Novel Recombinant Protein Constructs for Improved Insulin-like Growth Factor-1 Delivery

by

Adam Was

Submitted to the Department of Mechanical Engineering in Partial Fulfillment of the Requirements for the Degree of Bachelor of Science at the Massachusetts Institute of Technology

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Abstract

Four novel recombinant protein constructs were designed to improve the delivery of insulin-like growth factor-1 (IGF-1). The IGF-1 sequence was subcloned into a HisMax plasmid and expressed at low levels by transient transfection in 293 cells. Site-specific mutagenesis was used to insert all four construct sequences into the IGF-1 HisMax plasmid, and to insert a secretory sequence into all four construct plasmids. A stable cell line selection in 293 and CHO cells using Zeocin was attempted. The four construct sequences were then subcloned from the HisMax plasmids to TrcHis plasmids and expressed in bacteria for higher production efficiency. Ni-NTA purified protein was detected in three of the four constructs. These proteins may ultimately be useful for myocardial delivery of IGF-1 to facilitate cardiac repair.

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1 Introduction

Cardiovascular disease is a widespread problem. It transcends geographic, social, economic and gender boundaries to cause 16.7 million deaths per year worldwide. By 2010 cardiovascular disease will likely be the leading cause of death across the globe. However, more people survive than succumb to cardiovascular events. Every year 20 million people worldwide survive cardiovascular events. These survivors require rehabilitation and treatment to improve their conditions.

Treatment often comes in the form of therapeutic drugs to help the patient compensate for damaged myocardial tissue. Ultimately, we aim to repair injured myocardium. There are many compounds with reparative potential that currently cannot be delivered to achieve the required efficacy. Clinical implementation can be hampered by the inability to control the delivery of a drug at a specific site without systemic toxicity. The difficulties faced in delivering some drugs include rapid and widespread diffusion in the bloodstream, a short compound half-life or the need for local injection of the compound.

This study focuses on the delivery of one such potentially therapeutic cardiovascular reparative compound, insulin-like growth factor-1 (IGF-1). IGF-1 is a potential myocardial repair factor that would best be delivered to the specific target site to avoid systemic effects. Novel recombinant protein constructs were designed to test different methods of anchoring IGF-1 to a bioscaffold for myocardial delivery.

Additionally, this study investigates the potential of “smart” delivery of therapeutic compounds. Smart delivery implies responsive rather than passive medicine: introducing the drugs that are released only when they are needed. Medicines can be released in anticipation of or in response to a damaging event such as cardiovascular injury. This method of delivering medicine has the potential to compose the next wave of individualistic and tailored healing. In this study, recombinant protein constructs will also test the potential to deliver IGF-1 in such a fashion.
2 Background

2.1 Insulin-like Growth Factor-1

Insulin-like growth factor-1 (IGF-1) is a single chain polypeptide composed of 70 amino acids. The protein's main secondary structure is composed of three alpha helices. IGF-1 also contains three disulphide bonds (6-48, 18-61, 47-52), the importance of which will be explained later. IGF-1 is highly regulated by insulin-like growth factor binding proteins (IGFBPs). Six IGFBPs are primarily responsible for the inhibition of IGF-1 through interference with cell surface receptor binding.

The interaction between IGF-1 and the IGFBPs is important for IGF-1 receptor affinity and biological potency. Structural changes of human IGF-1 cause drastic changes to the proteins' affinity for IGFBPs and type 2 insulin-like growth factor receptor. Conversely, recombinant fusion protein analogues of IGF-1 can have an increase in biological potency induced by a change in IGFBP interaction.

IGF-1 has significant effects on the growth, differentiation and repair of the cardiovascular system. IGF-1 expression in the heart has been shown to increase the formation of myocytes and induce the division of cardiac stem cells. Furthermore, cardiac IGF-1 expression is associated with attenuated myocyte death and the delay of aging myopathy. Negative effects of both overproduction and underproduction of IGF-1 highlight its role in the regulation of cardiac development and function.

Numerous changes in the cardiovascular system are associated with aging, such as decreases in myocyte count and decreases in exercise capacity. These changes may be associated with a gradual decline in plasma IGF-1 as the body ages. IGF-1 is strongly associated with the reparation of cardiac and skeletal muscle. It enhances the skeletal muscle regeneration process of older animals in response to muscle injury. Treatment with IGF-1 also yields an increase of skeletal muscle, increased cardiovascular function as measured by various parameters, and even the alleviation of muscle wasting in several muscle diseases.

In addition to these benefits, a number of other positive effects of IGF-1 have been identified. These include increased bone marrow contribution to adult skeletal
muscle, increased immune function, improved learning and memory, and vascular protection.\textsuperscript{10,11} Thus, although this project was designed to facilitate myocardial repair, targeted delivery of IGF-1 could have other therapeutic uses beyond the heart.

2.2 Controlled Delivery of IGF-1

One method to improve the effects of IGF-1 is to modify portions of the IGF-1 protein in a way that increases its ability to activate growth factor receptors. Another approach to changing its biological potency is changing the interaction between IGF-1 and IGFBPs.\textsuperscript{12} Here I use a third approach, to alter the microenvironment in which cardiac cells are delivered, by using bioscaffolds made of self-assembling peptide fibers. Self-assembling peptides are oligopeptides composed of alternating hydrophobic and hydrophilic amino acids.\textsuperscript{13} These peptides are highly customizable and well suited for alteration of the cardiac microenvironment\textsuperscript{14}. When exposed to physiological osmolarity and pH they assemble like Lego blocks into 3D scaffolds in which cells are capable of growth and differentiation. Even unmodified self-assembling peptides have proven beneficial to the growth and survival of cells. The injection of such peptide fibers into the cardiac microenvironment has been shown to recruit endothelial cells and promote survival.\textsuperscript{15}

As long as the hydrophobic-hydrophilic alternation of amino acids within the peptide fibers is maintained, the oligopeptides allow the possibility of incorporating non-peptide elements or tethered larger proteins within the constructs.\textsuperscript{16} This capability greatly expands the potential to modify the cardiac microenvironment. One factor that can now be controlled is adhesion to the self-assembled bioscaffold. By engineering biological anchors into the peptide fibers, other proteins or compounds can grab hold of these attachment sites and become fixed to the bioscaffold. This can be accomplished by using a specific acceptor peptide sequence and biotin-streptavidin linkages, forming a biotin sandwich that links the peptide sequence to the scaffold.\textsuperscript{17}

This leads to the possibility of improving protein delivery by attaching the therapeutic compound to the bioscaffold. For a factor such as IGF-1, which acts at great distances but diffuses rapidly upon injection, this can be an especially attractive option. Attaching biotinylated IGF-1 to the self-assembling peptide RAD16-II through a biotin
sandwich has been successful in prolonging the delivery of IGF-1 to the myocardium. Furthermore, introducing IGF-1 with self-assembling peptides improved cardiac performance in vivo.\textsuperscript{18}

Self-assembling peptide gels provide the opportunity to control not just the location of a protein's delivery but its timing as well.\textsuperscript{19} A compound tethered to a bioscaffold can be released in a controllable fashion by mechanical or proteolytic stimuli. This enables the possibility of “smart” materials which react to changes in their environment.

Proteolytic cleavage has been established as a method for responsive delivery of compounds.\textsuperscript{20} A peptide conjugate was created with three sections: an anchor for attachment to a bioscaffold, a therapeutic protein, and an enzymatically cleavable linkage separating the two. When the appropriate enzyme is introduced it cleaves the linkage and released the therapeutic protein from the bioscaffold.\textsuperscript{21}

One of the enzymes used previously was matrix metalloproteinase II (MMP-2), a member of the extracellular matrix digesting family of enzymes.\textsuperscript{22} MMP-2 not only functions as a release mechanism for anchored therapeutic proteins, but it also has a role in coronary artery diseases and injured myocardium.\textsuperscript{23} For instance, injured rat myocardium was found to release MMP-2. Elevated levels of MMP-2 in human patients upon admission for myocardial infarction and unstable angina have also been reported.\textsuperscript{24} This increase of MMP-2 concentration in the face of damaged myocardium, combined with the ability of MMPs to recognize specific peptide sequences and act locally, makes MMPs good candidates for the smart trigger of enzymatic protein release.

2.3 Protein Construct Production

Protein folding occurs in a spontaneous but highly coordinated process, the end product of which is the native active protein. IGF-1 may not fold properly under many conditions, perhaps because the 47-52 disulfide bond is not thermodynamically stable in native IGF-1.\textsuperscript{25} Recombinant IGF-1 must therefore be produced in eukaryotic cells or in prokaryotic cells accompanied by post-production refolding to achieve the native conformation.\textsuperscript{26, 27}
Large scale production of IGF-1 in eukaryotic cells has been largely unsuccessful. Expression of IGF-1 in CHO cells (Chinese Hamster Ovary) has allowed increase to 300% of baseline production by one group. IGF-1 produced in yeast was confirmed to have the same disulfide arrangement as plasma IGF-1, but production levels were similarly low.

Conversely, production of IGF-1 recombinant protein constructs or Lac Z fusion proteins at milligram per liter levels has been achieved in *E. coli*. While expression quantities were high, the recombinant protein did require post-production reduction and oxidative refolding. The constructs were then further purified by reverse-phase high pressure liquid chromatography to separate the refolded isomers. The resulting protein was comparably bioactive, indicating a successful protein construct production and purification procedure.
3 Objective

The objective of this work is to produce four novel protein constructs. In order to achieve this aim, plasmids with the construct DNA sequences must be created and transformed into cells, which will then produce the protein constructs in relatively large quantities. The protein constructs must then be purified and altered to a biologically active form for future *in vitro* and *in vivo* experiments.
4 Procedure

4.1 Description of Protein Constructs

The generic protein construct which was created in this study is shown in Figure 1. As the figure shows, each construct has several components in common. Due to the plasmid vector into which the coding sequences of the constructs have been inserted, each construct contains a number of native components. All constructs have a His tag that enables purification via chelation with Nickel-NTA (Ni-NTA) beads. Each construct also has an anti-Xpress epitope section to which anti-Xpress antibody can bind for detection during a Western blot. The constructs also contain an enterokinase cleavage site at which the protein can be truncated to separate the aforementioned components from the rest of the molecule. Finally, each construct was designed to contain rat IGF-1.

