C_m of WFGF1 shifts to 4 to 6M after addition of 10X of heparin because heparin is known as protection of WFGF1 from heat. However, pFGF1 is not effected by heparin. Therefore, even in the presence of heparin, pFGF1 exhibits similar results that the secondary structure of the pFGF1is in unfolded form at high concentration of urea (4M). In addition, the tertiary structure of the pFGF1 would be lost at a ranging concentration between 3.2 to 4M. High melting concentration of urea (C_m) indicates that pFGF1 loses its secondary and tertiary structure at high concentration of urea (higher than WFGF1).

Method	Circular Dichroism	Fluorescence
FGF types	C_m	C_m
WFGF1 without heparin	2 M	1.2M
WFGF1 with heparin	6 M	4 M
pFGF1 without heparin	4 M	4.8 M
pFGF1 with heparin	4 M	3.2 M

Table 2: *This table summarizes the melting concentration point of urea (Cm) for pFGF1 and WFGF1 with and without heparin by suing two techniques: CD and fluorescence.*

A.



B.

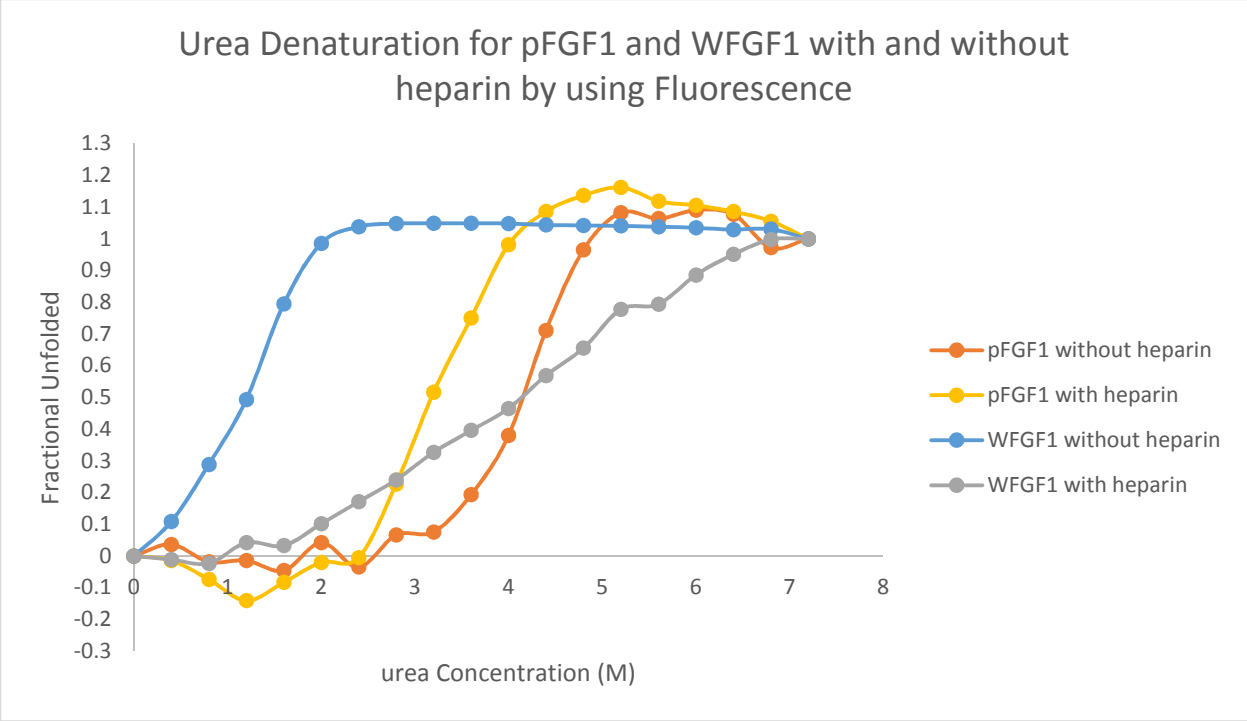


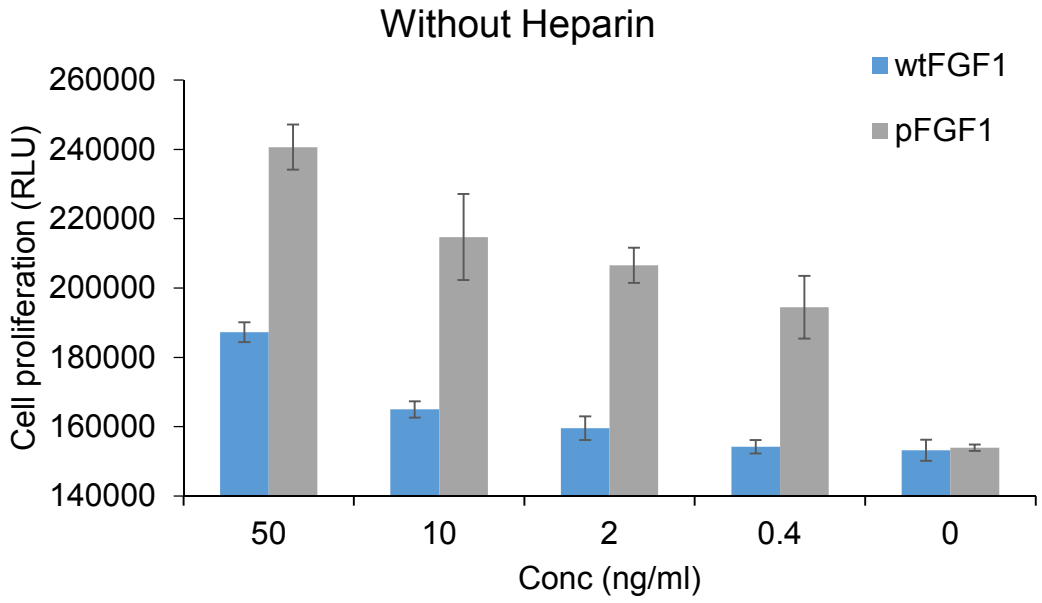
Figure 15: *Shows urea denaturation for pFGF1 and WFGF1 in the presence and absence of heparin through using two main techniques, involving CD and fluorescence. pFGF1 increase protein stability because WFGF1 is denatured by 1.2M of urea, while pFGF1 is denatured by 3.2 to 4.8M of urea.*

Even though pFGF1 does not change FGF1's secondary structure and beta barrel shape, pFGF1 thermally denature at high temperature and chemically unfolded in high urea concentration. In fact, these results show that pFGF1 is more stable than WFGF1.

