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13. SUPPLEMENTARY NOTES

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 that specifically target c-Raf activity.

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 final stage the project has achieved 80% 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?**

1). Our major activities 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. 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. After a series of different experimental approaches including altering the time course of treatments and peptide dose response we were unable to establish any solid conclusions. This prompted us utilize C2C12 skeletal myotube cultures as a more high throughput approach to help determine optimal working conditions in a skeletal muscle cell lines.

Utilizing gene expression analysis of the orphan hormone nuclear receptor genes NR4A1, 2 and 3, we were able to identify 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 thought to be involved in IGF-1 induced skeletal muscle hypertrophy and regulation of GLUT4 gene expression. Together the data suggesting a link to IGF-1 mediated signaling exists to GLUT4 expression through induction of NR4A family members in muscle which prompted us to focus 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 of NR4A2 expression. The Krebs-ringer buffer allowed for greater control over media ion concentration and osmolality compared to pre-formulated 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.

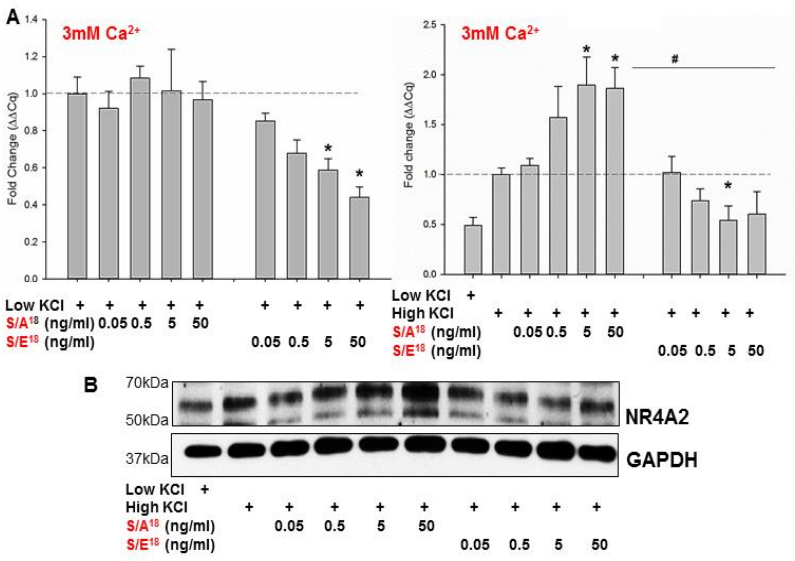

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Fig 1. A. Modulation of NR4A2 mRNA expression in C2C12 myotubes treated with S/A^{18}, S/E^{18} E-domain peptide variants at different doses following depolarization in Krebs-Ringer buffer (97mM KCl +/- 3mM Ca^{2+}) for 1hr (*P<0.05, vs either low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