The proteins differ in the section between IGF-1 and the native vector components, as shown in Figure 2. Each construct was designed to contain an “anchor” component that allows it to attach to a material. Each construct was also designed to contain a “linker” component that serves to distance the therapeutic IGF-1 molecule from the anchor. Two different anchoring methods and two different linkage methods were constructed, necessitating four different protein constructs.

The first anchoring method uses an acceptor peptide (AP) sequence. The AP sequence is the known target sequence for the bacterial enzyme biotin ligase. Because biotin binds very tightly to streptavadin, and materials such as self-assembling peptides can be biotinylated, the two biotin-containing materials can become linked through a tetravalent streptavadin. This process forms a “biotin sandwich”, shown in Figure 3, in which the construct is anchored to a material through a streptavadin molecule and a biotin molecule. Two of the four constructs have an AP sequence.

The second anchoring method uses a RAD16-II (RAD) sequence. RAD16-II is a type of self-assembling peptide. The RAD sequence was added to the other two constructs to determine whether a protein could anchor to a RAD16-II matrix by directly incorporating into it.
The first linker component is a matrix metalloproteinase II (MMP) cleavable sequence. The protein sequence is a substrate MMP-2 and separates the anchor component from the IGF-1 molecule. Two of the four constructs have a MMP-2 cleavable linker.

The second linker component is composed of the same amino acids as the MMP-2 cleavable sequence, but in a scrambled (SCR) order. The scrambled protein also separates the anchor component from the IGF-1 molecule and serves as a control to the MMP-2 cleavable sequence. The other two of four constructs have a scrambled linker sequence.

Therefore, four unique protein constructs were designed. One has the acceptor peptide anchor sequence and scrambled linker (AP-SCR). The second has the acceptor peptide anchor sequence and MMP-2 cleavable linker (AP-MMP). Another has the RAD16-II anchor sequence and scrambled linker (RAD-SCR). The final construct has the RAD16-II anchor sequence and MMP-2 cleavable linker (RAD-MMP).

4.2 Subcloning IGF-1 and Construct DNA Sequences into the HisMax Plasmid

A pcDNA4/HisMax® TOPO® TA plasmid with the 210 base pair DNA sequence for rat IGF-1 (rIGF-1) was obtained from another lab member, Dr. Michael Davis. This vector was formed by using the TOPO® expression kit with a rat IGF-1 DNA product. The rat IGF-1 DNA sequence was amplified by a typical PCR reaction with Taq polymerase, ensuring that each PCR product had a 3' deoxyadenosine (A) overhang. The rat IGF-1 PCR product was mixed with the pcDNA4/HisMax® TOPO® TA plasmid, incubated for 5 minutes, and transformed into TOP10 competent E. coli cells. The cells were allowed 1 hour of growth to allow expression of ampicillin resistance before selection by ampicillin. The insertion of rat IGF-1 DNA sequence into the vector was checked by plasmid isolation, gel electrophoresis and DNA sequencing.

The plasmid with rIGF-1 was used as a template for the insertion of protein construct DNA sequences by site-directed mutagenesis of large DNA sequences. Eight large oligonucleotide sequences were ordered for use with the QuikChange® Site-
Directed Mutagenesis Kit. The DNA sequence, translated peptide sequence and oligonucleotide pairs sequences for each construct are shown in section 8.1.

Each oligonucleotide has three sections: the coding sequence to be inserted by site-directed mutagenesis, an upstream overlapping sequence and a downstream overlapping sequence. Both overlapping sequences are identical to the 20 base pairs upstream and downstream of the coding sequence insertion point, respectively. For the insertion of the construct DNA sequences, the upstream overlapping sequence is the first 20 base pairs of IGF-1 and the downstream overlapping sequence is the 20 base pairs of plasmid before the beginning of IGF-1. The forward and reverse oligonucleotide sequence for each construct is mixed with the IGF-1 vector in preparation for a shortened PCR cycle. During the PCR cycle the vector is denatured, ligated and extended. Site-specific insertion of the coding sequence occurs on the rare instances when Taq polymerase incorporates one or both of the oligonucleotides into the vector.

Vectors containing the inserted sequence are selected by adding the restriction enzyme dpnI to the PCR reaction mixture. The dpnI enzymes digest all vectors without the inserted coding sequence. After an hour incubation period, plasmids from the digested reaction mixture are transformed into competent *E. coli* cells and grown for an hour. The transformed cells are plated and selected for ampicillin resistance. Colonies are grown overnight in preparation for a mini-prep, to isolate the vector DNA. Plasmid DNA was analyzed by gel electrophoresis with insertion sequence-specific primers in order to detect successful sequence insertion. Sequence insertion was confirmed by DNA sequencing.

### 4.3 IGF-1 Expression in 293 and CHO Cells

IGF-1 expression was tested in 293 and CHO cells by transient transfection. Because large quantities of IGF-1 plasmid were needed for transient transfection, 50 mL of bacteria containing the IGF-1 vector was grown. The IGF-1 plasmid was isolated from this growth with a maxi-prep kit.

293 cells were plated at a density of 400,000 cells per well in 6 well plates. The IGF-1 plasmid was added to the 293 cells at quantities of 0.5, 1.0 and 2.0 ug per well.
The reagent lipofectamine was used to emulsify and apply the IGF-1 plasmid. Cells were incubated for 24 hours before harvesting.

The harvested cell mixture was run and separated on two SDS-PAGE gels. One gel was stained with Coomassie blue solution. Protein in the other gel was transferred to a membrane for Western blot analysis. Anti-Xpress antibody was used to probe the gel for the IGF-1 protein construct.

Protein production in 293 and CHO 7 cells was also attempted through stable cell line selection. First a Zeocin death assay was performed by adding Zeocin to CHO cells at concentrations of 0, 50, 100, 200, 400, 600, 800 and 1000 ug per well. The Zeocin-treated cell media was replaced every 3 days, and cells were examined daily for death progress. Specifically, the lowest concentration at which all cells died was monitored for later use in stable cell line selection.

The production of a stable cell line was attempted by introduction IGF-1 plasmid to CHO cells at 1.0 ug per well in 6 well plates with 400,000 cells per well. The cells were incubated for a day before being exposed to Zeocin, a typically toxic agent, to which cells containing the HisMax plasmid are resistant. Zeocin was added to the cells at concentrations of 400 and 600 ug per well. Every three days the Zeocin was replenished and the cells were washed. Cells were examined daily by microscope for death progress and to detect Zeocin-resistant cells. Cells were treated for a period of four weeks.

4.4 Subcloning a Secretion Sequence Into Each Construct Plasmid

A secretion sequence was subcloned into each construct for easier protein purification in 293 and CHO cells. The secretion sequence allows protein to be harvested from the cell media since it targets the protein for secretion through the endoplasmic reticulum. The method used to subclone this secretion sequence into each construct vector was similar to the method used to subclone the construct sequences into the IGF-1 vector. The difference between the methods was the choice of oligonucleotides and original vector.

The original vector in the subcloning procedure for each construct was the vector created by subcloning the construct sequence into the IGF-1. Two sets of oligonucleotides and their reverse complements were used on the four vectors. One set
of oligonucleotides contained the secretion sequence flanked by the 20 base pairs prior to the construct coding sequence and the first 20 base pairs of the acceptor peptide (AP) sequence. The other set of oligonucleotides contained the secretion sequence, 20 base pairs upstream of the construct sequence and the first 20 base pairs of the RAD16-II (RAD).

Successful secretion sequence insertion was verified by PCR with secretion sequence-specific primers and gel electrophoresis. The correct sequence was further verified by DNA sequencing.

### 4.5 Subcloning Construct Sequences into the TrcHis Vector

Protein production was switched from eukaryotic 293 and CHO 7 cells to *E. coli* bacteria when we realized that protein production rates in the eukaryotic cells was too low. The construct DNA sequences were therefore subcloned again, from the eukaryotic pcDNA4/HisMax© TOPO® TA vector to the bacterial TrcHis vector. The subcloning procedure differed from previous subcloning procedures.

Three oligonucleotides were ordered for the procedure. One oligonucleotide, used on the subcloning of all four vectors, was the reverse complement of the last 18 base pairs of IGF-1. The other two oligonucleotides were the first 18 base pairs of the acceptor peptide and RAD16-II sequences, respectively. The construct sequences were isolated from the pcDNA4/HisMax© vector and amplified by running a PCR cycle with the reverse complement of IGF-1 oligonucleotide and the appropriate acceptor peptide or RAD-16II oligonucleotide. The PCR reaction products were run on an agarose gel electrophoresis to separate the various products. The DNA band of appropriate size and intensity was excised and purified by a modified mini-prep. This process was performed for each construct.

The purified DNA bands contained the total construct coding sequences, including IGF-1. These pieces of DNA were incorporated into the TrcHis vector by a method similar to that described above for the IGF-1 sequence and HisMax vector. For each construct the DNA band was mixed with the TrcHis vector, incubated for 5 minutes and transformed into Top 10 *E. coli* cells. The cells were incubated for 1 hour minutes
and plated onto LB-agar plates containing ampicillin in order to select for resistant colonies. These colonies were picked and grown in LB-amp media to ensure ampicillin resistance.

Colonies with ampicillin resistance were harvested for the plasmid using a mini-prep kit. This DNA was run in a PCR reaction with a construct-specific primer and a plasmid-specific reverse primer to ensure that the insertion was successful. Appropriate insertion and orientation was confirmed by DNA sequencing. DNA sequence traces were used to confirm successful sequences when ambiguities were present.

