Genetically Modifying Escherichia coli to Produce Insulin-like Growth Factor 1

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Abstract

Insulin-Like Growth Factor-1 (IGF-1) is a chemical messenger involved as a signaling molecule in the AKT pathway. The growth factor binds to a receptor on the outside of the cell and initiates reactions to synthesize proteins for maturation. Ultimately, the signals initiated by the presence of IGF-1 cause the fusion of mononuclear muscle cells into a multinuclear complex. Through this mechanism, the growth factor plays a large role in growth and development during the fetal and postnatal stages. As tissue engineers seek to discover which ingredients can produce the most effective muscle cells in vitro, the role of IGF-1 in muscular development has made it a target of much research. If a concrete link can be established between the presence of IGF-1 and greater, faster, or more efficient growth of muscular tissue, it would be a step toward cost-effective, easier, and more accessible muscular tissue construction. This would mean that not only would future research on muscular tissue be easier to accomplish, but engineered muscle grafts would be available to more people as well. As of 08/08/2023, Murine IGF-1 is sold at a price of $522 for 100 µg. Therefore, before experiments can be done to understand the effects of IGF-1 in greater detail, a renewable source of IGF-1 is necessary. To this end, this experiment will attempt to transform Murine IGF-1 cDNA into *E. coli*, which would allow the cells to synthesize the growth factor.

Background Information

Tissue Engineering is a science aiming to aid the recovery of the body after damage or to maintain normal function. 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. 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. 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. The insulin-like growth factor 1 peptide (IGF-1) is one such component needed.

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 ad nauseum. Cloning Recombinant Deoxyribonucleic Acid (DNA) is a technique used frequently to change the genetics of a target organism. This is generally done in order 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.

In order 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

Stock Sample Production and Storage.

IGF-1, pQE-16, pQE-60, and pQE-70 plasmids were Z-streaked onto Luria Broth (LB) plates and incubated at 37ºC for 24 hours. A colony from each plasmid was added to a solution of 5mL LB and 5µL ampicillin and then spun at 215 rpm and 37ºC for 24 hours. The populated LB plates and stock solutions were then stored at 4ºC. The formation of new stock with which to work was made easy by the ampicillin resistance gene included in the purchased plasmids. This is because the *E. coli* colonies that absorbed the plasmids had the ability to resist ampicillin that was placed on the LB plate, while the colonies that had not absorbed plasmids did not have that ability and could not survive as a result. Because of this, all that needed to be done to develop stocks of *E. coli* containing the

plasmid—and later, stocks of *E. coli* containing the activated gene for IGF-1—was streaking the *E. coli* onto LB plates with ampicillin and placing colonies removed from the plates into a solution of Luria Broth with ampicillin for further growth.

Primer Design.

Primers were designed for the IGF-1 plasmid that would create restriction sites matching each vector plasmid. The restriction sites were as follows:

Target Vector Plasmid	Upper Site	Lower Site
pQE-16	BamH1	BamH1
pQE-60	Nco1	BamH1
pQE-70	Sph1	BamH1

DNA Isolation.

The DNA was isolated by subjecting the newly created stock solutions to the Qiagen Miniprep process according to the manufacturer's instructions. The isolated DNA samples were quantified on an EPOCH spectrophotometer.

Polymerase Chain Reaction.

Several solutions were made with proportions of 10 µL of Master Mix, 3 µL of each of the proper upper and lower primers for the target vector plasmid (diluted to a concentration of 1:100), 3 µL of sterile water, and 1 µL of extracted IGF-1 DNA and divided into sets based on their target vector plasmid. These sets of solutions then underwent a Polymerase Chain Reaction (PCR) routine in which they were stored at a temperature of 95ºC for 1 minute, stored at a temperature of 55ºC for 1 minute, and then stored at a temperature of 72ºC for 1 minute for a total of 35 repetitions. The PCR sample sets were quantified on an EPOCH spectrophotometer.

DNA Digestion.

The success of the PCR routine was verified by performing gel electrophoresis on each set of PCR samples using 1% agarose gel and searching for DNA at the location that would indicate a length of approximately 500 bp. The gel and DNA at the target base pair length were extracted using the Qiagen QIAquick Gel Extraction Kit according to the manufacturer's instructions. The sets of extracted DNA were quantified on an EPOCH spectrophotometer. Each set of extracted DNA or target vector plasmids was digested overnight in the New England Biolabs (NEB) rCutSmart buffer at 37ºC in solutions of the following concentrations:

	IGF-1 Insert	Target Vector Plasmid
pQE-16	1 µg DNA (12.5 µL) BamH1 (1 µL) 10x NEB rCutSmart Buffer (1.5 µL)	1 µg DNA (2 µL) BamH1 (1 µL) 10x NEB rCutSmart Buffer (1.5 µL) Sterile Water (10.5 µL)
pQE-60	1 µg DNA (11.5 µL) BamH1 (1 µL) Nco1 (1 µL) 10x NEB rCutSmart Buffer (1.5 µL)	1 µg DNA (3 µL) BamH1 (1 µL) Nco1 (1 µL) 10x NEB rCutSmart Buffer (1.5 µL) Sterile Water (8.5 µL)
pQE-70	1 µg DNA (11.5 µL) BamH1 (1 µL) Nco1 (1 µL) 10x NEB rCutSmart Buffer (1.5 µL)	1 µg DNA (4 µL) BamH1 (1 µL) Nco1 (1 µL) 10x NEB rCutSmart Buffer (1.5 µL) Sterile Water (7.5 µL)

Calf Intestine Alkaline Phosphatase Treatment.

The digestion products were purified by performing gel electrophoresis on each set of samples using 1% agarose gel and extracting the gel and DNA at the target base pair length using the Qiagen QIAquick Gel Extraction Kit according to the manufacturer's instructions. The purified digestion products were quantified on an EPOCH spectrophotometer. The digestion products were combined within their sets to form one sample for each of the three inserts and one sample for each of the vectors. The 5' strand of each sample was dephosphorylated using Calf Intestinal Alkaline Phosphatase (CIP) according to the NEB protocol.

Ligation.

The dephosphorylated samples were quantified on an EPOCH spectrophotometer. Each pair of samples was ligated overnight at 16ºC in solutions of the following concentrations:

pQE-16	50 ng Vector DNA (1 µL) 37.5 ng Insert DNA (2 µL) 10x NEB T4 DNA Ligase Buffer (2 µL) Sterile Water (14 µL) T4 DNA Ligase - Added Last (1 µL)
pQE-60	50 ng Vector DNA (5 µL) 37.5 ng Insert DNA (1 µL) 10x NEB T4 DNA Ligase Buffer (2 µL) Sterile Water (11 µL) T4 DNA Ligase - Added Last (1 µL)
pQE-70	50 ng Vector DNA (1 µL) 37.5 ng Insert DNA (1 µL) 10x NEB T4 DNA Ligase Buffer (2 µL) Sterile Water (15 µL) T4 DNA Ligase - Added Last (1 µL)

A control was made for each sample by substituting the insert DNA for the same amount of sterile water.

After 12 hours, the reactions were inactivated by storing the samples at 65ºC for 10 minutes and then storing the samples on ice.

Transformation.

The Thermo Fisher Top10 Chemically Competent *E. coli* Cell Transformation Procedure was followed to produce two plates of each sample and one plate of each control and each plate was incubated at 37ºC for 24 hours. Colonies found on experimental plates with empty control plates were made into liquid and plated cultures. Cultures were stored at -20ºC for future experimentation.

Discussion

Results.

Figure 1: EPOCH spectrophotometer results (Steps 3, 4, 5, 6, 7)

	Average Purity (260/280)	Average Concentration (ng/µL)
3: pQE-16	1.829	477
3: pQE-60	1.815	570
3: pQE-70	1.805	460
3: IGF-1	1.750	40
4: pQE-16	1.750	85
4: pQE-60	1.740	92
4: pQE-70	1.840	88
4: IGF-1	1.810	87
5: IGF-1 (pQE-16)	1.800	32
5: IGF-1 (pQE-60)	1.750	39
5: IGF-1 (pQE-70)	1.690	52
6: pQE-16		19
6: pQE-60		17
6:pQE-70		7
7: pQE-16		22
7: IGF-1 (pQE-16)		6
7: pQE-60		4
7: IGF-1 (pQE-60)		20
7: pQE-70		21
7: IGF-1 (pQE-70)		16

At any step of the process, the necessary requirements to move to the next step were eventually achieved. At times, this required repeating the previous steps many times to obtain concentrations or purities high enough to move forward, but the necessary result was always achieved by following the previous steps. However, in the final step there was growth on both the experimental and control plates of the sample produced using pQE-70. When this step was repeated with closer attention paid to aseptic technique, there was once again growth on the plates produced with pQE-16 and pQE-60, but there was no growth on the plates produced using pQE-70. This was not deemed a large concern, though, as the success of the experiments using pQE-16 and pQE-60 meant that success with pQE-70 was not necessary for us to achieve our goal of developing a renewable source of IGF-1.

Previous Work.

