Genetically modifying *E. coli* to produce Insulin-like Growth Factor 1

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Background Information

Tissue Engineering is a science aiming to aid the recovery of the body after damage or to maintain normal function (National Institute of Health 2017). This makes the field a prime candidate for research and development. Because tissues are complex and differentiated from other cells found in the body, each application of this technique has different demands and requirements (Lawson). For the cellular components of skeletal muscle tissue, myofibers, regeneration is an attractive application of tissue engineering. This tissue is of critical importance to human motility, thus the refinement of techniques targeting this tissue has applications in injury recovery and potential gain of function growth for individuals without necessary skeletal muscle tissue. An engineered scaffold is used to both guide regeneration and deliver the needed components of regeneration (National Institute of Health 2017). However, this alone is not sufficient to induce the desired result of differentiated cellular tissue growth. Myofibers need many intracellular and extracellular vitamins, chemical messengers, enzymes, co-enzymes, nutrients, and resources (Lawson 2000). The insulin-like growth factor 1 peptide (IGF-1) is one such component needed (Schiaffino 2011).

The problem arises in obtaining this signaling peptide in sufficient quantity to use in tissue engineering. To this end, we will employ a technique to obtain IGF-1 in sufficient quantity from a renewable source using genetic manipulation of *Escherichia coli* bacteria. Cloning Recombinant Deoxyribonucleic Acid (DNA) is a technique used frequently to change the genetics of a target organism. This is generally done to obtain a new genotype of the organism that contains a genetic coding region not previously found in the organism. Bacteria are used because of their unique genetic structure known as plasmids. A plasmid is required because the circular structure naturally recombines in standard biochemical conditions. In addition, bacteria are cheap, replicate quickly, and are easy to grow in laboratory settings. *Escherichia coli* (*E. coli*) is a logical choice due to the ease of growth of the bacteria and it is cheaper and safer than other options.

For future use of IGF-1 in tissue engineering research at Hampden-Sydney College, a stable and sizeable source for this peptide was needed. Thus, a proposal for transforming *E. coli* with IGF-1 coding DNA using a modified, but established, technique of Cloning Recombinant DNA was desired. This would yield a safe, reliable, and cheap option for producing the needed quantities of the essential growth signal peptide.

Materials and Methods

The primary goal of the research was to produce IGF-1 for future use in tissue engineering of myofibers. This required a plasmid containing IGF-1 that can be transformed into *E. coli*. The first step of Cloning Recombinant DNA is to design primers used for Polymerase Chain Reactions (PCR). These primers were designed with restriction enzyme coding regions specific to each chosen vector plasmid to be used later in the procedure. The three separate vector plasmids were chosen to increase the chances of successful transformation of *E. coli*. They were QIAGEN's pQE-16, pQE-60, and pQE-70. These were chosen because of their protein upregulation transcription sites which would allow for inducing increased IGF-1 peptide production after successful transformation. For the pQE-16 plasmid vector, the upper primer was designed to use a BamH1 restriction enzyme site and the lower primer was also designed to use a BamH1 restriction enzyme site. For the pQE-60 plasmid vector, the upper primer restriction enzyme site chosen was Nco1 while the lower primer restriction enzyme site chosen was BamH1. An upper primer was designed to use a Sph1 restriction enzyme site and a lower primer was designed to use a BamH1 restriction enzyme site for the pQE-70 plasmid vector. These restriction sites were chosen to minimize the possibility of interfering with the upregulating transcription factors or other necessary components of the respective vector, for instance the Ampicillin resistance gene. Finally, a sequence of base pairs coding for six histone amino acids was added to the end of each of the lower primer to allow for future confirmation of IGF-1 peptide presence via fluorescent tagging. These plasmid primers and vectors were all designed using the software program SnapGene. The new primers were produced by and ordered from IDT Technologies. Autoclaved deionized water (diH_2O) was added to the ordered primers to bring them to a 100 uM solution.

Next stock Luria-Bertani agarose plates with ampicillin (LB-amp) were prepared for each vector plasmid, pQE-16, pQE-60, and pQE-70, and the insert plasmid containing IGF-1. The plates were z-streaked and incubated at 37 degrees Celsius overnight to produce single cell colonies. A single colony from each plate was used the next day to produce a stock solution of *E. coli* containing a single type of each plasmid in five mL of Luria-Bertani broth and five uL of ampicillin. These solutions were shaken at 215 rpm at 37 degrees Celsius overnight. The stock LB-amp plates were stored in a four-

degree Celsius refrigerator. The stock solutions were used to grow DNA for extraction via QIAGEN's QIAprep protocol. This protocol yielded plasmid DNA suspended in 30 uL of diH_2O. The four plasmids had their DNA concentrations quantified using a BioTek EPOCH spectrometer, which measures UV light absorption of a liquid sample to determine concentration. This step is necessary to ensure enough DNA is present for a successful PCR. The purified plasmid DNA in water was stored in a -20-degree Celsius freezer. This process was repeated until sufficient concentration for future work of was met.