Biological Activity of pFGF1:

Even though pFGF1does not bind to heparin, it shows cellular proliferation activity. In the absence of heparin, pFGF1 shows much higher biological activity than WFGF1. Nevertheless, pFGF1 shows less biological activity than WFGF1 in the presence of heparin because it lacks of heparin binding affinity (Figure 16). This shows that the penta FGF1 can activate the signaling cascade without the requirement of FGF1.

A.



B.

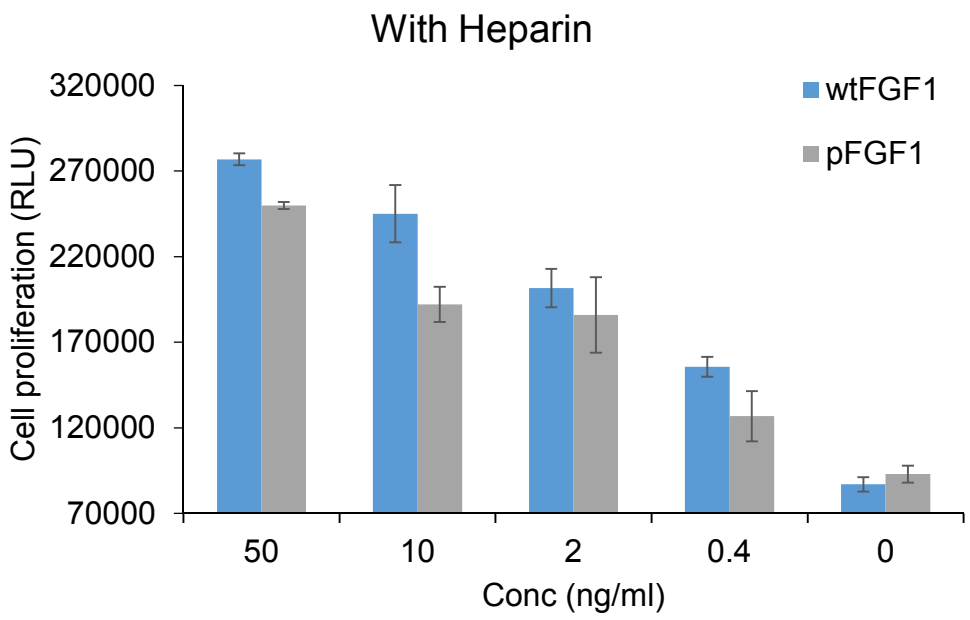


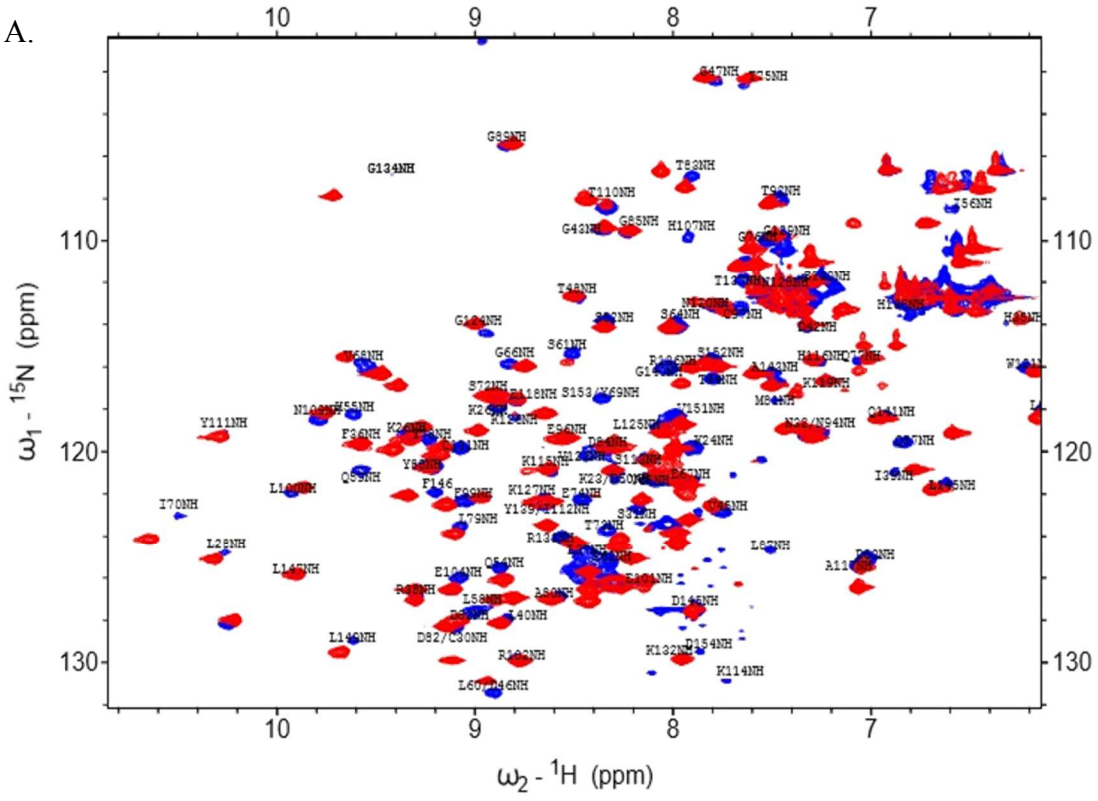
Figure 16: *Cellular proliferation activity for WFGF1 and pFGF1in the absence of heparin (graph A) and in the presence of heparin (graph B).*

