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Expression, purification, and characterization of recombinant basic fibroblast growth factor in pichia pastoris

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EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF RECOMBINANT BASIC FIBROBLAST GROWTH FACTOR IN *PICHIA PASTORIS*

by

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DEDICATION

This thesis is dedicated to the love of my life, Dr. Emily Vu.

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Abstract

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Wounds in the mouth, occurring after oral surgery, take time to heal. No ointment can be added to help with the healing process because mouth saliva will constantly wash it away. In order to combat this problem, we propose engineering a normal flora microbe to grow at the site of injury and secrete a recombinant growth factor to promote healing of the damaged tissue. Our goal is to have the yeast *Pichia pastoris* produce human basic fibroblast growth factor (bFGF), which aids in cellular proliferation. *P. pastoris* is a good choice for this application because not only is it considered generally recognized as safe (GRAS) by the FDA, but it is a eukaryote that is able to perform posttranslational modifications and secrete large amounts of recombinant protein.

Previous studies have shown that a strain of *P. pastoris* can be engineered to express bFGF from a methanol-sensitive promoter. The study also showed that the bFGF, which was purified from the yeast's extracellular medium, was able to promote the growth of NIH/3T3 cells (mice fibroblasts). Because we needed the *P. pastoris* to express the bFGF in glucose –based tissue culture medium in the presence of mammalian cells, we expressed the bFGF from the constitutive promoter *GAP* promoter. Along with optimizing and characterizing expression of bFGF, we also investigated the effect of the recombinant protein on mammalian cell growth

using both scratch ad MTS assays. In addition, the effects of the yeast being co-cultured with mammalian cells was studied. Our results provide a basis for how a recombinant protein can be clinically used to improve wound healing in the mouth using a yeast strain to produce and secrete a growth factor at the site of injury.

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Chapter 1: Introduction

Healing of Oral Injuries

Wounds in the mouth, whether from a surgery or an injury, take some time to heal. For example, the gums from teeth extractions take 1 week to initially close and up to 8 weeks for the extraction cavity to be filled with bone (Politis, Schoenaers, Jacobs, and Agbaje, 2016). Within that time frame, a host of problems can arise such as dry socket and infection (Politis, Schoenaers, Jacobs, and Agbaje, 2016). Dry socket is when the blood clot of an extracted tooth gets dislodged, dissolves, or never forms before the wound is healed, exposing underlying bone and nerves which is extremely painful. In addition, a bacterial infection can happen anytime there is an opening in our tissue, which would mean that the longer the wound is present, the more likely there will be a bacterial infection. Topical ointments that can promote healing are not effective when applied to the injured oral site because the constant production of saliva will simply wash it away, rendering the ointment ineffective. We will try to tackle this problem by creating a product containing a strain of yeast that will be able to adhere to the site of injury and constitutively secrete a recombinant growth factor to accelerate the healing of the damaged tissue so that no further complications can arise.

Recombinant Protein

The introduction of genetic engineering has opened the door to the world of recombinant proteins, such as antibodies, enzymes, and vaccines. These products are made in host cells, such as bacteria or yeasts, that normally do not produce these proteins. This process called heterologous protein expression has become a multibillion-dollar market (Gomes et al., 2012). Because of this, many advances in this technique have been made, but the basics for

heterologous protein expression start with identifying the gene that encodes for the protein of interest. Then, a cDNA is made from the respective mRNA. The cDNA is then inserted into a vector (bacteriophage, plasmid, or virus) which delivers the cDNA to a suitable host including bacteria (Baneyx, 1999), yeast (Cregg et al., 2000), and mammalian cell (Rosser et al., 2005) cells. That host will then express the desired protein from the cDNA that was transformed into it after transcription and translation. The proteins will then be purified and collected in order to use or sell. The steps of this process vary for each protein depending on the expression vector and host.

Bacterial Host Protein Expression System

The first system for foreign protein expression was done in bacteria, or more specifically, in *Escherichia coli* (*E. coli*) (Chen, 2011). This system is generally favored for it is easy to genetically manipulate, has a doubling time of only 30 minutes, and is generally easy to grow in lab conditions. Even though this protein expression system may be efficient, *E. coli* has its limitations on what kinds of proteins can be produced due to the fact that it is a prokaryote. Many prokaryotes have difficulty or are incapable of performing many complex post-translational modifications such as disulfide bond formation and glycosylation. This is mainly because prokaryotes do not have any membrane bound organelles that can make post-translational modifications like the endoplasmic reticulum (ER) or the Golgi apparatus (Choi et al., 2003). Some proteins require these modifications in order to be fully functional because post-translational modifications control charge, solubility, folding, correct cellular targeting, and immunogenicity (Walsh and Jefferis, 2006).

Another limitation to what *E. coli* can do lies in the purification of the desired protein. When *E. coli* produces the target protein, the protein generally is retained within the cell inside the cytoplasm (Sahdev et al., 2007). Not only is lysing the cells necessary, but many endogenous proteins from the *E. coli* will be present so many steps are required to isolate the foreign protein of interest. Not only will this system bring up the cost and time to purify the desired protein, but there will a significant loss of the protein during the many steps to purification (Sahdev et al., 2007). Recombinant proteins isolated from *E. coli* also contain endotoxins as it is a part of a Gram-negative cell wall. Endotoxins can lead to inflammation and other problems if the protein is taken internally (Suffredni et al., 1999).

Yeast Recombinant Protein Expression System

Yeast protein expression systems have been successful systems for many years with the development of genetic engineering tools (Gomes et al., 2018). There is a large variety of yeast expression hosts that are used such as *Saccharomyces cerevisiae* (*S. cerevisiae*), *Pichia pastoris* (also known as *Komagataella phaffii*), *Kluyveromyces lactis*, and *Yarrowia lipolytica* (Gomes et al. 2018). Yeasts are often used as hosts to express certain proteins because they are simple eukaryotes which can do post-translational modifications (Mu et al., 2007). Yeast cells are also physically robust, so they are easy to cultivate (Mu et al., 2007). Lastly, because yeasts do not secrete a lot of their own proteins, do not have endotoxins, and are not subject to bacteriophage contamination, recombinant protein purification does not require many costly and time-consuming steps (Cereghino and Cregg, 1999). Because of these reasons, yeasts are used as hosts to express a large variety of recombinant proteins such as chemicals, fuels, food, and pharmaceuticals (Gomes et al., 2018).

Pichia pastoris as a Recombinant Protein Expression System

The methylotrophic yeast (yeast that can reduce 1 carbon compounds as the carbon source for their growth) *Pichia pastoris* (*P. pastoris*) has several advantages as a host. Although *S. cerevisiae* is the dominant yeast species chosen for heterologous expression, *P. pastoris* has achieved a very high level of success in producing over 5,000 recombinant proteins, including industrial enzymes and biopharmaceuticals (Safder et al., 2018). These *P. pastoris* produced peptides are less likely to be immunogenic and inactive than those made in *S. cerevisiae* since *P. pastoris* does not hyperglycosylate its proteins (Ahmad et al., 2014). *P. pastoris* prefers respiratory growth over fermentation and does not produce ethanol. Because of the absence of this toxic alcohol, *P. pastoris* can reach a higher cell density than baker's yeast to produce more of the desired recombinant protein. In addition, *P. pastoris* does not secrete as many endogenous proteins as *S. cerevisiae*; therefore, purification of the secreted desired protein can be done much easier with *P. pastoris* (Faber et al., 1955). It was even found, in one instance, that up to 80% of the total protein secreted into the extracellular medium was the foreign recombinant protein (Montesino R. et al., 1998).

Another reason for using the yeast *P. pastoris* is because the members of the genus *Pichia* are found to be a part of the normal flora in the human mouth (Hunter et al., 1998). This is very important because introducing a new species of microorganism into an environment that normally does not have it can cause the new microbe to outcompete the ones that are already there, causing an overgrowth of the introduced species. This can result in many health problems. An example of this would be oral thrush, an overgrowth of the fungus *Candida albicans*. Oral thrush causes creamy white lesions, redness, burning, and soreness that may be severe enough to

cause difficulty eating (Singh et al., 2014). Therefore, introducing *Pichia* to the mouth should not be a problem since it is already part of the mouth's microbial ecosystem.

Lastly, another advantage of using *P. pastoris* is that the Food and Drug Administration (FDA) recognizes *P. pastoris* as GRAS (generally recognized as safe). *P. pastoris* was also originally developed and used as a source of high protein animal feed for livestock. Now, several products made by *P. pastoris* have been approved by the FDAfor uses as therapeutic proteins, such as Kalbitor®, a kallikren inhibitor (Ciofalo, 2006; Thompson, 2010). This is useful to our long-term goal because the yeast will need to be inside of the oral cavity to do its work and will need to be safe for human consumption.

Pichia pastoris **Promoters**

Since *P. pastoris* is a methylotrophic yeast, it has a very tightly regulated alcohol oxidase (*AOX1*) promoter which is used to provide a high level of heterologous proteins (Cregg et al. 1985). It is a methanol induced promoter that can be used to control the production of the desired recombinant protein. When in methanol, the promoter is transcribed about 1,000 times faster than if the yeast was grown in glucose (Cregg et al. 1985). A common application of this promoter is when the desired protein being made is toxic to the yeast cells (Yang et al., 2018). Thus, cells are grown in glucose to accumulate biomass but inhibit expression of the recombinant protein and then switched to methanol to trigger production of the desired protein. This way, the large biomass will be able to produce a large quantity of the recombinant protein. Although this promoter has many advantages, we will not be using the *AOX1* promoter in this project since methanol is not present in the human mouth and we want the growth factor to be

constitutively expressed and secreted. The promoter that will be used here is the glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter.

The *GAP* promoter is a strong and constitutive promoter, meaning it is transcribed at a consistent rate and leads to the production of a lot of target protein on many different nutritional media (Qin et al., 2011). The *GAP* promoter is active in the presence of glucose or glycerol and has been observed to be more efficient than the *AOX1* promoter for certain proteins like *Tribolium castaueum* carboxylesterase (TCE) (Zhang et al., 2009). We will be using the *GAP* promoter, which will be accompanied by a Zeocin™ resistance gene as well as a *MATα* secretion signal in the protein expression vector (pGAPZαB).

Protein Secretion

It is generally easier to purify a protein from the extracellular meddium than from the cytoplasm of a host cell, such as *P. pastoris*. This protein export eliminates the need to do high-cost and low yielding cell disruption to obtain the desired protein. To be delivered into the extracellular medium, a protein must transit the secretory system of the yeast. The protein secretory pathway of a eukaryote has a few key steps (Lodish et al., 2000). When proteins with ER targeting signals get synthesized by ribosomes, they are transported into the ER where N-glycosylation, folding, and cleaving occurs. From there, the proteins get sent to the Golgi apparatus to be further modified and packaged into secretory vesicles that bud out from this organelle. Finally, these vesicles fuse with the plasma membrane to release the protein. They are then either retained in the periplasm, integrated into the cell wall, or released into the extracellular matrix (Haguenaur-Tsapis, 1992) The protein follows this route only if it has a secretion leader attached. Many recombinant proteins made by *P. pastoris* get secreted out of

the yeast's cells by using a *S. cerevisiae MATα* prepro signal leader peptide (Lin-Cereghino and Cregg, 2000). The pre-region of the *MATα* signal leader is cleaved at the ER and then the remaining pro-region is removed in the Golgi (Kjeldsen, 2000). The protein then will be secreted out of the cell free of any extraneous peptides. The gene of interest in this project will be cloned into the pGAPZαB vector at the C-terminal end the *MATα* sequence in order for it to reach the extracellular medium. This vector also has the Zeocin™ selectable marker, the restriction sites *EcoRI* and *SalI*, and the *GAP* promoter.

Engineered Probiotic Organisms

Probiotics have been used for thousands of years to improve the body's overall health (Schrezenmeir and Vrese, 2001). Probiotics is an umbrella term for organisms and substances which contribute to intestinal microbial balance (Parker, 1974). In order for a microbe to be a probiotic, it must be certified GRAS by the FDA so that they are safe for human consumption. These are often administered orally and are an attractive alternative to drugs for intestinal diseases (Steidler, 2003). Lately, probiotics have been used as a drug delivery system for the digestive tract (Hudson et al., 2014). This is done by genetically modifying cells to produce proteins or chemicals that the probiotics typically do not make. This is beneficial because when they are in the human body, the engineered probiotic can make the necessary drug to treat the human's digestive tract ailments. This system is very attractive because it could permit lower drug dosage since the probiotic is interacting with the mucosal immune system itself (Hudson et al., 2014). Genetically engineered probiotics should also be less expensive than other delivery mechanisms such as nanoparticles and liposomes since bacteria and yeasts can be grown in large quantities easily (Hudson et al., 2014).

Heterologous protein expression has successfully been done with the yeast *Saccharomyces boulardii* (*S. boulardii*). *S. boulardii* is a probiotic used to treat antibiotic-induced diarrhea that is caused by *Clostridium difficile* overgrowth, inflammatory bowel disease, and other gastrointestinal disorders (McFarland, 2010). An example of a recombinant protein produced by *S. boulardii* was human lysozyme, which is beneficial for human gut health (Liu et al., 2016). This lab was able to make this protein using CRISPR-Cas9.*S. boulardii* was chosen over bacterial probiotics because *S. boulardii* is a eukaryote so it is capable of post translational modifications and producing more complex compounds. *S. boulardii* was also found to be more tolerant of higher temperatures and lower pH found in the digestive tract than other yeasts species such as *S. cerevisiae* (Edwards-Ingram et al., 2007). Lastly, *S. boulardii* does not colonize the gastrointestinal tract of human which is needed for accurate drug dosing and easy clearance of the yeast from the intestine (Blehaut at al., 1989). Our *P. pastoris* is similar in that it is GRAS and will be used as a drug delivery system to secrete the growth factor at the site of injury in the oral cavity.

Basic Fibroblast Growth Factor

Our goal is to use *P. pastoris* as a drug delivery system for basic fibroblast growth factor (bFGF), a 146 amino acid long eukaryotic protein that acts as a growth factor/signaling protein. Depending on the posttranslational modifications, the size of the protein can be 18, 22, 23, or 24 kDa in the human body. bFGF has a broad variety of functionality. These pleiotropic effects include being a known mitogen that influences chemotaxis, cell differentiation, proliferation, and tissue regeneration. Tissue regeneration includes that of cartilage, skin, cornea, eardrum, periodontal, and bone. Because bFGF is a eukaryotic protein, using bacteria to heterologously express bFGF would not likely be successful since the protein needs to be folded correctly as

well as receive post-translational modifications, which prokaryotes, like bacteria, cannot do reliably. The posttranslational modifications of bFGF are still quite a mystery. The only one identified is the demethylation of arginine which takes place in glycine-arginine blocks of the N-terminal (Patry et al., 1994).

bFGF is made by many tissues, but mostly in human adipocytes (NCBI, 2019). bFGF affects both proliferation and migration of cells. When bFGF binds to its receptor (bFGFR) of a fibroblast, the receptor will stimulate PI3-kinase which will then upregulate GTPase Rac1. Rac1 will then activate JNK by phosphorylating it which will result in lamellipodial extension (i.e. migration).