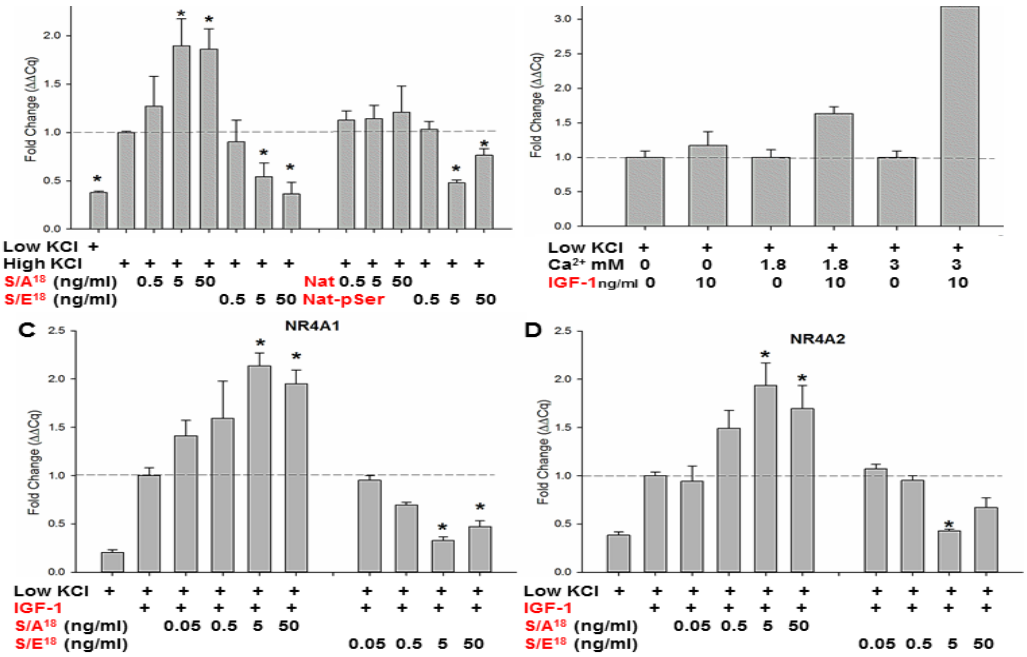

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Fig 2. A. Reciprocal modulation of NR4A2 gene expression treated with S/A^{18}, S/E^{18} peptides compared to Nat, Nat-pSer^{18} peptides following membrane for 1hr **B.** Effect of increasing extracellular Ca^{2+} on 10nM R^{3}IGF-1 stimulation of 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 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 within the E-domain peptide resides within a potential phosphorylation consensus motif. Consequently, we were able to demonstrate that Ser18 phosphorylation modifies peptide activity supporting our initial hypothesis. Consequently, based on these results, we reanalyzed the sequences surrounding Ser18 and its proximity to the polybasic motif within the MGF-domain to determine whether these 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 phosphorylation of Serine 473 and 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 ($Bad^{(S136)}$, $GSK\alpha/\beta^{(S21/9)}$, $FoxO1/3^{(T24/32)}$, $FoxO3a^{(T253)}$ $PRAS40^{(T246)}$), 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.

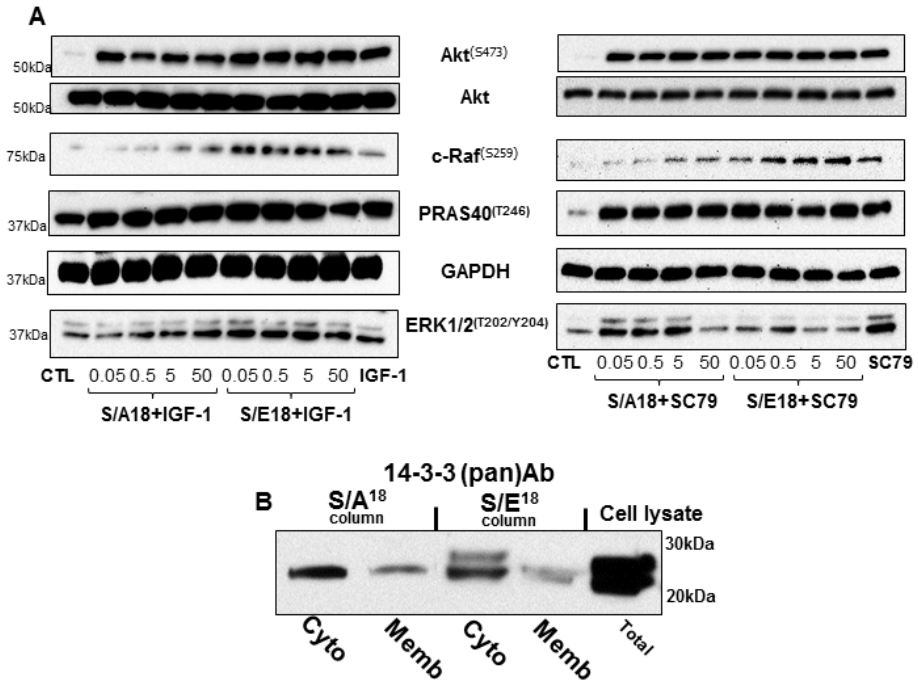

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Fig 3. A. Immunoblots showing phosphorylation of Akt consensus sites that interact with 14-3-3 binding proteins on c-Raf and PRAS40. C2C12 myotubes pretreated with different doses of S/A^{18} and S/E^{18} peptides (ng/ml) followed by 10nM R^{3}IGF-1 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. IGF-1R pathway regulation by 14-3-3 proteins is better understood in other cell types,

with 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.