The tertiary structure fold of pFGF1 is discernibly different from the wild type-FGF1:

All significant changes were determined by using two-dimensional nuclear magnetic resonance (2D NMR). The 2D NMR spectroscopy was preformed through using 1H-15N heteronuclear spin quantum coherence (1H-15N HSQC). HSQC is considered to be a finger print of protein backbone conformation. Every 1H-15N HSQC crosspeak indicates an amino acid residue in certain protein backbone. Thus, chemical shift perturbation through 1H-15N gives information about structural changes in protein backbones under atomic level because of ligand binding or mutations. In this context, heparin was not used in NMR experiment because heparin can be polydispersity, which generate unreliable date. Therefore, sucrose octa sulfate (SOS) which is analogues to heparin and promotes cellular proliferation activity as well as heparin was used. In this study, several structural changes occur in FGF1 due to five different mutations.

Through overlying the HSQC pFGF1 on the WFGF1, it was revealed that theses mutations can cause chemical shift perturbation to one mutational residue H107S and nearby amino acids including C30, H55, Q59, I70, K132, G134, L145 (Figure 17). The perturbed residues K132 and G134 which are in heparin binding pocket suggests that pFGF1 lost heparin binding affinity, and this result is compatible with ITC, heparin purification, and thermodynamic results. Moreover, some amino acids are disappeared after designing penta mutations such as S61, L87, and K114, S152/Y69. The same results were applied for WFGF1/pFGF1 with SOS because pFGF1 unable to bind to SOS (Figure 18).

A.



B.

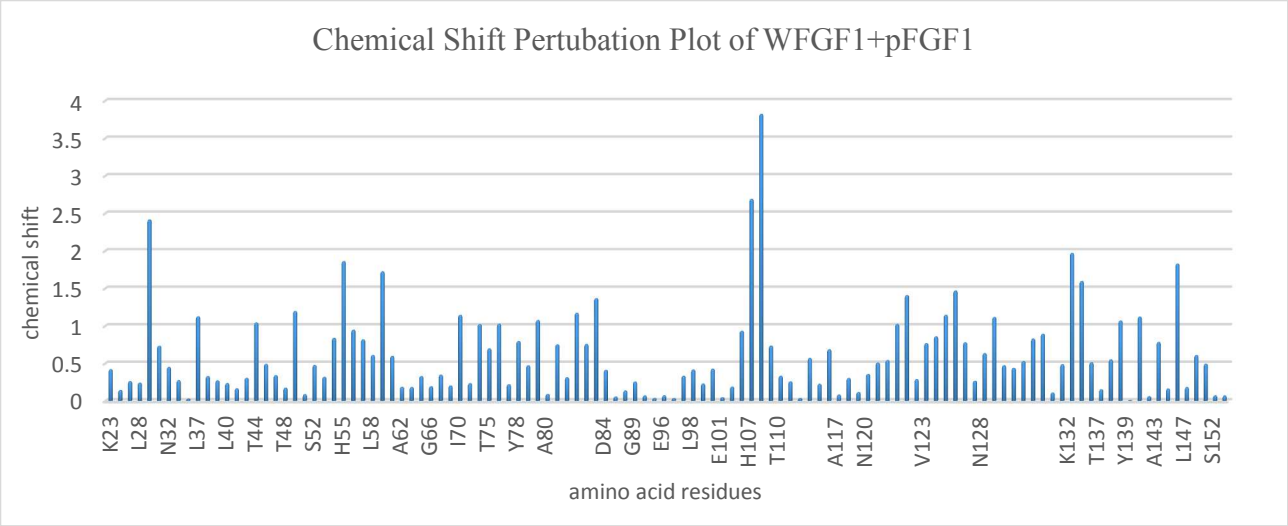
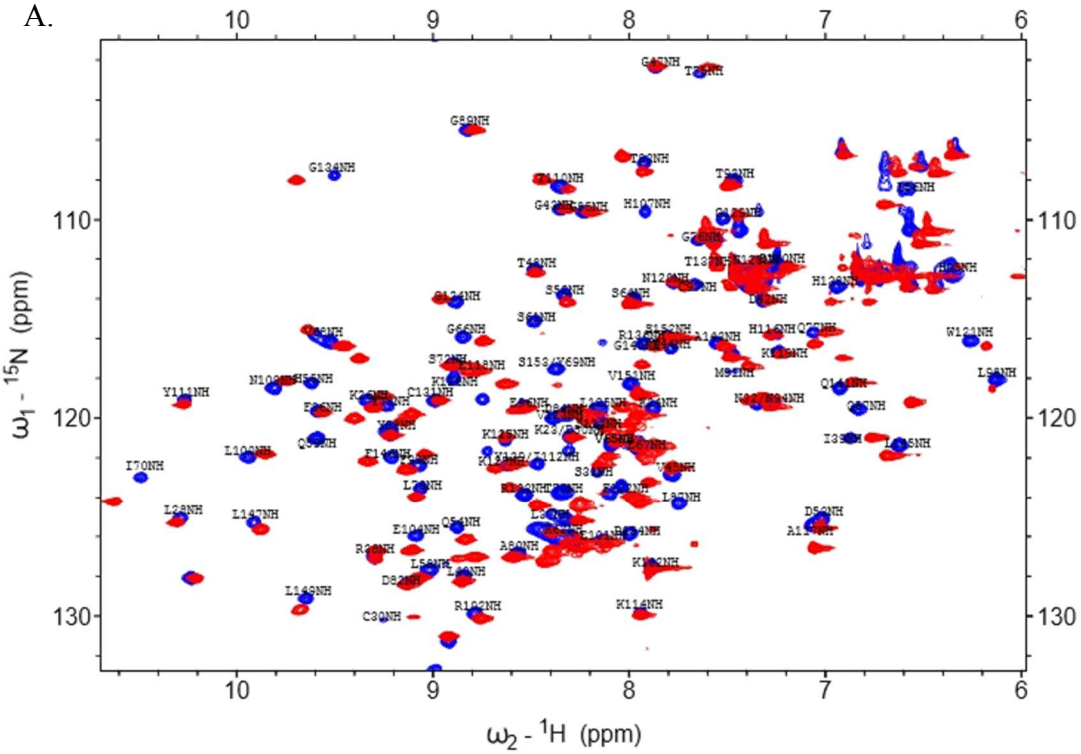
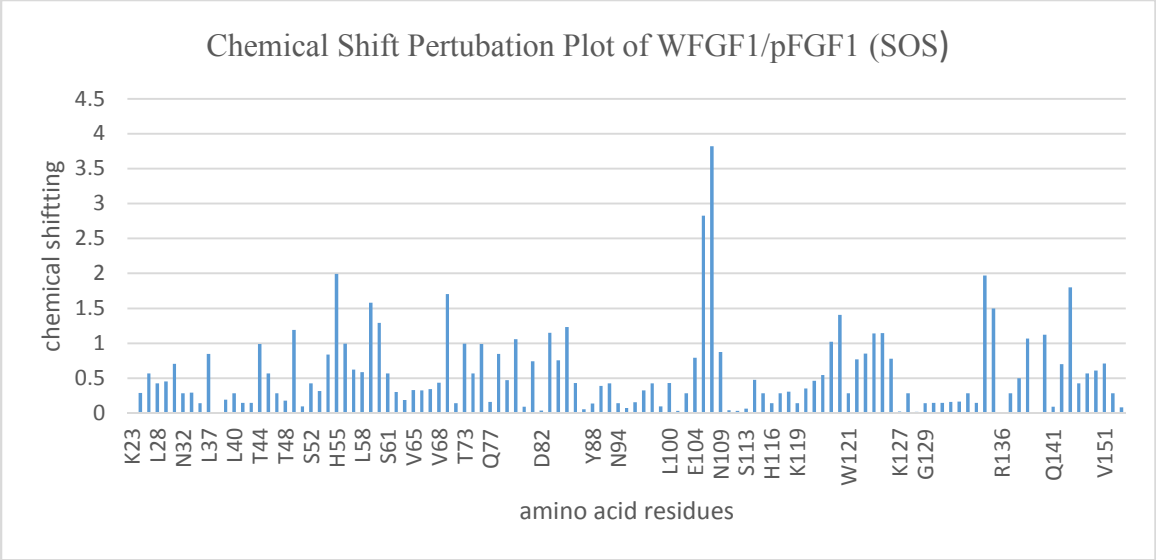


