1 Estradiol-17 β -induced bovine muscle growth

2
3
4
5 Role of G protein-coupled estrogen receptor-1 in estradiol 17 β -induced alterations in protein
6 synthesis and protein degradation rates in fused bovine satellite cell cultures¹

7 E. Kamanga-Sollo², K. J. Thornton³, M. E. White² and W. R. Dayton^{2*}

8 ²Department of Animal Science, University of Minnesota, St. Paul 55108

9 ³Department of Animal, Dairy and Veterinary Science, Utah State University, Logan, UT 84322

10
11
12
13
14 *Corresponding Author: William R. Dayton
15 348 Andrew Boss Laboratory
16 1354 Eckles Avenue
17 Department of Animal Science
18 University of Minnesota
19 St. Paul, MN 55108
20 Phone: 612-624-2234
21 Fax: 612-624-3677
22 E-mail: wdayton@umn.edu
23

24 ¹This material is based upon work that is supported by the National Institute of Food and
25 Agriculture, U.S. Department of Agriculture, under award number 2012-67015-19447 and by the
26 Minnesota Agricultural Experiment Station.

Abstract

In feedlot steers, estradiol-17 β (**E2**) and combined E2 and trenbolone acetate (TBA) (a testosterone analog) implants enhance rate and efficiency of muscle growth; and, consequently, these compounds are widely used as growth promoters in several countries. Treatment with E2 stimulates protein synthesis rate and suppresses protein degradation rate in fused bovine satellite cell (**BSC**) cultures, however the mechanisms involved in these effects are not known with certainty. Although the genomic effects of E2 mediated through the classical estrogen receptors have been characterized, recent studies indicate that binding of E2 to the G protein-coupled estrogen receptor (GPER)-1 mediates non-genomic effects of E2 on cellular function. Our current data show that inhibition of G protein-coupled estrogen receptor (GPER)-1, matrix metalloproteinases 2 and 9 (MMP2/9), or heparin binding epidermal growth factor-like growth factor (hbEGF) suppresses E2-stimulate protein synthesis rate in cultures bovine satellite cells (BSC) ($P < 0.001$) suggesting that all of these are required in order for E2 to stimulate protein synthesis in these cultures. In contrast, inhibition of GPER-1, MMP2/9, or hbEGF has no effect on the ability of E2 to suppress protein degradation rates in fused BSC cultures indicating that these factors are not required in order for E2 to suppress protein degradation rate in these cells. Furthermore, treatment of fused BSC cultures with E2 increased ($P < 0.05$) pAKT levels in fused BSC cultures compared to untreated control cultures, indicating that the pAKT pathway may play a role in E2-stimulated effects on cultured BSC. In summary, our current data show that active GPER-1, MMP2/9, and hbEGF are necessary for E2-stimulated protein synthesis but not for E2-simulated suppression of protein degradation in cultured BSC. Additionally, E2-treatment increases pAKT levels in cultured BSC.

49 **Key Words:** bovine, estrogen, G protein-coupled estrogen receptor-1, muscle, protein turnover,
50 satellite cell, pAKT

51

1. Introduction

Estradiol-17 β (E2) and combined E2 and trenbolone acetate (TBA) (a testosterone analog) implants enhance rate and efficiency of muscle growth in feedlot steers; and, consequently, these compounds are widely used as growth promoters in the United States [1, 2]. Although the positive effects of E2 on rate and efficiency of muscle growth are well established, the mechanisms involved in these effects are not well understood. Since our previous studies have shown that E2 treatment significantly stimulates protein synthesis and inhibits protein degradation in fused bovine satellite cell (BSC) cultures [3], it seems likely that these effects of E2 on protein turnover play a significant role in the ability of E2 to stimulate rate and extent of muscle growth.

Our previous studies have shown that the classical estrogen receptors (ESR) play a significant role in the ability of E2 to affect rates of protein synthesis and degradation in cultured BSC; however, studies in nonmuscle cells have also shown that a non-genomic mechanism involving binding of E2 to G protein-coupled estrogen receptor (GPER)-1 results in activation of matrix metalloproteinases 2 and 9 (MMP2/9) resulting in proteolytic release of heparin binding epidermal growth factor-like growth factor (hbEGF) from the cell surface. Released hbEGF binds to and activates the epidermal growth factor receptor (EGFR) resulting in increased proliferation [4-9]. We have recently reported that this mechanism also may play a role in E2-stimulated proliferation of cultured BSC [10]. In order to ascertain if these receptors must also be active in order for E2 to affect muscle protein turnover, we have evaluated the effects of inhibiting GPER-1, MMP2/9 or hbEGF on the ability of E2 to stimulate protein synthesis rate and decrease protein degradation rate in fused BSC cultures. Additionally, because treatment of many different cell types with E2 has been shown to activate the AKT pathway [11] and

75 activation of this pathway is known to increase skeletal muscle hypertrophy [12], we have
76 analyzed the effect of E2 treatment on pAKT levels in fused BSC cultures.