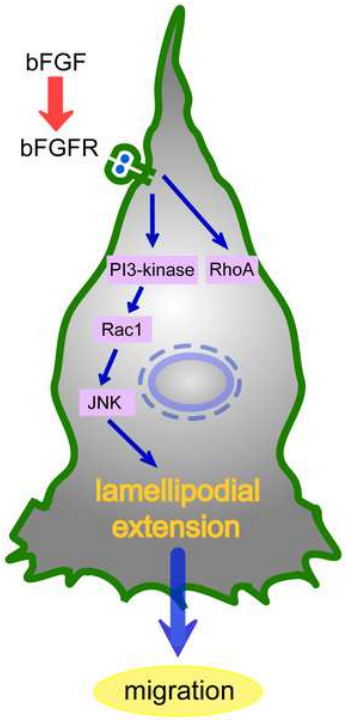


Figure 1: The cell signaling pathway for bFGF to cell migration in a fibroblast cell. (Kanazawa et al., 2010)

P. pastoris Made bFGF

Scientists have previously engineered a strain of *P. pastoris* that can produce the bFGF, but they did it using the *AOX1* promoter (Mu et al., 2008). They were able to clone the bFGF sequence into the pPICZαA expression vector and transform it into the yeast. It was found that the optimal bFGF production occurred in yeast cells growing at pH5 for 72 hours in a methanol concentration of 0.5% (Mu et al.,2008) 300 mg was purified from 2 L of cultivation broth (Mu. et al., 2008). The recombinant bFGF was then tested for biological activity in growing mammalian cell, specifically NIH/3T3 cells (mouse fibroblast cells) in an MTT assay. An MTT assay is a colorimetric assay that uses metabolic activity to measure viable cells. MTT is a tetrazolium dye that gets reduced by the cell's NAD(P)H-dependent cellular oxidoreductase enzyme to the insoluble form, formazan. Formazen is colored so the more cells there are, the more the tetrazolium dye gets reduced, the more formazan there is to observe. A plate reader is used to quantitate the absorbance of formazen. The higher the absorbance, the more viable cells there are. We will follow a similar strategy to express bFGF, except the *GAP* promoter will be used instead of the *AOX1* promoter.

Goal

The overall goal of this project is to create a strain of *P. pastoris* that will be able to co-exist with mammalian cells as well as secrete the protein bFGF constitutively to enhance the growth of these model animal cells. In order to do this, we will need to generate a heterologous expression of bFGF in *P. pastoris* and test the effects of the bFGF on mammalian cell cultures. We will first co-culture the yeast with the mammalian cells to see the overall effects of the total secretion products on the cells. Then, we will purify the bFGF protein using the cation exchange

and test the isolated bFGF on mammalian cells. This project aims to engineer a novel probiotic that will be used in the dental field one day to help accelerate the healing of oral tissue damage.

Chapter 2: Materials and Methods

Strains, Media, and Reagent

TOP10. The *Escherichia coli* (*E. coli*) TOP10 strain from Invitrogen Corp. (Carlsbad, CA.) was used for recombinant DNA manipulation and replication. TOP10 cells were cultured in Lennox Broth (LB) medium (0.5% yeast extract, 1% glucose, and 0.5% NaCl) supplemented with 25μg/mL kanamycin (kan) or plated on LB+kan plates. Bacterial cultures were incubated and shaken in a New Burnswick Scientific C25 Incubating Shaker (Edison, NJ) at 37°C. Bacterial plates were incubated at 37°C.

yJC100. The yJC100 *Pichia pastoris* (*P. pastoris*) strain, used in much of this work, is a derivative of the original wild type *P. pastoris* strain NRRLY11430 from North Regional Research Laboratories, US Department of Agriculture (Peoria, IL). This strain of yeast was used for protein expression and secretion of the basic fibroblast growth factor (bFGF). yJC100 was generally cultured in yeast extract peptone dextrose (YPD) medium (1% yeast extract, 2% peptone, and 2% glucose) supplemented with 100μg/mL Zeocin^{TM} (Zeo) or plated on YPD+Zeo plates. Yeasts were also cultured in Dulbecco's Modified Eagle Media (DMEM) from Biological Industries (Cromwell, Connecticut), a serum-free media with amino acids, vitamins, supplementary components, and glucose. Other media includes Dulbecco's Modified Eagle Media with fetal bovine serum (DMEM+ 10%FBS), Dulbecco's Modified Eagle Media with calf serum (DMEM+ 10%CS), buffered minimal dextrose (BMD, 20% 1M KPi Buffer, 10% Yeast Nitrogen Base with ammonium sulfate and without amino acids, 0.02% biotin, 10% dextrose), and buffered Dextrose-complex Medium (BMDY, 2% dextrose, 2% peptone, and 1% yeast

extract). Yeast cultures were incubated and shaken in a New Burnswick Scientific C25 Incubating Shaker (Edison, NJ) at 28°C. Yeast plates were incubated in at 30°C.

The pCMV3-Myc-bFGF plasmid was from Sino Biological Inc. (Beijin, China). The forward and reverse primers for the PCR were purchased from Sigma Genosys (Plano, Texas). The antibody mouseαbFGF and the mouseαMyc, antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, California). The antibodies mouse αJNK/pJNK/GAPDH were purchased at Cell Signaling Technology (Danvers, Massachusetts). All restriction enzymes and their respective buffers were purchased from MBI Ferments (Hanover, MD). Optical densities of yeast cultures were measured with Spectronic Genesys 2 (Spectronic Instruments Inc. Rochester, NY). The DNA and protein concentrations were measured at 260 nm and 280 nm, respectively, using the Nano Drop 2000c (Thermo Scientific, Wilmington, DE).

NIH/3T3. These mouse fibroblast stem cells were obtained from ATCC (Manassas, Virginia). These cells were used for the scratch and MTS activity assays to test the biological activity of bFGF. These cells are cultured in DMEM and DMEM+CS and incubated at 37°C with CO_2.

BioInformatics

Generation of DNA sequences, DNA primers, and restriction digest maps were performed on SnapGene (Chicago, Illinois).

Polymerase Chain Reaction (PCR)

Oligonucleotides were first designed with SnapGene and then synthesized by Sigma Genosys (Planto, Texas). Once the oligonucleotides were obtained, 5μL of each forward and

reverse primers were diluted to a concentration of 100ng/µL and added to 5µL of 10x PCR buffer, 10µL 5x buffer enhancer, 2µL dNTPs solution, 17.7µL of sterile MilliQ water, and 0.3µL of Taq DNA polymerase all in a 0.5 ml Eppendorf PCR tube, using the kit components provided by the MasterTaq kit from 5 Prime (San Francisco, California). PCR was carried out in a Applied Biosystems 2720 Thermal Cycler (Foster City, California). For the first cycle, the PCR tube was incubated 95°C for 5 minutes. Then, for cycles 2-35, the tube was incubated at 95°C for 1 minute, 53°C for 1 minute, and 72°C for 1 minute each cycle. Lastly, the tube was incubated at 72°C cycle for 10 minutes and left at 4°C cycle until the tube was taken out.

Plasmid Miniprep

Plasmid DNA was extracted and purified using the Zyppy Plasmid Miniprep Kit from Zymo Research (Irvine, CA). Using the overnight liquid culture of the transformed bacterial cells, 1.5 mL were transferred to a 1.5 mL Eppendorf tube, which was spun down in a centrifuge at 16,000*xg* for 2 minutes to pellet the cells. The supernatant was discarded and the pellet was resuspended in 600µL of sterile MilliQ water. 100µL of 7x Lysis Buffer was added and the tube was inverted 6-7 times. 350µL of cold Neutralization Buffer was added and mixed by inverting the tube 3-4 times (until all the blue turned yellow). The tube then was centrifuged again at 16,000*xg* for 4 minutes so that a pellet of cells, proteins, and chromosomal DNA formed at the bottom, leaving the plasmid DNA in the supernatant (~1000µL). The supernatant was transferred to a Zymo-Spin column/collection tube and spun in a centrifuge at 16,000*xg* for 30 seconds. The flow through that in the collection tube was discarded, and 200µL of Endo-Wash buffer was added to the spin column and centrifuged at 16,000*xg* for 30 seconds. The flow through was once again discarded, and 400µL of Zyppy Wash Buffer was added to the spin column and centrifuged at 16,000*xg* for 1 minute. Afterwards, the spin column was transferred

to a new, clean 1.5 mL Eppendorf tube with 30µL of Elution Buffer added to the column. The tube was centrifuged for 30 seconds to collect the eluted and purified plasmid DNA.

DNA Clean and Concentrate

Digested pDNA was cleaned and concentrated using the Zyppy DNA Clean and Concentrator Kit from Zymo Research (Irvine, CA). The digested DNA was added to a volume of DNA Binding Buffer that was 2x the volume of the sample. After the solution was transferred to a new Zymo-Spin column with a collection tube underneath, it was spun down in a centrifuge at 16,*000xg* for 30 seconds, and the flow through was discarded. 200µL of Wash Buffer was added to the column, spun down at the same speed for 30 seconds, and the flow through was discarded. The washing step was repeated two more times. The column was then transferred to a new, clean 1.5 mL Eppendorf tube and 20µL of Elution Buffer gets added to the column. The column is then spun at 16,000 *xg* for 30 seconds to elute the cleaned, concentrated DNA.

Restriction Enzyme Digest

The concentration of the DNA was measured using the Nanodrop 2000c. A specified amount, usually 500ng of DNA, was digested in a 1.5ml Eppendorf tube. 0.5µL of the restriction enzyme(s) are added to the DNA, along with the appropriate 10x restriction enzyme buffer (MBI, Fermentas, Hanover, MD, etc.). Sterile MilliQ water was added to bring the total volume up to 30µL. The reaction was then incubated in a 37°C water bath for 1-2 hours.

Agarose Gel Electrophoresis

Pre-made 1.2% agarose Flash gels (Lonza, Rockland, ME) were used for running and detecting DNA. 2µL of 5x DNA loading dye (50% glycerol, 0.25% bromophenol Blue, 0.35%

xylene cyanol, 0.01% SDS, 10 mM Tris pH 8.0, 1 mM EDTA pH 8.0) was added to 8µL of each DNA sample. 6µL of each sample was loaded into a well of the Flash gel along with 5µL of 1Kb Plus ladder from ThermoScientific (Waltham, Massachusetts). The proteins were electrophoresed at 275 V for 7 minutes and then visualized using the ChemiDoC™ XRS+ System from Bio-Rad (Hercules, CA).

DNA Sequencing

Approximately 600ng of all purified and concentrated DNA were sent out to Quintarabio (Richmond, CA) for sequencing in a new and clean 1.5ml Eppendorf tube.

Ligation

A 3:1 molar ratio of insert and vector was needed for a ligation. The insert, vector, and sterile water were mixed in a 1.5 mL Eppendorf tube and heated at 65°C for 2 minutes and then put on ice for 5 minutes. Next, 2µL of 10x Fermentas ligase buffer (Hanover, Maryland) and 1µL of Fermentas T4 DNA ligase (Hanover, Maryland) were added to the tube. Sterile water was added to bring up the final volume to 20µL. The reaction was spun down and mixed before letting it incubate at room temperature for at least 3 hours.

Bacterial Transformation

Using a heat shock technique, 50µL of competent bacterial cells (OneShot TOP10) Purchased from Invitrogen Corp. (Carlsbad, California) were put into a sterile 1.5mL Eppendorf tube. 5µL of plasmid DNA or ligation mix was added to the competent cells and incubated on ice for 30 minutes. The tube was then taken out and placed into a 42°C water bath for 30 seconds to heat shock the cells. After the heat shock, the tube was rapidly transferred onto ice for 1-2 minutes.

245µL of LB was added to the tube, which was placed in a 37°C water bath for 20-60 minutes. The transformation mix was then plated onto an LB+(appropriate antibiotic) plates and incubated overnight in a 37°C incubator.

Yeast Transformation

Using an electroporation technique, 40µL of electrocompotent *P. pastoris* cells and 4µL of linearized plasmid DNA were aliquoted into a cold 2.0mm electroporation cuvette and incubated on ice for 5 minutes. Afterwards, the cuvette was wiped down with a kimwipe and placed into the chamber of the BTX ECM 630 Electroporator from Harvard Apparatus (Brookline, MA). The pulse parameters were set at 200Ω, 1500V, 50µF, and 2.0mm. Immediately after pulsing, 50% sorbitol in YPD (500µL sorbitol and 500µL YPD) were added to the cuvette, and the transformation reaction was subsequently transferred to a 1.5mL Eppendorf tube. After the cells were allowed to recover at 30°C for 1 hour, they were plated on YPD+appropriate antibiotic and incubated for 3 days until colonies appeared.

bFGF Expression Plasmid Construction

A plasmid with the desired protein coding sequence was purchased. The purchased plasmid was then transformed into One Shot TOP10 cells to replicate the plasmid. A plasmid miniprep was done to extract the plasmids. The plasmids were then run through a PCR which replicated the desired protein's coding sequence as well as add on restriction sites on the ends. A restriction digest was done on both the vector the plasmid was designed to go into as well as the PCR product. The ligation protocol was then used to ligate the two pieces together.

Small Scale Expression in 96 Well Plates

Cells were grown in a sterile 96 deep well plate (DWP) format for enzymatic assays (Weis et al, 2004). Five hundred microliters of sterile water was added to the perimeter of the plate to prevent evaporation. Next, 250 µL of BMD 1X (buffered minimal dextrose 1%; 1 M K_2HPO_4 and 1 M KH_2PO_4 with pH 6.0, 10X YNB, 500X of 0.02% biotin, and 10X dextrose) liquid medium, along with any amino acid supplements if needed, was added into the wells that were designated to hold the cell cultures. Isolated colonies were selected from fresh plates and inoculated into their designated wells containing BMD 1X. Parafilm was wrapped around the edges of the DWP and the corners were taped to block evaporation. The DWP was placed into the 29^{o}C shaking incubator at 325 rpm. After 48 hours, 5 µL of cell culture from select random wells were added onto glucose test strips to confirm that all the glucose had been exhausted. Subsequently, the cells were induced with 250 µL of 2X BMDY, making the final dextrose concentration 1%. The plate was sealed with Parafilm and placed back into the shaking incubator with the same settings for 24 hours. On the next day, the cells were harvested into 1.5 mL microcentrifuge tubes. The OD_{600} of each sample were measured with a 1:20 dilution. The samples were extracted from the wells into 1.5 mL microcentrifuge tubes and spun down at maximum speed for 1 minute and either stored in the -80^{o}C freezer or used immediately.

