MECHANISM OF ANABOLIC STEROID STIMULATED BOVINE MUSCLE GROWTH: ROLE OF THE EPIDERMAL GROWTH FACTOR RECEPTOR

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ABSTRACT

Estradiol (E2) enhances muscle growth in a number of species; however, the mechanism by which E2 enhances muscle growth is not known. Treatment with 10 nM E2 stimulates proliferation and protein synthesis and inhibits protein degradation in cultured bovine satellite cells (BSC). This is particularly significant because satellite cells are the source of nuclei needed to support postnatal muscle fiber hypertrophy and are thus crucial in determining the rate and extent of muscle growth; however the mechanism responsible for these E2 induced effects is not clear. Studies in other tissues have suggested that E2 may stimulate proliferation by activating the G protein-coupled estrogen receptor (GPER)-1 (formerly known as G protein-coupled receptor 30). Activation of GPER-1 results in activation of matrix metalloproteinases which release heparin-binding epidermal growth factor (hb-EGF) from the cell membrane and released hb-EGF interacts with the Epidermal Growth Factor Receptor (EGFR) resulting in increased proliferation. As an initial step in determining if this mechanism is involved in the effects of E2 on bovine satellite cell cultures, we have shown that silencing EGFR expression utilizing a specific EGFR siRNA suppresses the ability of E2 to stimulate proliferation in BSC cultures. Additionally, treatment of BSC cultures with a specific MMP 2/9 inhibitor suppresses E2 stimulated proliferation ($p < 0.05$). Finally, treatment of BSC cultures with G15 or G36 (specific GPER-1 antagonists) suppresses E2 stimulated proliferation ($p < 0.05$). These data strongly suggest that E2 stimulated transactivation of EGFR via GPER-1 is at least partially responsible for E2 induced increases in proliferation in BSC cultures.

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Introduction

The utilization of growth enhancing technologies, specifically anabolic steroids, is a very common management practice in the beef industry today. According to the National Animal Health Monitoring System (NAHMS) (2000), 97% of feedlot cattle receive at least one steroid implant during their lifespan. The utilization of implants results in a significant reduction in production costs, showing an increase in value of $93 per animal (1). Growth enhancing technologies play a significant role in the increased efficiency of the beef industry, by streamlining the input of resources per unit of food produced. The animals require less days on feed and are more efficient with the nutrients they receive, resulting in less feed required per unit of gain (2). The effects of implants are far reaching, as in addition to increasing production efficiency and being economically beneficial they contribute to achieving the sustainability needs of the agricultural industry. Implant usage contributes to a decrease in emission of greenhouse gases per unit of food due to fewer animals necessary to produce the same quantity of food (3). Animals receiving implants produce 31% less CH_4 and N_2O compared to non-implanted animals due to decreased days on feed and dry matter intake necessary to achieve market weight (4). The use of growth enhancing technologies also contributes to sustainability by decreasing the land necessary to produce the same quantity of beef (2; 3). Implantation is a management practice that improves overall beef herd productivity and reduces the non-productive portion of the animal's life. There are many benefits to anabolic steroid implant use in the beef industry, however the mechanism that anabolic steroids utilize to enhance muscle growth is unknown. This literature review will

concentrate on the estrogenic effects in a multitude of tissues and how they contribute to elucidating estrogen's function in muscle growth.

Anabolic Steroid Implants

Steroid implants have been used as a beef management tool since the 1950s. According to the Center for Veterinary Medicine there are currently thirty-eight different implant products available to beef producers. Implants contain anabolic steroids that are slowly released over a period of time. They can be utilized in either gender, for a broad age range of cattle, and at different stages of production. The implants are either an estrogenic, androgenic, or combination of estrogenic and androgenic compounds. The estrogenic options are the natural hormone estradiol (E2) or a synthetic compound zeranol. There are eight available implants utilizing solely estrogenic activity. The androgenic compounds contain either the natural hormone testosterone propionate or the synthetic compound trenbolone acetate (TBA). There are only two available implants utilizing only androgenic activity. TBA is more active than testosterone propionate and is more commonly utilized in implants. There are twenty-eight implants that utilize a combination of estrogenic and androgenic activity (5). Currently in the industry about two thirds of the implants used are trenbolone acetate (TBA) and estradiol (E2) combination implants (2). The use of steroid implants as a management practice has been shown to increase efficiency of beef production.

Production Characteristics

Steers that received a TBA/E2 combination implant in addition to other growth enhancing technologies have a decrease in cost per kilogram of body weight gained. Implanted steers cost producers $1.12 per kilogram of weight gained as compared to non-implanted steers that cost $1.35 per kilogram of weight gained (4). Implants are most efficient during the first forty days after application as their benefits are most pronounced during that time period. However, they are still functional for up to 115 days following initial implantation (6). A TBA/E2 combination implant given to steers increases average daily gain (ADG) 18-36% and feed efficiency 8-34% over non-implanted steers (1; 6-9). In studies done in wethers a TBA/E2 implant induced an increase in ADG of 25% and in feed efficiency of 23% over non-implanted wethers (8). Estrogen alone increases feed intake as much as 12% (6). During the period from 0 to 40 days steers implanted with TBA/E2 exhibited an 82% increase in carcass protein accretion over control steers (6). An increase in daily carcass protein accretion was also seen in wethers implanted with TBA/E2 (8). The combination of TBA/E2 results in the greatest increase in protein accretion. Steers receiving TBA/E2 displayed 25% more protein accretion than steers implanted with E2 and 60% over steers implanted with TBA. Correlating with protein accretion, body weight gain was greatest for steers implanted with TBA/E2, followed by steers given E2, and lastly steers treated with TBA (10). Implantation increased carcass weight 7% over non-implanted steers. Although implantation has a positive impact upon production efficiency, it has an ambiguous impact upon carcass characteristics. A review of implantation studies showed there was a 7% decrease in fat cover, 5% decrease in marbling, and 17% decrease in carcasses

grading Choice or better (11). The decrease in fat and marbling of the animal may be due to energy going towards increased muscle production and sustained muscle growth instead of achieving a muscle growth plateau that allows the energy to contribute to fat accretion. Additionally, there are conflicting reports of implants resulting in higher Warner-Bratzler shear force values indicating that animals receiving implants have tougher meat (1). However, based upon consumer panels there is no difference in palatability between implanted and non-implanted meat products (11).