The studies conducted during the first year of this project we were able to establish two major conclusions with respect to the activity and site of action of the E-domain peptide. These were; 1), the E-domain peptides function as allosteric modulators of Ca^{2+}-dependent actions associated with IGF-1 stimulated signaling into the family of NR4A transcription factors. 2), 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.

In the final six months of the project we focused our attention on the E-domain peptide interaction surrounding c-Raf phosphorylation and the importance of Ser 259 phosphorylation in the regulation of c-Raf activity via 14-3-3 binding proteins. We examined whether activation of other kinases known to phosphorylate Ser 259 were influenced by the E-domain peptides. Focusing on the PKA pathway, and literature showing the importance of PKA activation leading to increases in NR4A gene expression, increases in extracellular Ca^{2+} influx, the release of Ca^{2+} from intracellular stores, the phosphorylation of c-Raf at Ser 259, stimulation of Glut4 activity and

linkage to beta-adrenergic receptor stimulation in striated muscle, we treaded C2C12 myotubes with forskolin to stimulate adenylyl cyclase activity. Analysis of NR4A1-3 gene expression profiles following forskolin and Formoterol (a long acting beta^{2}-adrenergic agonist) treatment, showed robust and significant increases in the expression of all NR4A genes. Treatments in the presence of the S/A^{18} and S/E^{18} E-domain peptide variants dose response, did not demonstrate evidence of reciprocal regulation since the S/A^{18} peptide did not augment expression. However there was a clear and significant inhibition of NR4A1-3 expression following treatment with the S/E^{18} E-domain peptide variant in response to both forskolin and formoterol **(Figure 4A&B)**.