A PCR was run using 10 uL of 2x MasterMix, three uL of 1:100 diluted upper primer, three uL of 1:100 diluted lower primer, three uL of diH_2O, and one uL of template IGF-1 DNA. MasterMix is a solution containing free nucleotides and the necessary proteins for DNA assembly. The run cycled from 95 degrees Celsius for one minute, to 55 degrees Celsius for one minute, and to 72 degrees Celsius for one and a half minutes for a total of four hours. This would produce an abundance of primed insert DNA. A negative control test PCR was also performed on each set of primers without the template DNA to check for possible primer dimers.

Gel electrophoresis was employed after PCR to check for successful amplification of the desired genetic product, a primed IGF-1 gene of about 500 base pairs. A one percent weight to volume ratio of agar to Tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer gel was made for the electrophoresis procedure. The wells of the gel were loaded with 5uL of each PCR product, 5 uL of diH_2O, and 4 uL of EZVision. When in need of a higher concentration following this step, wells would be loaded with only PCR product and EZVision. The electrophoresis chamber was filled with TAE buffer to protect DNA from damage caused by redox. The gel was run at approximately 120 V for no more than thirty minutes. Photographs of the gel were taken under UV light taking advantage of the EZVision. The preceding procedure was repeated until successful confirmation of desired genetic product.

Following confirmation, a razor blade was used to cut the desired genetic product out of the gel. The QIAquick gel extraction protocol was followed to obtain purified DNA in diH_2O from the gel. The samples then had concentration quantified in ng per uL using the spectrometer. This concentration was important in the next step.

Using the concentrations of the primed IGF-1 inserts for each specific vector plasmid, digestion reactions were planned to ensure no more than one ug of both the primed IGF-1 insert and the relevant vector plasmid. The reactions contained a maximum volume of 15 uL with various amounts of DNA and diH_2O added but always one and a half uL of 10x Cutsmart buffer, one uL of each relevant restriction enzyme. The digestion reactions were kept at 37 degrees Celsius overnight. Each digestion reaction product was run on an electrophoresis gel to confirm the length of the product matched the predicted length at approximately 500 base pairs. The same gel extraction procedure was performed to purify the digestion reaction products. These results were then quantified using the concentration found via spectrometer.

The purified digestion products then had their 5'-phosphate groups removed via a Calf Intestine Alkaline Phosphatase catalysis (CIP treatment) to aid in successful ligation by reducing self-annealing of the insert DNA. To do this, digestion reaction products were The CIP treated digestion products were then ligated following New England Biolabs procedure for T4 DNA Ligase. Each reaction called for two uL T4 DNA Ligase buffer and 1 uL T4 DNA Ligase. Importantly, the volume of added insert and vector for each reaction was variable based on the size of the insert, the size of the vector, and finally the amount of vector added determined by concentration of stock solutions made previously. The total volume of the ligation reaction was 20 uL for each pQE-16, pQE-60, and pQE-70. The ligation reactions were kept at 16 degrees Celsius until transformation procedure could be completed.

A transformation procedure for Invitrogen OneShot Top10 chemically competent *E. coli* cells was followed. Negative control transformations using only the vector plasmid and no insert DNA were also run on *E. coli* cells. Additionally, a second negative control using stock pUC-19 plasmid vector was implemented during transformation. The transformed cells were spread onto LB-amp agar plates and incubated overnight at 37 degrees Celsius. Growth of transformed *E. coli* was checked visually. Single colonies from these transformation plates were used to prepare liquid cultures in 500 mL Luria-Bertani broth and 5 uL ampicillin and z-streaked on new LB-amp agar plates. These samples were incubated overnight at 37 degrees Celsius. The liquid cultures were then used for further confirmation testing while the plates were stored in -20-degree freezer.

The liquid cultures underwent a QIAprep DNA extraction protocol. The extracted DNA was concentration quantified using the spectrometer. A PCR was run on the samples using the respective primers for each plasmid. Two PCRs were run: the first using IGF-1 as a positive control the second using pUC-19 as a negative control. EZVision was

added to the products of these reactions, and they were loaded into an agarose gel for electrophoresis to confirm presence of IGF-1 in transformed *E. coli* cells.