Role of Satellite Cells in Muscle Growth

During embryonic muscle development multipotent stem cells experience differentiation and determination to form muscle precursor cells that are dedicated to forming muscle. Muscle precursor cells proliferate and after expression of myogenic regulatory factors muscle precursor cells further differentiate into mononucleated cells known as myoblasts. Myoblasts are unable to undergo proliferation; however they fuse with each other to form multinucleated myotubes that develop into muscle fibers found in mature muscle. In meat-producing animals fiber formation is completed prior to birth. Muscle fibers are not able to proliferate and their nuclei no longer have the ability to divide. Consequently, the number of muscle fibers is fixed at birth and does not increase significantly during postnatal growth. Due to the fact that muscle fiber number is fixed at birth, hypertrophy of muscle fibers is necessary for postnatal muscle growth. In order to support postnatal muscle fiber hypertrophy, something crucial in the meat production industry, additional nuclei, beyond those present at birth, must be added to the muscle fiber in order to support critical cellular functions. However, since nuclei in the muscle fiber can no longer divide or undergo DNA synthesis, the new nuclei needed to maintain an

acceptable protein to DNA ratio and support muscle growth must come from outside the muscle fiber. Mononucleated satellite cells found in the "satellite niche" located between the basement membrane and the sarcolemma of each fiber are the source of these nuclei needed to support postnatal muscle growth. Satellite cells are very important as they contribute 60-90% of DNA present in muscle fibers of mature muscle (12).

Moss and Leblond (1971) ascertained the contribution of satellite cells' nuclei to muscle fibers during an *in vivo* study conducted in rats. Rat muscle was injected with ^{3}H-thymidine and at 1 hour all of the labeled nuclei were in satellite cells. There were no labeled nuclei in the muscle fiber confirming that the nuclei in the muscle fiber were not synthesizing DNA. Twenty-four hours post-injection 15% of the labeled nuclei had fused with the true muscle fiber. After 72 hours 65% of the labeled nuclei were incorporated in the muscle fiber, indicating that the satellite cells were fusing with muscle fiber, providing additional DNA (13). Satellite cells are able to proliferate increasing their numbers or differentiate to fuse with existing muscle fiber providing nuclei needed for hypertrophy. Whether they proliferate or differentiate is dependent upon the growth factor and hormone levels present in the satellite cell niche (14). The number of satellite cells decreases during postnatal muscle growth as differentiation and fusion take precedence over proliferation.

In newborn animals 30% of muscle nuclei are satellite cell nuclei. As the animal ages, the amount of available satellite cell nuclei decreases to 2-10% of muscle cell nuclei (12) and the majority of the remaining satellite cells enter a quiescent phase (G_0) in which they neither proliferate nor fuse with existing muscle fibers unless stimulated by exercise

or muscle injury. As a result, the number of nuclei available to add to fibers to support hypertrophy required for normal muscle growth is greatly reduced resulting in plateauing of muscle growth. The importance of satellite cells in postnatal muscle growth is shown by studies in which satellite cell numbers are dramatically reduced by radiation or genetic manipulation. Animals undergoing radiation or genetic manipulation exhibit postnatal muscle growth that is dramatically retarded (15; 16).

Quiescent satellite cells are activated in response to stimuli such as exercise or injury. Satellite cells express Pax7 which enables them to undergo proliferation or differentiation. Myogenic regulatory factors (MRFs) play an important role during embryonic development and their impact upon satellite cell activation is very similar to their effect on embryonic myogenic cells. Myoblast determination protein (MyoD) and Myogenic Factor 5 (Myf5) expression result in the activation of satellite cells in the quiescent phase (16) and the satellite cells undergo proliferation. Myoblasts produce myogenin and muscle-specific regulatory factor 4 (MRF4) which are necessary for myoblasts to undergo terminal differentiation and fuse into myotubes (16). MRFs are important not only in the activation of quiescent satellite cells but also in determining whether satellite cells will undergo proliferation or differentiation.

There are three facets that interact to achieve muscle growth; proliferation, protein synthesis and protein degradation. Proliferation provides additional satellite cells that can either continue dividing or fuse with existing muscle fiber to provide DNA needed to support protein accretion. Protein synthesis and protein degradation interact to result in protein accretion. An increase in protein synthesis is and a reduction in protein

degradation is ideal for optimal protein accretion. Skeletal muscle hypertrophy requires both increased protein accretion and increased nuclei number to support the additional protein present in the myofiber.

Effect of a combined TBA/E2 implant on satellite cell number and activity

As indicated in the previous section, satellite cells are necessary for postnatal muscle growth. Determining the impact that anabolic steroids exhibit on satellite cells will help elucidate the pathway involved in anabolic steroid enhanced muscle growth. Muscle from steers implanted with Revalor-S™ (an implant containing 120mg TBA and 24mg E2) contained 50% more satellite cells (7) than muscle from non-implanted steers. Additionally, cultured satellite cells from implanted animals exhibited greater proliferative response to growth factors such as Insulin-like Growth Factor-1(IGF-1) and Fibroblast Growth Factor (bFGF) than satellite cells from non-implanted animals (17). Satellite cell cultures isolated from TBA/E2 implanted steers contained 50% more myotube nuclei than corresponding cultures isolated from non-implanted steers (7). Cultured satellite cells isolated from non-implanted steers had a longer lag phase prior to proliferation than satellite cells isolated from steers that received a TBA/E2 implant, indicating that a larger proportion of the satellite cells in non-implanted muscle was in a quiescent state (7). These results indicate that implantation has an impact upon both satellite cell number and behavior.