Protein Concentrator

Th protein Centrifugal Filter Units (Milipore, Hayward, California) was washed with 3 mL of sterile water. It was spun at 1200 rpm for 10 minutes. After centrifugation, the flow through was discarded. 3 mL of protein was placed into the concentrator and spun again at 1200 rpm for 10 minutes. These steps were repeated until all of the samples were gone and the volume remaining was down to 500µL. The 500µL was transferred into a new and clean 1.5mL Eppendorf tube.

Spot Western Blot

75µL-200µL of sample were spotted onto nitrocellulose using a vacuum spot blot unit from Topac (Bristol, United Kingdom). The membrane was processed using the SNAP i d®. Protein Detection System from Millipore (Billerica, MA) following manufacturer's instructions. Briefly, the spotted nitrocellulose was allowed to dry in a 60 degree C oven for 5 minutes. Afterwards, the membrane was soaked in 1X PBS and placed on the blot holder, followed by a spacer sheet. The closed blot holder was placed onto the SNAP i.d®. apparatus attached to a vacuum. With the vacuum turned on, 30 mL of I-Block (ThermoFisher Scientific, St. Louis, MO) solution (1xPBS, 0.2% I-Block, 0.1% Tween 20) was added to the membrane. The vacuum was turned off before incubating the membrane with 5 mL of I-Block solution containing 10 or 2µL of the primary antibody, which was mouse anti-bFGF (Santa Cruz biotechnology, Santa Cruz, California) or mouse anti-Myc respectively. After 10 minutes of incubation with the primary antibody, the vacuum was turned on again, and the membrane was washed three times with a total of 100 mL wash buffer (1X PBS, 0.1% Tween 20). The membrane was then incubated in 5 mL of I-Block containing 25uL of the goat anti-mouse secondary antibody (Applied Biosystems, Foster City, CA) for 10 minutes with the vacuum off. Afterwards, the membrane was washed three times with wash buffer again, as previously described, before it was incubated for 5 minutes in a petri dish containing 20 mL of femtoLUCENT™ *PLUS*-AP 1X TBST (G-Biosciences, St. Louis, MO). A flat plastic-wrap surface was prepared where 1-2 mL of the femtoLUCENT™ *PLUS*-AP detection reagent was added dropwise onto the membrane. After 5 minutes of incubation at room temperature, the detection reagent was drained off. The membrane was placed into a plastic envelope or wrap and developed using the Bio-Rad

ChemiDoc XRS+ Imaging System (Hercules, CA) with exposure times of 3 minutes. Signals were quantified with Bio-Rad ImageLab software.

Western Blot Analysis

SDS-PAGE. 20μL of each protein sample was mixed with 4μL of 6x protein sample buffer (PSB). The sample then was heated at 100°C for 10 minutes with Surelocks on. 14% Tris-Glycine premade gels from Bio-Rad (Hercules, California) were locked in a running apparatus and placed in a running tank containing 1 liter of 1x SDS-PAGE gel running buffer (3 g/L Tris base, 14 g/L glycine, and 1 g/L SDS). Each well was washed with the SDS-PAGE running buffer to remove any impurities and bubbles. The heated samples were then spun down after they were cooled to room temperature. 20μL of each sample was loaded into its respective well as well as 5μL of PageRuler protein ladder from ThermoFisher Scientific (St. Louis, MO). The proteins were electrophoresed at 125 V for 50 minutes.

Membrane transfer. Once the gel was run, the manufacturer's instructions for the iBlot system (Invitrogen, Carlsbad, California) were followed.

Detection. The same steps described for as the spot western blot above were used for detection.

Scratch Assay

Starving the mammalian cells. DMEM (starve medium) was warmed up to room temperature. NIH/3T3 cells in 6 well plates are taken out and checked to see if they are confluent. The growth medium was aspirated from each well of the plate. The cells were washed

with PBS (2 mL/well). The PBS was aspirated after that. 2 mL of DMEM was added to each well. The plate was set back into the CO_2 37°C incubator for 6 hours.

Treatments. Starve: new DMEM (2mL) was added to the its respective well. 10%CS: new DMEM+10%CS (2mL) was added to its respective well. WT cells: 100K *P. pastoris* (yJC100) cells were added to DMEM (2mL) and placed in its respective well. Hope cells: 100K *P. pastoris* (Hope) cells were added to DMEM (2mL) and placed in its respective well. The plate was incubated again for another 6 hours in the same incubator.

Scratch. The plate of wells was taken out and brought into the hood. A 1000µL pipette tip was used to scratch three straight lines in each well from top to bottom. Under a microscope with a camera attached, pictures were taken of the scratches in each well. Cells were then put back into the 37°C CO_2incubator for 6 hours. The plate was then taken out and pictures were taken at the same place as the first set of photos to compare how far the cells have moved into the scratch. All images were analyzed using ImageJ program.

MTS Assay

The treatments were prepared first. For starve, new DMEM (50µL) was just added to the each appropriate well. For 10%CS, new DMEM+10%CS (50µL) was just added to each appropriate well. For both Hope and WT conditioned medium, both the strains were grown in DMEM for 24 hours in a shaking incubator at 27°C. Once the cultures were ready, the cultures were spun down at 4,000 rmp to form a pellet of yeast at the bottom. The supernatant was taken out and pushed through a sterile filter to get rid of any cells. The sterile supernatant was then used as the conditioned medium. For the Hope and WT cells, the pellets were resuspended in fresh DMEM and the OD was measured for each. This was used to calculate 10,000 cells to add

to the appropriate wells each. For WT and Hope C+C (conditioned medium + cells), 10K cells/well were calculated to add to the conditioned medium. 50µL of each will go into their respective wells. For commercial bFGF or isolated bFGF, 10ng/well would be calculated and added into DMEM. 50µL was added to the appropriate wells. The mammalian cells were taken out and used a cell counter to calculate 5,000 cells/50µL. 50µL of mammalian cells were added into each well. The cells proliferated for 24 hours in the 37°C incubator. The next day, 20µL of MTS reagent was added into each well and the plate was then covered in tinfoil. The plate incubated in the 37°C incubator for 2 hours. The plate was read using a plate reader. The software that recorded the readings was SoftMac Pro. Two readings were recorded for each wavelength: 490 nm and 650 nm.

Magnetic Myc-bead Purification kit.

The manufacturer's instructions for the ChromoTek Myc-Trap were followed.

Cation Exchange Purification

Appropriate buffers were made beforehand depending on what kind of protein was planned on being purified. The buffers are hooked up to the cation exchange column. The machine washed the loops on its own. While the machine was being washed, the superloop was taken out. The superloop got filled with the extracellular matrix that the protein trying to be purified is in. The superloop got put back into the machine and the machine ran the exchange. In the end, there were 47 fractions in 15ml conical.

Chapter 3: Results

The goals of this project were 1) develop a yeast that was capable of producing the human growth factor basic fibroblast growth factor (bFGF); 2) co-culture it with mammalian cells in order to determine its effects on proliferation and migration, and 3) purify the bFGF protein to confirm its cellular activity. We accomplished the first goal by transforming *P. pastoris* with the coding sequence of bFGF. The second goal of testing the effects of the yeast on mammalian cells was done using scratch and MTS assays. Lastly, the purification was done using cation exchange chromatography and the recombinant protein was tested using the MTS assay.

Construction of the pHML1 Expression Vector

The commercially available plasmid pCMV3-Myc-bFGF (Figure 2) contains the bFGF coding sequence as well as a c-Myc tag attached to its N-terminus. The pCMV3-Myc-bFGF was transformed into TOP10 cells for the purpose of amplifying the plasmid. Three colonies were grown in LB+Kan liquid media for minipreps the next day. A miniprep was done to extract and purify the plasmid DNA from all three cultures. Restriction digests were then performed on the purified DNA from all three samples digested with *KpnI* and *Xbal* restriction enzymes and run on an agarose gel electrophoresis to confirm the presence of the pCMV3-Myc-bFGF plasmid. The restriction enzymes cut the plasmid to yield two expected bands with sizes 500 bp and 3,000 bp (Figure 3).

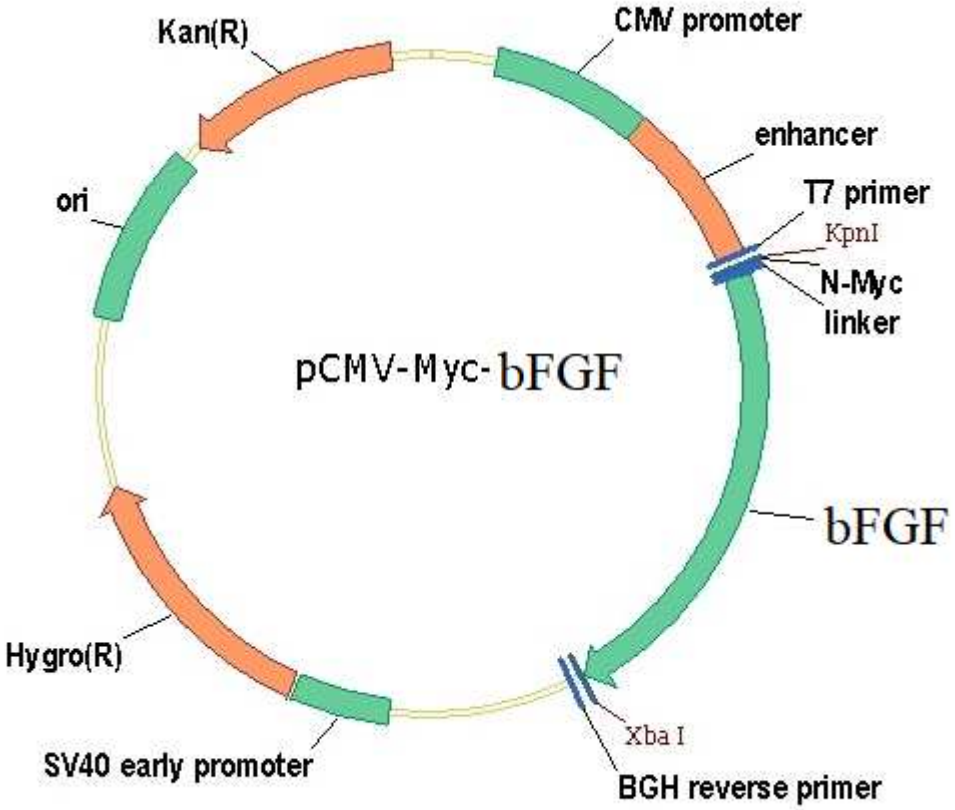


Figure 2: Plasmid map of pCMV3-Myc-bFGF

Table 1: Sequences for bFGF Forward and Reverse primers to do PCR

bFGF*EcoRI* Forward Primer	5'-GGGAATTCACATGGAGCAGAAACTCATC-3'
bFGF*SalI* Reverse Primer	5'GGGTCGACTTAGCTCTTAGCAGACATGG-3'

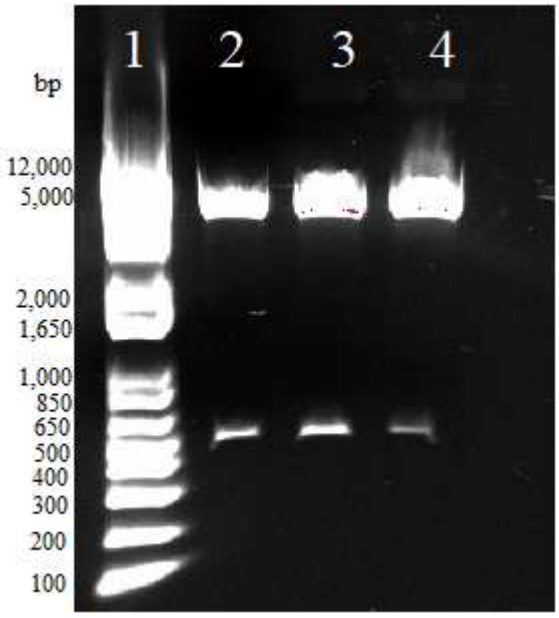


Figure 3: Digested pCMV3-Myc-bFGF using *KpnI* and *Xbal.* Lane 1: 1kb plus DNA ladder. Lane 2: Sample 1 of digested pCMV3-Myc-bFGF. Lane 3: Sample 2 of digested pCMV3-Myc-bFGF. Lane 4: Sample 3 of digested pCMV3-Myc-bFGF

A polymerase chain reaction (PCR) was run using the bFGF*EcoR*I forward primer and bFGFSalI reverse primer (Table 1) to amplify the c-Myc-bFGF coding sequence for subcloning. The PCR reaction and the primers (negative controls) were then electrophoresed by agarose gel electrophoresis to show the expected PCR product of ~500bp was present (data not shown).

To clone the c-Myc-bFGF coding sequence into the vector, pGAPZαB (Figure 4), both the PCR product and the pGAPZαB vector were digested with the restriction enzymes *EcoRI* and *SalI*. The band sizes for the bFGF PCR and pGAPZαB were ~500 bp and ~3100 bp respectively, as seen by agarose gel electrophoresis (Figure 5). Purified digested vector and insert were mixed together and ligated. The ligation mix pGAPZαB-bFGF was then transformed into STELLAR cells and selected on a LB+Zeocin^{TM} (Zeo). 6 transformants were chosen and cultured in LB+Zeo liquid medium. Minipreps were performed, and plasmid DNA was digested with the restriction enzymes *PstI* and *BsHtI* (*AgeI*). The desired plasmid was expected to give the

following band sizes: 604 bp and 2962 bp. An empty pGAPZαB vector was also digested with the same enzymes as a negative control (Figure 6). Agarose gel electrophoresis shows that sample 3 contained the correct band lengths while samples 5 and 6 have an extra band at 189 bp. Samples 1,2, and 4 have bands at 2962 bp and 189 bp while the negative control only displayed a band length at 2962. Sample 3 was selected to be grown in large quantities in LB+Zeo liquid media, and the plasmid found in sample 3 was renamed pHML1 (Figure 7). pHML1 was confirmed that the plasmid contained the DNA sequencing of bFGF in the proper orientation and reading frame. pHML1 contained the c-Myc-bFGF fused in frame to the alpha-factor secretion signal, under the control of the constitutive *GAP* promoter.