Results

The first results of the project were photographs of gel electrophoresis under UV light. The second set of results were purified DNA in diH_2O concentrations via quantification using the spectrometer. The third set of results was *E. coli* bacterial growth on LB-amp agar plates. Obtained results had the dual purpose of confirming success of the previous step and generating data for the next step. Examples of each form result are shown below.

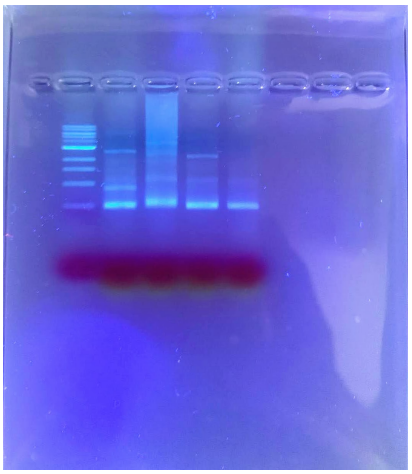

Figure 1: a photograph of a 1% agarose gel after electrophoresis. Wells from left to right are: one kilobase pair ladder, PCR-amplified post-transform pQE-60 vector plasmid DNA, positive control PCR-amplified IGF-1 stock DNA, PCR-amplified post-transform pQE-16 vector plasmid DNA, and negative control PCR-amplified pUC-19 DNA.

	Trial 1	Trial 2	Average
Sample 1	56.930	60.599	58.765
Sample 2	57.509	56.917	57.213

Figure 2: a table showing ng of purified IGF-1 template DNA to µL diH_2O concentrations produced via BioTek EPOCH UV/vis spectrometer.

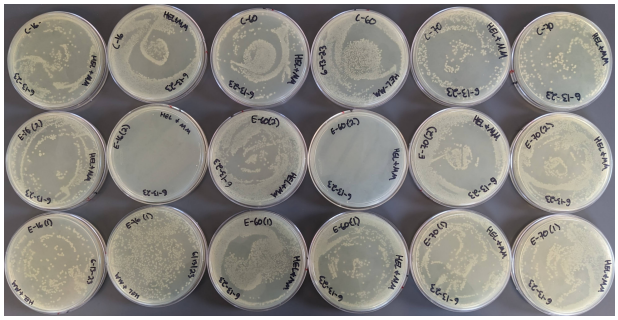

Figure 3: A set of LB-amp agar plates with transformed E. coli bacteria growing. Columns of plates from left to right are: two transformations using pQE-16 vector plasmids, two transformations using pQE-60 vector plasmid, and two transformations using pQE-70 vector plasmid. The rows of plates from top to bottom are: one control row without any insertion in vector plasmid, one row using IGF-1 insert from sample two, one row using IGF-1 insert from sample one.

Discussion

Prior research at Hampden-Sydney College had attempted to transform *E. coli* to produce IGF-1. However, quantification and presence tests from prior attempts were inconclusive. Primer design for PCR and plasmids was indicated most recently to be the source of prior difficulties. This indicated a need for careful, precise, and systematic precautions during the earliest stages of the project. Thus, this project concentrated on refining of the techniques used in prior attempts.

The refinement process led to choosing three distinct vector plasmids each with their own set of restriction enzyme sites. Likewise, constant verification via gel electrophoresis and DNA concentration quantification was employed. Despite these precautions, multiple failed attempts at various stages of the project required effective restarts of the procedure. The final verification of a successful transformation, bacterial growth in the presence of LB-amp media, only partly confirmed the desired results. This, in addition to near constant presence of human error during the project necessitated a double check on successful results. As seen above in Figure 1, the final gel photograph of the research indicated a positive result in the negative control pUC-19 well. This seeming contradiction was typical of results. Post-transformation *E. coli* would grow on LB-amp agar regardless of whether an insert was used in the transformation reaction or not as shown in Figure 3. As the results stand, success of the Cloning Recombinant DNA procedure cannot be confirmed.

In the future, research could be continued with the final products of the project. If successful, the

transformed *E. coli* would have been manipulated further to have their new IGF-1 coding DNA upregulated. The upregulated proteins could be tested using an SDS-Page gel electrophoresis protocol to confirm the presence of the desired peptide. Once done, this would yield an effectively renewable source of the valuable peptide. Further research on this project could then focus on scaffolds for myofibers and other applications in tissue engineering.

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