77

78

2. Materials and Methods

2.1 Bovine satellite cell isolation

Satellite cell isolation was done as described previously [13-15]. Yearling steers that had never received steroid implants were sacrificed by bolting followed by exsanguination. Using sterile techniques, approximately 1 kg of the semimembranosus muscle was dissected out and transported to the cell culture laboratory. Subsequent procedures were conducted in a sterile field under a tissue culture hood. After removal of connective tissue, the muscle was passed through a sterile meat grinder. The ground muscle was incubated with 0.1% Pronase in Earl's Balanced Salt Solution (EBSS) for 1 h at 37 °C with frequent mixing. Following incubation, the mixture was centrifuged at 1500 x g for 4 min, the pellet was suspended in phosphate buffered saline (PBS: 140 mM NaCl, 1 mM KH₂PO₄, 3 mM KCl, 8 mM Na₂HPO₄, pH 7.4) and the suspension was centrifuged at 500 x g for 10 min. The resultant supernatant was centrifuged at 1500 x g for 10 min to pellet the mononucleated cells. The PBS wash and differential centrifugation were repeated two more times. The resulting mononucleated cell preparation was suspended in cold (4 °C) Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS and 10% (v/v) dimethylsulfoxide (DMSO) and frozen. Cells were stored frozen in liquid nitrogen for use in future studies. Clonal analysis of satellite cell cultures established from these preparations showed that between 80 and 90% of the cells were able to establish colonies that contained fusion, indicating that between 80 and 90% of the cells in the preparations were myogenic.

2.2 Bovine satellite cell culture

Bovine satellite cell preparations were plated on 9.6 cm² culture dishes precoated with reduced growth factor basement membrane Matrigel (Corning, Tewksbury, MA) diluted 1:10 (v/v) with DMEM as described previously [16]. Media used in this study did not contain phenol red unless otherwise indicated. Plating density was empirically established so that all cultures were approximately 50% confluent after 72 h in culture. Cells were plated in DMEM containing 10% fetal bovine serum (**FBS**) and incubated at 37 °C, 5% CO₂ in a water-saturated environment. Cultures were fed at 72 h with DMEM containing 10% FBS and at 96 h with a fusion-promoting medium that consisted of DMEM with 3% horse serum and 1.5 mg bovine serum albumin (**BSA**)-linoleic acid/mL. At 120 h, cytosine arabinoside (final concentration 10⁻⁶ M) was added to the culture media. Fusion percentage at 144 h was typically 75-80%.

2.3 Protein synthesis rate

Protein synthesis rate in fused BSC cultures was determined as described previously [3]. At 144 h in culture, fused BSC cultures established as described in section 2.2 were rinsed and treated with serum free medium (**SFM**) (DMEM containing 250 µg insulin-like growth factor binding protein (IGFBP)-3-free fetuin/mL, 100 µg BSA-linoleic acid/mL (Sigma Aldrich, St. Louis, MO), and 10⁻⁹ M insulin) plus the indicated additions of E2 (Steraloids, Inc., Newport, RI), LR3-IGF-1 (GroPep Bioreagents Pty Ltd, Thebarton, SA, Australia, Cat# BU100) (an insulin-like growth factor (**IGF**)-1 analogue that binds normally to the IGFR-1 but has little or no affinity for IGF binding proteins) [17], the GPER-1-specific inhibitor G36 [18] (Azano Pharmaceuticals, Inc. Albuquerque, NM), the MMP-2/MMP-9 Inhibitor II (MMPI), [19] (Calbiochem, Billerica, MA, Catalog no. 444249) or the hbEGF inhibitor CRM197 [20] (Sigma-Aldrich). After 3 h of incubation, 3 µCi of ³H-phenylalanine were added to each culture, and incubation was continued for an additional 3 h. After this incubation period, medium was

removed, and cells were washed with DMEM and incubated overnight at 4 °C in 5% trichloroacetic acid (TCA). Cells were then washed 2 times with 5% TCA and lysed with 1 N NaOH containing 1% Triton X-100. Total protein was quantified using the Pierce® BSA Protein Assay Kit (ThermoScientific, Waltham, MA) on a BioTek Synergy 2 plate reader (BioTek, Winooski, VT), and ³H-phenylalanine was measured by counting an aliquot of the cell lysate in a Beckman LS-3801 liquid scintillation counter. For each individual assay, protein synthesis rate was calculated as ³H-phenylalanine incorporation/mg of protein for each well. All experiments contained triplicate determinations. To facilitate statistical analysis of combined data from multiple experiments, synthesis rates in each assay were normalized by expressing them relative to the synthesis rate in SFM-treated cultures included in each assay.