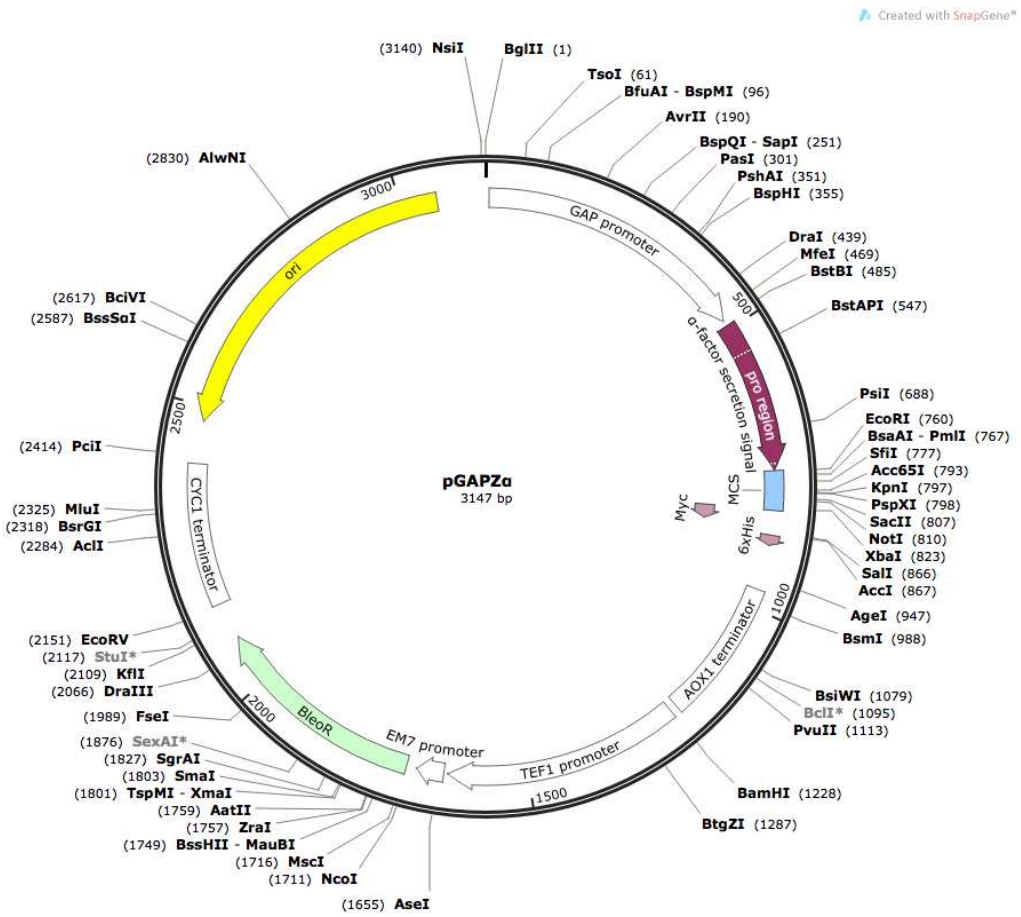


Figure 4: Plasmid map of pGAPZαB with restriction enzymes.

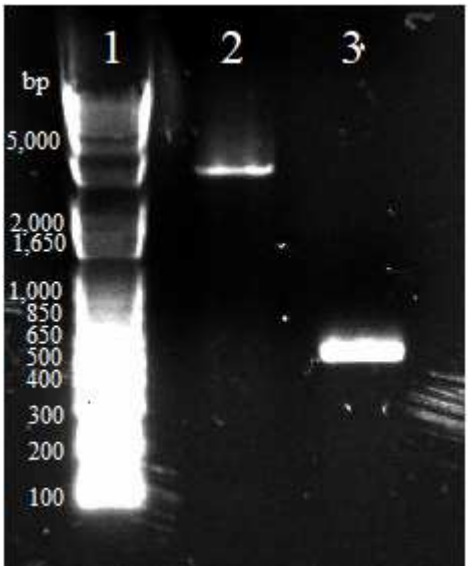


Figure 5: Restriction digest of pGAPZαB and bFGF PCR using *EcoR*I and *Sal*I. Lane 1: 1 kb DNA ladder. Lane 2: digested pGAPZαB. Lane 3: digested c-Myc-bFGF PCR.

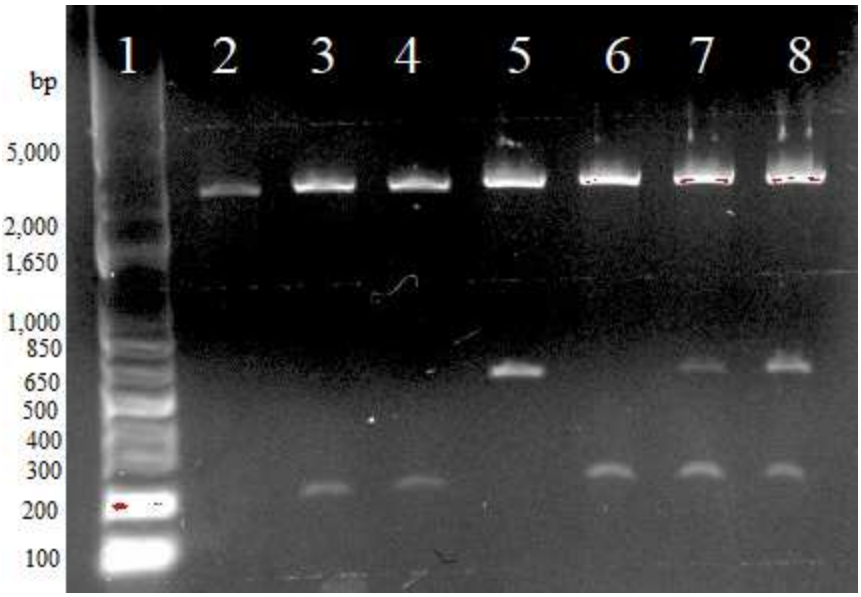


Figure 6: Restriction digest of pGAPZαbFGF from different cultures. Lane 1: 1 kb+ ladder. Lane 1: pGAPZαB alone as negative control. Lane 2: Sample 1 Lane 3: Sample 2. Lane 4: Sample 3. Lane 5: Sample 4. Lane 6: sample 5. Lane 7: Sample 6. Lane 8: Sample 7

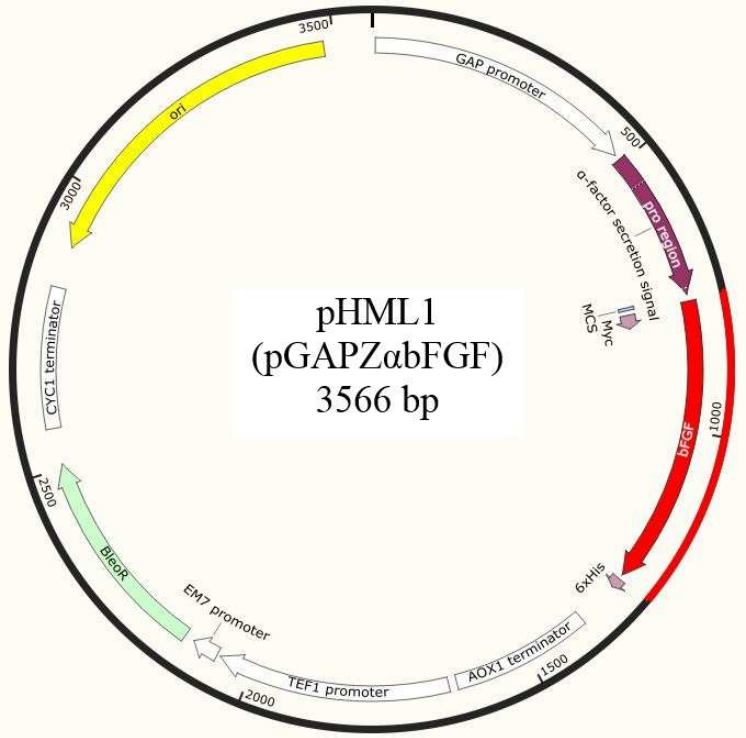


Figure 7: Plasmid map of pHML1 (pGAPZαbFGF) with a stop codon before the 6xHis.

Transformation of pHML1 into *P. pastoris*

To transform the plasmid into competent *P.* pastoris, pHML1 (pGAPZαbFGF) was linearized with the *Pag*I restriction enzyme. The linearized pHML1 DNA was purified and electrophoresed on a gel to show that it was Linearized and had the size of 3566 bp (data not shown). The wild type *P. pastoris* strain JC100 was transformed with linearized pHML1 by electroporation. Once transformed, the transformants were plated on 8 plates of YPD containing increasing concentrations of Zeocin^{TM} (100 µg/mL, 200 µg/mL, 300 µg/mL, 500 µg/mL, and 900 µg/mL, 1100 µg/mL, 1200 µg/mL, and 1300 µg/mL). Selection on increasing levels of antibiotic usually produces colonies with higher copy numbers of plasmid, which often (but not always) leads to higher expression of the recombinant protein encoded by the plasmid. The number of

colonies isolated on each plate are listed in Table 2. Twenty colonies total from plates 100, 300, 500, 900, 1200, and 1300 were streaked onto YPD+Zeo plates (100 μg/mL).

Expression of bFGF

All the transformed strains are yJC100 *Pichia pastoris* containing the pHML1 plasmid that were selected at a specific concentration of zeocin and denoted by the concentration of zeocin for selection. Thus, the second colony selected from the zeocin plate with 100 μg/mL zeocin was yJC100:pHML1-100B or simply called 100B. 20 colonies were then grown in a deep well plate (Figure 8) to determine which of the selected colonies were able to synthesize and secrete the bFGF growth factor. Uninoculated medium and a strain containing the empty vector were also grown as negative controls. A strain expressing pDT300, which produces GFP-c-Myc from a *GAP* promoter in high amounts (Li et al., 2010), was also grown as a positive control. Cultures were grown for 1 day in BMD and 1 day in BMDY before harvesting. Equal amounts of extracellular medium from each well was then spotted on a spot western and the mouse anti-Myc primary antibody followed by the goat anti-mouse antibody were used in the detection (Figure 9). Cultures 100B and pDT300 (positive control) showed the strongest signal; however, strains 300B, 500B, 500D, 900B, 900D, 1200A, and 1300A also showed some expression of a c-Myc tagged protein and were pursued for more in-depth analysis.

Table 2: The number of colonies that grew on each plate of varying zeocin.

Zeocin concentration	100 μg/mL	100 μg/mL	100 μg/mL	200 μg/mL	300 μg/mL	500 μg/mL	500 μg/mL	900 μg/mL	1100 μg/mL	1200 μg/mL	1300 μg/mL
#colonies	>100	19	90	60	39	30	13	19	0	1	1

Table 3: The number of colonies picked from each of the plates containing different levels of zeocin.

Zeocin concentrations	100µg/mL	300µg/mL	500µg/mL	900µg/mL	1200µg/mL	1300µg/mL
#colonies	3	3	6	6	1	1

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		100A			100B			100C			Pos.	
C		300A			300B			300C			pDT	
D		500A			500B			500C			300	
E		500D			Empty	500E			900A			
F		900B			Neg	900C			900D			
G		900E			pGAPZα	1200A			1300A			
H												

Figure 8: Growing the numbered colonies in triplicates in a 96 well deep well plate.

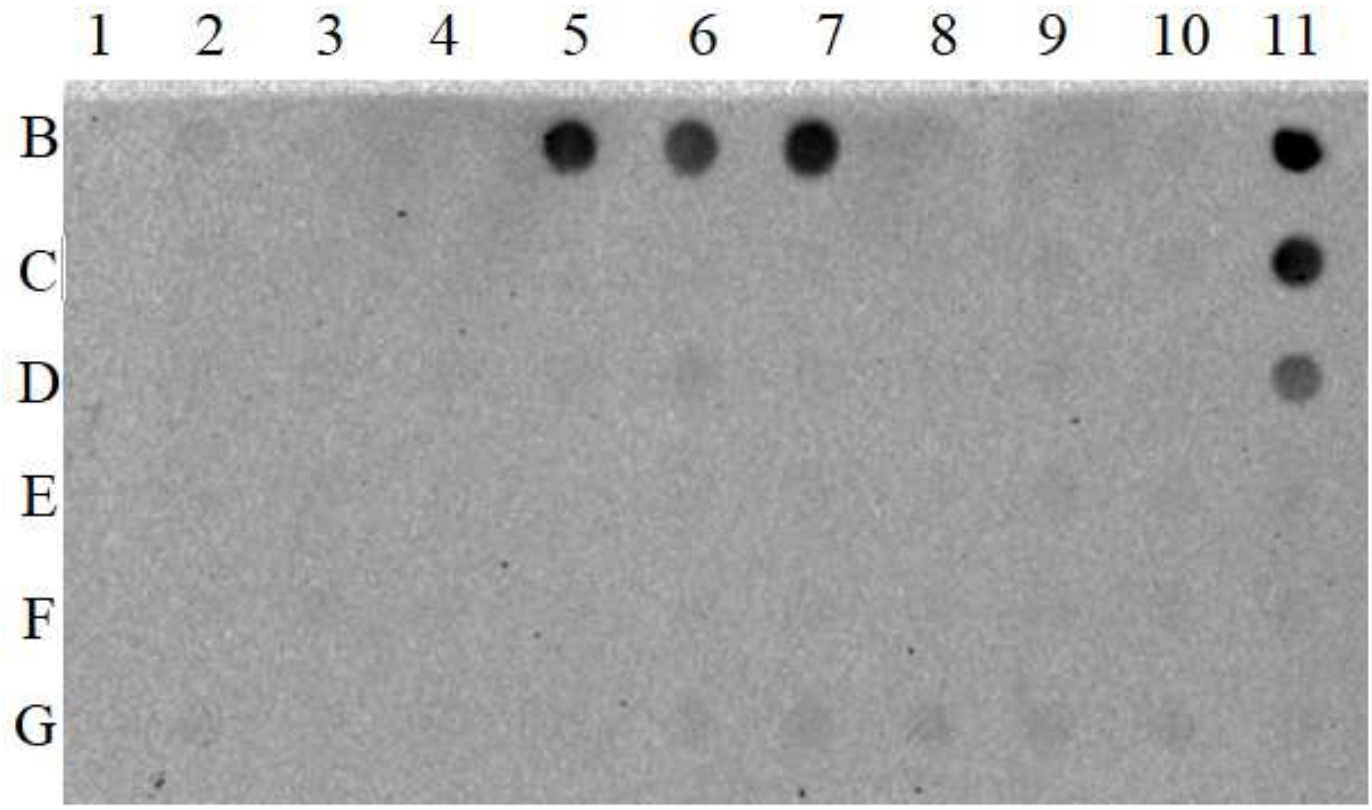


Figure 9: Spot western of the extra cellular matrix of the 96 well deep well plate.

