Cloning and Expression of Human Recombinant FGF-2 Isoforms

A Major Qualifying Project Report

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Abstract

Following discoveries about the role that human basic fibroblast growth factor (bFGF or FGF-2) plays in cell reprogramming events, this project endeavored to establish a mammalian cell platform for further study of these proteins and their functions. FGF2 is encoded by a single mRNA , which utilizes the discrete 5' start sites resulting in production of five FGF2 protein isoforms; a low molecular weight (18kDa) isoform that is ligand for the plasma membrane FGF tyrosine kinase receptors and four high molecular weight isoforms (22, 22.5, 24 and 34 kDa) that regulate cell function via nuclear-mediated actions. The five isoform sequences were originally cloned into separate pBluescript vectors. The sequences were then subcloned into a mammalian expression vector, and the protein was overexpressed in CHO-K1 cells where production and localization were verified by immunoblotting. We report production of two of the five protein isoforms as recombinant human proteins.

Acknowledgements

We would like to thank our project advisors, Professors Buckholt and Dominko for their continued support and guidance throughout our project. We would also like to thank Denis Kole and Allie Grella, as well as the rest of the Dominko Lab for their invaluable support. Lastly, to lab manager Abbie White for providing needed equipment and materials to support our project.

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Background

1.1 Introduction

The Fibroblast Growth Factor (FGF) family of growth factors is comprised of several different genes, all of which bind as ligands to FGF tyrosine kinase receptors. This family of proteins was first identified in 1973 in pituitary extracts (Armelin, 1973), and later it was observed that this same protein extracted from a cow brain caused 3T3 fibroblasts to proliferate in a bioassay (Gospodarowicz, 1974). This type of activity was later related to an observation from 1939, that the brain was an area with a high occurrence of fibroblast mitogenic activity. It was found that FGF when separated by isoelectric points fell into two major peaks: one acidic and one basic. These were then dubbed Acidic FGF (aFGF or FGF-1), and Basic FGF (bFGF or FGF-2) (Thomas, 1987). Since that point, there have been 22 FGF genes identified, which are dispersed throughout the genome in small clusters. Some FGFs even have multiple active loci, such as FGF-7 which has active segments in 16 locations throughout the genome (Ornitz and Itoh, 2001; Kelley et al., 1992). These facts support the idea that there have been both gene and chromosomal replication and translocation over the course of time (Ornitz and Itoh, 2001). These genes gain even more variable structures and functions via alternative splicing and internal ribosomal entry sites (IRES). FGF-2 and FGF-3 exhibit IRES activity, which utilizes 5' CUG alternative initiation sites to create 5' extensions of the protein (Keifer et al., 1994; Arnaud et al., 1999). Other FGFs also use sub-exon splicing to allow further varieties of the protein to be created (Ornitz and Itoh, 2001). The coding section of the DNA is typically comprised of 3 exons where exon 1 contains the translational start methionine; however there are notable exceptions, such as the FGFs with 5' CUG initiation sites. Fibroblast Growth Factors typically have a recognizable amino terminal secretion sequence, which means that they act in an exocrine-like manner. FGFs 9 (Miyake et al, 1998), 16 (Miyamoto et al., 1993), and 20 (Ohmachi et al., 2000) all lack known amino-terminal secretion signals, yet are secreted from the cell normally. FGFs 1 and 2 also lack an amino-terminal secretion signal, (with

the exception of the 18kDa FGF-2 Isoform) instead they have nuclear localization signals (Ornitz and Itoh, 2001). All of the various FGFs are expressed at different times throughout an organism's life cycle, and while some (FGF3,4,8,15,17,and 19) are expressed solely in the developmental stages, others seem to be expressed throughout the entire lifespan (FGF1,2,5,6,7,9-14,16,18, and 20-23) (Ornitz and Itoh, 2001). It has been shown by creating knockout animals that many different phenotypes can be observed by deleting single FGFs: some are immediately lethal (killing the animal before birth), while others have very subtle phenotypes (Ornitz and Itoh, 2001). The appearance of subtle non-fatal phenotypes is thought to arise because FGFs have similar and overlapping functions, and thus deletion of one could possibly be compensated for by another similar FGF. Overall, these genes and their protein products seem to play a fairly diverse role in the developmental stages of life, but based on many methods of regulation: alternative splicing, alternative initiation, secretion, and specificity. These proteins are capable of being tightly regulated, in order to prevent uncontrollable growth or another deleterious phenotype.

1.2 FGF-2

Basic Fibroblast Growth Factor 2 (bFGF-2 or FGF-2) was first discovered as a 146-amino acid protein that was isolated from the pituitary gland (Bohlen, et *al.,* 1984). FGF-2 cDNAs were cloned and the start codon (AUG) was located in the place to begin translation of a 155 amino acid protein (Abraham, et *al.,* 1986). Also, there was no in-frame start codons found upstream, so the aforementioned start codon is the predicted start site for translation (Abraham et al., 1986). On the other hand, FGF-2 proteins come in a variety of lengths, both shorter and longer than the predicted length from the cDNA sequence (Florkiewicz, et *al.,* 1989). The longer or higher molecular weight proteins (196, 201, and 210 amino acids) were found to be created through *in vitro* transcription and translation analysis. This revealed that CUG codons were used as the translation start site for these larger segments (Prats, et *al.*,1989). The shorter proteins were found to be from the result of proteolytic degradation (Klagsbrun, et *al.,.* 1987).

Other translation happens by internal ribosomal entry sites in the FGF-2 mRNA (Vagner, et *al.,* 1995). When the single FGF-2 cDNA is expressed in cells, a single AUG and three CUG start codons were observed (Figure 1) (Quarto et al., 1991). The 22, 22.5, and 24 kDa sizes of the protein are initiated at the CUG start codon (Quarto et al., 1991). The 18 kDa protein is initiated at the AUG start codon, which is farther downstream. This explains why the 18 kDa protein is so much smaller than the others (Quarto, et *al.,* 1991). More recently it was determined that the 34 kDa isoform has a CUG start codon that is located 86 nucleotides away from the FGF-2 mRNA 5' end (Arnaud et *al.,* 1999). Also, the 22, 22.5, and 24 kDa sizes of the protein are largely localized in the nucleus, while the 18 kDa size is localized in the cytoplasm (Bugler, et *al,.* 1991).