TBA/E2 implant impact on muscle IGF-1 mRNA and IGF-1 serum levels

The insulin-like growth factor system plays an important role in growth and development of numerous tissues and regulates overall growth. The IGF system is composed of two IGF ligands, insulin-like growth factor-1 (IGF-1) and insulin-like growth factor-II (IGF-2). Within the system there are three cell surface receptors that mediate IGF function, insulin-like growth factor receptor-1 (IGFR-1), insulin-like growth factor receptor 2 (IGFR-2), and the insulin receptor (IR). Additionally, the IGF system contains a family of six IGF-binding proteins that contribute to regulation of the IGF system by determining if IGF ligands are available to bind and activate the receptors (18). IGF-2 is primarily expressed prenatally and decreases after birth (18). In contrast IGF-1 expression is low prenatally and increases significantly as an animal matures (18). Insulin-like growth factor -1 stimulates muscle growth by increasing muscle protein synthesis rate (19) and decreasing muscle protein degradation rate (19). Additionally, IGF-1 stimulates proliferation and differentiation of muscle satellite cells. Consequently, adequate levels of IGF-1 are crucial to muscle growth.

Based on the positive effects of IGF-1 on muscle growth, it is significant that serum levels of IGF-1 were increased by anabolic steroid implantation. By day 6 steers implanted with TBA/E2 (Revalor-S™) exhibited an increase in serum IGF-1 of 32% over non-implanted steers and sustained the higher level for the 32 day trial (7). An additional study supported the impact of TBA/E2 upon serum IGF-1 levels; reporting that by days 14 and 28 there was a 53% and 85% increase, respectively, in serum IGF-1 levels in implanted steers over non-implanted steers (20). Steers receiving a TBA/E2 implant exhibited a 54% increase in serum IGF-1 over their pre-implant IGF-1 levels (20).

Due to the fact that the majority of circulating IGF-1 is synthesized in the liver, the effect of steroid implantation on liver IGF-1 mRNA expression was measured. Steers given a TBA/E2 combination implant exhibited a 69% increase in hepatic IGF-1 mRNA level (9). Wethers implanted with TBA/E2 showed a 150% increase in hepatic IGF-1 mRNA levels (8). This indicates that the increase in IGF-1 mRNA levels in the liver contributes to increased circulating IGF-1 levels in Revalor-S™ implanted steers.

However, the role of circulating IGF-1 in stimulating muscle growth is controversial because growth rates are not significantly impacted in rats in which liver IGF-1 production has been knocked out (21). In contrast, studies in mice overexpressing IGF-1 in muscle have shown that increased production of IGF-1 in muscle stimulates muscle growth via autocrine/paracrine mechanisms (22). Consequently, muscle IGF-1 mRNA expression in Revalor-S™ implanted steers was examined. Twenty-six days after implantation with Revalor-S™ steers exhibited longissimus muscle IGF-1 mRNA levels 3 times higher than on day 0 (9). Additionally, Revalor-S™ implanted steers exhibited 94% higher IGF-1 mRNA levels than non-implanted steers (9). Based on studies showing that overexpression of IGF-1 mRNA in muscle increases muscle growth rate (22), it appears likely that the increased IGF-1 mRNA levels in muscle of implanted steers plays a significant role in anabolic steroid induced muscle growth.

In order to examine the individual roles of TBA and E2 in Revalor-S™ stimulated muscle growth, steers were implanted with either TBA or E2 at the dosage present in the Revalor-S™ implant. Biopsies of the longissimus muscle showed that steers implanted solely with E2 exhibited an increase in IGF-1 mRNA levels by day 7 (23) and ultimately

expressed 71% higher levels of IGF-1 mRNA than steers that were not implanted (23). Steers implanted with TBA exhibited the same IGF-1 mRNA levels as steers that were not implanted (23).

Effects and mechanism of action of Estrogen in non-muscle systems

Estrogen is involved in a variety of body systems and functions including reproduction, immune, nervous, cardiovascular, and cancer and its physiological effects include growth, development, and homeostasis of a multitude of tissues. Additionally, E2 has been shown to stimulate proliferative activity in breast, uterus, tumor and non-tumor cells (24-26). The mechanism of E2 action in these non-muscle tissues has been extensively studied and results of these studies provide information about the potential mechanism of E2 enhance muscle growth in cattle. Consequently, in this section I will review the mechanism of action of E2 in a variety of non-muscle body systems.

Classic Estrogen receptor mechanism of action

Estrogen has two classical receptors, ER-α identified in the 1970's and ER-β identified in the mid 1990's. These two classical receptors interact with E2 and are responsible for some of the effects that E2 exhibits on cell function, especially the genomic effects (27). While ER-α and ER-β can move between the nucleus and cytoplasm the majority are found in the nucleus with ~5% found in the cytoplasm (28). Classical estrogen receptors can be activated in several ways. They can function as ligand-activated nuclear transcription factors producing genomic effects. Secondly, classical estrogen receptors may be activated in the cytoplasm through phosphorylation or other post-transcriptional

modifications producing rapid non-genomic effects (29). ER-α is the predominate estrogen receptor in most tissues (30).

Role of Epidermal Growth Factor Receptor (EGFR) in E2 action

E2 rapidly stimulates Epidermal Growth Factor Receptor (EGFR) activation in cancer cells (31; 32). Epidermal Growth Factor Receptor (EGFR) is a 170kDa tyrosine kinase receptor that is activated after binding with ligands such as Epidermal Growth Factor (EGF) or heparin-binding Epidermal Growth Factor (hb-EGF). Activation of EGFR ultimately results in DNA synthesis and cell proliferation. In MCF-7 breast cancer cells and COS-7 cells E2 and IGF-1 treatment increased phosphorylation of EGFR while not to the extent observed in cells treated with EGF (33). A specific EGFR tyrosine kinase inhibitor AG1478, which binds to the receptor and distorts its shape rendering it incapable of proper function, inhibited IGF-1 induced phosphorylation of EGFR in Rat-1, human embryonic kidney, and breast cancer cells (33; 34). AG1478 inhibited the phosphorylation of EGFR without reducing levels of EGFR protein in CNE2 cells (35). The lack of change in protein levels indicate that AG1478 inhibition happens at a post-translational level. In addition to affecting EGFR phosphorylation, AG1478 also inhibited serum induced cell proliferation of CNE2 nasopharyngel carcinoma cells (35). To further support the results from the AG1478 studies, knockdown of the EGFR with a siRNA suppressed the stimulation of proliferation induced by E2, IGF-1, and EGF (34; 36-38) in COS-7 and MCF-7 cancer cells. This accumulation of data conveys that suppression of EGFR also suppresses ER-α and IGFR-1 activity. This supports the model that EGFR is the downstream converging point for ER-α and IGFR-1 action.