Optimization of bFGF production

Another deep well plate assay was used to measure the expression of bFGF in different media and reassess the level of bFGF expression by the candidate strains. Eight colonies (100B, 300B, 500B, 500D, 900B, 900D, 1200A, and 1300A) were grown under three different conditions, BMDY pH5, BMDY pH6, and DMEM (Figure 10). These media were chosen for 2 reasons: 1) to see if a pH change could improve bFGF secretion in the standard yeast medium BMDY, which has been found in other studies (Eissazadeh et al., 2016) and 2) to determine if the strains could produce sufficient bFGF in DMEM, a medium in which future co-culturing experiments between the recombinant *P.pastoris* and mammalian cells will occur. The positive control of pDT300 and negative controls of media only and the negative pGAPZαB-transformed strain were also grown in each condition. The cell density of each sample was then measured using a spectrophotometer to see how each medium affected growth (Table 5). Both the BMDYpH5 and pH6 samples grew to a much higher cell density (about three times) than that of the cultures grown in DMEM, but the yeast strains still showed significant growth in the tissue culture medium.

A spot western was then conducted on supernatants of the wells using the mouse anti-Myc primary antibody and goat anti-mouse secondary antibody. Results for this spot western were not consistent because the duplicates did not show the same levels of expression for some of the wells (data not shown). From this spot western, only 100B and 1200A were able to express a reliably high level of bFGF in all conditions, and cultures grown in pH5 appeared to be more productive than pH6.

Western blot analysis were then run to explore and confirm these results (Figure 11 and 12). In BMDY medium, thepHML1-transformed yeast produced one bFGF band (size: ~25 kDa) while multiple, larger molecular weight bFGF bands were secreted in DMEM. The strain that was most consistent bFGF secretor in the different media was 100B.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		100B	100B	300B	300B	Pos	100B	100B	Pos	300B	300B	
C		500B	500B	500D	500D	Pos	500B	500B	Pos	500D	500D	
D		900B	900B	Neg	900D	900D	Neg	900B	900B	900D	900D	
E		1200A	1200A	Empty	1300A	1300A	Empty	1200A	1200A	1300A	1300A	
F		100B	100B	300B	300B	Neg	Pos	500B	500B	500D	500D	
G		Empty	900B	900B	900D	900D	Pos	1200A	1200A	1300A	1300A	
H												

Figure 10: Growing select colonies in duplicates in different media. Green: BMDY pH5. Orange: BMDY pH6. Blue: DMEM

Table 4: OD reading of the 96 well deep well plate. 100B, 500D, neg, and 1200A of each media.

Sample	Well #	OD (600nm)	Sample	Well #	OD (600nm)	Sample	Well #	OD (600nm)
100B	B2	12.06	100B	B7	13.8	100B	F3	4.3
500D	C4	12.2	500D	C10	13.9	500D	F11	4.8
Neg	D4	12.9	Neg	D7	12.3	Neg	F6	2.5
1200A	E2	13.6	1200A	E8	12.6	1200A	G8	5.2

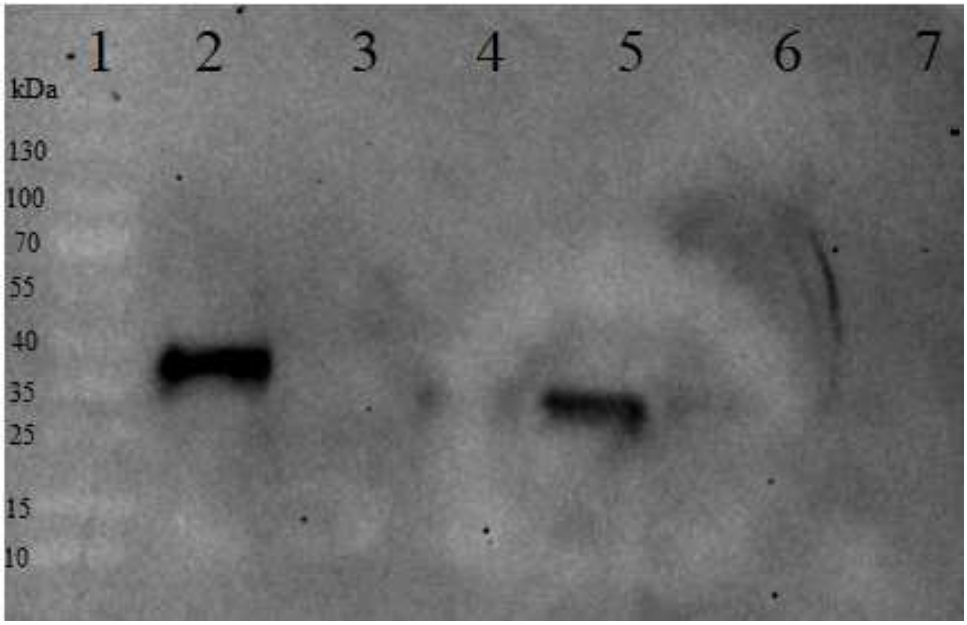


Figure 11: Western blot analysis of the extracellular matrix of the 96 well deep well plate. pH5 vs. pH6 Lane 1: PageRuler protein ladder. Lane 2: C6 (pos, GFP-c-Myc). Lane 3: D4 (neg). Lane 4: B3 (100B pH6). Lane 5: B7 (100B pH5). Lane 6: E3 (1200A pH6). Lane 7: E8 (1200A pH5).

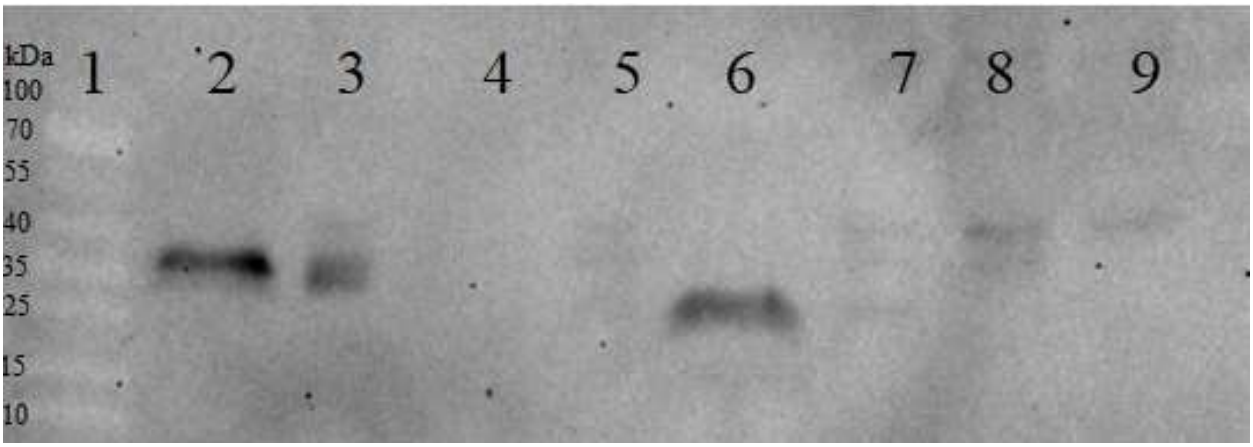


Figure 12: Comparison of bFGF expression in different strains and media. Lane 1: PageRuler protein ladder. Lane 2: C6 (BMDY pH5 pos). Lane 3: F7 (DMEM pos). Lane 4: D4 (BMDY pH5 neg). Lane 5: F6 (DMEM neg). Lane 6: B7 (BMDY pH5 100B). Lane 7: F2 (DMEM 100B). Lane 8: G3 (DMEM 900B). Lane 9: G10 (DMEM 1300A)

Cell extracts were then made from the twelve cultures (100B, 500D, 1200A, and negative from each growing conditions) that were grown in the deep well plate to test how much bFGF

was being produced intracellularly, compared to how much was being secreted. Only BMDY pH5 and DMEM cell extracts were electrophoresed on a western blot analysis because cells grown in pH5 medium secreted more bFGF than those in pH6 (Figure 13). The western blot was also ran with a positive control of the extracellular matrix (ECM) of a strain producing GFP-c-Myc. Along with detecting strong expression from the positive control, the western blot showed strong signals from BMDY pH5 100B, but weak signals from BMDY pH5 1200A and DMEM 100B. At least 2 bFGF proteins were present within the cells while only 1 bFGF protein was detected in the extracellular medium, suggesting the modifications occur to bFGF inside the cell during its secretion. Because strain 100B was the most consistent producer of bFGF both intracellularly and extracellularly on different media, we decided to concentrate our using strain 100B in the next stages of our study. Colony 100B was renamed Hope for obvious reasons.

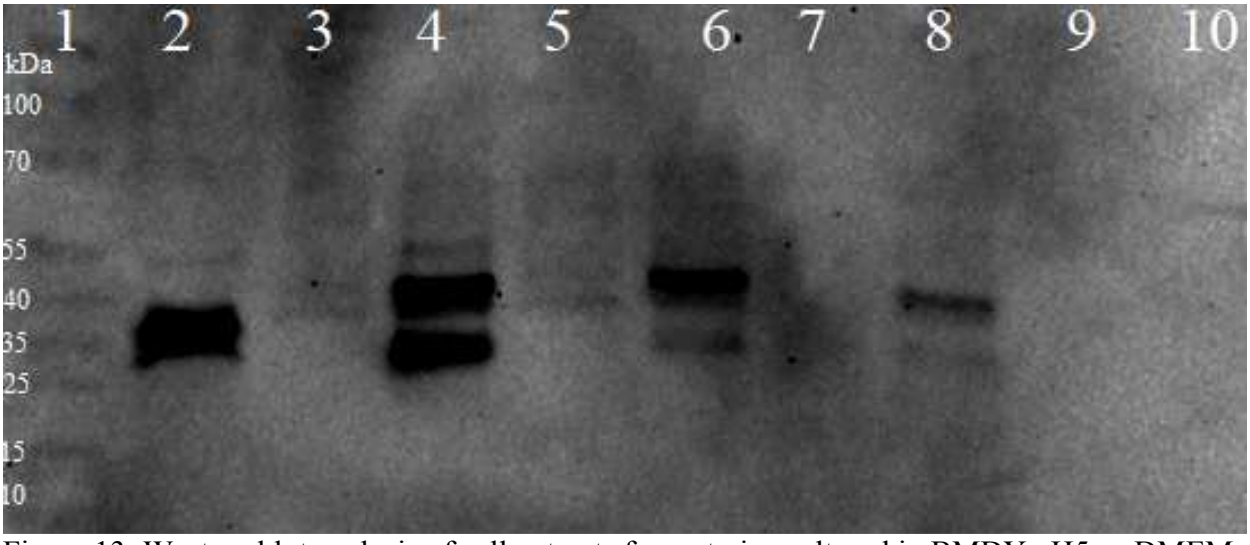


Figure 13: Western blot analysis of cell extracts from strains cultured in BMDY pH5 vs DMEM. Lane 1: PageRuler protein Ladder. Lane 2: P2F6 (pos control, GFP-c-Myc). Lane 3: BMDY pH5 neg. Lane 4: BMDY pH5 100B. Lane 5: BMDY pH5 500D. Lane 6: BMDY pH5 1200A. Lane 7: DMEM neg. Lane 8: DMEM 100B. Lane 9: DMEM 500D. Lane 10: DMEM 1200A.

Cultures of 100B were then grown under different conditions and for different amounts of time in order to find the best conditions to express the Myc-bFGF. The conditions were BMDY pH5, BMDY pH5+FBS, YPD, and BMDY pH5+Methanol+2% glucose. Each ECM were collected at 48 hours and 72 hours. A western blot analysis (Figure 14) was conducted using mouse anti-Myc primary antibody and goat anti-mouse secondary antibody with the Hope BMDY pH5 from the deep well plate as the positive control. Hope BMDY pH5 (pos), BMDY pH5+FBS 48 hrs, BMDY pH5+FBS 72 hrs, BMDY pH5+Methanol+2% glucose 48 hrs, and BMDY pH5+Methanol+2% glucose 72 hrs had very strong signals. BMDY pH5 48 hrs and BMDY pH5 72 hrs had weak bands. The addition of FBS, which is often supplemented to DMEM medium, caused the production of higher molecular weight bFGF bands (lanes 5 and 6). YPD 48 hrs and YPD 72 hrs did not have any bands. Thus, we determined that BMDY medium pH5 would be the best choice for the production of secreted bFGF.

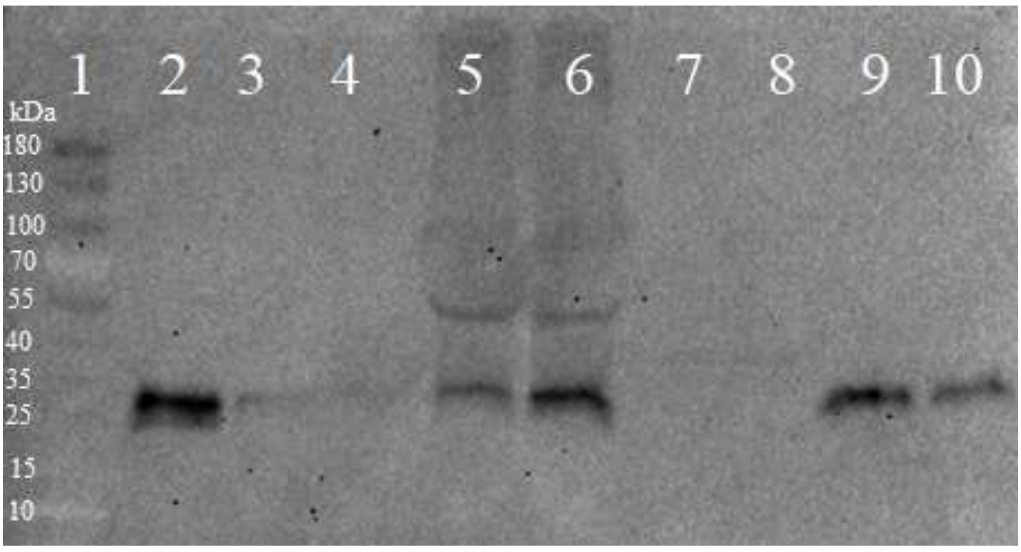


Figure 14: Western blot analysis of expressing bFGF in different conditions. Lane 1: PageRuler protein ladder. Lane 2: BMDY pH5 (pos). Lane 3: BMDY pH5 48 hrs. Lane 4: BMDY pH5 72 hrs. Lane 5: BMDY pH5+FBS 48 hrs. Lane 6: BMDY pH5+FBS 72 hrs. Lane 7: YPD 48 hrs. Lane 8: YPD 72 hrs. Lane 9: BMDY pH5+Methanol+2% glucose 48 hrs. Lane 10: BMDY pH5+Methanol+2% glucose 72 hrs.