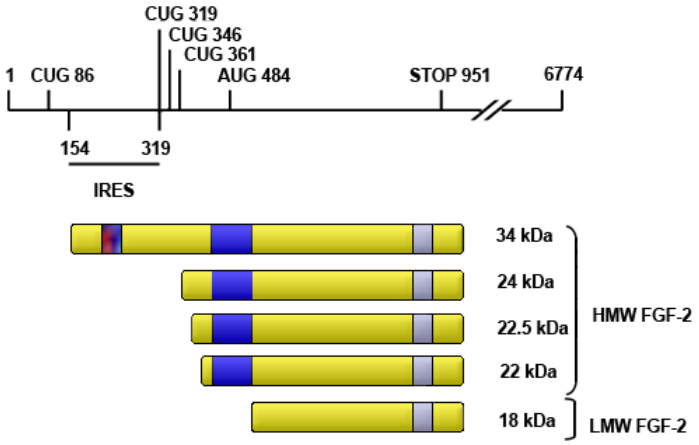

Fig 1: mRNA coding schematics of the five FGF-2 isoforms with start codons for each iosform (Atlas of Genetics).

A three-dimensional crystalline structure of 18 kDa FGF-2 has been created through the use of recombinant proteins (Ericksson, et *al.,* 1991). FGF-2 includes 12 anti-parallel beta sheets that are organized into a trigonal pyramidal structure (Zhu et *al.,* 1991). The receptor binding sites are believed to be residues 13 to 30 and 106 to 129 (Baird et *al.,* 1988). There are also two potential phosphorylation sites, serine 64 and threonine 112. Protein kinase A and protein kinase C are responsible for the phosphorylation of FGF-2, and they may be localized in the nucleus and at the cell surface (Feige and Baird, 1989).

A distinctive feature of the high molecular weight isoforms of FGF-2 is the amino terminal extension. The largest isoforms has a nine glycine – arginine reiterations (Quarto et al., 1991).

1.3 Cell Proliferation

FGF-2 has been shown to stimulate cell proliferation in an immature dental pulp cell model. These cells were isolated from mandibular incisors in rats. The cells were exposed to BMP-2, BMP-4, FGF-2, FGF-8, and NGF treatments and assayed from cell proliferation at four days in culture. Of the five treatments, FGF-2 yielded significantly higher proliferation. The treatments were performed a second time but with the use of β-Glycerophosphate, which is thought to induce growth in this cell type. The second treatment increased proliferation in all five samples but FGF-2 was still the only one to show significant increases in proliferation. Not only is FGF-2 effective in stimulating cell proliferation across varying cells types, it also can be paired with specific inducers to improve effectiveness (Nakao et *al,.* 2004).

1.4 Cell Migration

Cell migration requires all cells to be properly directed to specific locations. FGF-2 has been shown to play a crucial role in this function in certain functions, particularly in myelantation of the central nervous system (CNS). Oligodendrocyte precursors differentiate into the myelin-forming oligodendrocytes in the CNS. These cells originate at a specific location in the neural tube; therefore they must undergo migration to populate the entire CNS. One study showed that in the presence of FGF-2 cells underwent movement, but once FGF-2 was removed from the culture medium or FGF receptors were blocked, movement almost immediately stopped (Bribian, et. *al.,* 2006).

1.5 Early Embryonic Differentiation

The process by which a fertilized egg changes into a fully developed organism is known as differentiation. This is when the mass of totipotent cells differentiates into highly specified cells in the body (i.e. skin or bone cells). Totipotent cells are cells that have the ability to become any other

cell, unlike a cell that is hematopoietic, which can only differentiate into a blood cell. The main mystery surrounding totipotent cells is how they organize themselves into an embryo with a dorsal and a ventral side. Studies have shown that just before differentiation occurs, proteins can segregate themselves toward what will be the frontal and posterior ends of the embryo. A better understanding of these processes could lead to greater successes with in-vitro fertilization. Also, this process could in theory be used to make a cell revert to a state of higher potency and can thus be used for clinical research. Due to cells dependence on FGF-2 to maintain pluripotency FGF-2 could potentially play a role in steps of embryonic differentiation. (Said, et *al., 2010)*

1.6 Neuronal development

FGF-2 has been implicated in a variety of functions in the body, one of which is neurological development. Studies into these functions have yielded a great deal of information that may lead to some potential research avenues and therapies for various disorders. A prime example of this is a study from 2000, which presented a potential treatment to slow the rate of retinal degeneration due to retinitis pigmentosa, a type of eye disease leading to incurable blindness. In the study, transgenic rats were injected with an adenovirus containing the FGF-2 gene. These rats presented with a decreased rate of photoreceptor degeneration, as well as a slight increase in electroretinographic (ERG) responses, an electrical response in various parts of the eye, such as rods and cones. However, these ERG responses were not a significant improvement over the ERG responses from rats given an adenovirus without the FGF-2 treatment (Lau, *et al.,* 2000).

With regards to neurological function, FGF-2 is considered essential to developing the central nervous system. Studies have shown that in murine embryos, FGF-2 is found at high levels during neurulation, an early developmental stage of nervous system formation in utero. In addition, overexpression of specific FGF-2 isoforms has been implicated in regulating growth during spinal cord development. In an article from 1998 entitled “Impaired cerebral cortex development and blood pressure regulation in FGF-2-deficient mice”, scientists at the University of Heidelberg noted

that mice that lacked expression of FGF-2 resulted in widespread neuronal defects in the cerebral cortex, spinal cord, and hippocampal commissure, which is a part of the hippocampus that connects the other sections (Dono, *et al.,* 1998). All of this data suggests that FGF-2 is an integral protein in pre-natal nervous system development.

FGF-2's functions in the nervous system are also prevalent in fully developed organisms. FGF isoforms are found in astrocytes in the brain, which are necessary for healthy brain function and the regeneration of neurons, among other vital biochemical processes in the brain. FGF-2's specific function in these cells is to regulate levels of glial fibrillary acidic protein (GFAP), which is found in gray matter astrocytes. A study into FGF-$2^{-/-}$ single and FGF-$2^{-/-}$-FGF-$5^{-/-}$ mutants showed that FGF-2 is required for these astrocytes to differentiate. The most important finding from this study, however, is that FGF-2 plays a role in maintaining the blood brain barrier via GFAP and astrocytes. All of this information provides a strong foundation for understanding FGF-2's role in neurology, and a greater understanding of these FGF-2-associated pathways could lead to novel therapies for neurological disorders, as well as a potential way to repair some forms of brain damage (Reuss, *et al.,* 2003).

1.7 Angiogenesis

Although FGF-2 has a definitive link to the nervous system, one of the growth factor's major features is its ability to promote the growth of blood vessels, a process known as angiogenesis. In a study comparing murine blood vessels grown using FGF-2, vascular endothelial growth factor (VEGF)-A, and VEGF-C, it was found that FGF-2-based blood vessels had little or no vascular fenestrations, or openings in blood vessels allowing for rapid exchange of fluids between the circulatory system and surrounding tissues. In contrast, VEGF-A blood vessels were found to be highly fenestrated. This finding would suggest that FGF-2-based angiogenesis could be a potential method of creating blood vessels for vital organs. While scientists do not currently understand how exactly FGF-2 regulates angiogenesis, FGF-2 is currently undergoing clinical trials as a treatment for

ischemic heart disease (Cao et *al.,.* 2004).

