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PRINCIPAL INVESTIGATOR: Paul H. Goldspink, Ph.D.

CONTRACTING ORGANIZATION: Medical College of Wisconsin, Inc.
Milwaukee, Wisconsin 53226-3548

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Paul H. Goldspink, Ph.D.

E-Mail: pgoldspink@mcw.edu

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Medical College of Wisconsin, Inc.
Office of Research,
8701 Watertown Plank Rd,
PO Box 26509,
Milwaukee,
Wisconsin, 53226-3548

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14. ABSTRACT

The metabolic changes in diabetes are directly triggered by hyperglycemia and the rate limiting step in glucose uptake is the translocation of the insulin-sensitive glucose transporter protein-4 (GLUT4) in insulin sensitive tissues. The purpose of this was to test whether peptide analogs derived from the IGF-1 isoform expressed in muscle known as Mechano-growth factor can function as a bio-therapeutics to modulate of AS160 phosphorylation which is necessary to stimulate GLUT4 function. Using skeletal muscle cell based models, we aimed to define an optimal candidate peptide to test in animal based modes of diabetes. Skeletal muscle myotube cells undergoing membrane depolarization in the presence of 3 mM extracellular calcium are necessary to demonstrate peptide mediated actions. We have found by preventing phosphorylation within the 14-3-3 binding domain of the S/A^{18} peptide blocks Akt mediated inhibition of the Raf/ERK signaling branch following IGF-1 stimulation, whereas phosphorylation within the 14-3-3 binding domain of the S/E^{18} peptide may augment Akt signaling by shutting down Raf/ERK signaling. Consequently, we have defined that the MGF E-domain peptides act as modulators of branches of the IGF-1 signaling pathway through interactions with specific 14-3-3 binding proteins. Since Akt directly phosphorylates AS160 to stimulate GLUT4, we propose the S/E^{18} peptide may function as an optimal candidate for further testing.

15. SUBJECT TERMS

None listed

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1. **INTRODUCTION:**

Insulin resistance is an important risk factor in the pathogenesis of diabetes and inflammation has a crucial role in the development of insulin resistance. The metabolic changes in diabetes are directly triggered by hyperglycemia and the rate limiting step in glucose uptake is the translocation of the insulin-sensitive glucose transporter protein-4 (GLUT4) from an intracellular compartment to the sarcolemma within insulin sensitive tissues. This project was designed to test whether peptide analogs derived from the IGF-1 isoform expressed in muscle known as Mechano-growth factor can function as a bio-therapeutics to circumvent pro-inflammatory cytokine induced insulin/IGF-1 resistance and restore GLUT4 function in diabetes. Consequently, the aim was to examine the convergence of hormonal and inflammatory signaling in the regulation of AS160 which is necessary for GLUT4 translocation in skeletal muscles in the diabetic environment through the following specific aims.

2. **KEYWORDS:** Diabetes, GLUT4, Mechano-growth factor, E-domain peptides, Skeletal muscle.

3. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**

One the major goals of the project were to acquire L6 Glut4myc cell lines and begin testing Glut4 translocation with different MGF E-domain peptide variants. Our aim was to define which phosphorylation motifs in the phospho-null (S/A^{18}) MGF E-domain peptide regulate GLUT4 translocation with glucose uptake in GLUT4myc reporter skeletal muscle myotubes *in vitro*. Using a series of PCR and western blotting approaches we were to analyze the signaling

pathways that the E-domain peptides modulate to stimulate or inhibit Glut4 translocation. In conjunction with this, one milestone was to collect data for publication and begin preparing a manuscript for publication. At this stage the project has achieved 50% of this milestone.

The second major goal was to determine whether the phospho-null MGF E-domain peptide (S/A^{18}) stimulates GLUT4 translocation with glucose uptake in the skeletal muscles of GLUT4myc-epitope tagged reporter mice, *Ob/ob* diabetic mice and GLUT4myc diabetic mice *in vivo.* This goal was designed to test the efficacy of the top MGF E-domain candidate peptide in an animal model once we it was identified in our cell culture models. At this stage the project has achieved 0% of this milestone.