Role of G protein–coupled estrogen receptor (GPER)-1 in E2 action

Rapid estrogen action is not completely dependent on ER-α and ER-β as indicated by the fact that it takes place in SKBR3 breast cancer cells that lack mRNA for both of the classical receptors (39). Additionally, in ER-α and ER-β knockout mice there was still a response to estrogen. Further investigation revealed that G protein-coupled estrogen receptor (GPER)-1 was mediating estrogen function in these mice (28).

Numerous reviews of E2 action in non-muscle tissues and cancer cells have shown that, in addition to ER-α and ER-β, the G protein-coupled estrogen receptor (GPER)-1 contributes to rapid non-genomic estrogen response in numerous tissues (40; 41). These studies have shown that binding of E2 to GPER-1 results in transactivation of the Epidermal Growth Factor Receptor (EGFR) (28; 33; 34; 40; 42; 43). GPER-1 (previously known as G protein-coupled receptor 30 (GPR30)) is a member of the G-protein-coupled receptor (GPCRs) family which is the largest known family of signaling molecules with 900 members (41). GPER-1 is a seven transmembrane spanning receptor expressed on the cell surface. Estrogen binds to GPER-1 with high affinity and that binding activates GPER-1 releasing the Gβγ protein complex (44). These subunits of the GPER-1 stimulate the activity of matrix metalloproteinases (MMP) 2/9. Matrix metalloproteinase 2 and matrix metalloproteinase 9 are members of a family of zinc-dependent proteases. MMPs enable the proteolytic cleavage and release of heparin-binding Epidermal Growth Factor (hb-EGF) from the cell surface. Heparin-binding Epidermal Growth Factor is a peptide ligand in the EGF family. The cleaved hb-EGF is able to bind to Epidermal Growth Factor Receptor (EGFR) resulting in EGFR

transactivation which mediates downstream pathways, including the MAP kinase pathway (28; 33; 34; 40; 42; 43) which regulates proliferation and protein synthesis. GPER-1 transactivation of EGFR is crucial for the rapid, non-genomic response to E2 in cells.

The E2/GPER-1 mechanism of action is further supported by the following compilation of literature. Tamoxifen and ICI 182,780 are ER antagonists that exhibit a very high affinity for classical ERs and compete with E2 for a binding site resulting in inhibition of E2 stimulation. Surprisingly, treatment of COS-7 and MCF-7 breast cancer cells with these antagonists resulted in increased proliferation. Further studies revealed that in addition to binding to and inhibiting ER-α and ER–β activity, ICI 182,780 and tamoxifen are also able to bind to GPER-1; however, they function as GPER-1 agonists in bone and other tissues resulting in stimulation of activity instead of an inhibition of activity (28; 40; 42; 45-47). Treatment of MCF-7 breast cancer cells with these ER antagonists resulted in an increase in GPER-1 expression, and GPER-1 mediated EGFR phosphorylation (28; 40) resulting in increased proliferation. Consequently, it is likely that E2 binding to GPER-1 also results in increased proliferation rates via transactivation of EGFR.

The fact that E2 activates both the classical E2 receptors (ER-α and ER-β) as well as GPER-1 has presented a significant problem in defining the function of each class of receptor. However, the relatively recent discovery of two GPER-1 specific antagonists and an agonist that do not affect the activity of the classical E2 receptors has made it possible to study the role of GPER-1 independently of the classical E2 receptors. This

has led to additional research defining the complex relationship between estrogen and GPER-1. Both G15 and G36 are GPER-1 specific antagonists. The GPER-1 specific agonist, G1 has been found to weakly bind to ER at very high concentrations as does G15 (46). In uterine epithelial cells G1 stimulates proliferation and blocking GPER-1 with G15 reduced E2 induced proliferation by 50% (28). These results indicate that activation of GPER-1 may stimulate proliferation. Additionally, treatment of breast cancer cells and primary mouse mammary epithelial cells with a GPER-1 agonist leads to EGFR phosphorylation (48). When cells were treated with AG1478 (EGFR tyrosine kinase inhibitor), EGFR phosphorylation was inhibited resulting in a suppression of GPER-1 mediated mitogenic signaling in the ERK/MAP kinase pathways (44). This indicates that functional EGFR is necessary for GPER-1 activity. GPER-1 mediated EGFR phosphorylation was inhibited when MMP2/9 were inhibited, indicating that MMP2/9 are required in order for GPER-1 to stimulated EGFR phosphorylation (48). When the MMPs are inhibited the amount of proteolytically cleaved and free hb-EGF is reduced, indicating that MMPs are necessary for GPER-1 mediated release of hb-EGF, a crucial aspect of EGFR transactivation (42; 49; 50). The reduction in hb-EGF resulted in the EGFR phosphorylation being suppressed (42; 49; 50). In a breast cancer cell line overexpression of GPER-1 resulted in transactivation of EGFR via release of hb-EGF (48). These data support the hypothesis that receptor cross-talk between GPER-1 and EGFR involves both MMP2/9 and hbEGF and is responsible for various important biological functions such as gene expression and cancer cell proliferation (49).