In yet another role, FGF-2 has been shown to have the ability to induce hypotension in animals (Cuevas *et al.,* 1991), as well as hypertension under certain incidents of cellular stress. In the 1998 article from the University of Heidelberg, the FGF-2-deficient mice that were studied showed a lower than normal blood pressure, in addition to neuronal defects. However, subsequent tests on the cardiovascular system showed responsiveness, with the exception of an impaired baroreceptor reflex (Dono *et al.,* 1998). This led scientists to believe that the problem was coming from a dysfunctional autonomic nervous system, since the mice were already suffering various neurological defects, and baroreceptor reflexes are governed by the nervous system.

1.8 Regeneration and Stem Cell Pluripotency

The range of processes FGF-2 affects allows it to be applied to a variety of applications in research. Many recent discoveries for the applications of FGF-2 include bone healing, tissue regeneration, and maintenance of pluripotency. A clinical study running from 2006-2007 looked at alveolar bone height regeneration as affected by application of FGF-2. Patients suffered from 2- or 3-walled intrabony defects and were assigned to four groups, each receiving a separate treatment including controls and increasing dosage of recombinant FGF-2. The treatments were delivered during periodontal surgery to affected sites. The results showed major rate increases in regeneration between the control group receiving surgery with no FGF-2 administered and the group with the highest concentration of FGF-2 (Fujii, *et al.,* 2010). This result was one of many being discovered through the use of FGF-2 to restore bone and tissue mass. An even larger study published in 2010 yielded the same results, stating a percentage of bone fill, up to 69.14% (Kitamura, *et al.,* 2010). Bone fill is defined as the increase in alveolar bone height. FGF-2's stimulation of proliferation and pluripotenncy maintenance, as well as angiogenesis (Shimabukura *et al.,* 2005) and stimulation of other extracellular matrix production, created an environment for tissue regeneration to occur (Terashima *et al.,* 2005).

1.9 Maintenance of Stem Cell Pluripotency

The application of FGF-2 to stem cell growth media allows cells to maintain their pluripotent state. In a 2005 study, human embryonic stem cells were grown at concentrations of 40ng/ml and 100ng/ml FGF-2. At 40ng/ml ES cells were maintained with consistent centers of differentiation in the colonies; at 100ng/ml these centers were prevented. Cells cultured in 100ng/ml were maintained for 4-7 months and then successfully analyzed for OCT4 and NANOG, markers for undifferentiated cells. Later it was determined that expression of OCT4, SOX2, and NANOG all promote increased expression of FGF-2, creating a regulatory loop to maintain the pluripotent state (Levenstein et *al.,* 2006).

1.10 Osteogenesis

FGF-2 also can be used to increase bone-healing capacity in different parts of the body. The skull's frontal bone has been shown to possess great healing capacity and a highly activated FGF-domain. By exposing the less active parietal bone to FGF-2 it is able to mimic the superior healing of the frontal bone. After drilling holes in frontal and parietal skull bone of mice they were treated with FGF-2 soaked collagen sponges and monitored for FGF signaling and proliferation markers. After exposure to FGF-2, the parietal bone displayed increased bone regeneration, cell proliferation, and osteogenic progenitors as well as activated FGF-2 signaling pathways. The use of exogenous FGF-2 shows the ability to overcome pre-determined bone healing properties (Behr *et al.,* 2009).

1.11 Preserving organ viability

As well as possessing regenerative properties, FGF-2 has also been used as a supplement in serum-free media for storage of human and porcine corneas. Currently corneas are stored in fetal calf serum, but this has adverse effects due to bovine-related disease concerns and the inability to repeatedly produce standardized media. By using a serum-free media supplemented with FGF-2, the endothelial survival of corneas has a significant survival increase. Serum-free media

supplemented with FGF-2 yielded a reduced amount of damaged endothelial cells occurring over nine days of storage. After 22 days of storage, FGF-2 supplemented media still maintained a reduced damaged state. FGF-2 media also contained less apoptotic and dead cells, consistent with the effects of fetal calf serum and significantly better than serum-free media without FGF-2. The effect of FGF-2 in this media is purely protective; it prevents damage and death but was not shown to stimulate mitosis. It is an important contributor to improving organ storage and a viable alternative to serum based media (Rieck et *al,.* 2003).

1.12 Cloning and Expression of Human Recombinant FGF-2 Isoforms

With the discovery of the myriad of implications FGF2 has in the body, it has become prudent that this protein family be studied in depth. However, studying these proteins individually is hampered by the fact that they are all translated from the same mRNA. In this project, each of the unique isoforms were attempted to be cloned into separate recombinant expression vectors, as well as transfected into mammalian cell cultures to ensure recombinant protein production. In this project mammalian expression vectors for 22.5, 24, and 34 kDa isoforms were created and protein production of 22.5 kDa and 34 kDa isoforms was verified in CHO-K1 cells.

Results

2.1 Subcloning Plan

This project began with all 5 isoforms of FGF-2 (18, 22, 22.5, 24, 34 kDa) contained within individual pBlueScript bacterial vectors. The protein encoded in these sequences consisted of the full-length mature protein with a 6xHis-Tag at the N Terminus and a TEV protease cleavage site between that and the mature protein sequence. It was determined that the best way to express the FGF-2 isoforms in a useful way was to subclone the sequence into a mammalian expression vector directionally (Figure 2). The sequence contained EcoRI and Hind III restriction sites usable in the subcloning method. After pBS digestion with EcoRI and HindIII, the insert sequence, complete with 6xHis tag, TEV protease site, and sticky ends, would be recovered. This insert would then be ligated into the similarly digested mammalian expression vector pcDNA3.1(-) (Invitrogen). This ligation would yield a recombinant ligation product of pcDNA3.1(-) + FGF-2 with proper directionality.