- **What was accomplished under these goals?**

To date our major activities have focused on defining the optimal tissue culture conditions in skeletal muscle cell lines that show clear effects of the MGF E-domain peptides. Our initial objective was to recapitulate some of the events we observed in the skeletal muscles of mice related to AS160 phosphorylation in tissue culture models. AS160 is a critical step in the recruitment of GLUT4 to the plasma membrane. Mechanistic studies examining stimulation in skeletal muscle have shown that Akt phosphorylation inhibits the Rab GTPase activating protein domain of AS160. This prevents targeting of Rab in the GLUT4 storage vesicles, which favors GLUT4 exocytosis to the plasma membrane which is necessary for GLUT4 translocation. Based on studies and conditions reported in the scientific literature, we focused on the signaling events leading to AS160 phosphorylation and gene expression of known transcriptional regulators of GLUT4 expression and their modulation by the MGF E-domain peptides initially in the L6 GLUT4-myc reporter cells line as proposed.

While trying to define the actions of the E-domain peptides in the L6 GLUT4-myc reporter cell line we encountered data inconsistencies and a number of technical issues. After a series of experimental approaches including altering the time course of treatments and peptide dose response we were not able to establish any solid conclusions. This prompted us to analyze a number of potential tissue conditions in C2C12 skeletal myotube cultures to see if we could determine optimal working conditions in a skeletal muscle cell line with a higher through-put potential and focusing on gene expression read-outs.

Utilizing gene expression analysis of the orphan hormone nuclear receptor genes NR4A1, 2 and 3, we identified tissue culture conditions that clearly demonstrated E-domain peptide modulation of NR4A gene expression. While no endogenous ligand exists for the NR4A family, their immediate early gene expression profile is known to be regulated by several upstream pathways. Moreover, increased NR4A2 expression occurs in the heart with over-expression of the IGF-1R in mice and NR4A1 is involved in IGF-1 induced skeletal muscle hypertrophy and regulation of GLUT4 expression. Together the data suggesting a link to IGF-1 mediated signaling exists to GLUT4 expression through inductions of NR4A family members in muscle and prompted us to focused on the NR4A family as potential targets of peptide modulation.

Differentiating the C2C12 myotubes for 5 days before switching to Krebs-ringer buffer, permitted use to identify the need for membrane depolarization in the presence of 3mM extracellular Ca^{2+} to see clear signs of peptide modulation. The use of the Krebs-ringer buffer allowed for greater control over media ion concentration and osmolality compared to pre-formulated commercially available tissue culture media. Consequently, we were able to elicit changes in NR4A gene expression following membrane depolarization in 97mM KCl (High KCl) in the presence of 3mM Ca^{2+} compared to the absence of depolarization in 4.7mM KCl (low KCl) as shown in **Figure 1** for NR4A2 mRNA and protein expression. Under these conditions we were able to elicit opposing regulation (reciprocal regulation) of NR4A2 expression using the phospho-

null (S/A^{18}) and phospho-mimetic (S/E^{18}) MGF E-domain peptide variants. While the reciprocal regulation of expression was not evident in the absence of depolarization, the phospho-mimetic (S/E^{18}) MGF E-domain peptide still produced an inhibitory effect on NR4A2 expression.

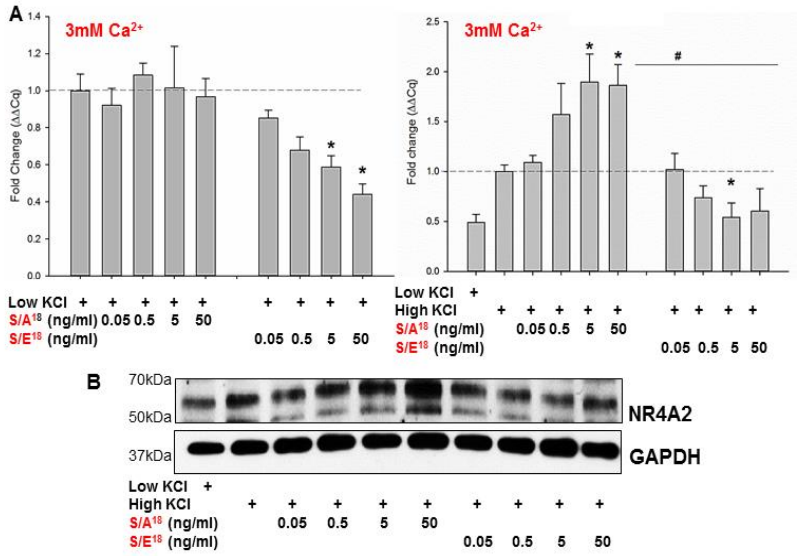

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Fig 1. A. Modulation of NR4A2 mRNA expression in C2C12 myotubes treated with S/A^{18}, S/E^{18} E-domain peptide variants at different doses following depolarization in Krebs-Ringer buffer (97mM KCl +/- 3mM Ca^{2+}) for 1hr (*P<0.05, vs either low KCl or high KCl, n=3). **B.** NR4A2 protein expression following depolarization.

