The Introduction of Insulin-like Growth Factor 1 into an Electrospun Scaffold

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Skeletal muscle tissue makes up most of the muscle mass in the human body and regulates the locomotion of bones. The system that makes up skeletal muscle involves a large amount of microscopic fibers called myofibers pulling on a tendon attached to a bone to cause a specific movement (1). Each myofiber is a single multi-nucleated cell that has matured by fusing many single nuclei myoblasts together (2). Myofibers are bundled together into a larger structure called a fascicle which can generate enough energy to pull a bone (2). Since skeletal muscle is readily used in the body, it can easily be damaged through sports and injuries. In addition to injury, skeletal muscle is also lost when undergoing tumor removal from tissue deeper in the body (2). While muscle is capable of healing from small injuries, a large loss of muscle mass results in severe scarring or the inability to function (1). As a result, damage to skeletal muscle tissue could cripple the life of someone with severe injuries (1).

Tissue engineering is a field of medicine that grows cell cultures with the intention of replacing the tissue lost in the human body (1). For more complex injuries, scaffolds can be used to give grafts the proper structure to fill in the missing tissue (1). Currently, the hope is to implement this process for skeletal muscle repair, but it has a set of challenges to overcome first (1). Since skeletal muscle has a specific structure, a successful replacement must replicate the structure muscle cells have in the body (1). In addition, myoblasts rely on nutrients and proteins in order to be healthy enough to grow and mature. In order for successful skeletal muscle to be made, it must contain a strong structure to withstand the environment and contain the necessary materials for myoblasts to undergo maturation, which can be done using proteins that signal muscle growth and maturation such as insulin-like growth factor 1 (IGF-1) (1).

IGF-1 is a protein found in the human body that acts as a signaling molecule in various types of cells in the body (3). Typically, it binds to the IGF-1 receptor that signals a response that varies depending on the type of cell (3). In skeletal muscle tissue, IGF-1 can promote muscle growth through protein synthesis when it is signaling through the AKT pathway (3). Since a high protein concentration is required to cause cell maturation, IGF-1 has been a point of interest in the tissue engineering of skeletal muscle tissue. By delivering IGF-1 to myoblasts, upregulated protein synthesis may occur which will cause the cells to undergo maturation (3). Thus, the goal of this research was to produce a scaffold through electrospinning that can give myoblasts the proper structural support and be used as a drug delivery system for IGF-1.

MATERIALS AND METHODS

In order to have an abundance of plasmid to work with, One Shot™ TOP10 Chemically Competent *E. coli* cells were transformed with an IGF-1 plasmid purchased from Sino Biological or a pQE 60 plasmid vector purchased from Qiagen. *E. coli* were transformed by adding 1-5 µL of the IGF-1 plasmid or the pQE 60 plasmid vector to vials containing the cells and were incubated first on ice for 30 minutes, then at 42˚C for 30 minutes, and finally at 37˚C after adding 250 µL of pure S.O.C. medium to each vial for 1 h. After incubation, the cells were spread on Luria-Bertani agar plates containing ampicillin (LB-amp) to confirm the growth of *E. coli* with the plasmid as the plasmids contain an ampicillin resistant gene. After growing overnight at 42˚C, the cells were stored at 4˚C. *E. coli* cells containing either the IGF-1 or pQE 60 plasmids were added to vials containing 5 mL of LB broth with 5 µL of ampicillin and placed on a shaker at 42˚C at 225 rpm to grow overnight. Stock cultures were created by adding 1 mL of the overnight cultures to vials with 1 mL of 50% glycerol for storage in the -80˚C freezer.

The remaining overnight *E. coli* plasmid cultures had the DNA extracted and purified using a QIAquick Spin Column Miniprep kit by following the manufacturer’s protocol (4). Each plasmid DNA sample was quantified separately for total DNA concentrations using an Epoch plate loaded into a spectrophotometer and Gen5 software. PCR was done on the *E. coli* transformed with the IGF-1 plasmid to increase the supply of the DNA by making a PCR solution containing 10 µL master mix, 3µL reverse NCO1 primer, 3µL forward BAMH-1 primer, 1 µL DNA, and 3 µL diH2O (Table 1).The PCR solutions were loaded into the thermocycler and run for 4 hours on a repeating process of 95˚C for 1 minute, 55˚C for 1 minute, and 72˚C for 1.5 minute. Products from the PCR were loaded into a gel made from 1 g agarose and 100 mL 1x TAE buffer and gel electrophoresis run to look for the presence of the IGF-1 DNA. The IGF-1 DNA samples were further purified by following the protocol from the QIAquick PCR Purification Kit and stored in the -20˚C freezer (5).