Previous iterations of this experiment have only used pQE-60 and have not yielded the anticipated results. The suspicion was that the primers inherited from previous researchers were annealing to each

other instead of the IGF-1 DNA, so a PCR reaction was done with a control group containing only the inherited primers. Since the results did not show any evidence of self-annealing, no single factor was able to be isolated as the reason for the lack of results. As such, it was decided that the course of action with the greatest chance of success was to begin the experiment from scratch and use three vector plasmids instead of just one.

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REFERENCES

Biolabs, N. E. (n.d.-a). *Ligation protocol with T4 DNA ligase (M0202)*. NEB. https://www.neb.com/protocols/0001/01/01/dna-ligation-with-t4-dna-ligase-m0202

Biolabs, N. E. (n.d.-b). *Protocol for dephosphorylation of 5' ends of DNA using quick CIP (NEB #M0525)*. NEB. https://www.neb.com/protocols/2019/06/04/protocol-for-dephosphorylation-of-5-ends-of-dna-using-quick-cip-neb-m0525

Corning Cell Culture Media, Sera, reagents, and single-use technology ... (n.d.). https://www.corning.com/media/worldwide/cls/documents/sellsheets/CLS-CG-BR-001.pdf

Free textbooks online with no catch. OpenStax. (2022, April 20). https://openstax.org/details/books/anatomy-and-physiology-2e

Hsu JR;, G. B. (n.d.). *Volumetric muscle loss*. The Journal of the American Academy of Orthopaedic Surgeons. https://pubmed.ncbi.nlm.nih.gov/21304045/

Mouse IGF-I recombinant protein. Mouse IGF-I Recombinant Protein (PMG0075). (n.d.). https://www.thermofisher.com/proteins/product/Mouse-IGF-I-Recombinant-Protein/PMG0075

Purslow;, L. (n.d.). *Differentiation of myoblasts in serum-free media: Effects of modified media are cell line-specific*. Cells, tissues, organs. https://pubmed.ncbi.nlm.nih.gov/10971037/

Qiagen. (n.d.). QIAprep Spin Miniprep Kit. https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=&cad=rja&uact=8&ved=2ahUKEwid_MGx5OyAAxU9LVkFHZ7KA7UQFnoECBAQAQ&url=https%3A%2F%2Fwww.qiagen.com%2Fus%2Fresources%2Fdownload.aspx%3Fid%3D56b0162c-23b0-473c-9229-12e8b5c8d590%26lang%3Den&usg=AOvVaw1rm7_dvmMHaRxrD1W3V2Oc&opi=89978449

Qiagen. (n.d.). QIAquick PCR Purification Kit Protocol. https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=&ved=2ahUKEwjwlty35OyAAxXVFlkFHSpHBbQQFnoECCIQAQ&url=https%3A%2F%2Fwww.qiagen.com%2Fus%2Fresources%2Fdownload.aspx%3Fid%3D95f10677-aa29-453d-a222-0e19f01ebe17%26lang%3Den&usg=AOvVaw29icUf9G50Hf6Z2JQwqx8B&opi=89978449

Recombinant mouse IGF1 protein, yeast, 50437-mnay - sino biological. Sino Biological. (n.d.). https://www.sinobiological.com/recombinant-proteins/mouse-igf1-50437-mnay

Schiaffino, S., & Mammucari, C. (2011, January 24). *Regulation of skeletal muscle growth by the IGF1-AKT/PKB pathway: Insights from genetic models - skeletal muscle*. BioMed Central. https://skeletalmusclejournal.biomedcentral.com/articles/10.1186/2044-5040-1-4

Taylor & Francis Online. (n.d.). The structure-function relationships of insulin-like growth factor 1 Ec in C2C12 cells. https://www.tandfonline.com/doi/full/10.1080/19336918.2017.1318240

Trauma facts and links. The American Association for the Surgery of Trauma. (2020, July 22). https://www.aast.org/resources/trauma-facts

U.S. Department of Health and Human Services. (n.d.). *Biomaterials*. National Institute of Biomedical Imaging and Bioengineering. https://www.nibib.nih.gov/science-education/science-topics/biomaterials

Witt R;Weigand A;Boos AM;Cai A;Dippold D;Boccaccini AR;Schubert DW;Hardt M;Lange

C;Arkudas A;Horch RE;Beier JP; (n.d.). *Mesenchymal stem cells and myoblast differentiation under HGF and IGF-1 stimulation for 3D skeletal muscle tissue engineering*. BMC cell biology. https://pubmed.ncbi.nlm.nih.gov/28245809/

University of California San Diego. (n.d.). Phage 101. UC Health - UC San Diego. https://health.ucsd.edu/news/topics/phage-therapy/pages/phage-101.aspx.