### 4.6 Small Scale Protein Construct Production in E. coli

The TrcHis plasmids for each construct were transformed into BL-21 E. coli cells for enhanced protein production. 1 µl of each plasmid was mixed with 50 µl of BL-21 cells on ice and incubated for 30 minutes. The cells were “heat-shocked” by exposing them to a 42 °C heat block for 45 minutes and then incubated at 37 °C for 1 hour. LB-agar plates containing ampicillin were used to select for cells that received the plasmid.

For each construct, colonies with the TrcHis plasmid were grown overnight in 2 mL of LB-ampicillin medium. 1 mL of overnight growth was used to inoculate 50 mL of LB-ampicillin medium. Bacteria were grown until for approximately 4 hours, until reaching a density such that the absorption by a spectrophotometer at 600 nm (OD$_{600}$) was equal to 0.6. IPTG was added at a final concentration of 1 mM to induce the bacteria. The solution was incubated with shaking for 4 more hours.

Bacteria were collected by spinning for 10 minutes at 5000 rpm. The pelleted bacteria were lysed by adding 8 mL of 6M guanidium lysis buffer and rocking for 15 minutes. Each solution was sonicated at power level 7 for three 10 second intervals. The lysed bacteria were then spun down for 15 minutes at 3000g. The supernatant was decanted and saved.

300 µl of nickel-NTA beads were added to the supernatant solution of each construct to bind the protein constructs. Each solution was rocked for 30 minutes to ensure complete binding. The beads were settled by spinning the solution for 5 minutes at 1000 rpm. The supernatant was aspirated and 4 mL of reducing binding buffer was added to the beads. Beads were rocked for 5 minutes before collection by spinning for 5
minutes at 1000 rpm. This process was repeated once, followed by two washes with reducing wash buffer.

4 mL of wash buffer was added to the beads one more time, but without spinning. Instead, the solution was poured into a filter column. The beads were allowed to settle before the wash buffer was drained from the column. 1 mL of denaturing elution buffer at pH was added to the column and allowed to sit for 5 minutes. The elution buffer, containing the protein construct, was collected in a tube. This was repeated three more times for a total of four 1 mL elution fractions.

24 μl of elution fraction for each construct was added to 8 μl of loading dye and boiled for 10 minutes at 95 °C. The mixture was spun and two separate 15 μl fractions of each purified construct were added to two separate 10% SDS-PAGE gels. The gels were also loaded with a positive control and protein size ladder. MED-SDS buffer was added to the gel box, which was run at 200V for 40 minutes. One gel was stained with Coomassie blue staining solution and the other gel was transferred to a membrane for Western blot analysis. The membrane was probed with anti-Xpress primary antibody and anti-mouse goat secondary antibody before exposure.

4.7 Large Scale Protein Production in E. coli

Large scale protein production in E. coli was attempted. The volume of LB media used was scaled up to 2 liters from 50 mL. Various parameters were changed in attempts to purify protein from large scale growths. In one experiment, the OD$_{600}$ value at which the bacteria were induced with IPTG was changed from 0.6 to 1.0. In another experiment, the volume of guanidium lysis buffer used to lyse the bacterial pellets was changed from 8 mL to 50 mL.

The BL-21 E. coli bacteria were checked to ensure that they each contained the appropriate vector. The bacteria were grown in 2 mL of LB and plated overnight on LB-agar plates containing ampicillin. Individual colonies were picked and grown overnight. For each construct, the overnight growth was pelleted, lysed and mini-prepped to acquire plasmid DNA. A restriction digestion analysis was performed on each construct plasmid by digesting with the restriction enzyme HindIII for 1 hour at 37 °C and visualizing with gel electrophoresis.
The location of the protein during the growth and purification process was investigated by removing fractions of media during each step of the purification. In one experiment each construct, and duplicates of the AP-SCR and AP-MMP constructs, were grown in 2 liters of LB and fractions were taken from the lysate supernatant, the lysate pellet, the media after the addition of nickel-NTA beads, the media after washing with binding buffer, the media after washing with wash buffer, and the elution. Every fraction except the media after washing with binding buffer was run on an SDS-PAGE. A Western blot was performed by transferring the protein to a membrane and probing with anti-Xpress antibody.

A similar experiment was performed to determine the level of protein construct solublization. Strains of bacteria containing each construct plasmid and a strain with the IGF-1 plasmid were grown in 150 mL of LB. Three 50 mL aliquots of induced bacteria were pelleted and lysed with 8 mL, 32 mL and 80 mL of 6 M guanidium lysis buffer. Fractions of each strain were taken from the lysate supernatant and pellet. A Western blot was run on each strain’s fractions using anti-Xpress antibody.

Another experiment was conducted by collecting lysate supernatant and pellet fractions of bacteria at pre-induction and post-induction stages. Strains of bacteria containing the AP-SCR, RAD-MMP and IGF-1 construct plasmids were grown in 100 mL of LB each. 50 mL of growth was harvested before induction at a concentration of OD_{600}, and the remaining 50 mL was harvested 4 hours after induction. The supernatant and pellet fractions were run on a Western blot with anti-Xpress antibody.

A final experiment was performed on strains containing the AP-SCR plasmid and a modified version of the IGF-1 plasmid, HB-IGF, as a control. Fractions were taken from the lysate supernatant, the lysate pellet, the media after adding beads, the media after washing with binding buffer, the media after the final wash with wash buffer, and the elution. The experiment was performed with another lab member’s reagents and solutions. A Western blot was performed on each fraction using the anti-Xpress antibody.
5 Results

5.1 Subcloning IGF-1 and Construct DNA Sequences into the HisMax Plasmid

The site-specific mutagenesis reaction had a high rate of failure. The reaction was repeated multiple times for each construct, and between 5 and 15 plasmids were sequenced per successful insertion. Visualization of bands by gel electrophoresis did not guarantee a successful sequence insertion.

5.2 IGF-1 Expression in 293 and CHO Cells

Low or nonexistent levels of IGF-1 expression by transient transfection were detected in 293 and CHO 7 cells. Figures 11 and 12 show a Western blots with anti-Xpress and anti-IGF-1 antibodies, respectively, that did not detect any protein. Figure 13 shows a Western blot with anti-Xpress that did detect low levels of protein. Produced protein was undetectable by Coomassie stains, but this was not surprising since this technique requires large amounts of protein for detection.

5.3 Stable Cell Line by Zeocin Selection

The effects of Zeocin on the cells during the death assay were apparent after only two days. There were far fewer cells in the wells that received 1000 or 800 ug of Zeocin than there were in wells receiving 0, 50, 100 or 200 ug of drug.

After five days there was clear cell death in all Zeocin-treated wells. The differences between the 400 to 1000 ug/mL concentration ranges were indistinguishable, with massive cell death permeating the well. The normal, untreated cells were confluent and growing well whereas the Zeocin treated cells were largely dead, clumpy, rounder and less connected.

One week into the Zeocin death assay there practically no living cells at concentrations 400 to 1000 ug/mL. There were very few living cells at the 50 to 200 ug/mL concentrations.
The stable cell line selection was performed using concentrations of 400 and 600 ug/mL of Zeocin. After three days of treatment with Zeocin there was already visible cell death at both concentrations. One week later there were many dead cells in the 600 ug/mL plates. There were still some living cells but it was difficult to distinguish the dying cells from the resistant cells. More living cells were visible in the 400 ug/mL plates but considerable cell death was still present. After two weeks practically all cells at both concentrations were dead. Stable cell line selection using Zeocin was eventually abandoned due to a lack of flourishing, Zeocin-resistant cells.

5.4 Subcloning a Secretion Sequence Into Each Construct Plasmid

The success rate for insertion of the secretory sequence into construct plasmids by site-specific mutagenesis was higher than the insertion of the construct sequences into the IGF-1 plasmid. Again, visualization of bands by gel electrophoresis - even bands of the appropriate length - did not correlate well with successful insertion of the secretory sequence as determined by DNA sequencing. The mutagenesis reaction was run twice before inserting the secretory sequence into all four constructs. 12 plasmids were sequenced, resulting in four successful insertions. The secretory sequence inserted into the AP-MMP construct contained an extra base pair in the middle of the sequence.

5.5 Subcloning Construct Sequences into the TrcHis Vector

Like the other subcloning experiments, the transfer of construct sequences from the HisMax plasmid to the TrcHis vector had a high failure rate. Figure 19 shows the construct sequences after being cloned out of the HisMax vector and separated by gel electrophoresis. This process occurred without failure.

Failure became more prevalent when transferring the construct DNA to the TrcHis vector. Two of the four constructs, AP-SCR and RAD-MMP, were successfully cloned on the first attempt. The other two constructs, AP-MMP and RAD-SCR, required multiple attempts and over 15 sequencings each before achieving a successful insertion. As before, visualization of DNA of appropriate length by gel electrophoresis was not a good indicator of insertion success as determined by sequencing.
5.6 Small Scale Protein Construct Production in E. coli

Small scale protein construct expression, purification and detection in E. coli was relatively unreliable. After successfully producing and detecting three of the four constructs by Western blot analysis with anti-Xpress, as shown in Figure 33, attempts to repeat this experiment failed partially or outright.

The experiment was varied slightly by inducing at OD600 = 1.0 instead of 0.6, or by doubling the volume of guanidium lysis buffer, but the experiments continued to fail by Coomassie staining and Western blotting with anti-Xpress antibody.

5.7 Large Scale Protein Production in E. coli

Various methods were attempted to express protein constructs on a large scale. Figures 35, 36, and 37 show attempts to grow the four constructs and take fractions at each purification step. The figures show that essentially all protein remained in the pellet.

The volume of guanidium lysis buffer used to lyse the pelleted bacteria was varied significantly in another experiment. As shown in Figures 38, 39, and 40, varying the volume of guanidium lysis buffer had little effect on the production and/or solublization of protein. Some construct protein was detectable in the lysate supernatant but most remained in the pellet.