2.4 Protein degradation rate

Protein degradation rates in fused BSC cultures were determined as described previously [3]. At 120 h in culture, BSC cultures established as described in section 2.2 were labeled for 24 h with 3 µCi of ³H-phenylalanine. At 144 h, the label was removed and cells were subjected to a 4-h chase in DMEM containing 1% IGFBP-3-free swine serum (SSS) (SSS was prepared by passing sera obtained from 6-wk-old male pigs castrated within 1 week of birth through an IGFBP-3 immunoaffinity column [21]) and 2 nM cold phenylalanine. At the end of this incubation, the chase medium was removed, the cells were washed, and test medium consisting of DMEM containing 1% SSS plus the indicated amounts of E2, LR3-IGF-1, G36, MMPI, and/or CRM197 were added to the cultures and allowed to incubate for 24 h. At the end of this incubation period, medium was quantitatively removed from the cells and 20% TCA was added to the medium to give a final concentration of 10% TCA. The TCA-insoluble material was collected by centrifugation; the pellet was then washed once with 10% TCA, and the wash was

combined with the soluble medium fraction. The pellet was solubilized in 1 N NaOH/1% Triton X-100 by heating at 42 °C for 30 min. The cells were precipitated with 10% TCA and solubilized in 1 N NaOH/1% Triton X-100 by heating at 42 °C for 30 min. Percentage protein degradation was calculated as (TCA soluble counts/total TCA soluble and insoluble counts) × 100 in each well. All individual experiments contained triplicate determinations. To facilitate statistical analysis of combined data from multiple experiments, degradation rates in each assay were normalized by expressing them relative to the degradation rate in SSS-treated cultures included in each assay.

2.5 Western blot analysis of pAKT

Cultures were grown and induced to fuse as described in section 2.2. At 144 h in culture, media was removed and cells were washed once with DMEM and then placed in fresh DMEM for 30 min. After this 30 min suppression period, previous media were removed and fresh DMEM containing either no treatment, 10 nM E2, 5ng/mL hbEGF or 25ng/mL LR3-IGF-I was added to individual cultures for 30 min. Culture plates were then immediately placed on ice, rinsed once with PBS and lysed in a total protein extraction buffer; 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid , 1 mM sodium fluoride, 1 mM polymethane sulfonyl fluoride , 1 mM sodium orthovanadate and 1% Nonidet p-40. Seventy five microliters of extraction buffer were used to pool cellular material from three 2-cm² culture wells. The samples were then centrifuged at 10,000 g for 10 min at 4 °C and the supernatant was removed and stored at -80 °C. Total protein was quantified using the Pierce® BSA Protein Assay Kit (ThermoScientific) on a BioTek Synergy 2 plate reader (BioTek). Samples were electrophoresed on 10% SDS polyacrylamide gels and then electrophoretically transferred to a nitrocellulose membrane. The level of pAKT was determined by incubation of the membrane

overnight at 4 °C with a rabbit anti-mouse pAKT antibody (Cell Signaling; Danvers, MA; Catalog no. 9271) (1:1000 dilution in TBS with 0.05% Tween 20 (TBST) containing 5% nonfat dried milk). The membrane was then rinsed 6 times for 5 min each in TBST after which time the membrane was incubated for 1 h at room temperature with HRP-conjugated goat anti-rabbit IgG (Cell Signaling; Catalog no. 7074) diluted 1:2,000. Membranes were then washed another 6 times for 5 min each and then placed in Western Sure Premium Chemiluminescent Substrate (LiCor, Lincoln, NE) and read on the C-Digit blot scanner (LiCor) using high sensitivity. Blots were then stripped using RestoreTM Western Blot stripping buffer (ThermoFisher Scientific, Grand Island, NY) as per the manufacturer's protocol and blotted for tubulin. Membranes were blotted as described above. In brief, membranes were incubated with a rabbit anti-human β -tubulin antibody (Cell Signaling) at a dilution of 1:2,000 in TBST + 5% BSA overnight at 4 °C. Membranes were then washed in TBST 6 times for 5 min each and incubated for 1 h in at room temperature with HRP-conjugated goat anti-rabbit IgG (Cell Signaling; Catalog no. 7074) diluted 1:4,000. Membranes were then washed another 6 times for 5 min each and then placed in Western Sure Premium Chemiluminescent Substrate (LiCor) and read on the C-Digit blot scanner (LiCor). All Western blot data obtained for pAKT level were normalized to Tubulin level.