Using KCl depolarization + 3mM Ca^{2+} conditions in the C2C12 myotubes, we tested whether phosphorylation at Ser18 with the MGF-domain peptide is a biologically relevant event by treating cells with stabilized native E-domain peptide and a phospho-Ser18 peptide varients (Nat-pSer^{18}). The phospho-peptide (Nat-pSer^{18}) treatment produced similar inhibitory actions as the phospho-memetic peptide (S/E^{18}), while the native peptide (Nat) did not enhance expression (**Figure 2A** below). These data support the conclusion that phosphorylation of Ser18 within the MGF E-domain peptide regulates it's the biological activity. Also, they indicate the need for membrane triggered signaling events within the cell and entry of extracellular calcium are necessary steps in the actions of the E-domain peptides.

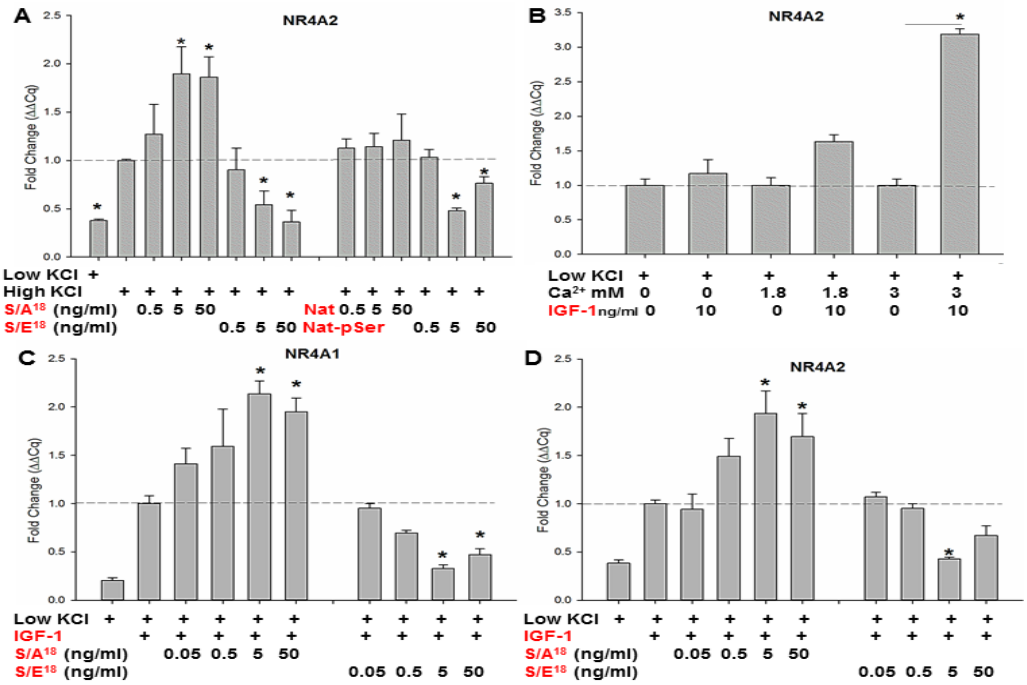

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Fig 2. A. Reciprocal modulation of NR4A2 gene expression treated with S/A^{18}, S/E^{18} peptides compared to Nat, Nat-pSer^{18} peptides following membrane for 1hr **B.** Effect of increasing extracellular Ca^{2+} on 10nM R^{3}IGF-1 stimulation of NR4A2 expression in 4.7mM KCl + 0, 1.8 & 3mM Ca^{2+}, Krebs-Ringer buffer for 1hr. **C&D.** E-domain peptide modulation of R^{3}IGF-1 stimulation of NR4A1 and 2 expression in 4.7mM KCl + 3mM Ca^{2+}, Krebs-Ringer buffer for 1hr (**P*<0.05, in **A** vs high KCl, **B** vs. Low KCl, **C&D** vs IGF-1, n=3).