Figures 41 and 42 show protein expression before and after induction with IPTG. In Figure 42 the film that was exposed for four hours, shows more protein in post-induction samples than pre-induction samples. Figure 41, the film that was exposed for 1 minute, shows that protein production was not ample. Protein production did not vary much based on the construct.

Figures 43 and 44 show protein expression of AP-SCR and a different control, HB-IGF, at various points during the purification process. Both figures show significant protein production in the HB-IGF control but negligible, if any, construct production in the AP-SCR bacteria.
6 Discussion

6.1 Subcloning IGF-1 and Construct DNA Sequences into the HisMax Plasmid

Figures 4 and 5 show the gel electrophoresis results of PCR reactions designed to amplify inserted sequences of construct DNA in the HisMax vector. In both figures, the top lanes show the result of a PCR reaction with the appropriate forward and reverse site-specific mutagenesis primers. Also in both figures, the primers used for the bottom wells were the appropriate forward mutagenesis primer and a BGH reverse primer. Bands of DNA were only expected when there was a successful insertion of construct sequence. The expected band length for successful construct insertions was approximately 275 base pairs for the top wells and approximately 400 base pairs for the bottom wells. A 215 and 360 base pair band of DNA was expected for IGF-1 in the top and bottom wells, respectively.

The data in Figure 4 therefore suggest that every plasmid had a successful insertion. In actuality, DNA sequencing showed that the RAD-MMP isolate in lane 8 was the only construct to have a successful insertion. The other 7 plasmids had no insertion at all.

Similarly, the electrophoresis data shown in Figure 5 does not accurately reflect the presence or absence of an insertion as determined by sequencing. Sequencing indicated that lanes 3, 4, 6 and 7 had successful insertions of constructs AP-SCR, AP-MMP, AP-MMP and RAD-SCR, respectively. A faint band in lane 4 on the top and lane 7 on the bottom is detectable. Very faint bands are also barely visible in lane 3 on top and lanes 4, 6, 7, and 8 on bottom. Again, all of the lanes with successful insertions of construct DNA have bands, but some lanes with bands, albeit very faint, are negative for an insertion by sequencing.

This lack of correlation between visible bands by gel electrophoresis and the presence of a successful insertion as determined by sequencing was a recurring problem with the site-specific mutagenesis procedure and data. Similarly, ampicillin-resistant colonies were not a reliable indicator of bands or a successful insertion.
This unreliability was probably caused by two factors. First, large oligonucleotides were used for the PCR reactions. This can result in non-specific binding of primers to the template plasmid, and therefore the amplification of non-construct DNA sequences. Second, the digestion of methylated DNA without a successful insertion by the restriction enzyme dpnl is not a failsafe process. Undigested plasmids without an insertion would therefore be transformed into bacteria and confer ampicillin resistance without the presence of an insertion.

Site-specific mutagenesis unreliability was handled by simply sequencing many plasmids. For the initial insertion of construct DNA into the IGF-1 vector, approximately one in four sequenced colonies was found to contain the insertion. This ratio falls within the range of success rates listed by others for site-specific mutagenesis using the QuikChange kit.

As shown in Figures 6, 7, 8, and 9, successful insertions of construct DNA in the correct orientation and sequence were found for the AP-SCR, AP-MMP, RAD-SCR, and RAD-MMP constructs, respectively. A second successful insertion of AP-MMP was found, as shown in Figure 10.

6.2 IGF-1 Expression in 293 and CHO 7 Cells

Production of IGF-1 in eukaryotic cells was ultimately far below the levels at which we needed to grow our protein constructs. During the expression and detection process we discovered that another group claiming increased IGF-1 production in CHO cells had increased expression by a mere 300%.

Figure 13 shows a Western blot with anti-Xpress antibody of harvested 293 cells after transient transfection with IGF-1 plasmid. While protein was detectable, the levels were too low to be seen on a Coomassie stain. As shown in figures 11 and 12, production of IGF-1 in 293 cells was sometimes completely undetectable by either anti-Xpress or ant-IGF-1 antibodies.

The stable cell line selection by treatment with Zeocin ultimately failed. The presence of flourishing cells in the absence of Zeocin indicated that the cells were capable of growing in the conditions in which they were plated. The death of all cells in the death assay showed that the Zeocin was working correctly. Concentrations of 400
and 600 ug/mL were chosen for the stable cell line selection because these were the lowest concentrations at which all cells were clearly killed in one week.

Since all the cells in the stable cell line selection eventually died, it is clear that they were not translating the incorporated plasmid at the level required to survive Zeocin treatment. Given that the cells died in approximately two weeks, compared to the one week required to kill all the cells at 400 and 600 ug/mL Zeocin in the death assay, it is possible that some cells achieved a small resistance to Zeocin that allowed them to survive a bit longer. It is more likely that the cells ultimately either did not incorporate the plasmid or were not capable of translating it at the level required to survive Zeocin treatment. Regardless, the stable cell line selection failed and would not work as a method of producing our protein constructs in large quantities.

The transient transfection method of producing protein in 293 or CHO 7 cells never achieved the full potential for which we had hoped. Similarly low levels of protein expression were found by other members of the laboratory using transient transfection with different plasmids. As described above, the cells do not readily take up plasmid and, when they do, do not produce large amounts of protein. At the levels of production we were achieving by transient transfection, unfeasible volumes of media would be needed to isolate the hundreds of micrograms of protein required. Given that fact that we expected to lose more protein during further purification steps, production of the protein constructs by transient transfection or stable cell line selection and production in eukaryotic 293 and CHO 7 cells was not feasible. This is why we decided to switch to production in bacteria, followed by post-expression modification and purification.

6.3 Subcloning a Secretion Sequence Into Each Construct Plasmid

A secretion sequence was inserted into each construct vector by site-specific mutagenesis in order to simplify the ultimate purification of protein from 293 and CHO cells. The secretion sequence directs cells to secrete the protein into the cell media, from which the protein could be harvested.

Figure 14 shows the gel electrophoresis to detect successful insertion of the secretory sequence. A secretory sequence-specific forward primer and BGH reverse primer were used in the PCR reaction to amplify the insert, which should be
approximately 526 base pairs in length. Therefore, a band should be present only when a successful insertion has occurred.

Bands appear in lanes 3, 4, 5, 6, 8, 14, 15, 17, and 19, of which bands 4, 5, 8, 17, and 19 are of approximately the correct size. However, the streaking in the gel makes determination of all band sizes difficult. This streaking was caused by accidentally using protein sample buffer instead of DNA loading buffer. Regardless, of these samples, only the isolates in lanes 4 (AP-SCR), 14 (RAD-SCR), and 19 (RAD-MMP) were shown to have a successful insertion of the secretory sequence by DNA sequencing. Again, the presence of a band in gel electrophoresis or the ampicillin resistance of a colony was not a reliable indicator of a successful sequence insertion. Instead, multiple sequencings per constructs had to be used to detect on successful insertion.

The successful insertions are shown in Figures 15, 16, 17, and 18. It should be noted that the secretory sequence inserted into the AP-MMP construct contains an extra base pair and does not match exactly. This is an example of another potential failure point of the site-specific mutagenesis protocol, whereby an incorrect sequence can be inserted.

Ultimately, the constructs with secretory sequences were unused because of the failure to produce sufficient levels of protein in 293 and CHO cells by transient transfection or stable cell lines.

6.4 Subcloning Construct Sequences into the TrcHis Vector

Sufficient levels of protein production in 293 and CHO cells were never achieved. For this reason it was decided to switch protein production from 293 or CHO cells to E. coli. Although expression of the protein constructs in bacteria would require more post-production modification due to the difficulty of making protein with disulfide bonds in bacteria, this was outweighed by the ability to make plentiful protein in E. coli. However, in order to produce the constructs in bacteria the construct coding sequences had to be subcloned from the HisMax vector to the bacterial TrcHis vector.

Figure 19 shows the construct sequences after PCR amplification of the HisMax vectors with the appropriate construct forward primer and IGF-1 reverse primer. The segments are expected to be approximately 275 base pairs in length. Each band is of the
appropriate length and is relatively strong, indicating the likely presence of the appropriate construct DNA coding segment.

The result of cloning these sequences into the TrcHis vector is shown in Figure 20. The PCR reaction was run with Xpress-specific forward primer and IGF-specific reverse primer, resulting in DNA segments that should be 350 base pairs and only appear if the construct DNA sequence was inserted correctly and in the correct orientation. The figure shows bands in lanes 3, 4, 5, 7, 8, 10, 11, and 12. Of these, only the DNA in bands 4 and 11 was confirmed to have the AP-SCR and RAD-MMP insertions, respectively, by DNA sequencing.

Figure 21 shows a gel with the PCR reactions using the same primers on more isolates. Although there are bands in lanes 2, 3, 4, 5, 6, 8, 9, and 10, none of these were positive for a successful insertion by sequencing. Ultimately the use of gel electrophoresis as a detection tool for successful insertion by site-specific mutagenesis was deemed ineffective, and the insertions for the remaining two vectors, AP-MMP and RAD-SCR, were found using sequencing alone.

Figures 24, 25, 26, 27, and 28 show the relevant sequences of the plasmids compared to the sequences of AP-SCR, AP-MMP, RAD-SCR, a second RAD-SCR, and RAD-MMP, respectively. The only differences between the sequences are a number of ‘N’ s in each plasmid, suggesting that the plasmid sequence is correct and the insertion was successful. However, the ‘N’ s represent bases that were not immediately determinable by the sequencing program, which means there is the potential for error. Therefore, the traces were checked by hand in order to ensure that the ‘N’ s represent the appropriate bases. The original traces and their corrected base pairs are shown in Figures 29, 30, 31, and 32. When the plasmid sequences are updated using the traces, they match the construct sequences exactly.