Potential role of IGF-1 and IGFR-1 in E2 mechanism of action

Insulin-like Growth Factor-1 (IGF-1) is a member of the insulin-like growth factor system which plays an important role in growth and development in most organs and tissues. ER-α regulates the IGF-1 pathway and in turn IGF-1 activates ER-α mediated signaling in a ligand independent manner (49). Insulin-like Growth Factor Receptor-1 (IGFR-1) is an 180kDa receptor that contains two α-subunits and two β-subunits. Insulin-like Growth Factor Receptor-1α (IGFR-1α), is a 85kDa subunit that is the extracellular portion of the IGFR-1 and the section that binds to IGF-1 (51). Insulin-like Growth Factor Receptor-1β (IGFR-1β), is a 95kDa subunit that crosses the cell membrane and contains the tyrosine kinase portion of the IGFR-1 (51). IGF-1 binding to IGFR-1 results in activation of the tyrosine kinase which begins a cascade of phosphorylation in other receptors. IGF-1 and IGFR-1 may be involved in the role of non-genomic E2 rapid response in cells. In breast and endometrial cancer cells, osteoblastic cells, and rat muscle cells E2 up regulated IGF-1 mRNA levels; however, treatment with ICI 182,780 (ER antagonist) inhibited the up regulation of IGF-1 mRNA levels (24; 25; 52). This indicates that functioning ER is involved in IGF-1 mRNA expression.

To further elucidate IGF/IGFR-1 role within different tissues AG1024, a specific inhibitor, was developed. AG1024 is a member of the tyrophostin family which is a family of synthetic protein tyrosine kinase inhibitors that inhibit receptor autophosphorylation. IGFR-1 is a member of the tyrosine kinase receptor family therefore functional tyrosine kinase of IGFR-1 is necessary for signaling function of the receptor. Although AG1024 is specific for IGFR-1β tyrosine kinase inhibition, due to the fact that the structure of the insulin receptor and IGFR-1 are quite homologous, AG1024

also is able to inhibit the insulin receptor tyrosine kinase although to a lesser degree than it inhibits the IGFR-1 tyrosine kinase (53). AG1024 resembles the phenolic group of tyrosine allowing AG1024 to compete effectively with IGF-1 for binding to IGFR-1. Following binding of AG1024 to IGFR-1 the receptor is distorted so it is incapable of autophosphorylation and the receptor is rendered non-functional (53). IGF-1 stimulates the phosphorylation of IGFR-1 and proliferation but those effects are abrogated by AG1024 (53). Treatment of MCF-7 breast cancer cells with AG1024 in addition to IGF-1 resulted in suppression of IGF-1 stimulation of EGFR phosphorylation (36; 37). Similarly, E2 stimulates IGFR-1 phosphorylation (30) and treatment of cells with AG1024 prevents that phosphorylation. This indicates that functional IGFR-1 is essential for both E2 and IGF-1 stimulated proliferation. In mouse fibroblast cells and MCF-7 breast cancer cells EGF did not stimulate phosphorylation of the IGFR-1, nor was its proliferative effects inhibited by AG1024 (36; 37; 53). Additionally, both IGF-1 and EGF were able to cause phosphorylation of EGFR (33) in COS-7 cells. Studies showing that IGF-1 is able to stimulate EGFR but EGF is not able to stimulate IGFR-1 suggest that EGFR is downstream of the IGFR-1. In breast cancer cells knockdown of IGFR-1 with siRNA did not impact expression of EGFR however it did suppress E2 stimulated phosphorylation of EGFR and E2-stimulated proliferation (31; 32). This implies that E2 stimulation of EGFR was mediated by IGFR-1. Studies on IGFR-1 silenced cells supported the conclusions of the AG1024 data showing that IGF-1 and E2 stimulated phosphorylation of EGFR and proliferation require functional IGFR-1 (51). This would support the theory that the IGFR-1 and EGFR are both part of the E2 pathway with EGFR being downstream of the IGFR-1.

Effects of Estrogen on muscle satellite cells in vitro

As described in the preceding section, the mechanism of estrogen action has been studied in a variety of non-muscle cells and systems. However, despite the fact that estrogen plays a crucial role in stimulating and sustaining postnatal muscle growth little information is available on the mechanism involved in E2 enhanced muscle growth in the bovine. However, recent *in vivo* studies have shown that an E2 stimulated increase in satellite cell proliferation may play a significant role in E2 enhanced muscle growth in the bovine. Consequently, elucidating the mechanism involved in E2 enhanced satellite cell proliferation should provide valuable information on the mechanism of E2 enhanced muscle growth. Bovine satellite cell culture provides a controlled environment that allows us to study the mechanism of E2 enhanced satellite cell proliferation.

Estrogen effect on IGF-1 expression in cultured BSC

As described previously, IGF-1 is a growth factor that plays an important role in growth and development. IGF-1 is locally produced in skeletal muscle and animals that overexpress IGF-1 in muscle show an increase in muscle hypertrophy and overall muscle mass (54). Consequently, it is significant that recent studies have shown that E2 implantation increases IGF-1 mRNA expression in bovine skeletal muscle (8). Due to bovine satellite cells grown in cell culture being able to produce IGF-1 and release it into the media, mimicking the action of skeletal muscle IGF-1 production *in vivo* (55), bovine satellite cell culture provides a controlled environment in which to study the mechanism involved in E2-stimulated IGF-1 mRNA expression.