Therefore, to determine whether IGF-1 stimulation of NR4A2 expression was also dependent on extracellular Ca^{2+} during depolarization, the $[Ca^{2+}]_o$ concentration was varied which resulted in the augmentation of IGF-1 mediated induction of NR4A2 (**Fig 2B**). Examining E-domain peptide modulation of IGF-1 meditated stimulation under optimal conditions, produced reciprocal regulation of NR4A1 and 2 expressions with the peptides and IGF-1 added together (**Fig 2C&D**). Thus, the influx of $[Ca^{2+}]_o$ and the concomitant increase in $[Ca^{2+}]_i$, possibility due to voltage gated events during membrane depolarization or in response to IGF-1 agonist stimulation, plays a

central role in excitation-transcription of the NR4A genes which are known to drive GLUT4 expression. The data indicates the E-domain peptides may function as allosteric modulators of Ca^{2+}-dependent aspects of with IGF-1 stimulation signaling into the transcriptional machinery.

IGF-1 and insulin signaling is mediated via PI 3-kinase, which generates phosphatidylinositol 3,4,5-trisphosphate, promoting activation of the AGC protein kinase, Akt. Several targets of PI3K/Akt signaling (e.g. TSC2, PRAS40, Bad) contain the minimal Akt phosphorylation consensus site (R*X*R*XX*(pS/pT), which sometimes creates binding sites for 14-3-3 proteins following Ser or Thr phosphorylation. Our initial analysis of linear motifs within the MGF E-domain first led us to hypothesize that Serine 18 was within a potential phosphorylation consensus motif. Consequently, we were able to demonstrate that Ser18 phosphorylation modifies peptide activity and supported our hypothesis. So based on these results, we reanalyzed the sequences surrounding Ser18 and its proximity to the polybasic motif within the MGF-domain to ask whether this sequences could potentially create an Akt consensus site (RRRKGS^{18}T) and induce a peptide/14-3-3 protein interaction following Akt activation. Probing deeper into the IGF-1 and insulin signaling pathway, we determine whether pretreatment with the E-domain peptides selectively modulate Akt specific phosphorylation of well-known Akt target proteins following Akt specific activation with a small molecule activator (SC79 4μg/ml), in C2C12 cells cultured in in low KCL+3mM Ca^{2+} *in vitro.* The data show that activation of Akt either by IGF-1 or SC79 was not influenced by pre-incubation with the MGF E-domain peptides. However, phosphorylation of the Akt consensus site at Serine 259 on c-Raf, is reciprocally modified by the presence of the E-domain peptides, whereas Akt consensus sites on other target proteins examined (Bad$^{(S136)}$, GSKα/β$^{(S21/9)}$, FoxO1/3$^{(T24/32)}$, FoxO3a$^{(T253)}$ PRAS40$^{(T246)}$), were not influenced (**Figure 3A** below, data shown for PRAS40$^{(T246)}$). In addition, the consequences of E-

domain peptide modulation of c-Raf Ser 259 phosphorylation and c-Raf activity was also evident in the phosphorylation and activation of ERK1/2 which lie downstream of cRaf in the Ras/Raf/MEK/ERK signaling branch of the IGF-1R pathway. Thus, these data indicate a potential mechanism exists whereby the E-domain peptides act as allosteric modulators of branches of the IGF-1 signaling pathway through interactions with specific 14-3-3 binding proteins that target the Akt phosphorylation site on c-Raf.