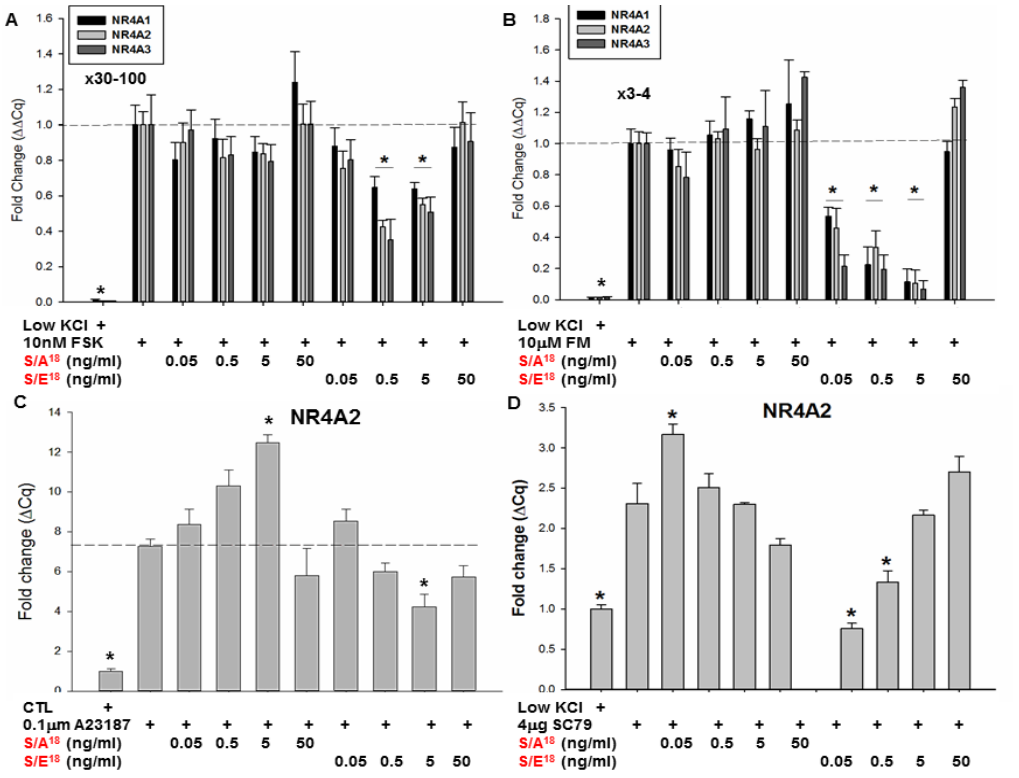

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Fig 4. A. Modulation of NR4A1-3 mRNA expression in C2C12 myotubes treated with S/A^{18}, S/E^{18} E-domain peptide variants at different doses following 10nM forskolin treatment for 1hr. **B.** NR4A1-3 mRNA expression following 10μM formoterol treatment for 1hr. **C.** NR4A2 mRNA expression following Ca2+ ionophore (A23187) treatment. **D.** NR4A2 mRNA expression following Akt activation with SC79 (*P<0.05, vs treatment, n=3).

Since both forskolin and formoterol lead to PKA activation and increase in intracellular Ca^{2+} levels, we examined whether raising intracellular Ca^{2+} alone by use of a calcium ionophore treatment, would be sufficient to induce reciprocal regulation of NR4A1-2 expression profiles. Calcium ionophore treatment alone produce a significant increase in NR4A1-3 expression which in the presence of 5 ng/ml of S/A^{18} and S/E^{18} E-domain peptides was reciprocal whereas other does did not alter the expression profile compared to the ionophore alone **(Figure 4C)**. Finally, considering the E-domain peptide modulation of c-Raf phosphorylation and the downstream ERK pathway we had previously shown in response to Akt activation, we examined whether stimulation of this pathway in isolation would result in the reciprocal regulation of NR4A1-3 expression. Using the small molecule activator of Akt (SC 79), an increase in NR4A2 expression was noted which was sensitive to reciprocal modulation with 0.05 and 0.5ng/ml of E-domain peptide treatments indicating that this Akt/cRaf/ERK signaling branch has direct input into the regulation of NR4A gene expression **(Figure 4D)**. These data also support our previous western blot analysis data using the Akt activator SC79, which shows that the S/A^{18} peptide blocks Akt phosphorylation of c-Raf Ser 259, which would be associated with enhanced activity of the c-Raf and ERK signaling whereas the S/E^{18} facilitates Akt phosphorylation of c-Raf Ser 259, thereby inhibiting its activity and its activation of ERK1/2 signaling.

Since our data showed that the E-domain peptides were targeting the c-Raf/ERK signaling branches to modulate signaling and gene expression profiles, we determined whether their activity was specific to Akt phosphorylation mediated events or whether PKA/Ca^{2+} mediated signaling was also targeted. Western blot analysis of c-Raf Ser 259 phosphorylation and other potential target proteins in response to forskolin treatment without and with extracellular Ca^{2+} present was examined. As shown in Fig 5, c-Raf phosphorylation and MEK1/2 which is immediately downstream were not altered by PKA activation with forskolin treatment either in the presence or absence of extracellular Ca^{2+} **(Figure 5A&B)**. However, ERK1/2 phosphorylation was

suppressed by PKA independent of extracellular Ca^{2+}, which was inhibited by the presence of the S/A^{18} E-domain peptide at lower doses but with not effect of the S/E^{18} peptide noted **(Figure 5C)**. Ribosomal protein S6 was included as a positive control which showed a calcium dependence phosphorylation by PKA which was inhibited by the highest dose of S/A18 peptide**(Figure 5D)**. Together, these data indicate that the E-domain peptide interact with their target c-Raf, to modulate its phosphorylation, activity and interaction with 14-3-3 binding proteins in an Akt specific manner.