Figure 17: *A. shows 1H-15N HSQC of the pFGF1(red) overlay on WFGF1 (blue). B. shows the chemical shift perturbation plot for pFGF1.*

A.



B.



C.

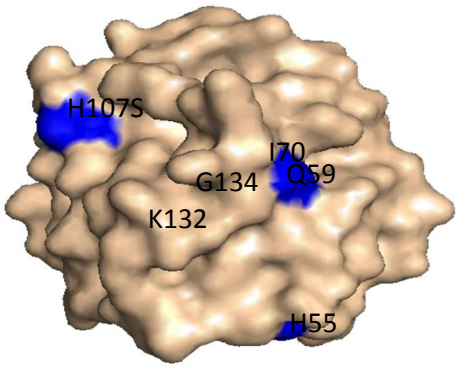


Figure 18: *A. shows 1H-15N HSQC of the pFGF1with SOS(red) overlay on WFGF1with SOS (blue). B. shows the chemical shift perturbation plot for pFGF1with SOS.C. Shows the structure of pFGF1. The most perturbed residues are highlighted in blue.*

<u>Studying pFGF1 stability through pFGF1 treatment with proteolytic enzyme</u>:

Trypsin, which is a member of serine proteases, cleaves the c-terminal end of positively charged amino acids such as arginine and lysine except for following of these amino acids by proline. Limited trypsin digestion is a significant technique to evaluate protein flexibility (Carstens, McKeehan, & Garcia-Blanco, 1998). To study the relationship between thermodynamic and urea stability, proteolytic susceptibility, and biological activity of pFGF1, pFGF1 was treated with 1mg/ml trypsin. After that, pFGF1was analyzed by SDS-PAGE and

densitometry scanning. The experiment was carried out at physiological temperature (37ºC) for 40 minutes. As shown in figure (19), after 40 minutes of incubation, WFGF1 is completely digested because it is susceptible to trypsin. Trypsin result is compatible with thermodynamic result that WFGF1 shows the lowest T_m as well as C_m . In contrast, penta mutant type, Q40P, S47L, H93S, K112N, R122E, remains unchanged even after they were incubated with trypsin for 40minutes. Having the same intensity of bands among different times indicates that pFGF1is extremely stable to proteolytic factors even in the absence of heparin. In previous work, triple mutations (Q40P, S47I, H93G) exhibit a high resistance against trypsin as well as K112N (Zakrzewska et al., 2005&2009). Therefore, pFGF is resistant to proteolytic cleavage because of replacement of positive charge residue (K 112) to (N) and the positive charge residue (R122) to the negative charge residue (E).