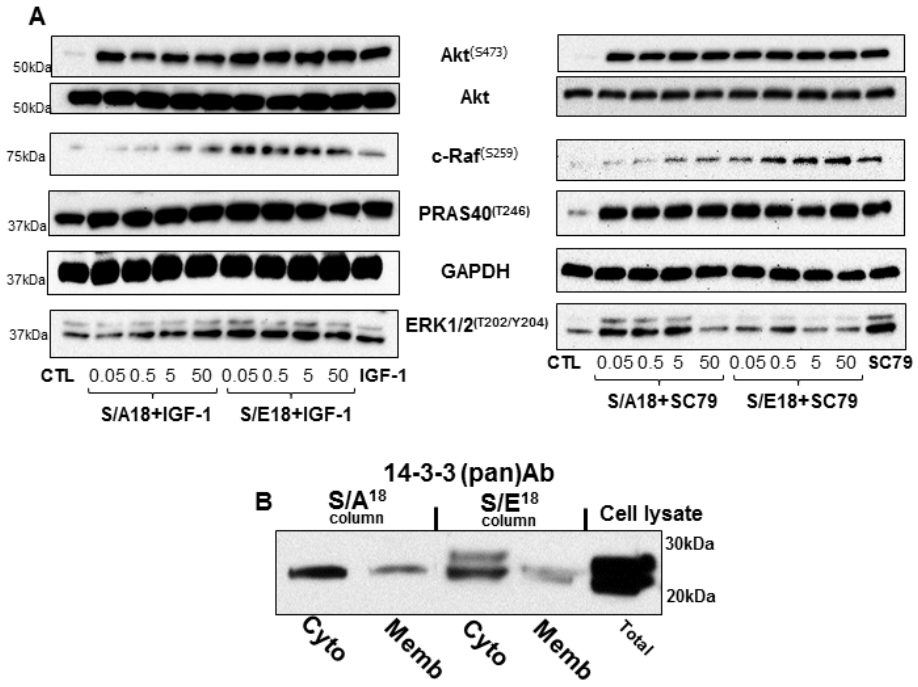

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Fig 3. A. Immunoblots showing phosphorylation of Akt consensus sites that interact with 14-3-3 binding proteins on c-Raf and PRAS40. C2C12 myotubes pretreated with different doses of S/A^{18} and S/E^{18} peptides (ng/ml) followed by 10nM R^{3}IGF-1 and Akt activator SC79 (4μg/ml), for 1hr in 4.7mM KCl + 3mM Ca^{2+} Krebs-Ringer buffer. **B.** Pull-down of 14-3-3 proteins with MGF E-domain peptide affinity columns in C2C12 myotube extracts.

Activation of the IGF-1R recruits' adaptor proteins to the membrane to stimulate two canonical branches of the IGF-1R signaling pathway via PI3K/Akt and Ras/Raf/MEK/ERK activation. Regulation of IGF-1R pathway by 14-3-3 proteins is better understood in other cell types, but little is known about which 14-3-3 isoforms and their substrate specificity in skeletal

myotubes. To determine whether a 14-3-3 protein/E-domain peptide interaction was occurring we cross-linked peptides to agarose affinity columns as “bait”, and incubating cytosolic and membrane fractions from IGF-1 stimulated C2C12 cells. A strong 14-3-3 interaction was detected in the cytosolic fraction with both S/A^{18} and S/E^{18} peptides, but more than one 14-3-3 isoform could be detected interacting with the S/E^{18} peptide when blotted with a pan 14-3-3 antibody **(Figure 3B).** Together these data indicate a potential mechanism exists whereby the E-domain peptides may act as modulators of branches of the IGF-1 signaling pathway through interactions with specific 14-3-3 binding proteins. Preventing phosphorylation within 14-3-3 binding domain in the S/A^{18} peptide blocks Akt mediated inhibition of the Raf/ERK branch, whereas phosphorylation within the 14-3-3 binding domain of the S/E^{18} peptide may augment Akt signaling by shutting down the Raf/ERK

What opportunities for training and professional development has the project provided?

Nothing to report

- **How were the results disseminated to communities of interest?**

  Nothing to report

- **What do you plan to do during the next reporting period to accomplish the goals?**
  - Now that we have a clearer indication of the mechanism of action and the optimal tissue cultures conditions, we will refocus our efforts on examining AS160 phosphorylation at Thr642, which is known to be an Akt specific consensus phosphorylation site that recruits

14-3-3 binding proteins. First, we will examine AS160 Thr642 phosphorylation in response to Akt activation with SC79, Akt activiation by IGF-1 and by insulin in C2C12 myotubes pretreated with the E-domain peptides. This will allow us to nominate which MGF E-domain peptide variant will be most likely to permit Aht mediated signaling to AS160 and GLUT4 translocation for final testing. Following, based on identification of the optimal peptide variant we will move the model back to the L6 GLUT4myc reporter cell line to examine GLUT4 translocation under conditions of membrane depolarization with and without 3mM Ca^{2+}. This will be followed by activating these cells with the Akt activator and via IGF-1 and insulin pathway stimulation.

- Following these in vitro experiments, we will start delivering the optimal E-domain peptide to the MCK-GLUT4myc transgenic mice Ob/ob diabetic mice and while other transgenic crosses and breeding are taking place for final analysis and competition of the study.
-

4. **IMPACT:**

The recognition that the MGF E-domain peptide modulates different branches of the IGF-1R signaling pathway is of great significance in skeletal muscle. Moreover, the identification of mechanism of action of the E-domain peptides involving 14-3-3 binding protein interactions with a Akt concensus phosphorylation sites, is entirely novel and has never been described before.