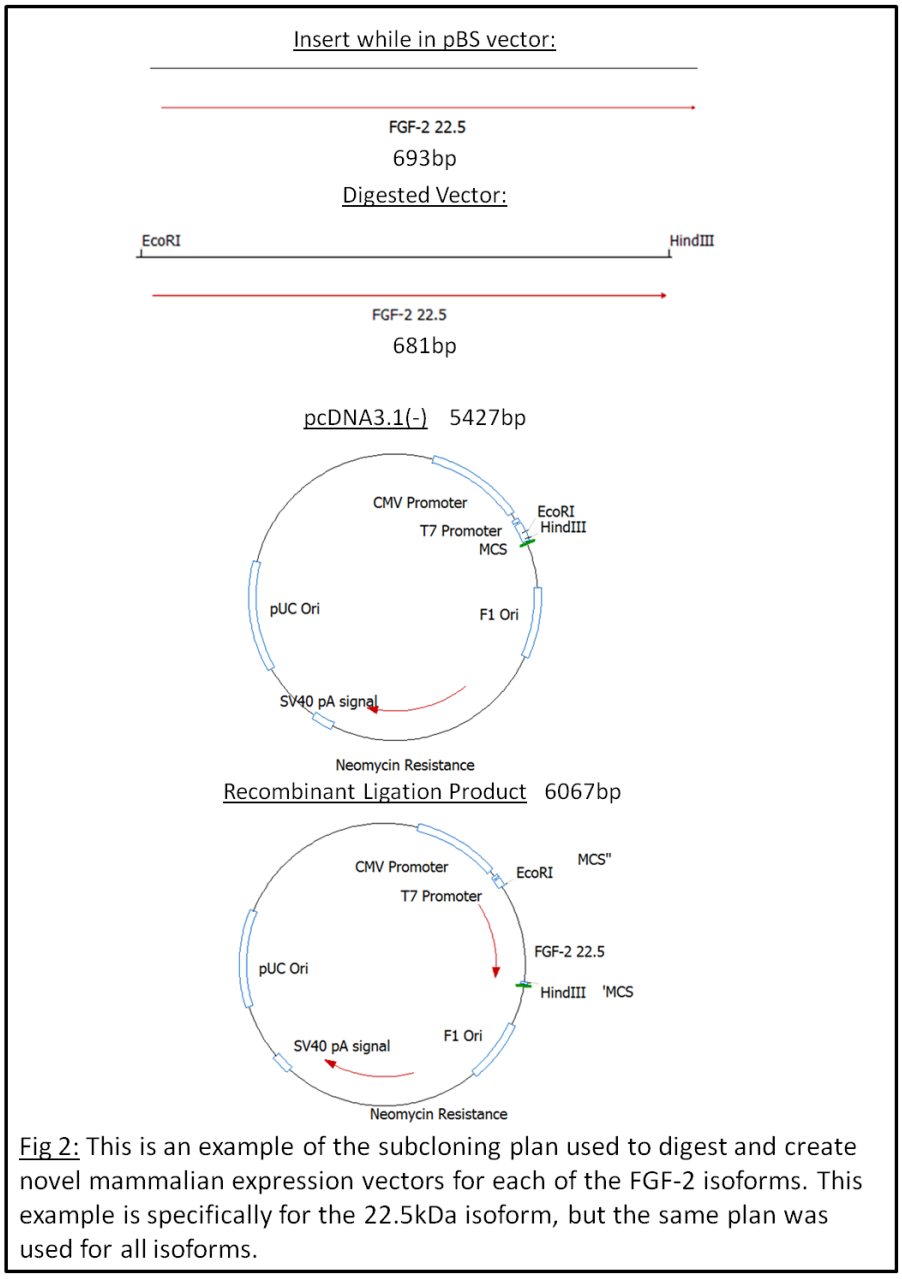

Fig 2: This is an example of the subcloning plan used to digest and create novel mammalian expression vectors for each of the FGF-2 isoforms. This example is specifically for the 22.5kDa isoform, but the same plan was used for all isoforms.

2.2 Isolation of Recombinant Isoform Specific cDNA

The initially supplied pBS vectors with FGF-2 (18, 22, 22.5, or 34 kDa) were digested with EcoRI and HindIII to cleave out the gene contained each vector's respective MCS (Figure 3). The digestions were then run on a 1% agarose gel. Each gel then had a band extraction and purification performed to recover the coding insert of each isoform in preparation for ligation into a

mammalian expression vector. The concentrations recovered from each extraction were: 18kDa - 60ng/µl, 22kDa - 23ng/µl, 22.5kDa - 25ng/µl, 24kDa – 23ng/µl, 34kDa – 59.9ng/µl.