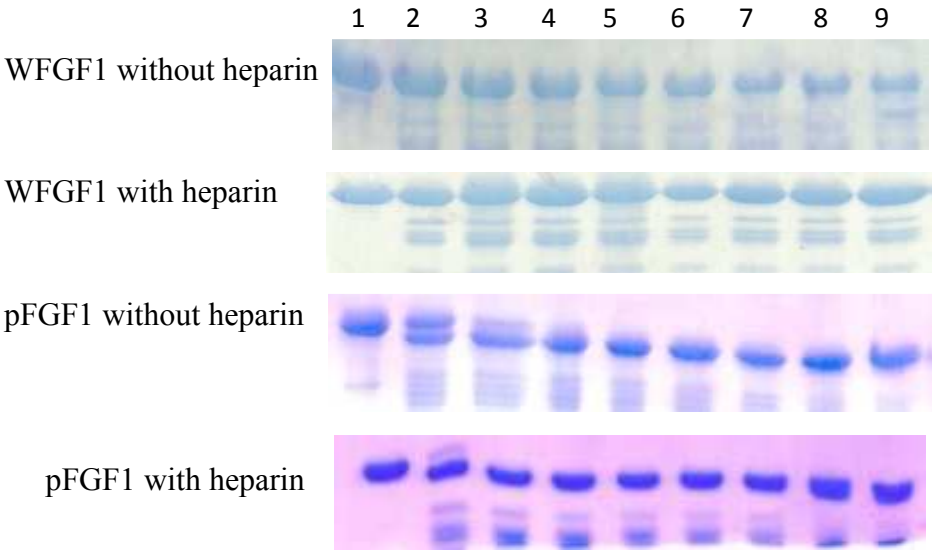


Figure 19: *SDS-PAGE analysis for trypsin digestion with limited time for both WFGF1 and pFGF1in the presence and absence of heparin. Every lane represent specific time for trypsin incubations, lane (1) zero time, lane (2) after 5minutes, lane (3) after 10 minutes, lane (4) after 15 minutes, lane (5) after 20 minutes, lane (6) after 25 minutes, lane (7) after 30 minutes, lane (8) after 35 minutes, and lane (9) after 40 minutes.*

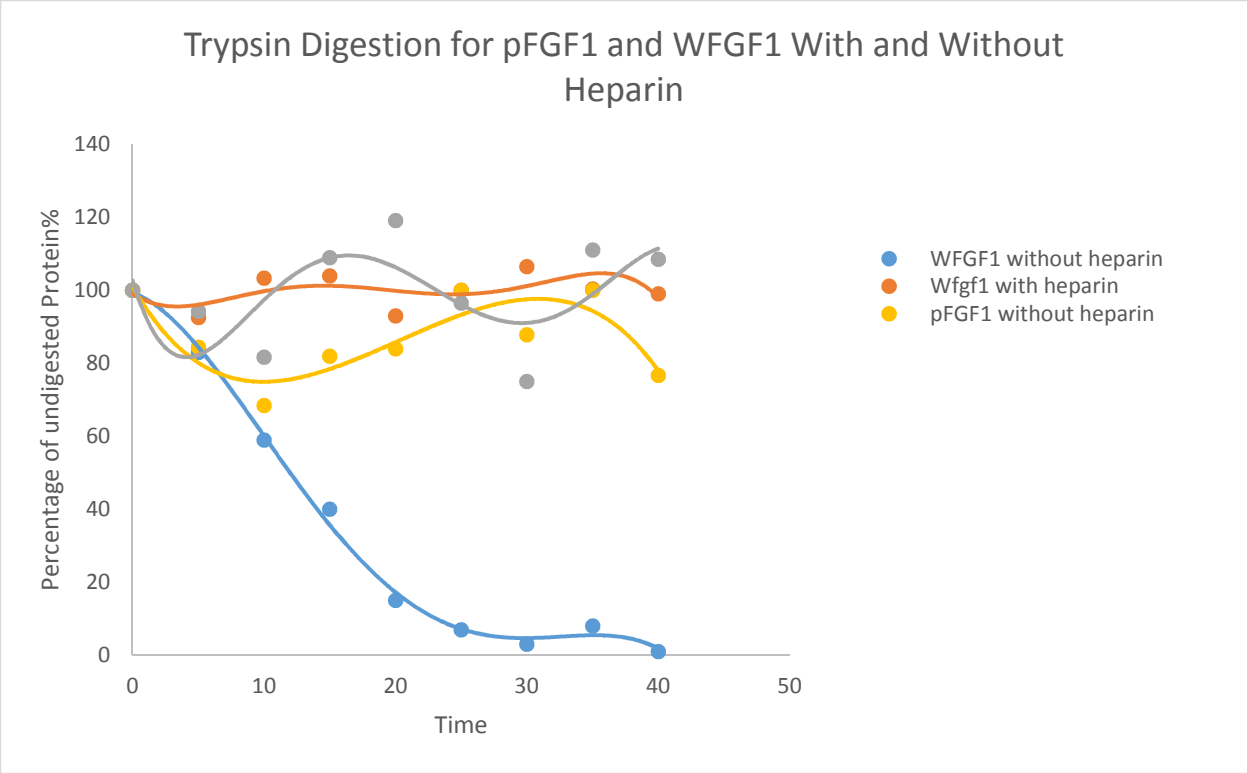


Figure 20: *Overly of trypsin digestion curves for WFGF1 and pFGF1 in the presence and absence of heparin. WFGF1 can easily degraded after 5 minutes of incubation while pFGF1 shows high stability and remain the same even when it was incubated with trypsin for 40 minutes.*

Proteolytic, thermodynamic, and chemical studies of pFGF1 show compatible results of pFGF1stability. The pFGF1 stability can be completely understood after running a molecular dynamic simulation of this mutant. pFGF1 is more stable than WFGF1because pFGF1 participates in making some hydrogen bonds and more salt bridges than WFGF1. For hydrogen bonds, Pro40 main chain is able to make two hydrogen bonds, one of which is with Gln43 main chain and the other one is with the side chain of Gln43. Also, the main chain of Leu47 is hydrogen bonded with the main chain of Tyr55. Moreover, Ser93 does not participate in making any hydrogen bonds, while Asn122 contributes in forming hydrogen bonds with Ser116- main chain. Moreover, the main chain of Glu122 is able to form two hydrogen bonds, one is with Gln127-side chain and the other is with Arg119- main chain whereas Glu122-side chain is

hydrogen bonded with Arg119-side chain. As mentioned previously, pFGF1 can make more salt bridges than WFGF1which enhance protein stability. Figure (21) shows salt bridges surface of pFGF1 which involves in making four more new electrostatic bonds (Asp 36-Arg24, Asp70-Arg119, Glu122-Lys118, Glu122-Arg119) beside the 8 bonds that can be seen in both WFGF1 and pFGF1. The sharing electrostatic interactions include Asp32-Arg24, Asp39-Arg24, Glu53-Lys128, Glu81-Lys100, Glu81-Lys101, Glu82-Lys101, Glu91-Lys128, and Glu104-Lys105.