***Table 1**: Gene Sequence of each primer*

NCO1 Sequence	GGGCCATGGGGGATGGGGAAAATCAG CAGCCTC
BAMH-1 Sequence	GGGGGATCCGGGCTTCTTGTGTTCTT CAAATGT

Next, digests were required to create staggered cuts on the IGF-1 gene and pQE 60 plasmid vector so that the IGF-1 gene could be inserted into the vector with a C-terminal end. IGF-1 purified DNA and pQE 60 plasmid vectors were individually digested by making 15 µL solutions made from 3 µL of each of the restriction enzymes NCO1 and BAMH, ~1000 ng of each plasmid, and the remaining volume was made up of autoclaved diH2O. These samples were incubated at 37˚C overnight. The IGF-1 digests were tested for the presence of IGF-1 using gel electrophoresis to make sure the digests still contained the desired DNA.

Digestion samples were purified using the QIAquick PCR Purification Kit as the residues in the digest can be washed away by the buffers in the kit. As an additional step to make sure the pQE 60 plasmid vector was cut properly for IGF-1, it was phosphorylated using calf intestinal alkaline phosphatase in an overnight incubation at 37˚C (6). Once this was completed, ligations were required to insert the IGF-1 gene into the plasmid vector. Using the Biolabs ligation protocol, 20 µL ligation solutions were then set-up using 2 µL T4 DNA Ligase Buffer (10x), 1 µL T4 DNA Ligase, 50 ng of phosphorylated pQE 60 plasmid vector, 37.5 ng IGF-1 digest, and the remaining volume filled with diH2O (7). To ensure the ligation formed properly, ligation and control solutions were loaded into an incubator at 20˚C overnight to ensure the samples stayed at room temperature; as stated in the Biolabs protocol (M0202).

Chemically competent *E. coli* cells were transformed as described previously with the pQE60 containing IGF-1 plasmids. To test for the presence of the pQE60 containing IGF-1 plasmid in *E. coli*, crude DNA samples were made using a sample of transformed *E. coli* cultures. These samples were run in the thermocycler under the same program as before and gel electrophoresis performed. Overnight cultures were made from the pQE60 containing IGF-1 transformed *E. coli* cells and stored in the -80˚C freezer in solutions made of 1 mL *E. coli* culture and 1 ml 50 % glycerol. Plates containing the pQE60 containing IGF-1 transformed *E. coli* cultures were stored at 4˚C.

Next, expanded cultures of pQE60 containing IGF-1 transformed *E. coli* were made to express the IGF-1 protein. Two expanded cultures were made using 500 mL of LB broth with 500 µL ampicillin and a sample of *E. coli* transformed with pQE60 containing IGF-1. A spectrophotometer was used to test the concentration of each culture to ensure that the *E. coli* concentration was in the O.D. range of 0.400-0.600. One culture was induced with a 1 mM isopropyl-β-D-thiogalactoside (IPTG) solution and incubated at 37˚ C at 200 rpm for 4 hours; the other culture was not induced, but incubated under the same conditions to act as a control. The expanded cultures were centrifuged at 17,000 rpm in conical tubes to collect the cell pellets. Crude lysis was done to each pellet using liquid nitrogen to flash freeze and a mortar and pestle ground the frozen pellet into a fine powder. The powder was placed in Eppendorf tubes and centrifuged at 1,400 rpm for 20 minutes. The supernatant was separated from the cellular debris and transferred into clean Eppendorf tubes. The supernatant of both the induced and uninduced cultures was tested for IGF-1 protein expression in a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and run using an electrophoresis set up from Bio Labs.