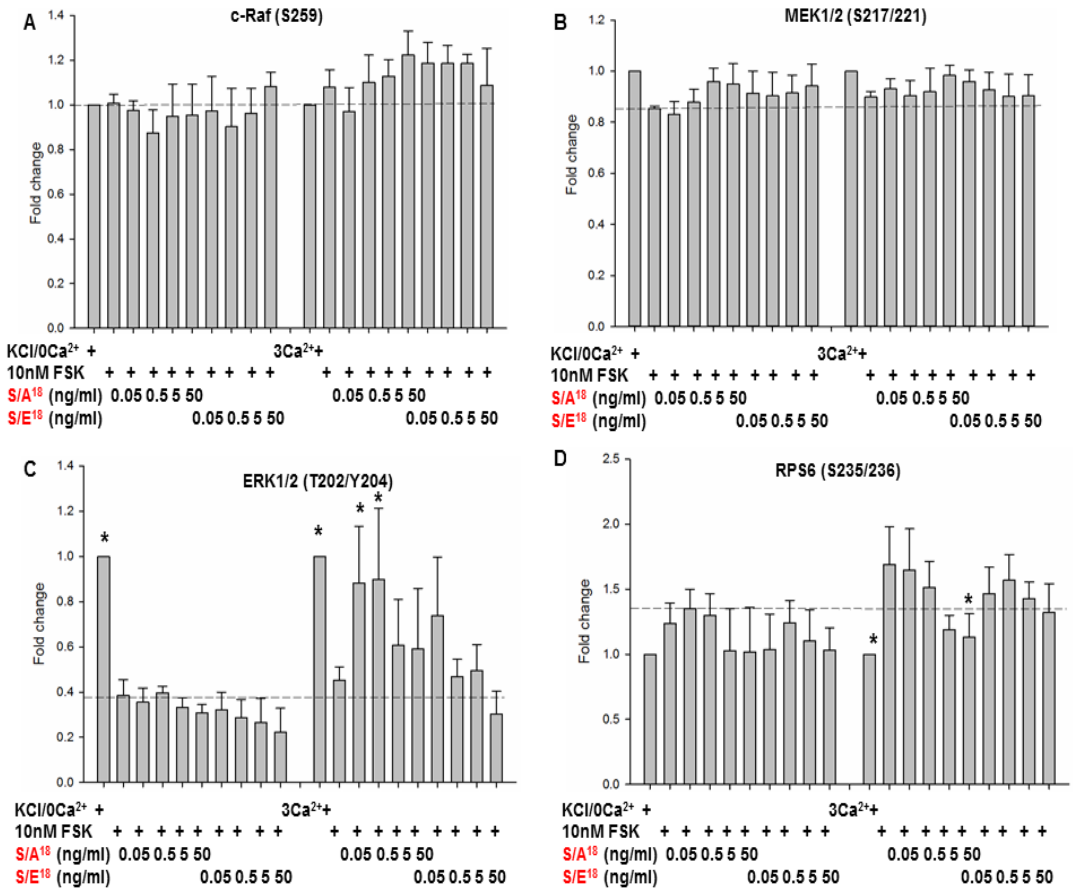

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Fig 5. A. Modulation of c-Raf phosphorylation C2C12 myotubes treated with S/A^{18}, S/E^{18} E-domain peptide variants at different doses following 10nM forskolin treatment for 1hr. **B.** MEK1/2 phosphorylation. **C.** ERK1/2 phosphorylation. **D.** Ribosomal protein S6 phosphorylation (**P*<0.05, vs treatment, n=3).

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?**
- Nothing to report

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 modulating Akt stimulated specific 14-3-3 binding protein interactions on a target protein c-Raf, within the AGC 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.
  -

- **What was the impact on other disciplines?**
  - Our data examining Akt specific 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. Identification of specific kinases and specific 14-3-3 interactions 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 specifically in response to Akt activation could help fold the regulatory domain back onto the catalytic domain and maintain c-Raf in an autoinhibited state and serve as a pharmacologic inhibitor of cancer promoting pathways in other cell types.
  -
- **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**
  - Nothing to report.
- **Changes that had a significant impact on expenditures**
  - Nothing to report.

- **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.
-

- **Other Products**

  The MGF E-domian peptide could be developed as a pharmacologic inhibitor of cancer promoting pathways.

-

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