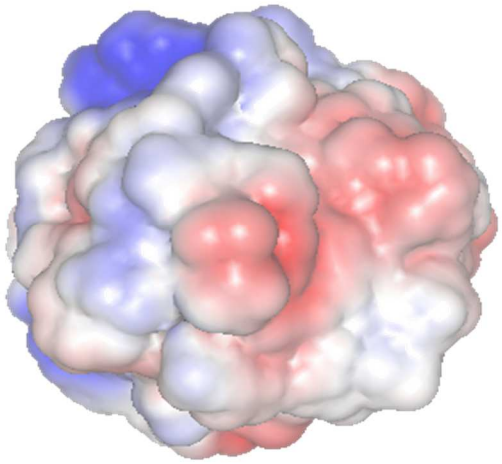


Figure 21: *PyMOL image showing the electrostatic surface for pFGF1 (PDB1RG8).*

Purification of truncated pFGF1 by using Nickle -Sepharose column:

Because pFGF1is resistance to trypsin, truncated pFGF1 (tr-pFGF1) was purified to be biophysically and biochemically characterized. Pure truncated pFGF1(tr-pFGF1) was isolated on the nickel-Sepharose column through using 50mM ammonium sulfate/ imidazole gradients with various concentrations from: 20 mM, 100mM, 250mM, and 500mM.Pure tr-pFGF1 was achieved at 20Mm of imidazole due to structural changes of the pFGF1 after trypsin.

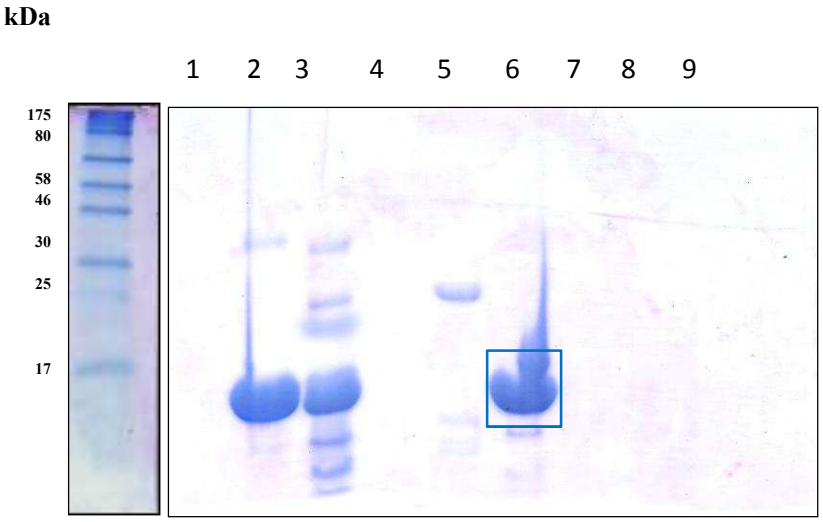
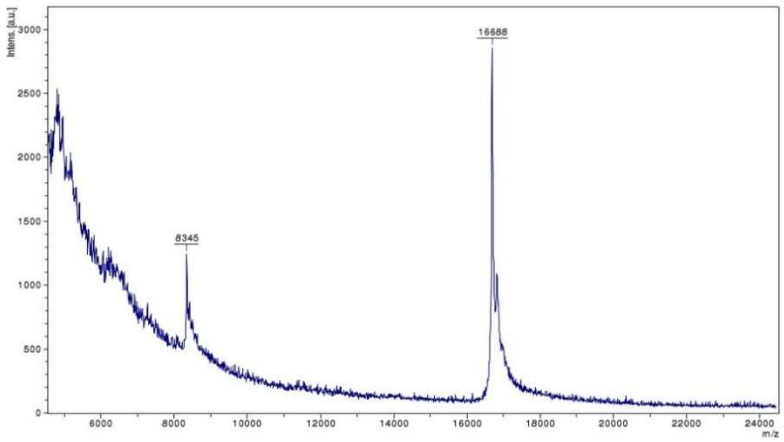


Figure 22: *SDS analysis for tr-pFGF1 purification on nickel column. lane (1): pure pFGF1, lane (2): digested pFGF1 by trypsin, lane (3): flow through, lane (4): ammonium sulfate buffer wash, lane (5): 20mM of Imidazole, lane (6): 100 mM of Imidazole, lane (7): 250mM of Imidazole, lane (8): 500 mM of Imidazole, lane (9): 8M of urea wash. The blue box band indicates pure tr-pFGF1 which matches the FGF1's molecular weight (~1k6Da). The band in lane (4) at 23 kDa shows trypsin band.*

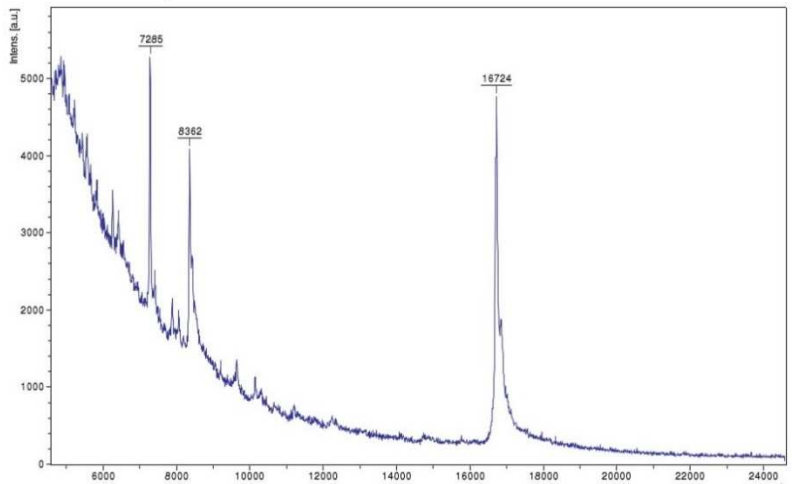
Determining the molecular mass of pFGF1and tr-pFGF1:

Mass spectroscopy is a technique that can be used to give a direct measurement of a protein's molecular weight based on their ionic charges and mass. In this study, WFGF1, pFGF1, and tr-pFGF1 were measured to understand the proteolytic effects on pFGF1.The molecular weight of pFGF1is 16724Da which is close to the molecular weight of WFGF1 is 16688 Da. However, the molecular weight of the tr-pFGF1 is 15582 Da which means that pFGF1 lost ~1100Da. We do not know if cleavage site weather would be cut at the C or N terminal end of pFGF1. That can be discovered in future work.

A.



B.



C.

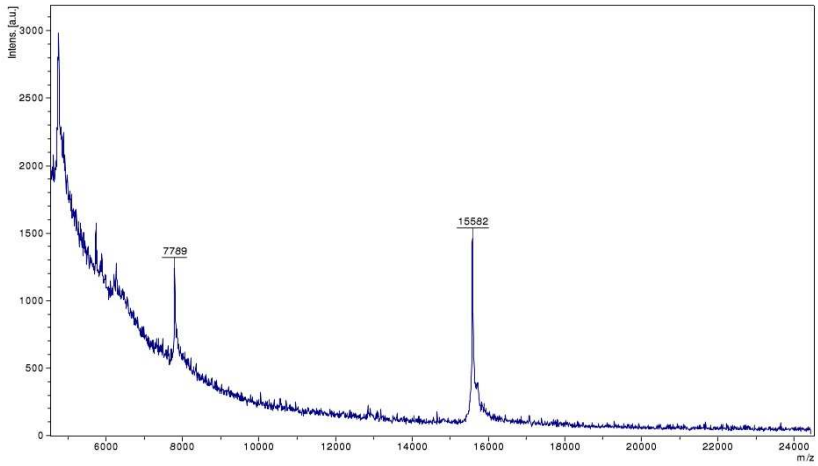


Figure 23: *A) Mass spectroscopy for WFGF1. B) Mass spectroscopy for pFGF1. C) Mass spectroscopy for truncated pFGF1 (tr-pFGF1).*

Cell proliferation activity for tr-pFGF1:

Though tr-pFGF1 is lost part of its structure, it shows biological activity. In the absence of heparin, tr-pFGF1 could promote cellular proliferation activity less than pFGF1. Further biological, physical characterization studies will be performed on tr-pFGF1 in the future.

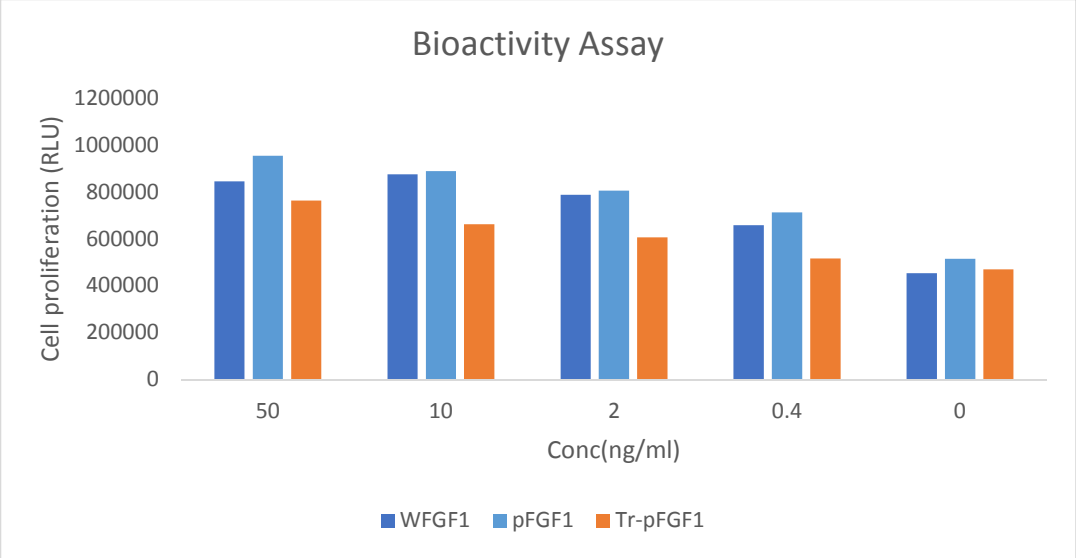


Figure 24: *Shows the biological activity of WFGF1, pFGF1, and tr-pFGF1 in the absence of heparin. Even though tr-FGF1 lost a part of C-terminal end, it is still showing biological activity.*

Discussion:

FGF1 promotes downstream signaling pathways after binding to heparin or HS. After determining the crystal structure of FGF1and FGF2/FGFR complex with heparin, it was proven that heparin is considerably important in complex formation (Schlessinger et al., 2000). However, several studies have shown that heparin is significant in the formation of FGFR/FGF complex, promoting signaling pathways as well as enhancing the biological activity of FGF (Delehedde et al., 2002).