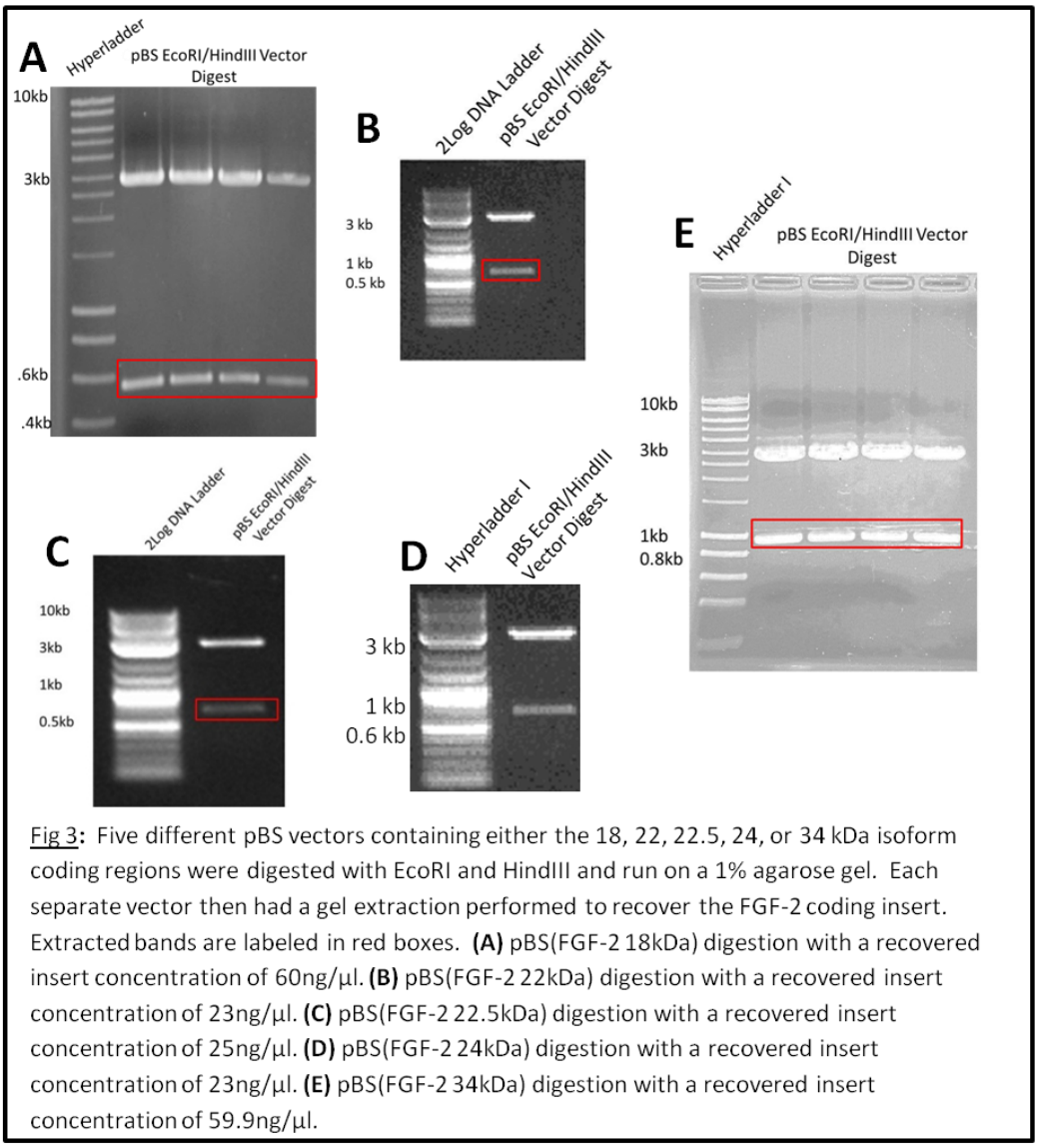

Fig 3: Five different pBS vectors containing either the 18, 22, 22.5, 24, or 34 kDa isoform coding regions were digested with EcoRI and HindIII and run on a 1% agarose gel. Each separate vector then had a gel extraction performed to recover the FGF-2 coding insert. Extracted bands are labeled in red boxes. **(A)** pBS(FGF-2 18kDa) digestion with a recovered insert concentration of 60ng/µl. **(B)** pBS(FGF-2 22kDa) digestion with a recovered insert concentration of 23ng/µl. **(C)** pBS(FGF-2 22.5kDa) digestion with a recovered insert concentration of 25ng/µl. **(D)** pBS(FGF-2 24kDa) digestion with a recovered insert concentration of 23ng/µl. **(E)** pBS(FGF-2 34kDa) digestion with a recovered insert concentration of 59.9ng/µl.

Each pBS vector, cut with EcoRI and HindIII, was then run on a single 1% agarose gel in order to demonstrate the increase in size of each isoform's coding region. Due to gel smiling, the migration of the bands was altered but the size differences are still apparent (Figure 4).

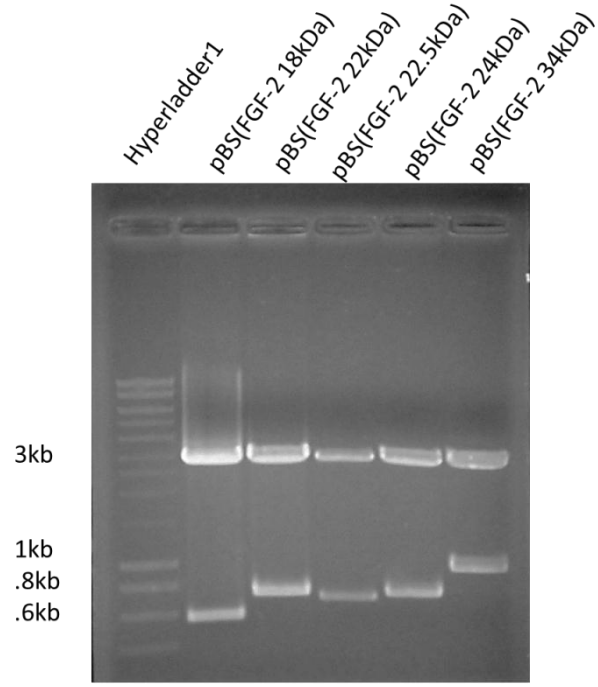

Fig 4: All of the pBS vectors containing the 5 different FGF-2 isoforms were digested in parallel with EcoRI and HindIII, and were run on a 1% agarose gel. The bands don't all run to the expected areas because the gel smiled on the right side(from lane 4-6).

2.3 Isolation of pcDNA3.1(-) Linearized Vector

After each isoform's insert was isolated and extracted from its pBS vector, it was necessary to ligate the insert into pcDNA3.1(-) as described in the subcloning method. In order to maximize use of materials pcDNA3.1 (-) was transformed into DH5α. The vector contains the gene for ampicillin resistance, therefore transformed DH5α was grown under the selective pressure of ampicillin. These liquid cultures were then miniprepped to extract the plasmid. The purified

plasmid was then prepared for ligation by digestion with EcoRI and HindIII in order to maintain directionality for insertion (Figure 5).

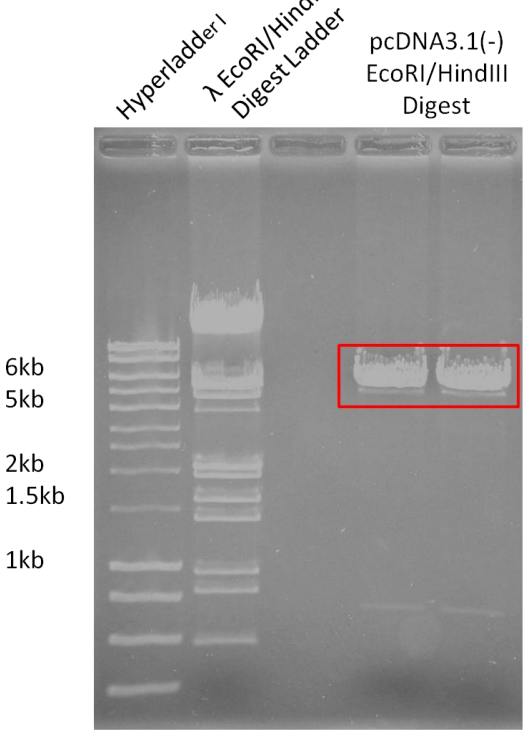

Fig 5: pcDNA3.1 was amplified in DH5α, and then digested with EcoRI and HindIII to prepare it for a directional ligation with the inserts recovered from gel extraction of the pBS vector digests. A λ EcoRI/HindIII Digest ladder was also used for finer reference to the band size. The bands in the red box were gel extracted, and yielded a concentration of 66.6ng/µL.

2.4 Creation of Mammalian Expression Vector

Once both the insert and vector had been digested and gel extracted, they were ligated and subsequently transformed into chemically competent DH5α. Colonies which grew on an antibiotic-containing LB+agar plate were liquid cultured, and their plasmid was miniprepped. The extracted plasmid was double-digested with EcoRI and HindIII to ensure the identity of the plasmid the transformants contained (Figures 6, 7, 8).