Purification

Magnetic Myc-bead Purification kit. We first attempted to purify the c-Myc-bFGF from ECM of 100B (well B3) and the GFP-c-Myc of pDT600 (well C6) from the deep well plate in Figure 10. This was done by using beads that had a conjugated bFGF antibody. However, the bFGF protein was found in the elution only after boiling the beads (Figure 15) in a western blot analysis using an anti-Myc antibody. Interestingly, which were the 2x Myc peptides, supposed to compete the protein off the anti-myc antibody portion of the magnetic bead, but it had no effect on eluting the protein.

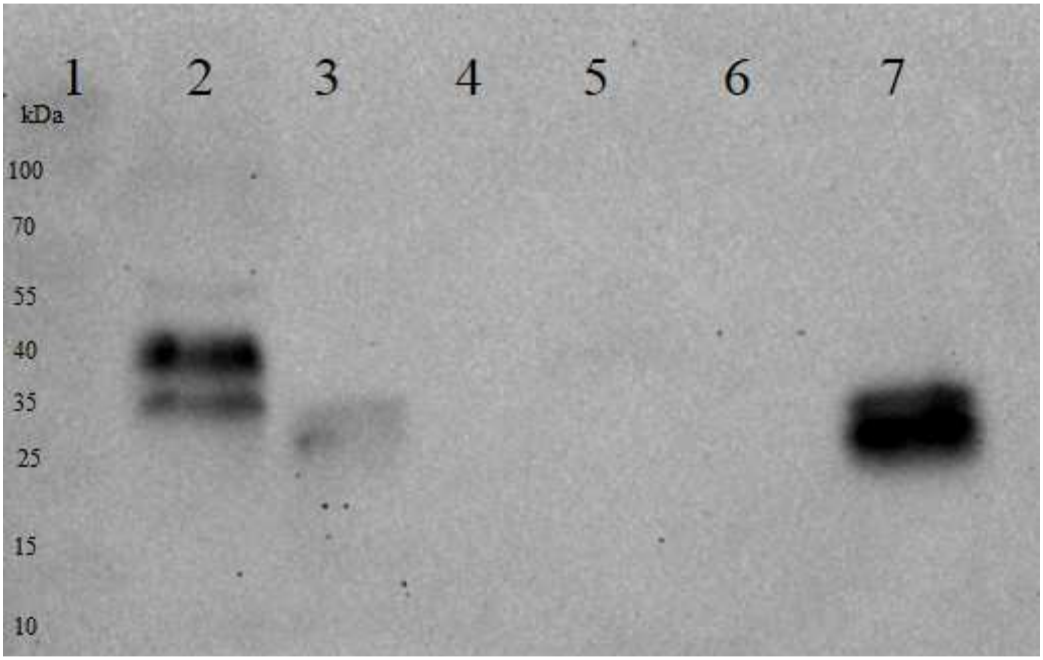


Figure 15: Western blot analysis of magnetic Myc-bead purification. Lane 1: Protein ladder. Lane2: Positive control P2F7 (GFP-c-Myc). Lane 3: Crude lysate before purification. Lane 4: Flow Through. Lane 5: First Wash. Lane 6: Elution with 2x Myc peptides. Lane 7: Boiled off protein

Cation exchange chromatography. Because the Myc-magnetic beads purification method was unsuccessful, we resorted to using a cation exchange column with a buffer of pH4. Because bFGF has a pI of 9.2, it should be highly positively charged in pH4 buffer. This chromatography suggests that the protein would only elute off in high salt-later fractions. The ECM of the 100B strain was loaded onto a cation exchange resin and recombinant proteins were eluted off in several fractions of increasing salt concentration. Using spot western analysis, it was found that the strongest signals of bFGF were in fractions 35-43 (Figure 16); these fractions (total volume=27 mL) were concentrated down to 500μL using a protein concentrator (molecular weight cutoff=10kDa) to create an enriched sample of recombinant bFGF. A spot western analysis was done to determine the amount of bFGF in the enriched sample using commercial bFGF at varying concentrations as a standard (Figure 16). It was estimated that the enriched sample's concentration was about 1ng/μL of bFGF. Based on this quantitation, we were able to use approximately the same amount of this enriched bFGF as the commercial bFGF to compare their effects in MTS assays.

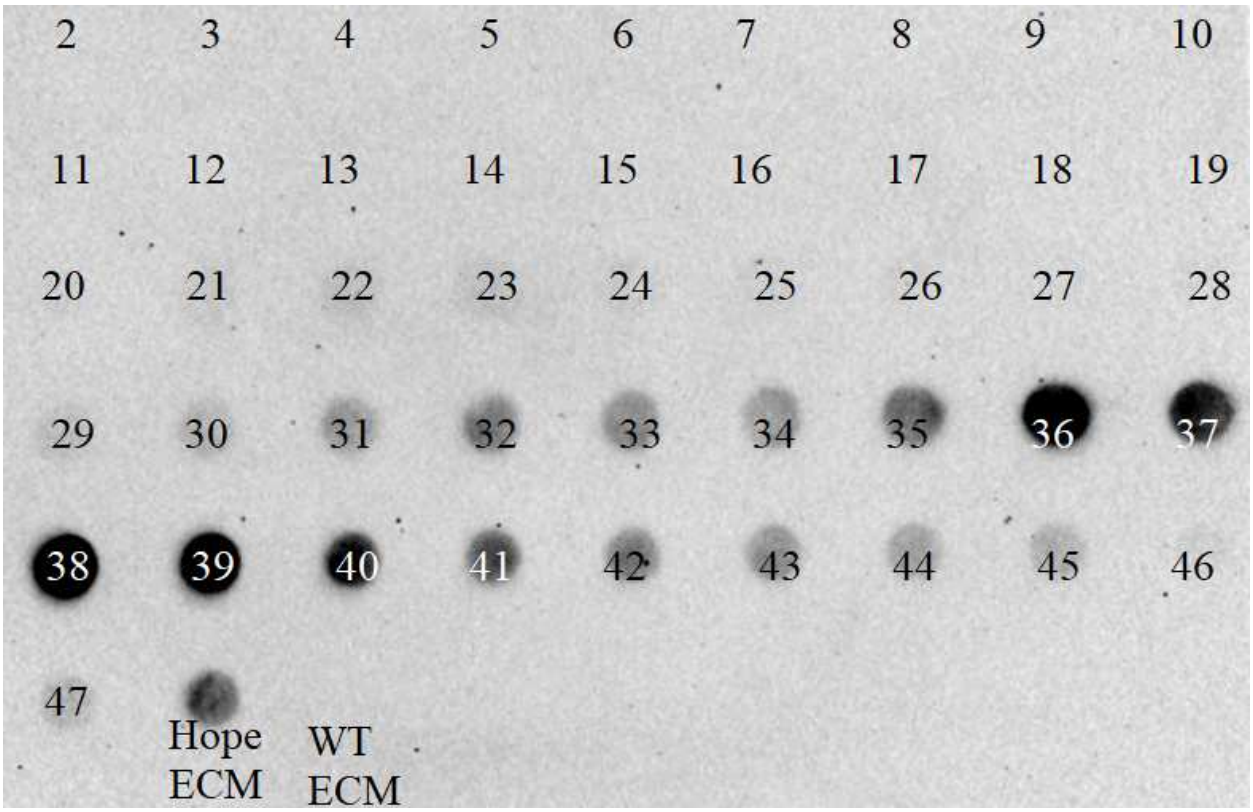
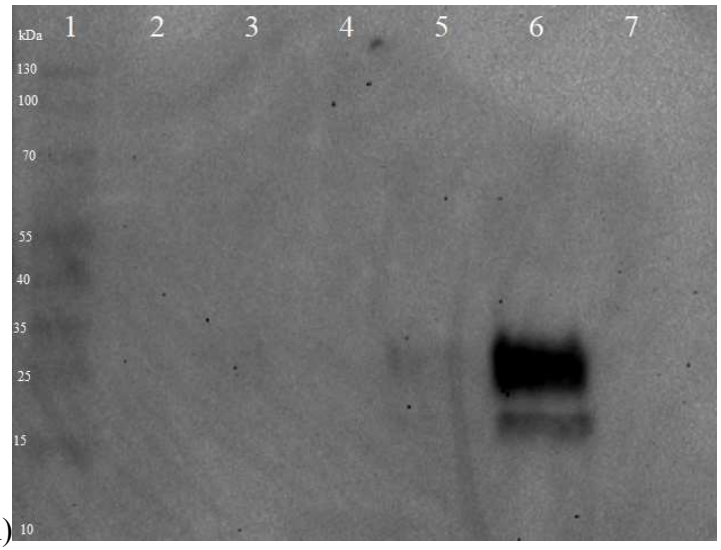


Figure 16: Spot western of cation exchange fractions along with the Hope's ECM as a positive control and WT (untransformed cell) ECM as a negative control.

In a western blot analysis, the bFGF was shown to be concentrated from its initial levels in the starting ECM (lane 3) to its level in fraction 38 (lane 5) and in the enriched concentrated sample (lane 6) (Figure 17). As shown by SDS-PAGE analysis and silver staining, the enriched bFGF was fairly concentrated and a lot purer than it was before (Figure 16B). It went from 624 mg of total protein in the starting ECM (Lane 3) to 1.08 mg of protein in the enriched fraction (Lane 6). However, it is not clear if the bFGF band is visible.

A)



B)

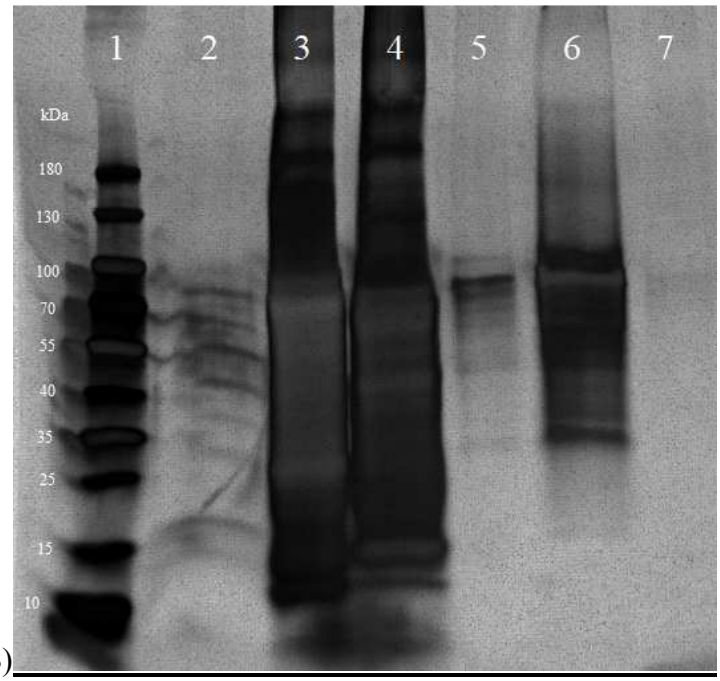


Figure 17: A) Western blot analysis of the cation exchange fractions. Lane 1: PageRuler protein ladder. Lane 2: commercial bFGF as a positive control (100 ng). Lane 3: Hope's ECM. Lane 4: Fraction 10 (negative control). Lane 5: unconcentrated fraction 38. Lane 6: concentrated fractions 35-43 (enriched bFGF). Lane 7: Flow through of concentrator. B)Silver stain of the fractions from the cation exchange. Lane 1: PageRuler protein ladder. Lane 2: commercial bFGF (positive contrl,100ng). Lane 3: 100B ECM before cation exchange. Lane 4: Fraction 11 (negative control) Lane 5: Fraction 38 (highest signal in Figure 16). Lane 6: Enriched bFGF Lane 7: Flow through of protein concentrator.

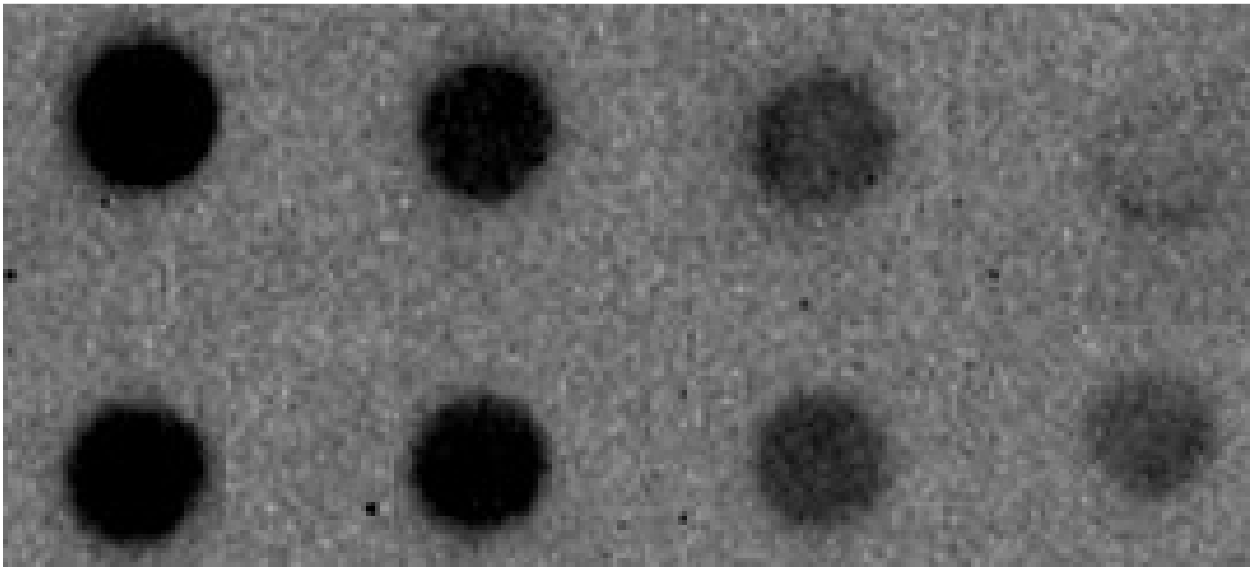


Figure 18: Spot western of commercial bFGF and enriched bFGF from the cation exchange chromatograhy. Top row: commercial bFGF concentrations (20ng, 15ng, 10ng, and 5ng). Bottom row: Enriched bFGF volumes (20µL, 10µL, 5µL, and 2.5µL)

Activity Assays

MTS assay. An MTS assay, which measures cell proliferation, was conducted with 5,000 NIH/3T3 cells in each colored well with different treatments (Figure 19) in order to determine the effects of the different treatments compared to the commercial bFGF, which is made in bacteria. NIH/3T3 cells were grown in the presence of DMEM supplemented with nothing (starve) or with chemicals (10%CS, commercial bFGF, enriched bFGF), conditioned extracellular medium from yeast cells, yeast cells, or combination of conditioned extracellular medium from yeast cells and the yeast cells (C+C). There were three negative controls, which contained no NIH/3T3 cells, designated as "only". Conditioned medium is DMEM based where the yeast have grown for 24 hours before sterile filtering. Thus, this medium was used only for the assay. The Hope conditioned media was also spotted on a spot western alongside known concentrations of commercial bFGF to measure the concentration of bFGF produced by Hope *P.*

pastoris cells in the conditioned media (Figure 20). It was found that the concentration of bFGF in the Hope conditioned media was 0.04ng/µL.