2.6 Statistical analysis

Statistical analysis was completed using the MIXED procedure of SAS (Version 9.4; SAS Institute Inc, Cary, NC). All data are presented as the least squares mean $\pm$ standard error of the mean. Data from multiple assays performed on cells isolated from different animals were combined. Each assay was replicated at least three times and each assay contained cells from a different steer. Preliminary analyses indicated that there were no effects ($P > 0.05$) observed for

193 either the assay number or the different animals; as such these two factors were included as
194 random variables in the model. The model included treatment as a fixed effect and assay number
195 and the animal the BSC were isolated from as random effects. When treatment differences were
196 found to be significant ($P < 0.05$), least squares means were separated using Tukey-Kramer
197 adjustments ($P < 0.05$).
198

3. Results

3.1 Effect of inhibiting GPER-1 activity on E2-stimulated protein synthesis and E2-suppressed protein degradation in fused BSC cultures

Both E2 and LR3-IGF-1 stimulated protein synthesis rate and suppressed protein degradation rate in fused BSC cultures ($P < 0.01$) (Fig. 1-3). In order to assess the effect and specificity of G36 inhibition of GPER-1, we analyzed the effect of G36 treatment on the effects of both E2- and LR3-IGF-1 treatments on rates of protein synthesis and degradation in fused BSC cultures. Inhibition of GPER-1 by treatment of BSC cultures with 20 nM G36 completely suppressed the stimulatory effect of E2 on protein synthesis rate ($P < 0.01$) (Fig. 1A) but had no effect on LR3-IGF-1-stimulated protein synthesis rate (Fig. 1A). In contrast G36 treatment had no effect on the ability of either E2 or LR3-IGF-1 to suppress protein degradation rate in fused BSC cultures (Fig. 1B). Treatment with G36 did not affect either protein synthesis rate (Fig. 1A) or protein degradation rate (Fig. 1B) in control cultures incubated in SFM or SSS, respectively.

3.2 Effect of inhibiting MMP2/9 activity on E2-stimulated protein synthesis and E2-suppressed protein degradation in fused BSC cultures

Inhibition of MMP2/9 activity by treatment with 10 μ M MMPI significantly ($P < 0.001$) suppressed both E2- and LR3-IGF-1-stimulated protein synthesis rates in fused BSC cultures (Fig. 2A). In contrast, inhibition of MMP2/9 activity did not affect the ability of either E2 or LR3-IGF-1 to suppress protein degradation rate in fused BSC cultures (Fig. 2B). Treatment with MMPI did not affect either protein synthesis rate (Fig. 2A) or protein degradation rate (Fig. 2B) in control cultures incubated in SFM or SSS, respectively.

222 *3.3 Effect of inhibiting hbEGF activity on E2-stimulated protein synthesis and E2-suppressed*
223 *protein degradation in fused BSC cultures*

224 Treatment with 1 µg CRM197/mL completely inhibited E2-stimulated increases in
225 protein synthesis rate ($P < 0.01$) but had no effect on the LR3-IGF-1-stimulated increase in
226 protein synthesis rate in fused BSC cultures (Fig. 3A). Treatment of fused BSC cultures with
227 CRM197 had no effect on the ability of either E2 or LR-3-IGF-1 to suppress protein degradation
228 rate (Fig. 3B). Treatment with CRM197 did not affect either protein synthesis rate (Fig. 3A) or
229 protein degradation rate (Fig. 3B) in control cultures incubated in SFM or SSS, respectively.

231 *3.4 Changes in pAKT protein level in fused BSC cultures after E2 treatment*

232 Fused BSC cultures treated with E2, hbEGF or LR3-IGF-1 for 30 min demonstrate
233 increases ($P < 0.05$) in pAKT protein level when compared to control cultures receiving no
234 treatment (Fig. 4). Protein synthesis assays were completed concurrently with the Western blot
235 analyses to ensure that E2, hbEGF and LR3-IGF-1 treated cultures demonstrated increases in
236 protein synthesis rate (data not shown). These data indicate that the AKT pathway is activated by
237 E2 treatment in fused BSC cultures.