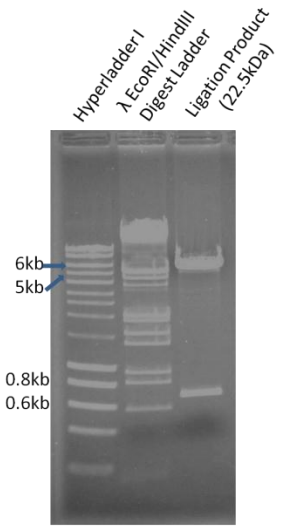

Fig 6: The ligation product was digested with EcoRI and HindIII in order to verify its identity and that it contained the desired insert. A λ EcoRI/HindIII digest ladder was used as an additional size reference for the verification digestion. The desired 681 bp insert and 5386bp vector are clearly visible in the "Ligation Product (22.5kDa)" Lane.

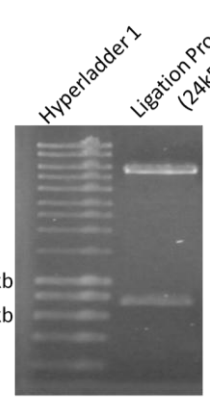

Fig 7: The ligation product of pcDNA3.1(-) and FGF-2 24kDa insert was digested with EcoRI and HindIII in order to verify its identity and that it contained the desired insert. The desired 708bp insert and 5386bp vector are clearly visible in the "Ligation Product" lane.

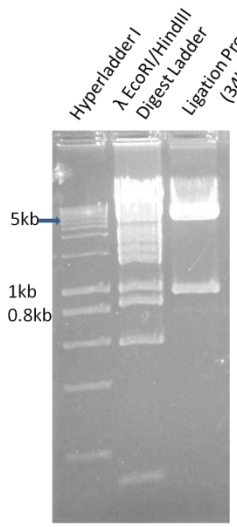

Fig 8: The ligation product was digested with EcoRI and HindIII in order to verify its identity and that it contained the desired insert. A λ EcoRI/HindIII digest ladder was used as an additional size reference for the verification digestion. The desired 942bp insert and 5386bp vector are clearly visible in the "Ligation Product (34kDa)" Lane.

2.5 Protein Production in Mammalian Cells

After the recombinant plasmid was generated, its identity and functionality needed to be tested. This was achieved by transfecting the plasmid into an easily transfectable mammalian cell line: CHO-K1 to be specific. After 3 days of being incubated with the lipid transfection reagent and recombinant plasmid, protein production was tested for by immunoblotting lysates from selected treatments (Figure 9. The ratios refer to µg plasmid: µL Transfection reagent).

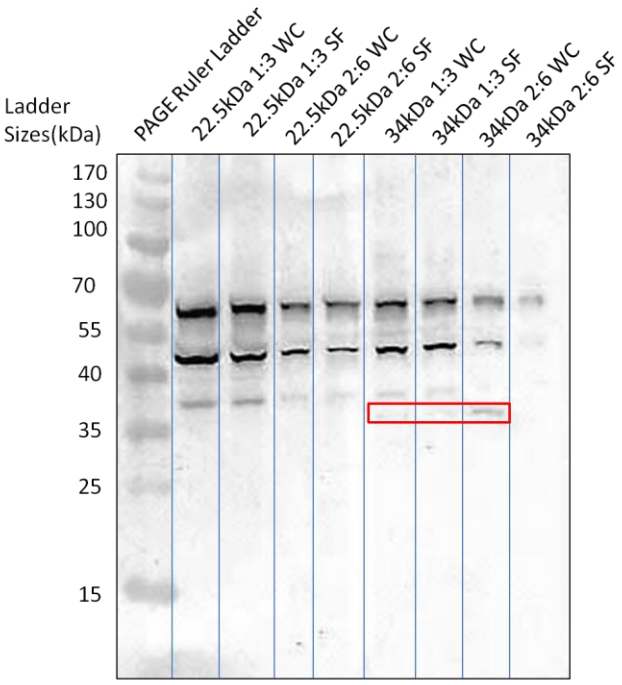

Fig 9: Whole cell and soluble fractions of all transient transfection s were Immunoblotted and probed with Ms-Anti-6xHis as the 1° antibody and Rb-Anti-Ms-AP as the 2° antibody. The area in the box contains light bands indicative of the 34kDa isoform with TEV and 6xHis tag intact. This signal is not present in the 34kDa 2:6 SF lane most likely because the blot appears to not have transferred completely, or the lane was not loaded completely.

After the transient transfections were tested, the cells were kept under antibiotic selection and were eventually immunoblotted again to verify the presence of His-tagged protein being constitutively expressed in all samples. This immunoblot for stable transfection was performed on both the 22.5kDa and 34kDa isoforms of recombinant FGF-2(Figures 10 and 11, respectively).

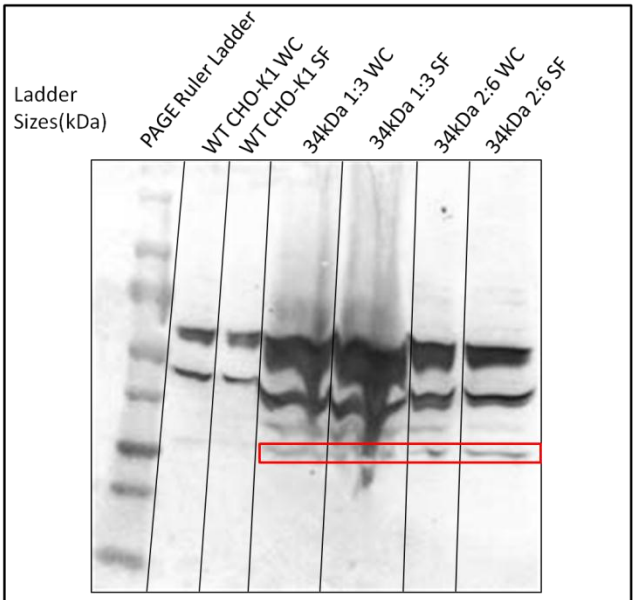

Fig 10: Extracts from 34kDa transfected cells were immunoblotted after 3 weeks of antibiotic selection. WT CHO-K1 cells were harvested and processed as a negative control. The boxed area indicates the band of 34kDa FGF-2, which ran at about 36kDa because of the non-endogenous TEV cleavage site and 6xHis tag. The 1° AB was Ms-Anti-6xhis, and the 2° AB was Rb-Anti-Ms-AP.

After constitutive overexpression of the proteins was confirmed, it was decided to begin performing loading controls and probe with alternative antibodies (Figure 11) such as Anti-FGF-2 antibody. Anti-Actin was used as a loading control to ensure that all lanes were loaded equally.