- **What was the impact on the development of the principal discipline(s) of the project?**
  - Our understanding of muscle cells respond to insulin in diabetes is constantly evolving. Our results to date regarding the role of 14-3-3 binding protein interactions add another level of regulation that has not been fully explored and may serve as a therapeutic target for the development next generation treatments for diabetes.

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- **What was the impact on other disciplines?**
  - Our data examining Akt target proteins identified clear effects on c-Raf activation. Over activation of the Ras-Raf-MEK-ERK pathway has been strongly implicated in the development of cancer. These kinases present new opportunities for the development of novel anti-cancer drugs designed to be target-specific and probably less toxic than conventional chemotherapeutic agents. A number of drugs inhibiting Ras, Raf or MEK are currently under clinical investigation. The ability of the MGF E-domain peptide variants to modulate c-Raf Ser259 phosphorylation by Akt Phosphorylation could help fold the regulatory domain back onto the catalytic domain and maintain c-Raf in an autoinhibited state. In addition to Akt, other kinases phosphorylate on Ser 259 on c-Raf, implying that the E-domain peptides could function as allosteric modulators of c-Raf activity and serve as a pharmacologic inhibitor of cancer promoting pathways in other cell types.
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- **What was the impact on technology transfer?**

  Nothing to report.

- **What was the impact on society beyond science and technology?**

  Nothing to report.

5. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**

  Nothing to report.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

- We encountered initial delays in using and defining the optimal conditions for L6 GLUT4myc reporter skeletal muscle cell lines. We found these cells grew slower and did not differentiate particularly well. We resolved these issues by testing a variety of conditions using the C2C12 skeletal cell lines which allowed a higher throughput approach and allowed us to optimize conditions to demonstrate the peptide effects.
-
- A second significant delay was encounter by the variability we experienced in specific activity of commercially available cytokines such as TNF-alpha. The initial lot purchased from Sigma (Lot 025M4034V) show excellent activity, unfortunately in follow-on experiments requiring the purchase of newly produced lots (Lot 047M4020V), we found the specific activity was significantly reduced. Purchase and testing TNF-alpha from other venders still resulted in lots with poor activity compared to our original lot. At this point we are waiting for Sigma to synthesize a new lot which when it becomes available will be tested for activity against what remains of our original lot.

- **Changes that had a significant impact on expenditures**
  - The greatest impact on expenditures was the purchase of pre-formulated tissue culture media and supplements and commercially available cytokines such as TNF-alpha that were either inconsistent in their biological activity or no suitable to elicit the biological effects that had been reported and initially hypothesized.
  -

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
- Nothing to report.
- **Significant changes in use or care of human subjects**
- Nothing to report.
- **Significant changes in use or care of vertebrate animals.**

- Nothing to report.
- **Significant changes in use of biohazards and/or select agents**
- Nothing to report.

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**

  Nothing to report.

- **Journal publications.**

  Nothing to report.

- **Books or other non-periodical, one-time publications.**

  Nothing to report.

- **Other publications, conference papers, and presentations.**

  Nothing to report.

- **Website(s) or other Internet site(s)**.

  Nothing to report.

  Technologies or techniques

  Nothing to report.

- **Inventions, patent applications, and/or licenses**.
- Nothing to report.

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  - **Other Products**

    In addition to Akt phosphorylate of c-Raf Ser 259 implies that the S/A18 peptide could be developed as a pharmacologic inhibitor of cancer promoting pathways.
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7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	Paul H Goldspink, PhD
Project Role:	PI
Nearest person month worked:	4
Contribution to Project:	Dr. Goldspink directed the overall experimental approach and performed work in the area of cell culture conditions optimization, cell signaling and protein-protein interactions.
Funding Support:	No change
Name:	Rebekah L. Gundry, PhD
Project Role:	Co-investigator
Nearest person month worked:	1
Contribution to Project:	Dr. Gundry directed the experimental approach in the area of mass spectrometry protein identification. She is performing protein ID studies to examine E-domain peptide signaling pathway protein interactions.
Funding Support:	No change

Name:	James Pena
Project Role:	Research Specialist
Nearest person month worked:	8
Contribution to Project:	James performed work in the area of cell culture western blotting, PCR analysis.
Funding Support:	No change

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  - Nothing to Report.
- **What other organizations were involved as partners?**
  - Nothing to Report.

8. **SPECIAL REPORTING REQUIREMENTS**

Nothing to report

9. **APPENDICES:**

Nothing to report