A scaffold to deliver the expressed IGF-1 protein to myoblasts was made using an electrospinning technique. A 10% (w/v) solution of gelatin dissolved in 2,2,2 tri-fluoroethanol (TFE) was made and mixed overnight. Next, microbial transglutaminase (mTG) was added to the solution in the varying amounts of 5 g, 2.5 g, and 1.25 g to act as a cross-linker (8). The solution was vortexed for 5 minutes and then loaded into a 5 mL syringe. The electrospinning apparatus was set up in a ventilation hood. Aluminum foil was carefully wrapped tightly around the mandrel that was attached to a motor. The syringe was then placed into a syringe pump 15 cm from the mandrel and connected to a voltage box with a constant voltage of 15 kV. The solution was pumped at a constant rate of 5 mL/hr. The mandrel rotated at a ~2500 rpm until the gelatin solution was either fully dispensed or stalled due to cross-linking. Spitting from the syringe was dealt with by increasing the voltage to 17 kV. After electrospinning, the mandrel and motor were disconnected, and the foil was gently pulled off. Four samples from each of the four scaffolds were cut and three fields of view at 400x magnification from each sample were observed to determine fiber alignment and average diameter.

RESULTS

Transformed *E. coli* with either the IGF-1 plasmid or the pQE60 plasmid vector grew on LB-amp plates indicating that the plasmids had been successfully taken up by the cells. Following DNA purification, the measured concentration of each DNA sample was 49.485 ng/µL for the IGF-1 plasmid and

24.16 ng/μL for the pQE60 plasmid vector. These results show that the DNA was successfully purified and was in a high enough concentration to be used in upcoming experiments. Figure 1 displays the gel electrophoresis results of the purified IGF-1 DNA with a 1 kilobase pair (kbp) ladder. IGF-1 has a length of ~480 bp and lanes 2-6 have bands at ~500 bp. This further confirms that the *E. coli* cells have been transformed with the IGF-1 plasmid.

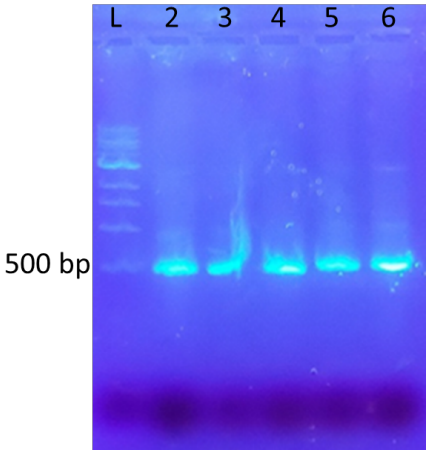

***Figure 1**: Gel electrophoresis of the purified PCR product showed the presence of the 480 bp IGF-1 DNA. The far left lane was the 1kb ladder (L) and lanes 2-6 contained IGF-1.*

Following digestion, the concentration of the IGF-1 sample was 160.88 ng/μL, ascertaining that the reaction contained a high amount of the IGF-1 gene. Digestion solutions of IGF-1 were tested using gel electrophoresis. Lanes 3-5 and 7-8 in Figure 2 have bands at ~500 bp showing that the IGF-1 DNA was still present. After purification, the digestions of IGF-1 and pQE 60 yielded DNA concentrations of 169.985 ng/μL and 46.638 ng/μL respectively. These concentrations were high enough to be used to make ligation solutions.

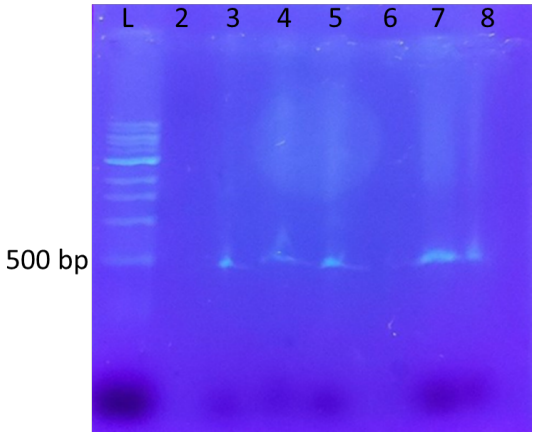

***Figure 2**: Gel electrophoresis results following digestion of the IGF-1 plasmid established that the IGF-1 DNA was still present because lanes 3-5 and 7-8 have bands at ~500 bp. The far left lane was the 1 kb ladder (L).*

Following ligation, transformation of the *E. coli* with the pQE60 containing IGF-1 plasmid was proven successful when the cells grew on LB-amp plates. The results from the gel electrophoresis shown in Figure 3 have two bands for each PCR sample. The first band was at ~500 bp signifying the presence of the IGF-1 gene. The second band shows the formation of primer dimer by-products. The expanded cultures for the expression of IGF-1 had measured absorbance values of 0.422 for induced and 0.502 for uninduced cultures. These values established that there was enough transformed *E. coli* to express a large quantity of the IGF-1 protein. Figure 4 displays the results of the crude cell lysis of all proteins expressed in the induced and uninduced cultures. However, the induced cultures have darker bands from 10 kilodaltons (kDA) to slightly above 25 kDa compared to the uninduced cultures. This provides some evidence that the IGF-1 protein was expressed as it has a molecular weight that ranges from 12-32.5 kDa.