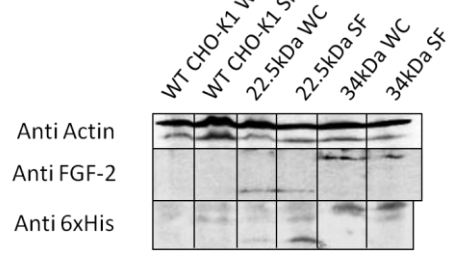

Fig 11: Extracts from 34kDa transfected cells were immunoblotted after 3 weeks of antibiotic selection. WT CHO-K1 cells were harvested and processed as a negative control. The boxed area indicates the band of 34kDa FGF-2, which ran at about 36kDa because of the non-endogenous TEV cleavage site and 6xHis tag. The 1° AB was Ms-Anti-6xhis, and the 2° AB was Rb-Anti-Ms-AP.

2.6 Enrichment of Recombinant FGF-2 via Nickel Column Enrichment

A nickel resin was used to preferably associate with the 6xHis tagged region of the protein, and then the resin was washed, and eventually the proteins associated with the resin were eluted by introducing increasing concentrations of imidazole to the buffers used. Imidazole competes for binding to the nickel in the resin, allowing for the proteins to be collected in the elution. The resulting fractions were immunoblotted to observe their purity (Figure 12).

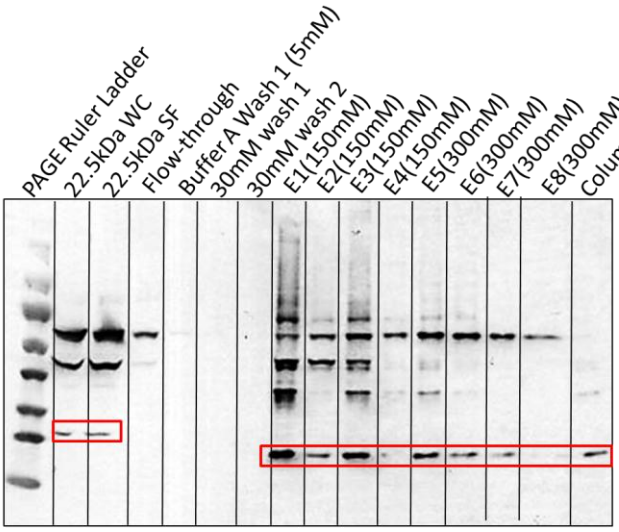

Fig 12: Recombinant FGF-2 22.5kDa was enriched with a nickel-resin pulldown. The washes and elutions were performed with WX buffer which contained the shown molarity of Imidazole, and the column strip used WX with 1M imidazole. The boxed areas represent the protein of interest: FGF-2 22.5kDa isoform.

Discussion

The five pBluescript constructs containing cDNA for each individual FGF-2 isoform (18, 22, 22.5, 24, 34kDa) were successfully amplified under ampicillin antibiotics in E. *coli* and then isolated. The isolated vectors were digested with EcoRI and HindIII and from the digest we were able to isolate the FGF-2 cDNA from the vectors. The isolated inserts were then ligated into pcDNA3.1(-). The 22.5, 24, and 34kDa cDNA were successfully ligated in the vector and amplified in E. *coli* under ampicillin selection. This was confirmed by restriction enzyme double digest and gel electrophoresis. We attempted to ligate the 18kDa and 22kDa isoform cDNA into the mammalian expression vector but transformation with the ligation reaction yielded no transformant colonies.

Only the completed 22.5 kDa and 34 kDa mammalian expression vectors were transfected into mammalian CHO-K1 cells. The transfection was performed using a lipid transfection reagent. Initially the cells were allowed to grow without antibiotics to allow the transfection reagent and vectors to be fully incorporated, but after three days the antibiotic G418 was added. This allowed for selection of only cells which were expressing neomycin resistance (signaling vector integration). The cells were cultured for three weeks at varying concentrations of antibiotics, proving that the cells had correctly taken up our genes of interest.

In immunoblots of stably transfected CHO-K1 cells, the expression of 22.5 and 34 kDa FGF-2 isoforms were examined. Whole cell fractions and soluble fractions of both cell lines were probed using anti-FGF-2 and anti-6xHis. CHO-K1Probing with each of these antibodies had a separate goal in the experiment of determining correct plasmid function. The anti-actin was to ensure that all lanes were loaded with an equal amount of protein so that all lanes were comparable to each other. The anti-his was to show whether recombinant protein was being produced, since wild type protein does not have a 6xHis tag. Finally, the anti-FGF2 was to see whether CHO-K1 cells endogenously produce FGF-2, and if this was the case to see whether plasmid treatment resulted in overexpression.

Part of this project's goal was to establish a mammalian cell platform for expression of individual isoforms. In order for this to be successful on a larger scale proper purification techniques must be established. Through nickel column enrichment we were able to show that the 6xHis tag is functional in a purification scenario. The enrichment of the 22.5 kDa isoform shows that the recombinant protein binds tightly to the resin because of the lack of FGF-2 in the flow-though. This enrichment also shows that the protein has a fairly high affinity for the resin, since during the various wash steps, no protein was eluted. As higher concentrations of imidazole are added to the resin the imidazole competes for binding, releasing the isoform from the column for collection. The successful protein enrichment suggests that a larger scale purification with a full column would result in protein purification rather than enrichment due to the larger concentration of recombinant protein as well as ability to be more specific with elution buffer concentrations.

The purpose of this project was to create a base for more study. Continuations of this project would absolutely include creation of the 18 kDa and 22 kDa mammalian expression vectors as well as transfection of the remaining vectors into CHO-K1 cells and validation of protein production. Additionally, transfection into a primary cell line (Fibroblasts) would allow for various immunoprecipitation assays to determine protein binding information such as chromatin immunoprecipitation (ChIP) and protein complex immunoprecipitation (Co-IP). Studies could also be done to examine exogenous activation of known membrane proteins (FGFR1) and protein localization. The range of activities associated with FGF-2 creates an even broader range of research venues for this protein. Once the actions of the individual isoforms can be identified therapy and research currently being done with FGF-2 may be significantly improved.

Materials and Methods

Vectors Used

The two vectors used included pBluescript (figure 13) and pcDNA3.1 (figure 14). pBluescript contains the ampicillin resistance gene and a lacZ gene. The lacZ gene was used as a reporter for proper ligation into the MCS. pcDNA 3.1(-) from Invitrogen was selected as the mammalian expression vector because of its strong CMV promoter and MCS orientation, as well as ampicillin and neomycin resistance genes used for bacterial and mammalian growth selection.