4. Discussion

We have previously shown that treatment of fused BSC cultures with E2 increases protein synthesis rate and decreases protein degradation rate; however, the mechanisms by which E2 accomplishes these effects are not well defined. The ESR1 and ESR2 antagonist ICI 182 780 suppresses E2-induced alterations in protein synthesis and degradation in fused BSC cultures [3]. Additionally, JB1, a competitive inhibitor of IGF-1 binding to the type 1 insulin-like growth factor receptor (IGFR-1), suppresses E2-induced alterations in protein synthesis and degradation [3]. These data show that classical E2 receptors (ESR1 and ESR2) and IGFR-1 may play a role in the effects of E2 on protein synthesis and degradation rates in fused BSC cultures. We have recently shown that the GPER-1, MMP2/9 and EGFR play a role in E2-stimulated proliferation in BSC cultures [22]. Here we examine whether they must also be active in order for E2 to alter protein synthesis and protein degradation rates in fused BSC cultures.

Our study shows that when GPER-1 activity is inhibited by G36, the E2- stimulated increase in protein synthesis rate is completely inhibited indicating that GPER-1 is required in order for E2 to stimulate protein synthesis rate in fused BSC cultures. In contrast, G36 has no effect on the ability of LR3-IGF-1 to stimulate protein synthesis rate in fused BSC cultures indicating that inhibition of GPER-1 does not have a general, non-specific inhibitory effect on BSC protein synthesis. Treatment of fused BSC cultures with G36 has no effect on the ability of either E2 or LR3-IGF-1 to suppress protein degradation in fused BSC cultures indicating that active GPER-1 is not required in order for E2 to suppress protein degradation in these cells.

Treatment with the hbEGF inhibitor CRM197 suppresses E2-stimulated protein synthesis in fused BSC cultures indicating that active hbEGF is required in order for E2 to stimulate protein synthesis in these cultures. In contrast, treatment with CRM197 does not suppress LR3-

IGF-1-stimulated protein synthesis, indicating that CRM197 does not have a general, non-specific effect on BSC protein synthesis. Treatment with CRM197 does not affect the ability of either E2 or LR3-IGF-1 to suppress protein degradation in fused BSC cultures indicating that active hbEGF is not required for either E2 or LR3-IGF-1 to suppress protein degradation rate in these cultures.

Numerous studies have shown that MMP2/9 are involved in proliferation and migration of a number of cell types including muscle satellite cells [23]. Studies in nonmuscle cells and in smooth muscle cells have shown that activated MMP2/9 proteolytically release hbEGF from the cell surface. Released hbEGF binds to the EGFR causing EGFR transactivation which mediates downstream pathways that regulate cell proliferation [4-9]. In light of our data showing that hbEGF stimulates protein synthesis rate in fused BSC, the fact that MMP2/9 inhibition suppresses E2-stimulated protein synthesis rate in fused BSC is consistent with the hypothesis that E2 binding to GPER-1 activates MMP2/9 resulting in proteolytic release of membrane bound hbEGF and subsequent activation of the EGFR. However, unlike the results obtained when GPER-1 or hbEGF activity is specifically inhibited, inhibition of MMP2/9 not only inhibits E2-stimulated protein synthesis in fused BSC but also inhibits LR3-IGF-1-stimulated protein synthesis rate. These data raise the possibility that the role of MMP2/9 in E2-stimulated protein synthesis in fused BSC may not be limited to proteolytic release of hbEGF. This possibility is supported by studies showing that MMPs are involved in extracellular matrix alterations involved in proliferation and migration of satellite cells [23]. Consequently, although our data show that active MMP2/9 is necessary for E2-stimulated protein synthesis in fused BSC cultures, they do not conclusively establish that the specific role of MMP2/9 is proteolytic release of hbEGF. Additionally, the fact that MMPI treatment does not affect the ability of either E2 or

LR3-IGF-1 to suppress protein degradation in fused BSC cultures indicates that active MMP2/9 are not required in order for either E2 or LR3-IGF-1 to suppress protein degradation rate in these cultures.