As a quality control check, a restriction enzyme digestion analysis was run on the bacteria containing the plasmids above. The results of this digestion are shown in Figures 22 and 23, which contain bands just under 5000 base pairs long in each lane. These results indicate that there is an approximately 4700 base pair plasmid with a HindIII cleavage site in each construct strain. TrcHis has a HindIII cleavage site and is approximately 4700 base pairs long with the inserted construct sequences. These results,
combined with the sequencing and trace data above, indicate that the bacterial strains do indeed contain the TrcHis vector with the inserted construct sequences.

6.5 Small Scale Protein Construct Production in E. coli

Protein was expressed on a small scale by growing E. coli carrying the TrcHis vector with the inserted construct sequences in 50 mL of LB-ampicillin media. Figure 33 shows the results of a Western blot of Nickel-NTA purified protein with anti-Xpress antibody. Bands are present in lanes 1, 2, 5, 6, 7, and 8 between 14 and 18 kDa.

This figure shows a number of things regarding bacteria production of the protein constructs. First, the bacteria are indeed capable of producing a protein that is detectable by anti-Xpress antibody. Second, the Ni-NTA column purification is effective. The protein shown in Figure 33 is pure, evidenced by the fact that there are very few, if any, other bands in each lane. This suggests that the protein constructs did indeed bind to and then elute from the Ni-NTA beads. The protein constructs are expected to be approximately 14 kDa with the tag from the TrcHis vector, which corresponds to the size of the bands in the figure. Not shown is the accompanying Coomassie stain without any bands, indicating that while the bacteria are producing the protein constructs, 50 mL of media still does not produce significant amounts of protein.

Figure 33 also highlights a number of uncertainties. It is unclear why lanes 1 and 2, the AP-SCR construct, contain double bands, or why there are no bands in lanes 2 and 3, the AP-MMP construct. In order to ensure that these discrepancies were not an aberration, the experiment was repeated. Figure 34 shows the results of a Western blot with anti-Xpress antibody on the most recent, “new” elutions in lanes 1-4 and the “old” elutions in lanes 5-8. The new elutions raised more questions than they answered, as only one of the four constructs appeared in lane 4 and the other lanes were blank.

Given the confirmed presence of a TrcHis vector with the inserted construct sequences, it is unclear why a double band showed up in the AP-SCR construct, why the AP-MMP construct failed give a band, and why the repeated experiment largely failed. However, since the expression of protein was established on the smaller scale of 50 mL growth, troubleshooting and production of the protein was moved to larger scale grow-
ups. Possible reasons for the failure of the aforementioned experiments will be discussed in the next section.

6.6 Large Scale Protein Production in E. coli

The protein constructs were blank in half of the lanes analyzed during the small scale expression. In order to determine at which step of the Ni-NTA purification process the protein was being lost or modified, fractions were removed from each step for later analysis. This was done for bacterial growth of each construct, plus doubles of the troublesome AP-SCR and AP-MMP constructs. The fractions were analyzed by Western blot with anti-Xpress antibody.

The results are shown in figures 35, 36, and 37. The only lanes that contained protein in every Western blot and each strain were lanes 3 and 9, the lysate pellet fraction. The supernatant of the RAD-SCR strain appeared to have some protein construct, as did the RAD-MMP lysate supernatant, supernatant after adding beads, supernatant after washing with wash buffer, and elution.

The results from figures 35, 36, and 37 are unreliable for a number of reasons. First, the gels are smeared, making detection of protein difficult to discern. Furthermore, the positive controls are blank in each gel. However, the positive controls were likely just old and undetectable because they continued to be blank in later experiments in which other, fresher positive controls were detected. The blank positive controls are therefore probably not important. Although the gels are smeared it is still clear that there is protein in the pellet but not in other fractions. Therefore we determined that the constructs were likely in the pelleted fraction of the larger grow-ups.

In an attempt to solublize the protein in the pellet and get it into the supernatant, increased volumes of guanidium lysis buffer were used to lyse the cells. 1x, 4x and 10x the suggested volume of guanidium lysis buffer was used on bacteria containing each construct, and IGF-1 as a control. The cell lysate supernatant and pellet were isolated and analyzed by Western blot with anti-Xpress antibody.

Figure 38, 39, and 40 show the results of the Western blots. Again, the data are hard to interpret because the gels are messy. The results of the gel showing 1x guanidium lysis buffer in particular are barely interpretable due to the extensive
smearing. The underlying reason behind the smeared gel is unclear, but it is likely caused by poor running buffer, not enough running buffer or overheating the gels. All three factors were controlled for in future experiments by mixing new running buffer to an exact volume, adding excess running buffer and packing the gel box as much as possible with ice water. One of these modifications largely corrected the smearing problem.

The 4x gel in figure 39 shows that the protein construct may be present in lanes 2, 4, 6, 8, and 10, which contain the pellets, but is not present in the lysate supernatant. Darker portions of bands are visible in the 15 kDa region of lanes 2, 4, 6, and 10, whereas there are no bands in the 15 kDa region of any supernatant lanes. The only protein bands in the supernatant are in lanes 3 and 5, are smaller than 10 kDa in size and are certainly not the produced protein construct. Therefore, if there is any protein construct in the samples lysed with 4x volume of guanidium lysis buffer, it is in the pellet, not the supernatant.

The 10x gel in figure 40 shows a similar result to that in figure xx. There is no protein in the 15 kDa range of supernatant lanes, and there is very little if any in pellets, lanes 2, 8, and 10, in particular.

The results from increasing the volume of guanidium lysis buffer in order to solublize the protein constructs from the pellet are unclear due to smeared gels and bands of odd shapes and placement. However, the data suggest that the volume of lysis buffer used to lyse the cells and solublize the protein is not causing the expression and purification problem, otherwise proteins would have been seen in the 15 kDa range in the supernatant lanes at higher volumes of lysis buffer when none were present at lower volumes. Instead, no appropriately sized protein was seen in the supernatant lanes at all, and the expression problem persists.

It was also hypothesized that the production problem was caused by the induction of the plasmid and the density of bacteria at which the cells are harvested. Uninduced versus induced fractions of lysate and pellet from the constructs AP-SCR and RAD-MMP, and IGF-1 as a control, were used to test this hypothesis.

Figure 41 shows the corresponding Western blot using anti-Xpress. After a one minute exposure no strong bands are apparent, despite a strong band from the positive
control. Only one very faint band is seen in the induced pellet from RAD-MMP. This blot indicates that the induction of bacteria with IPTG is not causing the expression problem.

Figure 42 shows the same Western blot exposed for four hours. Protein can now be seen in lanes 4, 7, 8, 9, 13, and 14, corresponding to the induced pellets of each strain, the induced supernatant of RAD-MMP and IGF-1, and the uninduced pellet of RAD-MMP. Furthermore, the protein in the AP-SCR and RAD-MMP lanes is around 17 kDa in size, which is approximately the correct size. As expected, more protein is detected in the induced than uninduced lanes, given the additional time to grow and produce protein.

However, the protein is present in such small concentrations that it was only detectable after 4 hours of exposure, while the positive control was strongly visible after 1 minute. It is therefore hard to draw definitive conclusions from this data, although the data doesn’t show much about the effect of induction anyway. One reason the protein was present in such low concentrations may have been the method of sonication. Typically the 50 mL tube is placed in a water bath which is then sonicated, but for this experiment a probe sonicator in direct contact with the lysate was used to help lyse the cells. The probe sonicator was very powerful and resulted in a finely crushed pellet that was smeared along the side of the tube rather than collected at the bottom after spinning. It is also possible that the sonicator was too powerful and had a detrimental effect on the protein. Nevertheless, this experiment offers no solution to the protein production problem because induction with IPTG is likely not the reason for bad expression.

A final experiment was conducted as a quality control and to make sure that the procedure being followed was appropriate. 2L of AP-SCR and 2L of another lab member’s constructs, HB-IGF, were grown and purified while collecting fractions at each step. The lab member’s solutions and procedure was followed exactly.

Figure 43 shows the results of the Western blot with anti-Xpress after a 1 minute exposure. There is ample protein in all HB-IGF lanes and none in the AP-SCR lanes. The protein in lane 9 shows that there are significant amounts of protein in the cell lysate. The presence of protein in lanes 11, 12, and 13 shows that protein is still present – and being lost - throughout the purification stages, but that it is also getting more pure. The strong band in lane 14 at approximately 16 kDa, especially juxtaposed to the IGF-1 band.
in lane 15 at 14 kDa, strongly indicates the presence of HB-IGF in the elution. This is further corroborated by the fact that the lab member repeated the experiment one week before and found essentially identical results.

On the other hand, there is a complete lack of protein in the AP-SCR lanes, even the pellet. Figure 44 shows the same Western blot after being exposed for one hour, and in this picture there is protein detectable in the AP-SCR lanes. However, protein is even detected in the lanes containing protein ladder, which means that the concentrations of protein in the AP-SCR lanes is very small, and it may not be the AP-SCR construct.

6.7 Final Discussion

These experiments, especially the last, indicate that there are still issues with the protein construct production and expression. Indeed, it is problematic when an experiment cannot be replicated or the same protocol run on two different constructs yields such vastly different results.

There are a number of potential reasons that protein has been difficult to produce and detect in bacteria. The first is the difficulty and confusion involved when scaling up protocols. The production of bacteria was scaled from 50 mL to 2 L. A series of experiments were run on 2 L volumes of bacteria that were doomed to fail because the volume of guanidium lysis buffer used to lyse the cells was not scaled up appropriately, and therefore any protein constructs were never solublized into the lysate supernatant. The last experiment also taught a lesson in the importance of checking and rechecking buffers and reagents. The pH of some buffers was surprisingly found to have been drastically changed, such as the elution buffer, the pH of which had increased by 2 since making the solution. In a pH-dependent purification scheme such as the Ni-NTA beads and column, a change in pH such as this can and likely did have drastic effects on the purification of protein.