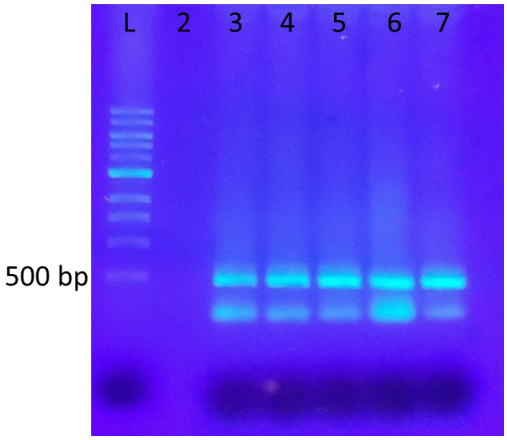

***Figure 3**: Results from the gel electrophoresis confirms that the ligation of the pQE60 vector plasmid with IGF-1 was successful as lanes 3-7 have bands at ~500 kb. The second band indicates the formation of a primer dimer. The far left lane is the 1 kb ladder.*

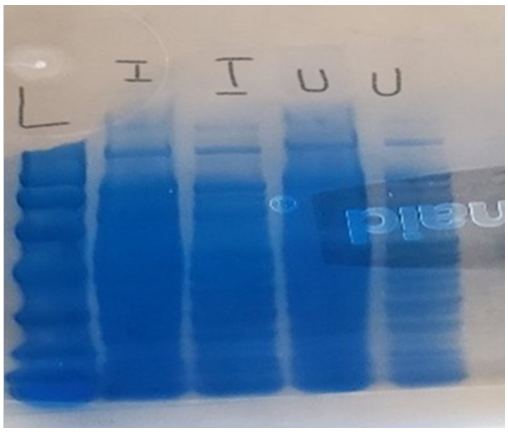

***Figure 4**: SDS-PAGE gel electrophoresis results with the induced (I) samples in lanes 2-3 and uninduced (U) samples in lanes 4-5. An SDS-PAGE standard ladder (L) was in lane 1.*

Several 10% (w/v) gelatin in TFE solutions were made using 5 g, 2.5 g, or 1.25 g mTG. During the electrospinning process, solutions containing the 5 g and 2.5 g mTG cross-linked inside the syringe and failed to fully electrospin the entire solution. Only the solution containing the 1.25 g mTG was able to electrospun the full 5 mL without cross-linking inside the syringe. All scaffolds had small amounts of beading and some fiber alignment in areas unaffected by the humidity. Figure 5 displays a region of the gelatin scaffold cross-linked using 1.25 mTG. Since only the 1.25 g mTG solutions electrospun the full 5 mL solution, these scaffolds were further tested using light microscopy. Some fiber alignment was observed, but not all fibers were aligned in the same direction. The average fiber diameter was 1.10 μm ± 0.28 μm and fiber diameters ranged from a minimum of 0.89 μm to a maximum of 2.08 μm.