In media containing 10% fetal bovine serum (FBS), Estrogen treatment stimulated IGF-1 mRNA levels in cultured bovine satellite cells (BSCs) in a concentration dependent manner. IGF-1 mRNA level was 1.9 times higher in BSC cultures treated with 0.01nM E2 over control cells. IGF-1 mRNA levels were up to 3.5 times higher in BSCs treated with 1nM E2 than in control BSCs (56). Treatment of cells with E2-BSA (E2-BSA is not able to enter the cell so in order to express an effect it must bind to a cell surface receptor) resulted in a 100% increase in IGF-1 mRNA (57). As ~95% of ER is located in the nucleus this indicates that E2 is not stimulating IGF-1 mRNA expression by binding to ER-α but rather by interacting with a cell surface receptor such as GPER-1. Additionally, ICI 182,780 (ER antagonist) was not able to inhibit the E2 stimulation of IGF-1 mRNA and actually resulted in a 93% increase in IGF-1 mRNA expression (57). ICI 182,780 behaves as a GPER-1 agonist, stimulating GPER-1 activity which could contribute to the increase in IGF-1 mRNA levels. These data establish that ER-α and ER-β are not involved in E2 stimulated IGF-1 mRNA expression in cultured BSC. Additionally, because ICI 182,780 is a GPER-1 agonist, the ability of this ER-α and ER-β antagonist to stimulate IGF-1 mRNA expression in cultured BSC suggests that E2 activation of GPER-1 might play a role in E2 stimulated IGF-1 mRNA expression in these cells. To further determine GPER-1 potential role a GPER-1 specific agonist, G1, was utilized to determine the GPER-1 role independent of the classical ER. BSCs treated with G1 exhibited a 100% increase in IGF-1 mRNA levels (57) implying that GPER-1 is most likely involved in stimulating IGF-1 mRNA expression. Additionally, it has been determined that muscle cells contain both mRNA and protein for GPER-1 (40; 58; 59). These data suggest that GPER-1 is involved in the IGF-1 mRNA increase and may be

mediating the E2 stimulated increase of IGF-1 expression which contributes to E2 stimulated muscle growth.

Estrogen effect on proliferation

In 1% SSS and 2% SSS, conditions under which E2 did not stimulate IGF-1 or IGFR-1 mRNA levels, E2 was still able to stimulate proliferation in BSCs (47; 51; 57). This suggests that an increase in proliferation isn't a result of increased IGF-1 or IGFR-1 expression. Treatment of cultured BSCs with ICI 182,780 (ER antagonist) resulted in suppressed E2 stimulated proliferation (47). Additionally, in BSC cultures in which ER-α was silenced E2 stimulation of proliferation was completely suppressed (51), indicating that ER-α is required for E2 stimulated BSC proliferation.

Treatment of bovine satellite cells with AG1024 (IGFR-1 tyrosine kinase inhibitor) resulted in the suppression of E2 and LR3-IGF-1 (IGF-1 analog) stimulated proliferation (51). Additionally, IGF-1 and E2 stimulated proliferation was suppressed in IGFR-1 silenced bovine satellite cells (51). This further supports that the function of IGFR-1 is necessary for E2 stimulated proliferation of bovine satellite cells. The role of GPER-1 in E2 stimulated BSC proliferation was assessed by treating BSC with G1. Treatment with G1 did not stimulate BSC proliferation indicating that activation of GPER-1 alone was not sufficient to stimulate BSC proliferation (57; 60).

Estrogen effects on protein synthesis and protein degradation in BSC cultures

In addition to an increase in proliferative activity, E2 also affected protein synthesis and protein degradation in BSCs under culture conditions in which neither IGF-1 nor IGFR-1

expression was increased. Fused bovine satellite cell cultures treated with E2 exhibited a concentration dependent increase in protein synthesis, with 10nM E2 resulting in a 1.7 fold increase in protein synthesis over control BSCs (60). Fused BSCs treated with E2 also exhibited a 70% decrease in protein degradation (60).

ICI 182,780 suppressed the E2 stimulated increase in protein synthesis and E2 stimulated decrease in protein degradation in bovine satellite cells. This indicates that ER-α is necessary for E2 to affect protein synthesis and degradation in bovine satellite cells grown in culture (60). Treatment of bovine satellite cell cultures with G1 had no effect on protein synthesis or protein degradation rates, indicating that E2 binding to GPER-1 was not sufficient to increase protein synthesis or decrease protein degradation in BSCs (60).

Proposed mechanism of Estrogen action

Although increased IGF-1 expression very likely plays a role in E2 stimulated BSC proliferation, based upon the compilation of literature detailing the estrogen mechanism of action in both non-muscle and muscle systems described in this review, we hypothesize that activation of both ER-α and GPER-1 initiate other pathways by which E2 can stimulate BSC proliferation. In addition to binding and activating ER-α, estrogen also can bind to and activate GPER-1. When GPER-1 is activated the Gβγ protein complex is released and these subunits of the GPER-1 stimulate the activation of matrix metalloproteinase (MMPs). MMPs enable the release of heparin-binding Epidermal Growth Factor (hb-EGF) from the cell surface. The hb-EGF then is able to bind to epidermal growth factor receptor (EGFR) resulting in EGFR transactivation which mediates downstream pathways (28; 33; 34; 40; 42; 43). Although the role of ER-α in

E2-stimulated BSC proliferation is not known (and will not be addressed in this thesis), E2-ER-α can reportedly form a complex with specific intracellular signaling molecules and this complex can interact with the intracellular subunit of IGFR-1 (26; 61) resulting in activation of the receptor which may interact with GPER-1 to stimulate BSC proliferation.

This thesis will delve into the transactivation of EGFR and EGFR's role in estrogen's mechanism of action in bovine skeletal muscle. In bovine satellite cells (BSCs) EGFR will be silenced utilizing siRNA and the impact of eliminating this receptor on E2, IGF-1, and hb-EGF stimulated proliferation in BSC cultures will be determined . Additionally, EGFR, ER-α, and IGFR-1 mRNA and protein levels will be examined in control and EGFR silenced BSC cultures. To further investigate the complex mechanism of estrogen stimulated proliferation in BSC cultures, the role of GPER-1 and MMPs will also be studied. The effect of inhibiting MMP 2/9 on E2 and IGF-1 stimulated BSC proliferation will be determined. Finally, GPER-1 will be inhibited and its impact upon E2 and IGF-1 stimulated BSC proliferation will be ascertained. This proposed plan of action will assist in elucidating estrogen's mechanism of action in bovine skeletal muscle.