In this paper, we hypothesized that heparin is considered to be a protection factor of unstable FGF1 from proteases, heat, and urea under physiological conditions. FGF1 is considerably unstable because it can be easily denatured after being exposed to low temperatures ranging from 35ºC to 45 ºC and low urea concentration between 1.2M to 2M at natural pH. An issue can be drawn from previous information that FGF1 can be partially denatured at physiological conditions, so FGF1 is easily cleaved by proteases such as trypsin. The addition of heparin is important since it increases the stability of FGF1, and that was well proven in earlier studies (Gospodarowicz, & Cheng, 1986; Pellegrini et al., 2000). Because the biological activity of FGF1 is performed in assays taking more than 12 hours, it is still unknown whether heparin is effective in cellular signaling pathways or is protective against denaturing agents. In a previous study, Zakrzewska and his coauthors introduced single points and multiple mutations that developed FGF1 stability and improved biological activity (2005). When FGF1 has high stability, it can be useful in treating several diseases, especially as angiogenic or mitogenic factors. FGF1 has shown to inhibit cell death and participate in protecting the brain after stroke (Reuss, & and Halbach, 2003; Cuevas et al.,1998). Furthermore, FGF1 is useful in repairing spinal injuries (Zakrzewska et al., 2005). In this study, we introduced five mutations, two of them Q40P and K112N were previously described as stabilizing mutations and the remaining three S47L, H93S, and R122E are new (Zakrzewska et al., 2005&2009). The pFGF1 increases the T_m of FGF1 by ~20 ºC and C_m by ~2M, to adjust the absence of heparin, through providing fundamental protection from urea, heat, and trypsin.

Biophysical and biochemical features of pFGF1, (Q40P, S47L, H93S, K112N, R122E), were studied. PFGF1 completely lost affinity for heparin as shown in the ITC experiments (Figure 13) due to the engineering of two mutations in heparin binding pocket K112N and

R122E. In earlier study, it was proven that K112N reduces affinity for heparin (Zakrzewska et al., 2009). Therefore, we imply that the combination of K122N and R122E is responsible for lacking of heparin binding affinity. NMR results provide agreement about this hypothesis because the most perturbed residues are K132 and G134 in the heparin binding pocket. This means that N112 and E122 participate in moving the heparin binding pocket from its original location.

The thermodynamic and urea denaturation data were analyzed by assuming two states of denaturation. The denaturation experiment was performed using CD and fluorescence simultaneously. Both techniques provided an excellent agreement that there is a two-state model of pFGF1. The data that has been provided in table (1&2) demonstrates that introducing of five mutations contributes to protein stability. The increase of protein stability causes by additive or corporative of the side chains of mutations, and this is can be seen in the pFGF1(Zakrzewska et al., 2005). The side and main chain of pFGF1 is important in forming hydrogen bonding as well as creating four new salt bridges (Asp 36-Arg24, Asp70-Arg119, Glu122-Lys118, Glu122-Arg119).

Biological studies were performed on pFGF1 (Figure 17). It was shown that pFGF1 shows higher biological activity than WFGF1 because increasing protein stability contributes in prolonged ability to promote cell proliferation even though heparin affinity was totally lost (Zakrzewska et al, 2009).

It was well studied and shown that the proteolytic resistance of proteins is considerably related to their thermodynamic stabilities (Hubbard,1998). FGF1 is usually unfolded at 35-45ºC, and can be simply degraded by proteases. By using trypsin, it was shown that the susceptibility of pFGF1 to trypsin was strongly affected by its thermodynamic stability. Even though pFGF1

was treated with trypsin and incubated for 40 minutes, the SDS-PAGE analysis shows similar band thickness. The trypsin digestion results of pFGF1 are in a good agreement with thermodynamic and urea denaturation study as well as mitogenic activity assay.

Conclusion and future work:

FGF1 is a mitogenic factor which has been used for diseases treatment including stroke. The fundamental issue with FGF1 is that it is prone to thermal and proteolytic degradation at a natural pH and under physiological conditions. To increase FGF1 stability and biological activity, penta mutations were introduced to pFGF1. Two of them are in the heparin binding pocket. Our results have shown that (pFGF1) is unable to bind to heparin. Therefore, a nickel-Sepharose column was used to purify pFGF1 instead of heparin because of engineering 6XHis tag in the N-terminal of pFGF1. After obtaining pure protein, it was biophysically and biochemically characterized through using valuable techniques. The secondary structure of pFGF1 remains unchanged. However, there are some significant changes in the tertiary structure due to these mutations. Biological study of pFGF1 shows higher mitogenic activity than WFGF1 even though pFGF1 is lack of heparin binding. Thermodynamic and proteolytic studies prove that pFGF1 is more stable than WFGF1. Because pFGF1 is resistance to trypsin, truncated pFGF1 (tr-pFGF1) was purified. Through determining the biological activity and molecular mass of tr-pFGF1, it was shown that tr-pFGF1 lost part of its structure and exhibits mitogenic activity. In the future, tr-pFGF1 will be more physically characterized to understand if pFGF1 is cleaved at its C or N terminal end.

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Chapter 3: FGF1 is a Novel Treatment for Wound Healing

Conclusion:

In short, fibroblast growth factors (FGFs) which mitogenic factors consist of 23 genes that encode specific poly peptides in not only humans but also mice. The molecular weight of FGF family ranges from 17 kDa to 34 kDa. FGF is considerably significant in eliciting several biological processes in embryonic and adult organisms. In this thesis, penta mutations Q40P, S47L, H93S, K112N, R122E were introduced in FGF1 to increase protein stability and enhance its biological activity. We worked on FGF1 because of several reasons. First, natural wild type variant of FGF1 is prone to thermal denaturation and proteolytic degradation, and its biological activity is heparin dependent. Second, FGF1 is a universal ligand which has the ability to bind to all four FGFRs. Third, FGF1 has conserved amino acid called the heparin binding pocket. This pocket can bind to heparin through electrostatic interactions. Finally, FGF1 is secreted under the non-classical secretion pathway by multiples cellular types. Though pFGF1 does not bind to heparin, it shows higher biological activity than WFGF1. The pFGF1 shows higher thermal stability than WFGF1, and it is resistance to proteolytic cleavage. In the future, further biochemical and biophysical characterizations will be done on pFGF1 to specify whether pFGF1 is cleaved at its C or N terminal end.