However, these types of procedural errors can be expected to affect and experiment or two at a time, but not to cause widespread failure in experiments as was found throughout this thesis. The level of difficult encountered with producing and purifying these protein constructs indicates a deeper rooted problem. One possibility is the presence of rare codons in the sequence of the constructs. The IGF-1 sequence in all
four constructs and the IGF-1 and HB-IGF-1 vectors has four rare arginine codons. The two AP constructs have no more rare codons, but the RAD constructs have an additional four rare arginine codons. It is possible that these rare codons are making it more difficult to express the constructs, although this does not account for the success in producing HB-IGF and the failure to produce AP-SCR.

The most likely explanation for why the protein constructs are difficult to express and detect is that they are not expressed well by the bacteria or are toxic. This would especially explain the difficulties in expressing large scale volumes of protein, and the low levels of expression throughout the experiments.
7 Conclusion

The results from this study support several conclusions. Site-specific insertion of large DNA segments by the QuikChange mutagenesis kit was successful in inserting the construct DNA into the IGF-1 HisMax vector and the secretory sequence into the construct HisMax vectors. This is supported by the gel electrophoresis and DNA sequencing data showing inserts of appropriate size, sequence and orientation.

Low levels of IGF-1 protein constructs were expressed in 293 and CHO cell, as indicated by Western blot analysis. However, other Western blots and Coomassie stains indicate that the cells do not produce protein in significant quantities by transient transfection and do not readily accept the plasmid for stable cell line selection.

Amplification of DNA segments by PCR was successful in cloning the construct DNA sequences out of the construct HisMax vectors, as shown by gel electrophoresis. Furthermore, the gel electrophoresis, DNA sequencing, and DNA trace data supports the fact that these DNA segments were inserted into TrcHis vectors. Therefore, the ability to subclone coding sequences of DNA from one vector to another was demonstrated.

Small scale growths of bacteria containing the TrcHis construct vectors showed that the protein constructs can be expressed in *E. coli*, purified by a Nickel-NTA column and detected by Western blot analysis. However, replication of these experiments can be difficult.

Large scale growths of bacteria containing the TrcHis construct vectors eliminated a number of supposed problems with protein production and detection. It was determined that the construct protein was remaining in the cell lystate pellet and that increased volumes of guanidium lysis buffer had little effect in solublizing this protein.

A number of potential reasons for difficult protein expression, purification and detection were listed, including buffers, incorrect procedures and rare codons. Ultimately the data support the conclusion that the protein constructs are either not expressed well in bacteria or are toxic to the cells, and that alternative methods of production need to be explored.
8 Acknowledgements

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9 Supplemental Material

*DNA and Protein Sequences*

**IGF-1**

Rat IGF-1 DNA Sequence (210 base pairs):

```
GAGCCAGAGACCCTTTGCGGGCTGAGCTGGTGGACGCTCTTCAGTTCGTGT
GTGGACCAAGGGGCTTTTTACTTCAACAAGCCCACAGGCTATGGCTGCCCAT
TCGGAGGGCACCACAGACGGGCATTGTGGATGAGTGTGGTTGCTTCCGGAGCTGT
GATCTGAGGGGTGGAGATGTACTGTGCTTCGCCCTGAAGGCTACAAAGTCAG
CT
```

Rat IGF-1 Translated Protein Sequence (70 amino acids):

```
GPETLGCAELVDALQFVGPRGFYFNKPTGYGSSIRRAPQTGIVDECCFRSDLR
RLEMYCAPLKPDKSA
```

**Construct Components**

Acceptor Peptide DNA Sequence (45 base pairs):

```
GGCCTGAACGACATCTTCGAGGCCCAGAAGATCGAGTGGCACGAG
```

Acceptor Peptide Translated Protein Sequence (15 amino acids):

```
GLNDIFEAQKIEWHE
```

RAD-16II DNA Sequence (48 base pairs):

```
AGAGCCAGAGCCGACGCCACGCCAGAGCCAGAGCCACGCGACGCCAGCCACGCC
```

RAD16-II Translated Protein Sequence (16 amino acids):

```
RARADADARARADADA
```

Scrambled Linker DNA Sequence (18 base pairs):

```
GGAATCGTGCGACCTCTG
```
Scrambled Linker Translated Protein Sequence (6 amino acids):
GIVGPL

MMP-2 Cleavable Linker DNA Sequence (18 base pairs):
CCTGTGGGACTGATCGGA

MMP-2 Cleavable Linker Translated Protein Sequence (6 amino acids):
PVGLIG

**AP-SCR**

Acceptor Peptide/Scrambled Linker (AP-SCR) DNA Sequence:
GGCCTGAACGACATCTTCTGAGGCCCAGAAGATCGAGTCGACGAGGGAATCGTGGGACCTCTGGGACCAGAGACCCTTTG

AP-SCR Protein Sequence:
GLNDIFEAQKIEWHEGIVGPL

AP-SCR “Forward” Oligonucleotide Sequence:
ACGATAAGGTACAGGCCCTTGGCCTGAACGACATCTTCTGAGGCCCAGAAGATCGAGTGGCACGAGGGAATCGTGGGACCTCTGGGACCAGAGACCCTTTG

AP-SCR “Reverse” Oligonucleotide Sequence:
GCAAAGGGTGCTCTGTCAGGGTCCCACGATTCCTTCTCGTGCCACTCGATCTCTGGGCCTCAGGATGTCGTTCAGGCCAAGGGCCTGATACCTTATCG

**AP-MMP**

Acceptor Peptide/MMP-2 Cleavable Linker (AP-MMP) DNA Sequence:
GGCCTGAACGACATCTTCTGAGGCCCAGAAGATCGAGTCGACGAGGGAATCGTGGGACCTCTGGGACCAGAGACCCTTTG

GACTGATCGGA
AP-MMP Protein Sequence
GLNDIFEAQKIEWHEPVGLIG

AP-MMP “Forward” Oligonucleotide Sequence:
ACGATAAGGTACAGGCCCTTTGGCCTGAACGACATCTTTCGAGGCCCAGAAGAT
CGAGTGGCAGCGAGCCTGTGGGACTGATCGGAGGACCAGAGACCCTTTTG

AP-MMP “Reverse” Oligonucleotide Sequence:
GCAAAGGGTCTCTGGTCTCCGATCGATCCACAGGCTCGTGCACCCTCGATCT
CTGGGCTTCGAAAGATGTCGTTCAGGCCAAGGGCCTGTACCTTATCGT

RAD-SCR
RAD16-II/Scrambled Linker (RAD-SCR) DNA Sequence:
AGAGCCAGAGCCGCAGCCGACGCCAGCCGACGCCGACGCCGACGCCGAGAGCC
ATCGTGGGACCTCTG

RAD-SCR Protein Sequence:
RARADADARARADADAGIVGPL

RAD-SCR “Forward” Oligonucleotide Sequence:
ACGATAAGGTACAGGCCCTTTGGCCTGAACGACATCTTTCGAGGCCCAGAAGAT
CGAGTGGCAGCGAGCCTGTGGGACTGATCGGAGGACCAGAGACCCTTTTG

RAD-SCR “Reverse” Oligonucleotide Sequence:
GCAAAGGGTCTCTGGTCTCCGATCGATCCACAGGCTCGTGCACCCTCGATCT
CTGGGCTTCGAAAGATGTCGTTCAGGCCAAGGGCCTGTACCTTATCGT

RAD-MMP
RAD16-II/MMP-2 Cleavable Linker (RAD-MMP) DNA Sequence:
AGAGCCAGAGCCGACGCCGACGCCAGAGCCAGAGCCGACGCCGACGCCCCT
GTGGGACTGATCGGA

RAD-MMP Protein Sequence:
RARADADARARADADAPVGLIG

RAD-MMP “Forward” Oligonucleotide Sequence:
ACGATAAGGTACAGGCCCTTAGAGCCAGAGCCGACGCCGACGCCAGAGCCAGAGCCGACGCCGACGCCAGAGCCGACGCCGACGCCGACGCCGACGCCCCT
GTGGGACTGATCGGAGGACCAGAGACCCTTTGC

RAD-MMP “Reverse” Oligonucleotide Sequence:
GCAAAGGGGTCTCTGGTCCTCCGATCAGTCCCACAGGGGCGTCGGCGTCGGCTCTGGCTCTGGCGTCGGCGTCGGCTCTGGCTCTAAGGGCCCTGTACCTTATCGT

Secretion Sequence

Secretion Tag DNA Sequence (53 base pairs):
AAGCTGCTGCCGTCGGTGGTGTGAAGCTCTTTCTGGCCGCAGTGTTGTCCGCG

Secretion Tag “Forward” Oligonucleotide Sequence:
GGACGGCCTCCGAAACCATGAAAGCTGCTGCCGTCGGTGGTGTGAAGCTCTTTCTGGCCGCAGTGTTGTCCGCGGGGGGTTCTCATCATCATCAT

Secretion Tag “Reverse” Oligonucleotide Sequence:
ATGATGATGATGAGAACCCCCCGCGGACAAACTGCGGCCAGAAAGAGCTTTC
ACACCACCAGCGCAGCAGCTTCATGGTTTCGGAGGCCGTCC
10 Figures
Figure 1. Total protein construct, by component.

<table>
<thead>
<tr>
<th>Anti-Xpress Enterokinase</th>
<th>6x His Tag Epitope</th>
<th>Cleavage Site</th>
<th>AP or RAD Anchor</th>
<th>SCR or MMP Linker</th>
<th>IGF-1</th>
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</thead>
</table>
**Figure 2.** Four different protein constructs.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Acceptor Peptide</th>
<th>Scrambled Linker</th>
<th>IGF-1</th>
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<tbody>
<tr>
<td>AP-SCR</td>
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<tr>
<td>AP-MMP</td>
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<tr>
<td>RAD-SCR</td>
<td>RAD16-II</td>
<td>Scrambled Linker</td>
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<tr>
<td>RAD-MMP</td>
<td>RAD16-II</td>
<td>MMP-2 Cleavable</td>
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Figure 3. Biotin Sandwich

<table>
<thead>
<tr>
<th>Self-assembling Peptide Gel</th>
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<tbody>
<tr>
<td>Biotin</td>
</tr>
<tr>
<td>Streptavidin</td>
</tr>
<tr>
<td>Acceptor Peptide</td>
</tr>
</tbody>
</table>
Figure 4. First gel electrophoresis of potentially inserted construct sequences in IGF-1 HisMax vector.