Both proliferation of satellite cells and protein synthesis and degradation in developing muscle fibers play crucial roles in muscle growth and development in beef cattle [24]. Our previous studies [25, 26] and results of the current study indicate that active GPER-1, MMP2/9 and hbEGF are required to support E2-mediated increases in both proliferation of mononucleated satellite cells and protein synthesis rate in fused BSC, while E2-mediated decreases in protein degradation rate in fused BSC does not require activity of these factors. We believe it is significant that E2 regulation of two dissimilar processes such as proliferation rate in unfused proliferating satellite cells and protein synthesis rate in fused BSC requires active GPER-1, MMP2/9 and hbEGF while the effect of E2 on protein degradation rate in fused BSC does not require these factors. Further studies are required to identify the intracellular signaling pathways activated by E2 to elicit the observed effects on these important parameters of muscle growth. As an initial step toward this goal, the present study found that treatment of fused BSC cultures with E2 increases pAKT level suggesting that the AKT pathway is involved in E2-mediated effects on fused BSC. The PI3K/AKT pathway has been shown to be involved in E2 mediated increases in cardiomyocyte growth [11] . Additionally, the AKT intracellular signaling pathway is known to be a crucial regulator of skeletal muscle hypertrophy in several different species [27, 28]. Our current results are the first evidence that the AKT pathway may be involved in E2-mediated alterations in fused BSC cultures. Additional studies are required to further elucidate the role of specific intracellular signaling pathways on E2-mediated proliferation in mononucleated BSC and on protein synthesis and degradation rates in fused BSC.

307

308 In summary, our results show that active GPER-1, hbEGF and MMP2/9 are required for
309 E2-stimulated protein synthesis rate in fused BSC cultures. These results are consistent with our
310 hypothesis that binding of E2 to GPER-1 results in activation of MMP2/9 resulting in proteolytic
311 release of cell surface bound hbEGF which then binds to and activates EGFR resulting in
312 increased protein synthesis rate. In contrast neither active GPER-1, hbEGF nor MMP2/9 appear
313 to be required in order for E2 to suppress protein degradation rate in fused BSC cultures. It also
314 appears that the AKT intracellular signaling pathway is activated after E2 treatment of fused
315 BSC cultures.

316

317

318

REFERENCES

- [1] Hunt DW, Henricks DM, Skelley GC, Grimes LM. Use of trenbolone acetate and estradiol in intact and castrate male cattle: effects on growth, serum hormones, and carcass characteristics. *J Anim Sci.* 1991;.69:2452-2462.
- [2] Johnson BJ, Anderson PT, Meiske JC, Dayton WR. Effect of a combined trenbolone acetate and estradiol implant on steroid hormone levels, feedlot performance, carcass characteristics and carcass composition of feedlot steers. *J Anim Sci.* 1996;74:363-371.
- [3] Kamanga-Sollo E, White ME, Hathaway MR, Weber WJ, Dayton WR. Effect of estradiol-17beta on protein synthesis and degradation rates in fused bovine satellite cell cultures. *Domest Anim Endocrinol.* 2010;39:54-62.
- [4] Filardo EJ, Quinn JA, Bland KI, Frackelton AR. Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Mol Endocrinol.* 2000; 14:1649-1660.
- [5] Filardo E, Quinn J, Pang Y, Graeber C, Shaw S, Dong J, Thomas P. Activation of the novel estrogen receptor G protein-coupled receptor 30 (GPR30) at the plasma membrane. *Endocrinology.* 2007;148:3236-3245.
- [6] Prossnitz ER, Arterburn JB, Sklar LA. GPR30: A G protein-coupled receptor for estrogen. *Mol Cell Endocrinol.* 2007; 265:138-142.

- 339 [7] Roudabush FL, Pierce KL, Maudsley S, Khan KD, Luttrell LM. Transactivation of the
340 EGF receptor mediates IGF-1-stimulated shc phosphorylation and ERK1/2 activation in
341 COS-7 cells. *Sci Signal*. 2000;275:22583.
- 342 [8] El-Shewy HM, Kelly FL, Barki-Harrington L, Luttrell LM. Ectodomain shedding-
343 dependent transactivation of epidermal growth factor receptors in response to insulin-like
344 growth factor type I. *Mol Endocrinol*. 2004;18:2727-2739.
- 345 [9] Prossnitz ER, Barton M. The G-protein-coupled estrogen receptor GPER in health and
346 disease. *Nat Rev Endocrinol*. 2011;7:715-726.
- 347 [10] Kamanga-Sollo E, Thornton KJ, White ME, Dayton WR. Role of G protein-coupled
348 estrogen receptor-1, matrix metalloproteinases 2 and 9, and heparin binding epidermal
349 growth factor-like growth factor in estradiol-17beta-stimulated bovine satellite cell
350 proliferation. *Domest Anim Endocrinol*. 2014;49C:20-26.
- 351 [11] Patten RD, Pourati I, Aronovitz MJ, Baur J, Celestin F, Chen X, Michael A, Haq S,
352 Nuedling S, Grohe C, Force T, Mendelsohn ME, Karas RH. 17beta-estradiol reduces
353 cardiomyocyte apoptosis in vivo and in vitro via activation of phospho-inositide-3
354 kinase/Akt signaling. *Circ Res*. 2004;95:692-699.
- 355 [12] Lai KM, Gonzalez M, Poueymirou WT, Kline WO, Na E, Zlotchenko E, Stitt TN,
356 Economides AN, Yancopoulos GD, Glass DJ. Conditional activation of akt in adult skeletal
357 muscle induces rapid hypertrophy. *Mol Cell Biol*. 2004;24:9295-9304.