MANUSCRIPT

Introduction

The utilization of anabolic steroids is a very common management practice in the beef industry today. According to the National Animal Health Monitoring System (NAHMS) (2000), 97% of feedlot cattle receive at least one steroid implant during their lifespan. The utilization of implants results in a significant reduction in production costs, showing an increase in value of $93 per animal (1). The effects of implants are far reaching, as in addition to increasing production efficiency and being economically beneficial, they contribute to achieving the sustainability goals of the agricultural industry (3; 4; 62). However, there are concerns about steroid hormone residue building in the environment and possible negative health concerns about eating meat from anabolic steroid implanted animals. Therefore, it is important to elucidate the mechanism of action of anabolic steroids on muscle growth in order to enhance understanding and to potentially devise new methodologies that may achieve similar positive growth enhancing effects without the negative concerns of steroid hormone use.

A trenbolone acetate (TBA) and estrogen (E2) combination implant given to steers increases average daily gain (ADG) 18-36% and feed efficiency 8-34% over non-implanted steers (1; 6-9). In order to achieve this increase in production characteristics, postnatal muscle growth must be increased. Satellite cells are the source of nuclei needed to support postnatal muscle growth (12). Estrogen is able to stimulated bovine satellite cell (BSC) proliferation (63). In proliferating cells where ER-α was silenced LR3-IGF-1(IGF-1 analog) and heparin-binding Epidermal Growth Factor (hb-EGF) were able to increase proliferation whereas the E2 stimulation of proliferation was completely

suppressed (51), indicating that ER-α is required for E2 stimulated BSC proliferation. However, it has been shown that rapid estrogen action is not completely dependent on ER-α and ER-β. The G protein-coupled estrogen receptor (GPER)-1 also mediates estrogen function (26). The role of GPER-1 in E2 stimulated BSC proliferation was assessed by treating BSCs with G1. Treatment with G1 did not stimulate BSC proliferation indicating that activation of GPER-1 alone was not sufficient to stimulate BSC proliferation (57; 60).

However, based upon the compilation of literature detailing the estrogen mechanism of action in non-muscle and muscle systems, we hypothesize that activation of both ER-α and GPER-1 is necessary in order for E2 to stimulate BSC proliferation. In addition to binding and activating ER-α, estrogen also can bind to and activate GPER-1. In cancer cells when GPER-1 is activated the Gβγ protein complex is released and these subunits of the GPER-1 stimulate the activation of matrix metalloproteinase (MMPs). The MMPs enable the release of heparin-binding Epidermal Growth Factor (hb-EGF) from the cell surface. The hb-EGF then is able to bind to epidermal growth factor receptor (EGFR) resulting in EGFR transactivation which mediates downstream pathways that regulate cell proliferation (28; 33; 34; 40; 42; 43). The objective of this study is to determine if EGFR is required for E2 stimulated proliferation in BSCs and further contribute to elucidating estrogen's mechanism of action in bovine skeletal muscle.

Materials and Methods

Bovine Satellite Cell Isolation

Satellite cells were isolated from yearling crossbred steers that had never received an anabolic steroid implant and had been on full feed for a 3 week period. Steers were euthanized using procedures approved by the University of Minnesota Institutional Animal Care and Use Committee. Using sterile technique, approximately 1 kilogram of semimembranosus muscle was dissected and transported to the cell culture laboratory. Connective tissue was removed from the muscle and the muscle was passed through a sterile meat grinder. The ground muscle was incubated with 0.1% pronase in Earl's balanced salt solution for 1 hour at 37˚C with mixing at every 10 minute interval. From the time the animal was harvested until muscle is mixed with pronase only 30 minutes are allowed to pass. Following the incubation period the mixture was centrifuged at 1,500 x g for 4 minutes, the pellet was re-suspended in PBS (140mM NaCl, 1 mM KH_2PO_4, 3mM KCl, 8 mM Na_2HPO_4, pH 7.4), and the suspension was centrifuged at 500 x g for 10 minutes. The resultant supernatant was centrifuged at 1,500 x g for 10 minutes to pellet the mononucleated cells. The PBS wash and differential centrifugation was repeated two more times. The resulting mononucleated cell preparation was suspended in 4˚C Dulbecco Modified Eagle Media (DMEM) containing 10% Fetal Bovine Serum (FBS) and 10% (vol/vol) dimethylsulfoxide (DMSO) and frozen at -80˚C. Cells were stored frozen in liquid nitrogen for future use.

^{3}H-thymidine incorporation assays

BSCs were grown on 2cm^{2} well plates. Prior to plating of cells the wells were coated with 250µl BD Matrigel Matrix (0.95mg/mL) + DMEM. Cells were plated in DMEM containing 10% fetal bovine serum (FBS) at a plating density (cells isolated from 0.16g muscle tissue/cm^{2}) empirically established so that cultures were approximately 50% confluent when proliferation rate was determined. At 72 hours media was removed, cells were washed once with warm DMEM and test media (0.5mL) containing DMEM plus 1% insulin-like growth factor binding protein (IGFBP)-3-free swine serum (prepared by passing sera obtained from 6 week old male pigs castrated within 1 week of birth through an IGFBP-3 immunoaffinity column) (51) containing 10nM E2 (Steraloids Inc, Newport, RI; catalog no. E0950-000) or other growth factors to be tested was applied. At 93 hours ^{3}H-thymidine was added to the media on the cells at a concentration of 1mCi/mL and incubated for 3 hours. Following the incubation media was removed and cells were washed 3 times with cold DMEM. Cells were fixed with 1mL of cold 5% trichloroacetic acid (TCA) overnight at 4˚C. Unincorporated ^{3}H-thymidine was removed when 5% TCA was removed and cells were washed twice with cold 5% TCA. Incorporation of ^{3}H-thymidine into cellular DNA was determined by dissolving the cell material in 0.5M sodium hydroxide (NaOH) for 30 minutes. Cell material was then placed in scintillation vials and counted in a scintillation counter. All experiments contained triplicate measurements.