Lane Key:
1. AP-SCR 2
2. AP-SCR 3
3. AP-MMP 1
4. AP-MMP 2
5. RAD-SCR 1
6. RAD-SCR 2
7. RAD-MMP 1
8. RAD-MMP 2
9. IGF-1
10. Negative control
Figure 5. Second gel electrophoresis of potentially inserted construct sequence in IGF-1 HisMax vector.

Lane Key:
1. 1kb Ladder
2. AP-SCR 4
3. AP-SCR 5
4. AP-MMP 3
5. AP-MMP 4
6. AP-MMP 5
7. RAD-SCR 3
8. RAD-SCR 4
9. RAD-SCR 5
10. HB-IGF
11. IGF-4
12. IGF-5
13. Negative Control
14. 1kb Ladder
Figure 6. AP-SCR DNA sequence in HisMax construct.

```
> _ AP-SCR  63 nt vs.
> _ 1-5     63 nt
scoring matrix: , gap penalties: -12/-2
100.0% identity; Global alignment score: 252

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</table>
```

47
Figure 7. AP-MMP DNA sequence in HisMax construct.

```
> AP-MMP
63 nt vs.
> 2-3
63 nt

scoring matrix:      gap penalties:  -12/-2
100.0% identity;   Global alignment score: 252

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<td>-</td>
<td>GGA</td>
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```
Figure 8. RAD-SCR DNA sequence in HisMax construct.

> RAD-SCR
> 3-3

scoring matrix: , gap penalties: -12/-2
100.0% identity; Global alignment score: 264

744525 AGAGCCAGAGCCGACGCCGACGCCAGAGCCAGAGCCGACGCCGACGCCGGAATCGTGGGA
AGAGCCAGAGCCGACGCCGACGCCAGAGCCAGAGCCGACGCCGACGCCGGAATCGTGGGA

744525 CCTCTG
CCTCTG

_
Figure 9. RAD-MMP DNA sequence in HisMax construct.

> RAD-MMP
> 4-2

scoring matrix: , gap penalties: -12/-2

100.0% identity; Global alignment score: 264

```
10  20  30  40  50  60
390513 AGAGCCAGAGCCGACGCCGACGCCAGAGCCGACGCCGACGCCGACGCCGACTG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
       AGAGCCAGAGCCGACGCCGACGCCAGAGCCGACGCCGACGCCGACGCCGACGCCGACTG

10  20  30  40  50  60
390513 ATCGGA
       ::::::
       ATCGGA
```
**Figure 10.** Second AP-MMP DNA sequence in HisMax construct.

```
> AP-MMP  63 nt vs.  63 nt
> 2-5
scoring matrix, gap penalties: -12/-2
100.0% identity
Global alignment score: 252

328478 GCCCTGAACGACATCTTGAGGCCCAAGATACTGAGGTGGCACGACTGACTGATC
                  :------------------------------------------------------
                  GGCCTGAACGACATCTTGAGGCCCAAGATACTGAGGTGGCACGACTGACTGATC

328478  GGA
: : :
: : :
     - GGA
```
Figure 11. Blank Western blot analysis with anti-Xpress antibody of 293 cells transiently transfected with IGF-1 plasmid.

Lane Key:
1. Dual Color Ladder
2. 0 ug plasmid / well
3. 0.5 ug plasmid / well
4. 1 ug plasmid / well
5. 2 ug plasmid / well
Figure 12. Blank Western blot analysis with anti-IGF-1 antibody of 293 cells transiently transfected with IGF-1 plasmid.

Lane Key:
1. Dual Color Ladder
2. 0 ug plasmid / well
3. 0.5 ug plasmid / well
4. 1 ug plasmid / well
5. 2 ug plasmid / well
Figure 13. Western blot analysis with anti-Xpress antibody of 293 cells transiently transfected with IGF-1 plasmid.

Lane Key:
1. No plasmid
2. IGF-1 1
3. IGF-1 2
4. HB-IGF 1
5. HB-IGF 2
6. Positive control
Figure 14. Gel electrophoresis of potentially inserted secretory sequences into HisMax construct vectors

Lane Key ("s" represents secretory sequence)
1. 1kb ladder
2. AP-SCR
3. AP-SCRs 1
4. AP-SCRs 4
5. AP-SCRs 5
6. AP-MMP
7. AP-MMPs 2
8. AP-MMPs 4
9. AP-MMPs 5
10. 1kb ladder
11. 1kb ladder
12. RAD-SCR
13. RAD-SCRs 1
14. RAD-SCRs 3
15. RAD-SCRs 5
16. RAD-MMP
17. RAD-MMPs 3
18. RAD-MMPs 4
19. RAD-MMPs 5
20. Negative Control
21. 1kb ladder
Figure 15. Secretory sequence in AP-SCR HisMax construct.

>._ Secretory Sequence 53 nt vs. 
>._ 1-4s 53 nt
scoring matrix: gap penalties: -12/-2
100.0% identity; Global alignment score: 212

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| 840141| AAGCTGCTGCCGCGGTCGGTGATGAAGCTCTTTCTGCCGCAGTGTTGTCCCG
| - | AAGCTGCTGCCGCGGTCGGTGATGAAGCTCTTTCTGCCGCAGTGTTGTCCCG

57
Figure 16. Secretory sequence in AP-MMP HisMax construct.

> Secretary Sequence
> 2-3a

scoring matrix: , gap penalties: -12/-2
98.1% identity; Global alignment score: 200

```
365019 AAGCTGCTGCGCTCGGGTG-GTGTGAAGCTTTTCTGCCGCAGTGTTGTCCGCG
   :::::::::::  :::::::::::  :::::::::::  :::::::::::  :::::::::::
- AAGCTGCTGCGCTCGGGTG-GTGTGAAGCTTTTCTGCCGCAGTGTTGTCCGCG
```

58
Figure 17. Secretory sequence in RAD-SCR HisMax construct.

>`_ Secretory Sequence_  
>`_ 3-30_  

Scoring matrix: , gap penalties: -12/2  
100.0% identity;  
Global alignment score: 212

<table>
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</tbody>
</table>

59
Figure 18. Secretory sequence in RAD-MMP HisMax construct.

>`_ Secretory Sequence_` 53 nt vs. `_ 4-5s_` 53 nt

scoring matrix: , gap penalties: -12/-2

100.0% identity; Global alignment score: 212

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</tbody>
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60
Figure 19. Gel electrophoresis to detect construct sequences subcloned out of HisMax constructs.

Lane Key
1. 1kb ladder
2. AP-SCR
3. AP-MMP
4. RAD-SCR
5. RAD-MMP
6. Negative Control
7. 1kb ladder
Figure 20. First gel electrophoresis to detect insertion of construct sequences into TrcHis vector.

Lane Key: ("b" denotes TrcHis vector, bacterial vector)
1. lkb ladder
2. AP-SCRb 1
3. AP-SCRb 2
4. AP-SCRb 3
5. AP-SCRb 4
6. AP-SCRb 5
7. AP-MMPb 1
8. AP-MMPb 2
9. AP-MMPb 3
10. AP-MMPb 4
11. RAD-SCRb 1
12. RAD-SCRb 2
13. RAD-MMPb 1
14. RAD-MMPb 2
15. RAD-MMPb 3
16. lkb ladder
Figure 21. Second gel electrophoresis to detect insertion of construct sequences into TrcHis vector.

Lane Key: ("b" denotes TrcHis bacterial vector)
1. 1kb ladder
2. AP-SCRb 2
3. AP-SCRb 4
4. AP-SCRb 5
5. AP-MMPb 2
6. AP-MMPb 3
7. AP-MMPb 4
8. RAD-SCRb 1
9. RAD-SCRb 2
10. RAD-SCRb 3
11. RAD-MMPb 1
12. RAD-MMPb 2
13. RAD-MMPb 4
14. Negative Control
15. 1kb ladder
Figure 22. First gel electrophoresis of TrcHis plasmid restriction enzyme digestion.

Lane Key:
1. 1kb ladder
2. AP-SCR 1-1
3. AP-SCR 1-2
4. AP-SCR 1-3
5. AP-SCR 1-4
6. AP-SCR 2-1
7. AP-SCR 2-2
8. AP-SCR 2-3
9. AP-SCR 2-4
10. AP-MMP 1-1
11. AP-MMP 1-2
12. AP-MMP 1-3
13. AP-MMP 1-4
14. 1kb ladder
Figure 23. Second gel electrophoresis of TrcHis plasmid restriction enzyme digestion.

Lane Key:
1. 1kb ladder
2. AP-MMP 2-1
3. AP-MMP 2-2
4. AP-MMP 2-3
5. AP-MMP 2-4
6. RAD-SCR 1
7. RAD-SCR 2
8. RAD-SCR 3
9. RAD-SCR 4
10. RAD-MMP 1
11. RAD-MMP 2
12. RAD-MMP 3
13. RAD-MMP 4
14. 1kb ladder
Figure 24. AP-SCR DNA sequence in TrcHis construct.

> - AP-SCR
> - lb-5

scoring matrix: , gap penalties: -12/-2
96.8% identity; Global alignment score: 244

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</table>
Figure 25. AP-MMP DNA sequence in TrcHis construct.

> ap-MMP
63 nt

> 2bh-4
63 nt

scoring matrix: , gap penalties: -12/-2
95.2% identity; Global alignment score: 240

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:::

__

GGA
Figure 26. First RAD-SCR DNA sequence in TrcHis construct.

> _RAD-SCR_  66 nt vs. _3h21_  66 nt

scoring matrix: , gap penalties: -12/-2
84.8% identity; Global alignment score: 224

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<td>_ CCTCTG</td>
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</table>
Figure 27. Second RAD-SCR DNA sequence in TrcHis construct.

>__ RAD-SCR
>__ 3r27

scoring matrix: , gap penalties: -12/-2

83.3% identity; Global alignment score: 220

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227734 AGAGCCAGACCGAGCCGAGACCCGAGACCAGACCGAGACCGAGCCGAGGGAATCGTGGA
227734 AGAGCCAGACCGAGCCGAGCCNCCNNNNNNNGACCGAGCCGAGCCGAGGAATCGTGGA

227734 CCTCTG

_   _   _   _   _   _   _   _

_   _   _   _   _   _   _   _

69
Figure 28. RAD-MMP DNA sequence in TrcHis construct.