358 [13] Frey RS, Johnson BJ, Hathaway MR, White ME, Dayton WR. Growth factor
359 responsiveness of primary satellite cell cultures from steers implanted with trenbolone
360 acetate and estradiol-17b. *BAM*. 1995;5:71-79.

361 [14] Hathaway MR, Hembree JR, Pampusch MS, Dayton WR. Effect of transforming growth
362 factor beta-1 on ovine satellite cell proliferation and fusion. *J Cell Physiol*. 1991;146:435-
363 441.

364 [15] Hathaway MR, Pampusch MS, Hembree JR, Dayton WR. Transforming growth factor
365 beta-1 facilitates establishing clonal populations of ovine muscle satellite cells. *J Anim Sci*.
366 1994;72:2001-2007.

367 [16] Johnson BJ, Halstead N, White ME, Hathaway MR, DiCostanzo A, Dayton WR.
368 Activation state of muscle satellite cells isolated from steers implanted with a combined
369 trenbolone acetate and estradiol implant. *J Anim Sci*. 1998;76:2779-2786.

370 [17] Francis GL, Ross M, Ballard FJ, Milner SJ, Senn C, McNeil KA, Wallace JC, King R,
371 Wells JRE. Novel recombinant fusion protein analogues of insulin-like growth factor
372 (IGF)-I indicate the relative importance of IGF-binding protein and receptor binding for
373 enhanced biological potency. *J Mol Endocrinol*. 1992;8:213-223.

374 [18] Dennis MK, Field AS, Burai R, Ramesh C, Petrie WK, Bologna CG, Oprea TI, Yamaguchi
375 Y, Hayashi S, Sklar LA, Hathaway HJ, Arterburn JB, Prossnitz ER. Identification of a
376 GPER/GPR30 antagonist with improved estrogen receptor counterselectivity. *J Steroid*
377 *Biochem Mol Biol*. 2011;127:358-366.

378 [19] Tamura Y, Watanabe F, Nakatani T, Yasui K, Fuji M, Komurasaki T, Tsuzuki H, Maekawa
379 R, Yoshioka T, Kawada K, Sugita K, Ohtani M. Highly selective and orally active
380 inhibitors of type IV collagenase (MMP-9 and MMP-2): N-sulfonylamino acid derivatives.
381 J Med Chem. 1998;41:640-649.

382 [20] Mitamura T, Higashiyama S, Taniguchi N, Klagsbrun M, Mekada E. Diphtheria toxin
383 binds to the epidermal growth factor (EGF)-like domain of human heparin-binding EGF-
384 like growth factor/diphtheria toxin receptor and inhibits specifically its mitogenic activity. J
385 Biol Chem. 1995;270:1015-1019.

386 [21] Kamanga-Sollo E, Pampusch MS, Xi G, White ME, Hathaway MR, Dayton WR. IGF-I
387 mRNA levels in bovine satellite cell cultures: Effects of fusion and anabolic steroid
388 treatment. J Cell Physiol. 2004;201:181-189.

389 [22] Kamanga-Sollo E, Thornton KJ, White ME, Dayton WR. Role of G protein-coupled
390 estrogen receptor-1, matrix metalloproteinases 2 and 9, and heparin binding epidermal
391 growth factor-like growth factor in estradiol-17beta-stimulated bovine satellite cell
392 proliferation. Domest Anim Endocrinol. 20014;49C:20-26.

393 [23] Montarras D, L'honore A, Buckingham M. Lying low but ready for action: the quiescent
394 muscle satellite cell. FEBS J. 2013;280:4036-4050.

395 [24] Dayton WR, White ME. MEAT SCIENCE AND MUSCLE BIOLOGY SYMPOSIUM--
396 role of satellite cells in anabolic steroid-induced muscle growth in feedlot steers. J Anim
397 Sci. 2014;92:30-38.