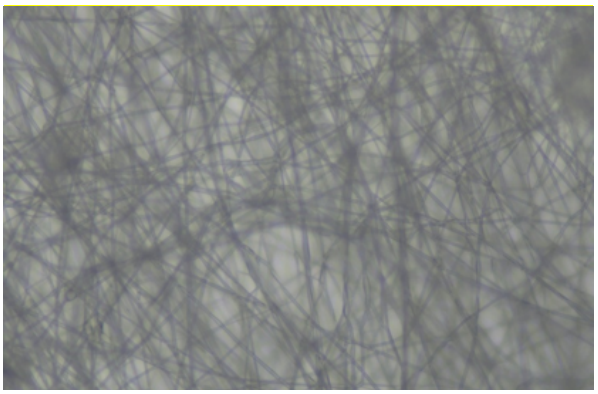

***Figure 5**: An electrospun 10% w/v gelatin scaffold cross-linked using 1.25 mTG at 400x magnification.*

DISCUSSION

The first goal of the project was to transform *E. coli* with IGF-1 so that the bacteria would replicate and express the IGF-1 protein. This process started with the transformation of *E. coli* with either the IGF-1 plasmid or the pQE 60 plasmid vector and plating on LB-amp plates. These plates displayed cell growth, which proved successful transformation with either the IGF-1 plasmid or the pQE 60 plasmid vector as the cells containing these plasmids gained ampicillin resistance. The next step was to amplify the IGF-1 plasmid DNA using PCR and test using gel electrophoresis to further confirm that the product was IGF-1. Figure 1 verifies the presence of IGF-1 as it shows bands near 480 bp with respect to the 1kb ladder. Next, digestion was done with the expectation of making specific cuts on both the IGF-1 plasmid and pQE 60 plasmid vector with the DNA remaining intact and prepared for ligation. The IGF-1 digest was proven successful using another gel electrophoresis set-up that demonstrated the presence of IGF-1 at ~480 bp (Figure 2). This was important to the experiment as it established that the samples post digest were still IGF-1.

After the IGF-1 and pQE 60 digests were purified, ligations were made with the goal of inserting the IGF-1 gene into the pQE 60 plasmid vector. The pQE60 containing IGF-1 plasmid was then inserted into *E. coli* and growth on LB-amp plates confirmed a successful transformation. The presence of the IGF-1 gene was further corroborated using gel electrophoresis as seen in Figure 3. The bands at ~500 bp verified the presence of the IGF-1 gene while the bands below 500 bp suggest primer dimer by-product formation. Since the creation of the pQE60 containing IGF-1 plasmid was proven, expanded cultures were made and induced with the purpose of expressing the IGF-1 protein. Once the absorbance values indicated a large enough cell number, protein expression was induced and a crude cell lysate obtained. Figure 4 shows the SDS-PAGE gel electrophoresis results of all proteins expressed in the induced and uninduced cell cultures. This experiment was completed in order to prove expression of the IGF-1 protein before moving on to purification. Since this was done on an impure sample, the supernatant was not purely IGF-1 protein and has a slightly darker region from 10 kDA to above 25 kDa (Figure 4). It was most likely that the bands in the uninduced lane were from other proteins of similar size being expressed by the cell. However, this step was important to the project as it provided evidence that the IGF-1 protein was most likely being expressed and previous experiments were successful.

Initially, early cross-linking in the 10% gelatin solutions occurred when electrospinning using 5 g and 2.5 g mTG. This meant that the full 5 mL of solution

was not electrospun and the resulting scaffolds were thin and fragile. This forced a smaller amount of mTG to be used and it was determined that 1.25 g mTG was an appropriate amount to hold off crosslinking for 1 hour in order to electrospun the full 5 mL solution. The importance of cross-linking with an enzyme was to ensure the fibers did not dissolve and maintained their structure after being subjected to an aqueous environment. This would be important when adding the IGF-1 protein to the scaffold and during cell culture experiments. After solving the crosslinking issue, the motor was unable to reach higher speeds without off balancing the entire set up. This resulted in the motor speed being reduced to ~2500 rpm and led to less fiber alignment. Despite the lower speed, the diameter of the fibers were very similar. In addition, the amount of beading reduced with each new scaffold made.

CONCLUSION AND FUTURE WORK

The goal of this project was to express and purify IGF-1 from *E. coli* and implement it into an electrospun gelatin scaffold to deliver the IGF-1 protein to myoblasts. Gel electrophoresis of transformed *E. coli* post-ligation demonstrated the presence of the pQE60 plasmid vector containing the IGF-1 gene. It was also shown that IGF-1 was the product within the induced supernatant by darker regions from 12-32 kDa on the SDS-PAGE gel. In addition, an electrospun 10% (w/v) gelatin scaffold cross-linked with 1.25 g mTG was successfully made and had an average fiber diameter of 1.10 μm ± 0.28 μm.

For future work with IGF-1, the protein must be purified and tested again using an SDS-PAGE gel to better establish that the product is the IGF-1 protein with more accuracy Also, the issue with fiber alignment in the electrospinning of the scaffold can be resolved by spinning the mandrel at a faster rate (9). However, this can only be done by fixing the balancing issue with the motor and mandrel. The set up may be insulated with rubber to help handle the energy produced at a higher speed. Once there is purified IGF-1 protein and an aligned scaffold, then IGF-1 can be added to the scaffold and tested to see if the protein is present. Then, it will be ready for maturation testing with myoblast cell cultures.

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