	1	2	3	4	5	6	7	8	9	10	11	12
A		Starve	10% CS	bFGF	WT cond.	Hope cond.	Enriched bFGF	WT C+C	Hope C+C	WT cells	Hope cells	Starve only
B												No 3T3 cells
C												No 3T3 cells
D	Hope cells only											WT cells only
E	No 3T3 cells											No 3T3 cells
F												
G												
H												

Figure 19: 96 well plate with 5K NIH/3T3 cells in each well with a treatment. Starve: Cell treatment with DMEM only. 10% CS: Cell treatment with DMEM+10% calf serum. bFGF: Cell treatment with DMEM+10ng commercial bFGF. WT cond.: Wild type conditioned media, Cell treatment with DMEM that WT was growing in for 24 hours and then had the yeast taken out.. Hope cond.: Hope conditioned media, Cell treatment with DMEM that Hope was growing in for 24 hours and then had the yeast taken out. Enriched bFGF: Cell treatment with 10ng of enriched bFGF in DMEM. WT C+C: Cell treatment with WT conditioned media and 10,000 WT yeast cells added in DMEM. Hope C+C: Cell treatment with Hope conditioned media and 10,000 Hope yeast cells added in DMEM. WT cells: Cell treatment with only 10,000 WT yeast cells in DMEM. Hope cells: Cell treatment with only 10,000 Hope yeast cells in DMEM. Starve only: DMEM only with no cells. WT cells only: 10,000 WT cells in DMEM with no 3T3 cells. Hope cells only: 10,000 Hope cells in DMEM with no 3T3 cells.

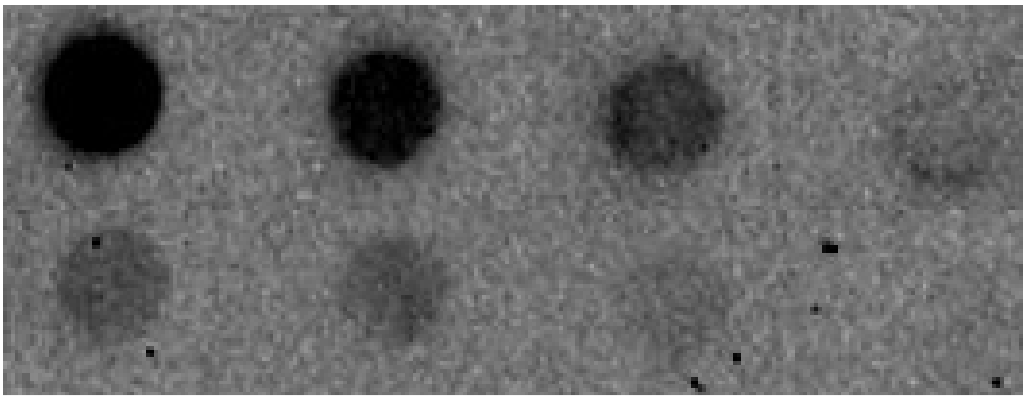


Figure 20: Spot western to estimate bFGF concentration in the Hope conditioned medium. Top row: commercial bFGF concentrations (20ng, 15ng, 10ng, and 5ng). Bottom row: Hope conditioned media volumes (200µL, 100µL, 50µL, and 25µL).

The results of the MTS assay (Tables 5 and 6, Figures 21 and 22) show that 10% CS helped the NIH/3T3 cells proliferate greatest compared to cells treated with starve medium (DMEM only). Commercial bFGF (0.1ng/µL) did the second best with about an 80% increase of proliferation compared to that of starve. Hope conditioned media (0.04ng/µL), enriched bFGF (0.1ng/µL), and Hope C+C gave about the same effect (~60% increase of proliferation compared to starve medium) when it came to accelerating the proliferation of the NIH/3T3 cells. WT conditioned media, WT C+C, WT cells and Hope cells all helped proliferation slightly (~10% increase) more than the starve medium. Thus, the Hope cells alone did not cause the same increase as the Hope conditioned medium and cells or the Hope medium alone.

Table 5: The average absorbance of each treatment in 490nm and 650 nm (normalize) along with the difference in the two readings and the standard deviation.

	Avg 490nm	Avg 650nm	Difference (490nm-650nm)	Standard Dev
Starve	0.2530	0.0429	0.2101	0.0111
10% CS	0.5215	0.0800	0.4416	0.0521
Commercial bFGF	0.4413	0.0540	0.3873	0.0306
WT Cond	0.2762	0.0450	0.2312	0.0157
Hope Cond	0.3826	0.0520	0.3306	0.0351
Enriched FGF	0.3890	0.0552	0.3338	0.0225
WT C+C	0.2800	0.0466	0.2334	0.0212
Hope C+C	0.4019	0.0558	0.3461	0.0315
WT Cells	0.2767	0.0467	0.2300	0.0204
Hope Cells	0.2761	0.0473	0.2288	0.0179
Starve alone	0.1424	0.0390	0.1034	0.0006
WT alone	0.1413	0.0398	0.1015	0.0023
Hope alone	0.1406	0.0377	0.1029	0.0008

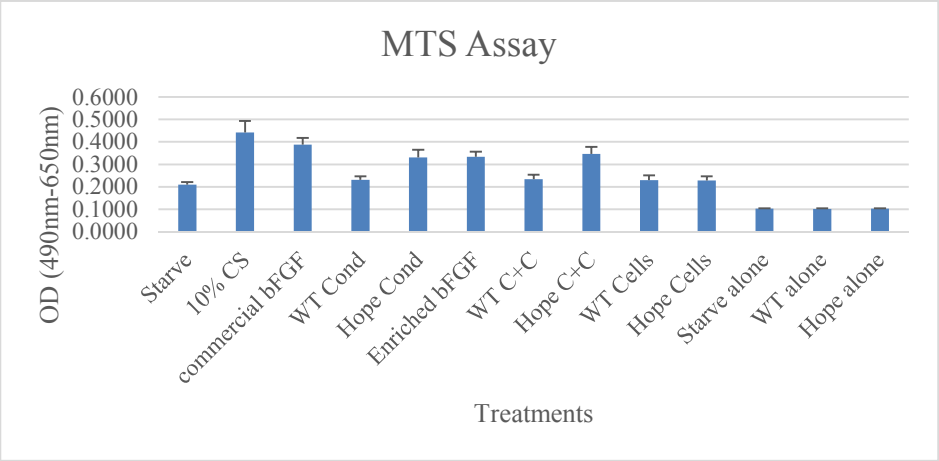


Figure 21: The graph representing the data from Table 5.

Table: 6: % difference in proliferation of NIH/3T3 cells compared to NIH/3T3 cells in starve medium alone (baseline)

	% difference from starve
Starve	0.00
10% CS	110.17
Commercial bFGF	84.36
WT Cond	10.05
Hope Cond	57.35
Enriched bFGF	58.87
WT C+C	11.07
Hope C+C	64.75
WT Cells	9.47
Hope Cells	8.89

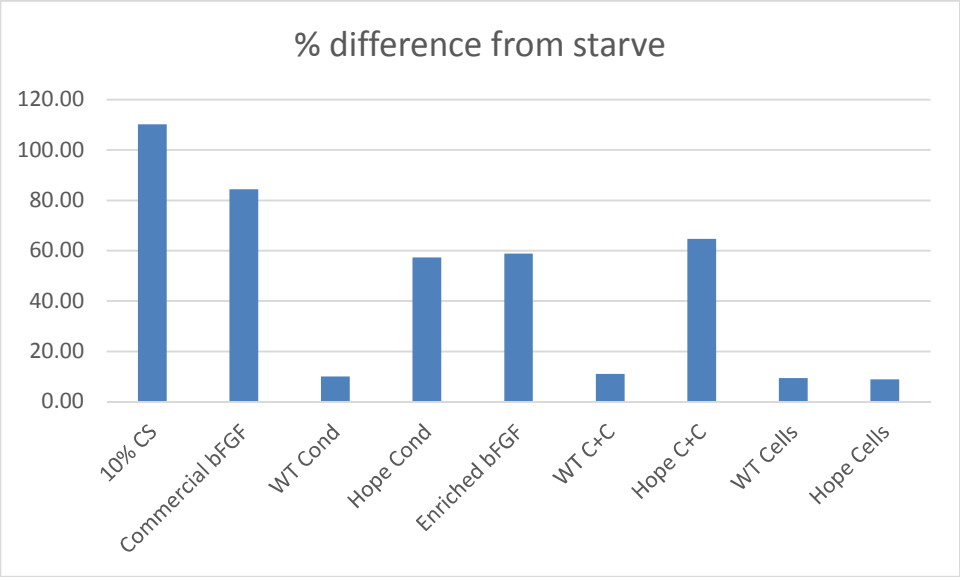


Figure 22: Graph depicting the data from Table 6.

Scratch Assay. The scratch assay was conducted using different treatments like that of the MTS assay. The treatments were starve (DMEM only), 10% CS (DMEM+10% calf serum), bFGF (10ng of commercial bFGF in DMEM), WT (wild type, untransformed *P. pastoris* co-cultured with the NIH/3T3 cells in DMEM), Hope (Hope cells co-cultured with the NIH/3T3 cells in DMEM), WT conditioned media (WT grown in DMEM for 24 hours and had the yeast

taken out), and Hope conditioned media (Hope grown in DMEM for 24 hours and had the yeast taken out). The scratch assay showed that 10% CS affected the mammalian cells the most by having the cells migrate into the scratch 25% of the way in 5 hours. Conditioned Hope media had the same effect as Hope cells, but the NIH/3T3 cells only moved in ~10% of the scratch. WT helped the cells migrate about 3% into the scratch. Lastly, both starve and WT conditioned media helped the cells migrate very little (Table 7 and Figure 23). This assay shows that Hope is able to positively affect the migration of the NIH/3T3 cells.

Table 7: The scratch assay with different treatments. Listed are how wide the initial scratch was at the start and after 5 hours of treatment. The difference is taken and the percent change is calculated.

	start	5 hours	difference	% change
Starve	75.6	74.5	1.1	1.5%
10% CS	78.8	58.9	19.9	25.3%
Hope cond.	79.2	70.4	8.9	11.2%
WT cond.	72.0	71.8	0.2	0.3%
Hope cells	114	103.6	10.4	9.1%
WT cells	114.6	112.3	2.3	2.0%

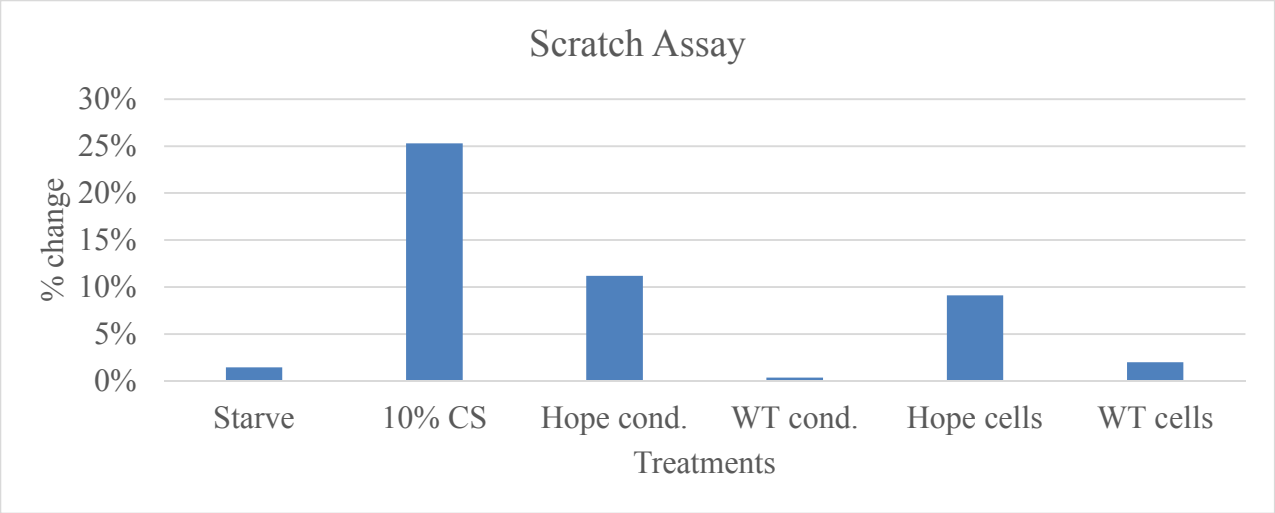


Figure 23: Percent change in how wide the scratch is after 5 hours. Graph of data on tabe 7.

Phosphorylated JNK

JNK is a downstream target that gets activated by phosphorylation during cell motility when bFGF binds a bFGFR (bFGF receptor). High levels of phosphorylated JNK correlates to cell motility so we expected to see high levels of pJNK in the samples where the cells were treated with some form of bFGF (commercial or Hope). The 5 treatments used were starve (DMEM only), 10% CS (DMEM+10% calf serum), bFGF (10ng of commercial bFGF in DMEM), Hope conditioned media (DMEM that had Hope growing in it for 24 hours and then had the yeast removed through sterile filter), and WT conditioned media (DMEM that had WT growing in it for 24 hours and then had the yeast removed through sterile filter). A GAPDH western blot analysis was performed to ensure equal loading and all the data were normalized to GAPDH signal to compare levels of JNK and pJNK. The JNK level checked against the pJNK level in western blot analysis confirm that the rise in pJNK was not form having more JNK to begin with. Based on signals normalized to GAPDH (Figure 24), JNK levels were roughly equal throughout all of the treatments (Figure 25), but the pJNK western blots showed that 10% CS,

bFGF, and Hope conditioned medium treatments caused an increased amount of phosphorylated JNK compared to the starve and WT (Figure 26). The Hope made bFGF gave us the expected results.