```
> RAD-MMP 66 nt vs. 4b-1 66 nt
scoring matrix: , gap penalties: -12/-2
93.9% identity; Global alignment score: 248

10  20  30  40  50  60
710943 AGAGCCAGACCCGACGCCAGCCAGCCAGCCAGCCAGCCAGCCAGCCAGCCAGCCACTG
- NNNAGCNNGAGCCACGCCAGCAGCCAGCCAGCCAGCCAGCCAGCCAGCCAGCCAGCCAGCCAGCCACTG

710943 ATCGGA

- ATCGGA
```
Figure 29. Original and corrected traces of AP-SCR in TrcHis vector.
Figure 30. Original and corrected traces of AP-MMP in TrcHis vector.
Figure 31. Original and corrected traces of RAD-SCR in TrcHis vector.
Figure 32. Original and corrected traces of RAD-MMP in TrcHis vector.
Figure 33. Initial small scale growth of protein constructs and detection by Western blot.

Lane Key:
1. AP-SCR
2. AP-SCR
3. AP-MMP
4. AP-MMP
5. RAD-SCR
6. RAD-SCR
7. RAD-MMP
8. RAD-MMP
9. SeeBlue Ladder
Figure 34. Repeated small scale growth of protein constructs and detection by Western blot.

Lane Key:
1. AP-SCR New
2. AP-MMP New
3. RAD-SCR New
4. RAD-MMP New
5. AP-SCR Old
6. AP-MMP Old
7. RAD-SCR Old
8. RAD-MMP Old
9. SeeBlue Ladder
Figure 35. First Western blot analysis of AP-SCR and AP-MMP purification fractions.

Lane Key:
1. SeeBlue ladder
2. AP-SCR 1 supernatant
3. AP-SCR 1 pellet
4. AP-SCR 1 supernatant after adding beads
5. AP-SCR 1 supernatant after final wash with wash buffer
6. AP-SCR 1 elution
7. SeeBlue Ladder
8. AP-MMP 1 supernatant
9. AP-MMP 1 pellet
10. AP-MMP 1 supernatant after adding beads
11. AP-MMP 1 supernatant after final wash with wash buffer
12. AP-MMP 1 elution
13. Positive control 3
14. Positive control 4
15. Dual color ladder
Figure 36. Second Western blot analysis of AP-SCR and AP-MMP purification fractions.

Lane Key:
1. SeeBlue ladder
2. AP-SCR 2 supernatant
3. AP-SCR 2 pellet
4. AP-SCR 2 supernatant after adding beads
5. AP-SCR 2 supernatant after final wash with wash buffer
6. AP-SCR 2 elution
7. SeeBlue Ladder
8. AP-MMP 2 supernatant
9. AP-MMP 2 pellet
10. AP-MMP 2 supernatant after adding beads
11. AP-MMP 2 supernatant after final wash with wash buffer
12. AP-MMP 2 elution
13. Positive control 3
14. Positive control 4
15. Dual color ladder
Figure 37. Western blot analysis of RAD-SCR and RAD-MMP purification fractions.

Lane Key:
1. SeeBlue ladder
2. RAD-SCR 1 supernatant
3. RAD-SCR 1 pellet
4. RAD-SCR 1 supernatant after adding beads
5. RAD-SCR 1 supernatant after final wash with wash buffer
6. RAD-SCR 1 elution
7. SeeBlue Ladder
8. RAD-MMP 1 supernatant
9. RAD-MMP 1 pellet
10. AP-MMP 2 supernatant after adding beads
11. AP-MMP 2 supernatant after final wash with wash buffer
12. AP-MMP 2 elution
13. Positive control 3
14. Positive control 4
15. Dual color ladder
Figure 38. Western blot analysis of all constructs and IGF-1 using 1x volume of guanidium lysis buffer.

Lane Key:
1. AP-SCR Lysate Supernatant
2. AP-SCR Lysate Pellet
3. AP-MMP Lysate Supernatant
4. AP-MMP Lysate Pellet
5. RAD-SCR Lysate Supernatant
6. RAD-SCR Lysate Pellet
7. RAD-MMP Lysate Supernatant
8. RAD-MMP Lysate Pellet
9. IGF-1 Lysate Supernatant
10. IGF-1 Lysate Pellet
11. DualColor Ladder
12. Positive control 1
13. Positive control 2
14. Positive control 3
15. SeeBlue Ladder
Figure 39. Western blot analysis of all constructs and IGF-1 using 4x volume of guanidium lysis buffer.

Lane Key:
1. AP-SCR Lysate Supernatant
2. AP-SCR Lysate Pellet
3. AP-MMP Lysate Supernatant
4. AP-MMP Lysate Pellet
5. RAD-SCR Lysate Supernatant
6. RAD-SCR Lysate Pellet
7. RAD-MMP Lysate Supernatant
8. RAD-MMP Lysate Pellet
9. IGF-1 Lysate Supernatant
10. IGF-1 Lysate Pellet
11. DualColor Ladder
12. Positive control 1
13. Positive control 2
14. SeeBlue Ladder
15. Positive control 3
Figure 40. Western blot analysis of all constructs and IGF-1 using 10x volume of guanidium lysis buffer.

Lane Key:
1. AP-SCR Lysate Supernatant
2. AP-SCR Lysate Pellet
3. AP-MMP Lysate Supernatant
4. AP-MMP Lysate Pellet
5. RAD-SCR Lysate Supernatant
6. RAD-SCR Lysate Pellet
7. RAD-MMP Lysate Supernatant
8. RAD-MMP Lysate Pellet
9. IGF-1 Lysate Supernatant
10. IGF-1 Lysate Pellet
11. DualColor Ladder
12. Positive control 1
13. SeeBlue Ladder
14. Positive control 2
15. Positive control 3
Figure 41. 1 minute exposure of Western blot analysis of induced and uninduced fractions of AP-SCR, RAD-MMP fractions, and IGF-1 control.

Lane Key:
1. AP-SCR Uninduced Lysate Supernatant
2. AP-SCR Uninduced Lysate Pellet
3. AP-SCR Induced Lysate Supernatant
4. AP-SCR Induced Lysate Pellet
5. SeeBlue Ladder
6. RAD-MMP Uninduced Lysate Supernatant
7. RAD-MMP Uninduced Lysate Pellet
8. RAD-MMP Induced Lysate Supernatant
9. RAD-MMP Induced Lysate Pellet
10. SeeBlue Ladder
11. IGF Uninduced Lysate Supernatant
12. IGF Uninduced Lysate Pellet
13. IGF Induced Lysate Supernatant
14. IGF Induced Lysate Pellet
15. Positive Control
Figure 42. 4 hour exposure of Western blot analysis of induced and uninduced fractions of AP-SCR, RAD-MMP fractions, and IGF-1 control.

Lane Key:
1. AP-SCR Uninduced Lysate Supernatant
2. AP-SCR Uninduced Lysate Pellet
3. AP-SCR Induced Lysate Supernatant
4. AP-SCR Induced Lysate Pellet
5. SeeBlue Ladder
6. RAD-MMP Uninduced Lysate Supernatant
7. RAD-MMP Uninduced Lysate Pellet
8. RAD-MMP Induced Lysate Supernatant
9. RAD-MMP Induced Lysate Pellet
10. SeeBlue Ladder
11. IGF Uninduced Lysate Supernatant
12. IGF Uninduced Lysate Pellet
13. IGF Induced Lysate Supernatant
14. IGF Induced Lysate Pellet
15. Positive Control
Figure 43. 1 minute exposure of Western blot analysis of AP-SCR and HB-IGF purification fractions.

Lane Key:
1. SeeBlue Ladder
2. AP-SCR lysate supernatant
3. AP-SCR lysate pellet
4. AP-SCR supernatant after adding beads
5. AP-SCR supernatant after washing with binding buffer
6. AP-SCR supernatant after last wash with wash buffer
7. AP-SCR elution
8. SeeBlue Ladder
9. HB-IGF lysate supernatant
10. HB-IGF lysate pellet
11. HB-IGF supernatant after adding beads
12. HB-IGF supernatant after washing with binding buffer
13. HB-IGF supernatant after last wash with wash buffer
14. HB-IGF elution
15. IGF-1 positive control
Figure 44. 4 hour exposure of Western blot analysis of AP-SCR and HB-IGF purification fractions.

Lane Key:
1. SeeBlue Ladder
2. AP-SCR lysate supernatant
3. AP-SCR lysate pellet
4. AP-SCR supernatant after adding beads
5. AP-SCR supernatant after washing with binding buffer
6. AP-SCR supernatant after last wash with wash buffer
7. AP-SCR elution
8. SeeBlue Ladder
9. HB-IGF lysate supernatant
10. HB-IGF lysate pellet
11. HB-IGF supernatant after adding beads
12. HB-IGF supernatant after washing with binding buffer
13. HB-IGF supernatant after last wash with wash buffer
14. HB-IGF elution
15. IGF-1 positive control
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18 Mike Davis’ unpublished works


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