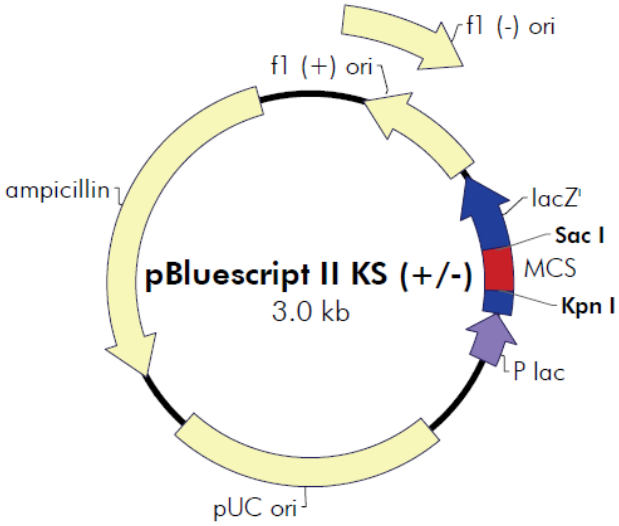

Fig 13: pBluescript schematic detailing origins of replication, ampicillin resistance gene, lacZ, and the multiple cloning sites.

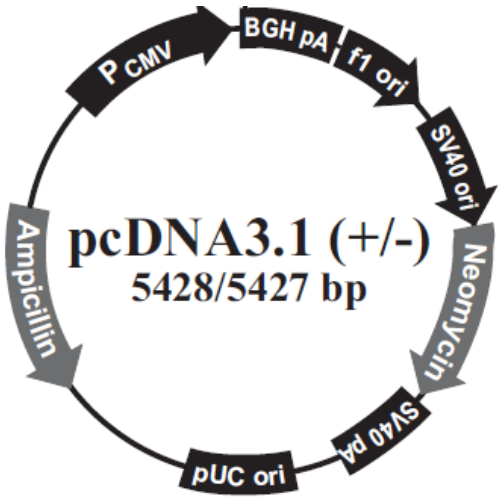

Fig 14: pcDNA3.1 (+/-) schematic detailing promoters, origin of replications, ampicillin, and neomycin resistance.

Cell culture

For propagation of the plasmid DNA for the subcloning process, the DH5α strain of E. coli was used. These were grown in LB or SOC Media, or on LB or SOC agar plates. Ampicillin was used in the media and agar to select for E. coli which contained the DNA of interest. The mammalian cell line used was the adherent CHO-K1 line. The CHO-K1 cells were grown in 50% DMEM/50% Ham's F-12 (D/F12), with varying concentrations of FBS (between 5 and 10%). Initially no antibiotics were used, but after transfections, G418 was used to select for transfected cells. Varying concentrations were used: 500µg/mL and 750µg/mL for transfections, and 100µg/mL for maintaining selection after protein production was confirmed.

Vector Construction

In order to construct the pcDNA3.1(-) mammalian expression vector, the directionality of the insertion was considered a top priority. Both the vector and gene of interest containing bacterial vector were digested with EcoRI and HindIII from NEBiolabs. These digests were analyzed

by gel electrophoresis on a 1% agarose gel in 1xTAE. The fragment was then ligated using the Fermentas Rapid DNA Ligation Kit following manufacturer directions. The resulting reaction was transformed into competent DH5α (Either NEBiolabs NEB5-α or Inoue competent cells). These were then plated on appropriate plates containing 50μg/mL of ampicillin.

Restriction Digest

Restriction digests were performed by combining the following into a PCR tube: 1μg of DNA, 1 μl EcoR1, 1 μL HindIII, 1 μl BSA, 1/10 final volume NEB Bufferx2, and DNA quality water (NEBiolabs) to adjust final volumes as needed. The reaction was then allowed to occur over 60 minutes at 37°C and sizes of restriction fragments verified by agarose gel electrophoresis.

Competent Cells

Competent cells used were NEB5α (New England Biolabs) and Inuoe competent cells. Inuoe competent cells were prepared according to the protocol outlined on pages 1.112-1.114 of Molecular Cloning: A Laboratory Manual, Volume 1 (Sambrook, et *al.* 1989).

Transient and Stable Transfections of CHO-K1

The transient and stable transfections were started in a 6 well plate with $9.6x10^4$ cells per well, and when they were between 50-90% confluent, transfection was performed with Biorad Transfectin lipid transfection reagent according to the manufacturer's protocol, except that the final volume of serum free media used per well was 100μL. The cells were left in the transfection media for 3 days and one well of each treatment was harvested, lysed and immunoblotted as described below. The remaining wells had the media replaced, and then were split into additional wells, and 500μg/mL G418 was used to select for transfected cells. After 2 weeks of selection at this level, half of each treatment remained under 500μg/mL G418 treatment, while the other half was increased to 750μg/mL G418. After the third week of antibiotic selection, one well of each treatment was transferred into a 10cm tissue culture dish. These were used to continue the treatments, and to create cells for analysis. After verification of protein production, the antibiotic treatment was reduced to 100μg/mL G418 to maintain the lines.

Preparation of cell lysates and Immunoblotting

Whole cell fractions were prepared via 3 rounds of 10 bursts of sonication on low power (because the pellets were small). The soluble fraction was prepared by centrifuging the whole cell fraction at max RCF of the centrifuge for 15 minutes, and then sampling the clarified supernatant. Samples of these two fractions were combined with 4:1 with 5xBME protein loading buffer. Samples were loaded into a 15% SDS acrylamide gel, electrophoresed at 170V in Tris-glycine running buffer (0.025M Tris 0.2 M Glycine + 1% SDS) using Fermentas PAGE Ruler as a size marker. The proteins were transferred to a PVDF membrane in a semi-dry electrophoretic transfer cell using Towbin transfer buffer (0.025M Tris 0.2M Glycine buffer with 20% methanol). The membrane was blocked using 5% milk in Tris Buffered Saline(0.14M NaCl, 3mM KCl, 0.025M Tris Base) +0.1% Tween (TBST). The membrane was then incubated overnight with the primary antibody (Anti-His from Clontech (1:5000 dilution), Anti-Actin from Sigma (1: 5000), or Anti-FGF2

from AbCam (1:1000)) with rotation at 4°C. The following day the membrane was washed in TBST and then incubated for 1.5-2hrs with the alkaline phosphatase-conjugated secondary antibody: anti-rabbit, anti-mouse, or anti-goat (all at a 1:5000 Dilution, and all from Abcam). The membrane was washed again first with TBST and then with diH_2O, and incubated with AP substrate. The color-producing reaction was stopped by immersing the membrane back in the diH_2O prior to visualization. Chemmilluminescence images were acquired using Kodak 4000MM imager.

Nickel Resin Enrichment

Nickel resin (General Electric) was used to enrich samples of the recombinant protein from soluble fractions isolated from the transgenic CHO-K1 cells. The soluble fraction was incubated with 250 µL of equilibrated nickel resin (because a small scale protocol was used), and then was washed and treated with various concentrations of imidazole which competes with his-tagged proteins to bind to the nickel resin. These elutions were collected, and then were subsequently immunoblotted using an anti-6xhis antibody as the primary antibody.

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