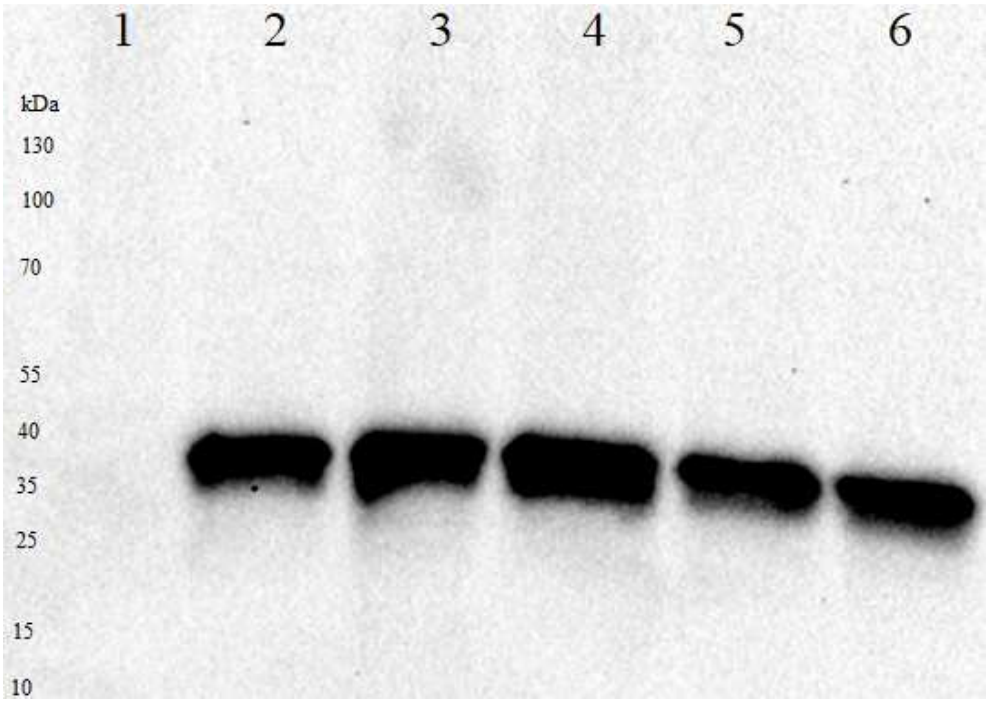


Figure 24: Western blot analysis with anti-GAPDH antibody: Lane 1: PageRuler protein ladder. Lane 2: Starve. Lane 3: 10% CS. Lane 4: WT conditioned medium. Lane 5: commercial bFGF. Lane 6: Hope conditioned medium.

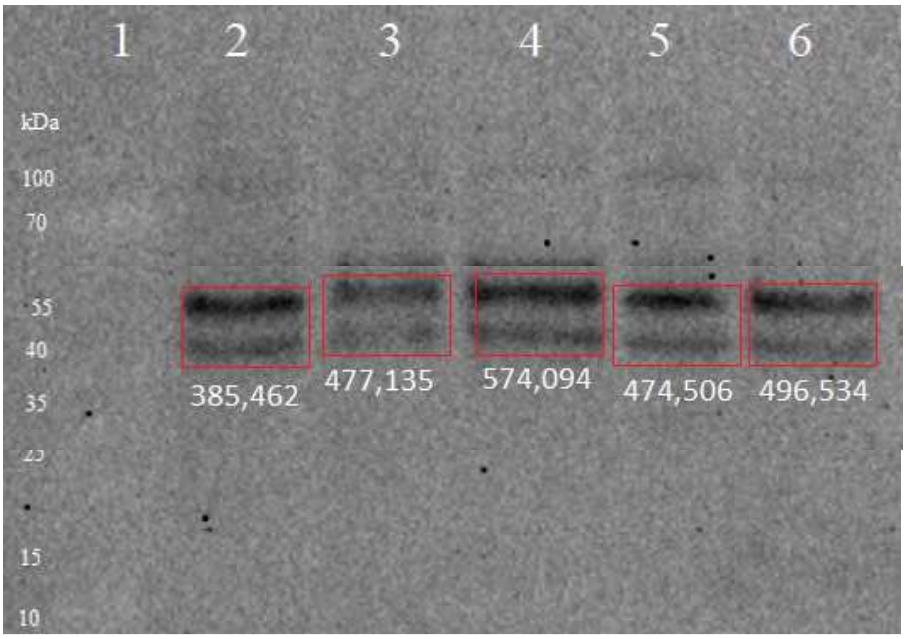


Figure 25: Western blot analysis with anti-JNK antibody: Lane 1: PageRuler protein ladder. Lane 2: Starve. Lane 3: 10% CS. Lane 4: WT conditioned medium. Lane 5: commercial bFGF. Lane 6: Hope conditioned medium.

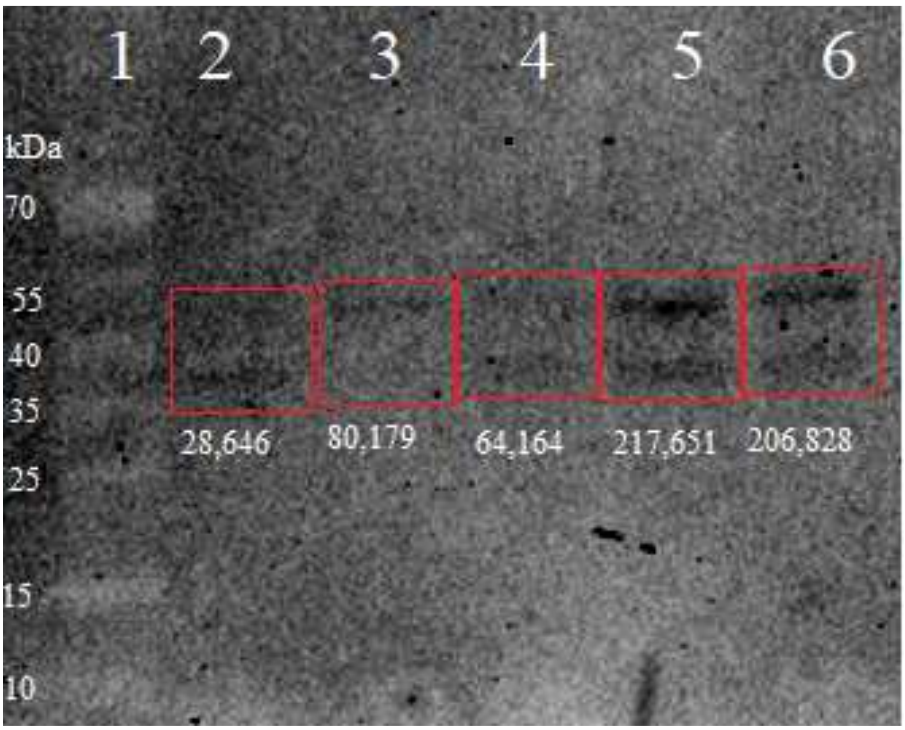


Figure 26: Western blot analysis with anti-pJNK antibody: Lane 1: PageRuler protein ladder. Lane 2: Starve. Lane 3: 10% CS. Lane 4: WT conditioned medium. Lane 5: commercial bFGF. Lane 6: Hope conditioned medium. The numbers underneath each box represents how much pJNK there are in that lane.

Table 8: Ratio of p-JNK to JNK

	Starve	10%CS	WT	Commercial bFGF	Hope
p-JNK/JNK	**0.07432**	**0.16804**	**0.11177**	**0.45869**	**0.41654**

Chapter 4: Discussion

Accelerating the healing of wounds inside the mouth is a difficult task. Because the constant production of saliva will wash away any medicinal ointment placed on the site of injury. A possible solution could be placing a microbe that will attach onto the injured site in order to constitutively express and secrete a growth factor to promote healing of the damaged tissue. Such microbes, which have been developed previously for the treatment of intestinal diseases, are known as engineered probiotics (Steidler, 2003). Many of these probiotics, like *Saccharomyces boulardii*, have not been studied enough to easily genetically modify the yeast. *Pichia pastoris*, on the other hand, has been studied for many years and techniques have been developed to genetically modify this yeast. Not only that, but *P. pastoris* is a great recombinant protein expressor. Past studies have found it possible to make a strain of *P. pastoris* that is able to express bFGF that has a regenerative effect on the NIH/3T3 mouse fibroblast cells (Mu et al., 2007). This strain had the bFGF DNA coding sequence under the control of the methanol induced promoter, *AOX1* (alcohol oxidase), so the bFGF was not expressed constitutively. In this project, we have also selected the yeast, *Pichia pastoris*, to make and secrete the basic fibroblast growth factor (bFGF) constitutively using the *GAP* (glyceraldehyde-3-phosphate dehydrogenase) promoter since the yeast would need to produce bFGF in the mouth in the future. We hypothesized the bFGF secreted by our recombinant strain of *P. pastoris* will increase both migration and proliferation of NIH/3T3 mouse fibroblast cells. In this study, the recombinant bFGF's effects on migration were tested on scratch assays where a plate of NIH/3T3 cells is scratched and the migration of the cells into the scratch is observed. The effects of the recombinant bFGF on proliferation were tested in MTS assays where equal numbers of NIH/3T3 cells were added to wells with different treatments and quantified after the treatment.

Purification

We originally tried a magnetic Myc-bead purification, but we were only able to elute the protein off the beads by denaturing the bFGF protein in a 100°C heat block. This was not useful because we needed functional protein to test in our scratch and MTS assays. Using the Myc peptides from Chromotek (München, Germany) as a competitor to elute our protein did not work because the c-Myc-bFGF was bound onto the magnetic beads too strongly and the Myc peptides had a hard time competing the bFGF protein off. This left us with having to perform a cation exchange chromatography to purify our recombinant protein. Because other proteins eluted off of the resin with the bFGF, the fractions were not pure bFGF. However, it became enriched to a high degree after concentrating the proteins from fractions 35-43. The 60 mL of ECM used in the cation exchange went from 624mg of total protein to just 1.08mg in the enriched fraction.

Functional Activity Studies

The MTS showed that the proliferation of the NIH/3T3 cells had been positively affected by the Hope strain's recombinant bFGF whether it was present in the conditioned media or as the isolated/concentrated protein. As controls, starved medium and the yeast cells alone did not increase absorbance in the MTS assay. The starved media was used as a baseline control to see how the cells would grow with just DMEM, which contained just the essentials to live. The 10% CS contained growth factors and hormones in it so it was expected to have the highest proliferation rate (>100% growth increase compared to DMEM). The enriched bFGF, Hope conditioned medium, Hope conditioned medium with *P. pastoris*, and commercial bFGF all displayed about the same amount of proliferation (~60% growth increase compared to DMEM). The wild type (WT) conditioned medium (from untransformed *Pichia pastoris* cells), WT

conditioned medium with *P. pastoris*, WT cells, and Hope cells showed about the same growth as just DMEM (~10% growth increase), meaning, they had little effect on the mammalian cell proliferation. While the Hope conditioned medium and Hope conditioned medium with *P. pastoris* induced a large increase in proliferation, the Hope cells showed little effect on the NIH/3T3 cells, which suggested that the Hope strain was not producing enough of the bFGF in the 24-hour window that the MTS assay lasted. This could be because there was not enough of the bFGF producing yeast in the assay to make sufficient bFGF to have an effect. This co-culturing to improve the effects of proliferation will need to be repeated with a higher concentration of Hope cells. The scratch assays showed that the Hope medium (both conditioned and with the cells) induced migration compared to that of the WT and starve (baseline). This is promising because the migration of cells is essential wound healing.

Throughout the project, the bFGF found made by the Hope strain was always found to be heavier than what bFGF's predicted mass. A possible reason is that it has some additional post translational modifications such as glycosylation, which would not be found on the bacterially-produced commercial bFGF. Even though the protein is heavier than the commercial bFGF, the activity of the recombinant bFGF is not hindered by it. The recombinant bFGF works almost as as well at the commercial bFGF in the MTS assay.

Migration of fibroblast cells is very important because they are involved in the wound healing. It has been shown that the activation of the protein JNK by phosphorylation is essential to a cell's movement (Kanazawa,2010). This correlation was observed in our western blots. The treatments that helped the cells migrate faster, such as conditioned Hope medium, were associated with higher levels of phosphorylated JNK than the treatments that did not help the NIH/3T3 cells migrate. This shows that *P. pastoris* made bFGF does trigger the cascade to

activate or phosphorylate the JNK in order for the NIH/3T3 cell to migrate just like the commercial bFGF.

Future Work

As stated; before in the MTS assay, the Hope strain co-culture did not produce a significant effect on the growth of NIH/3T3 cells. This could be due to the fact that there were not enough of the yeast in the well to make sufficient bFGF to see an effect. Co-culturing with NIH/3T3 cells will have to be optimized so that a high amount of bFGF is released by the Hope cells.

Another limitation of this project was in our early work that the human cell lines were difficult to grow and maintain for the activity assays. We had to resort to using mouse fibroblast cells (NIH/3T3) cells, as in the Mu et al., 2007 study. Now that it has been shown that the bFGF secreted from the Hope strain can positively affect the proliferation and migration of mouse fibroblast cells, the experiments can be repeated on more relevant human tissue culture cells such as human dental pulp cells. Both the MTS and scratch assays should be used to determine how the recombinant bFGF will affect the human cells.

Another idea for future work could be to improve the bFGF production in the yeast through mutating the *Matα* secretion signal to enhance the secretion. Multiple copies of the plasmid transformed into *P. pastoris* would be expected to give higher protein secretion (Aw, Polizzi, 2013), but it seemed like the more copies it had (higher zeocin resistance), the less efficient it was at expressing the bFGF protein. A possible reason could be that the cell is making too much bFGF to secrete out efficiently, causing a “traffic jam” of the recombinant protein in the secretory organelles (Aw, Polizzi, 2013).

Conclusion

This study has allowed us to make a strain of *Pichia pastoris* that is able to express and secrete bFGF. The bFGF made has been observed to cause a positive effect on mouse cells' migration and proliferation. There is great potential with this project. With further improvements, modifications, and testing, a *P. pastoris* product can one day exist for dentist to use in their practices to help heal the wounds of the mouth.

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