- [25] Kamanga-Sollo E, Thornton KJ, White ME, Dayton WR. Role of G protein-coupled estrogen receptor-1, matrix metalloproteinases 2 and 9, and heparin binding epidermal growth factor-like growth factor in estradiol-17beta-stimulated bovine satellite cell proliferation. *Domest Anim Endocrinol.* 2014;49:20-26.
- [26] Reiter BC, Kamanga-Sollo E, Pampusch MS, White ME, Dayton WR. Epidermal growth factor receptor is required for estradiol-stimulated bovine satellite cell proliferation. *Domest Anim Endocrinol.* 2014;48:48-55.
- [27] Schiaffino S, Mammucari C. Regulation of skeletal muscle growth by the IGF1-Akt/PKB pathway: insights from genetic models. *Skelet Muscle.* 2011;1-4.
- [28] Lai KM, Gonzalez M, Poueymirou WT, Kline WO, Na E, Zlotchenko E, Stitt TN, Economides AN, Yancopoulos GD, Glass DJ. Conditional activation of akt in adult skeletal muscle induces rapid hypertrophy. *Mol Cell Biol.* 2004;24:9295-9304.

Figure Legends

Figure 1: Effect of G36 (GPER-1 inhibitor) on E2- or LR3-IGF-1-induced alterations in protein synthesis rate (**A**) and protein degradation rate (**B**) in fused bovine satellite cell cultures. Protein synthesis and protein degradation rates were determined as described in the Materials and Methods (Section 2.3 & 2.4, respectively). To facilitate pooling multiple assays for statistical analysis, data from individual assays were normalized to control cultures in the same assay. Triplicate determinations were performed for each assay and normalized data from 4 separate assays for protein synthesis and 6 separate assays for protein degradation done on cells from three different animals, were statistically pooled to yield the data shown. Bars containing no letters in common are significantly different ($P < 0.01$).

Figure 2: Effect of MMP2/9 inhibitor (MMPI) on E2- or LR3-IGF-1-induced alterations in protein synthesis rate (**A**) and protein degradation rate (**B**) in fused bovine satellite cell cultures. Protein synthesis and protein degradation rates were determined as described in the Materials and Methods (Section 2.3 & 2.4, respectively). To facilitate pooling multiple assays for statistical analysis, data from individual assays were normalized to control cultures in the same assay. Triplicate determinations were performed for each assay and normalized data from 6 separate assays for protein synthesis and 4 separate assays for protein degradation done on cells from three different animals, were statistically pooled to yield the data shown. Bars containing no letters in common are significantly different ($P < 0.01$).

Figure 3: Effect of CRM197 (hbEGF inhibitor) on E2- or LR3-IGF-1-induced alterations in protein synthesis rate (**A**) and protein degradation rate (**B**) in fused bovine satellite cell cultures. Protein synthesis and protein degradation rates were determined as described in the Materials and Methods (Section 2.3 & 2.4, respectively). To facilitate pooling multiple assays for statistical

analysis, data from individual assays were normalized to control cultures in the same assay. Triplicate determinations were performed for each assay and normalized data from 4 separate assays for protein synthesis and 4 separate assays for protein degradation done on cells from three different animals, were statistically pooled to yield the data shown. Bars containing no letters in common are significantly different ($P < 0.05$).

Figure 4: A) Representative western immunoblot showing pAKT levels in fused cultures treated in DMEM and 5 ng/mL hbEGF, 10 nM E2 or 25 ng/mL LR3-IGF-1 for 30 min. Cultures were grown as described in the Materials and Methods (section 2.2) for 144 h. Cultures were then rinsed once with DMEM, placed in DMEM for a 30 min deprivation period and then placed into treatment medias for an additional 30 min. After this treatment period, protein was collected and stored at -80 °C. Later, proteins were separated on a 10% SDS-PAGE gel and then transferred to a nitrocellulose membrane. Membranes were blotted for pAKT (Cell Signaling, Catalog No. 2128), stripped and then blotted again for tubulin (Cell Signaling; Catalog no. 7074). Images were obtained using the C Digit Plus (Licor). **B)** Graph of pAKT level in bovine satellite cell cultures treated as described in A. All pAKT values were normalized to the tubulin values and to facilitate pooling multiple assays for statistical analysis, data from individual assays were normalized to the pAKT levels in DMEM-treated cultures (control) for each assay. Results are reflective of the average of eight different assays. Assays were performed utilizing cells isolated from six different animals. Bars containing no letters in common are statistically different from one another ($P < 0.01$).

Figure

A

Fig. 1B

Fig. 2a

Fig. 2B

Fig. 3A

Fig. 3B

Fig. 4