To assess the effects of GPER-1 inhibitors on proliferation of cultured BSC, bovine satellite cells were plated in DMEM/10%FBS as described above. At 72 hours media

was removed, cells were washed once with warm DMEM and incubated with 0.5mL DMEM/1% IGFBP-3-free swine serum or DMEM/1% IGFBP-3-free swine serum plus 20nM of the specific GPER-1 inhibitors G15 (Azano Pharmaceuticals, Inc. Albuquerque, NM) or G36 (Azano Pharmaceuticals, Inc. Albuquerque, NM) for 30 minutes. Following the 30 minute incubation period media was removed and BSC cultures were treated with 0.5mL DMEM/1% IGFBP-3-free swine serum or DMEM/1% IGFBP-3-free swine serum containing 25ng LR3-IGF-1/ml or 10nM E2 +/- 20nM G15 or G36. At 93 hours ^{3}H-thymidine was added to the media on the cells and the rest of the protocol was conducted as stated previously.

For MMP2/9 inhibitor assays, Bovine satellite cell preparations were thawed and plated in DMEM/10% FBS as described above. At 72 hours media was removed, cells were washed once with warm DMEM and cultures were incubated with 0.5mL DMEM/1% IGFBP-3-free swine serum or DMEM/1% IGFBP-3-free swine serum plus 10μM MMP2/9 inhibitor (MMP-2/MMP-9 Inhibitor II, Calbiochem, Billerica, MA, Catalog no. 444249) for 30 minutes. Following the 30 minute incubation period media was removed and cultures were treated with 0.5mL DMEM/1% IGFBP-3-free swine serum or DMEM/1% IGFBP-3-free swine serum containing 25ng LR3-IGF-1/ml or 10nM E2 +/- 10μM MMP2/9 inhibitor. At 93 hours ^{3}H-thymidine was added to the media on the cells and the rest of the protocol was conducted as stated previously.

Small interfering RNA knockdown of EGFR

Silencing of EGFR expression in BSC cultures was achieved utilizing custom-designed small interfering RNA (siRNA) specific for bovine EGFR (Qiagen, Valencia, CA 5'-

CUA CGG ACU CCA ACU UCU ATT-3' (sense strand)) and Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA). In every experiment controls included cells treated with a non-specific siRNA with no homology to any known mammalian gene (Allstars siRNA; Qiagen Ca#1027280). Bovine satellite cell preparations were thawed and plated on culture dishes precoated with reduced growth factor basement membrane Matrigel (Becton Dickson, Franklin Lakes, NJ) at 0.19mg/mL diluted in DMEM. Satellite cells obtained from 0.1g muscle tissue/cm^2 were plated per cm^2. Cells were plated in 10% Fetal Bovine Serum in DMEM and incubated at 37˚C, 5% CO_2 in a water saturated environment. At 72 hours cells were washed once with warm DMEM and DMEM + 10% FBS without any antibiotics was put on the cells. Transfection complex was formed by diluting Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) and EGFR siRNA or Allstars siRNA in Opti-MEM I Reduced Serum Media (Invitrogen, Carlsbad, CA) and incubating for 10 minutes at room temperature. Following this incubation, the transfection complex was added to the media on the cells (200µl/mL of culture media) to yield a final level of 1.5µl Lipofectamine/mL media and 2 nM EGFR or Allstars siRNA in the culture media. This transfection media was incubated on the cells for 6 hours, and then removed and replaced with fresh DMEM/10% FBS with antibiotics for 18 hours. At 96 hours cells designated for proliferation received 0.5mL test media consisting of DMEM/ 1% IGFBP-3 free swine serum (SSS), 10nM E2 (Steraloids Inc, Newport, RI; catalog no. E0950-000), 25ng LR3-IGF-1/mL (GroPep BioReagents Pty Ltd, Thebarton, SA Australia; Catalog no.Bu100; an IGF-1 analogue that binds normally to IGFR-1 but has little or no affinity for IGF binding proteins) (51), 5ng hb-EGF/mL (R&D Systems, Minneapolis, MN; Catalog no. 259-HE), or 10% FBS, and at 120 h proliferation rate was

determined using the ^{3}H-thymidine incorporation protocol. At 96 hours cells designated for isolation of RNA or protein received 1mL 1% SSS and at 120 h RNA and protein were isolated as described below.

Quantitative RT-PCR sample preparation and RNA isolation

RNA was isolated from bovine satellite cells with the use of Absolutely RNA Microprep Kit (Agilent, Santa Clara, CA). After a phenol chloroform extraction of the cell lysate, RNA was isolated according to the manufacturer's recommended protocol. Samples were treated with DNase while bound to the fiber matrix during the isolation process. RNA was then reverse-transcribed to produce first-strand complementary DNA (cDNA).

Quantitative RT-PCR

Quantitative RT-PCR was utilized to measure the quantity of EGFR, IGFR-1β, and ER-α mRNA relative to the quantity of cyclophilin mRNA in total mRNA. Complementary DNA was generated from 1.0µg RNA utilizing Taq-Man Reverse Transcriptase Reagents (Applied Biosystems, Carlsbad, CA) and the manufacturer's recommended protocol. Random hexamers were used as the primers in cDNA synthesis. The relative quantity of the desired cDNA was measured utilizing SYBR Green PCR Master Mix (Applied Biosystems), forward and reverse bovine primers (described in Table 1), and 1µL of cDNA mixture. Assays were performed in the GeneAmp 7,300 Sequence Detection System (Applied Biosystems, Grand Island, NY) with the use of thermal cycling parameters recommended by the manufacturer (40 cycles of 15 seconds at 95˚C and 1 minute at 60˚C).

Western immunoblots

Cultured cells were lysed in tracking dye (20% SDS, 1M Tris-HCl pH 6.8, Glycerol, 0.4% Bromophenol blue, Water, β-Mercaptoethanol) or lysis buffer (50mM Tris-HCl pH8, 150mM NaCl, 1% Igepal, 1mM EDTA, 1mM NaF). The samples in tracking dye were boiled and centrifuged to remove insoluble fragments. The samples in lysis buffer were used solely for a BCA protein assay to determine protein concentration. Utilizing the protein concentration an approximation of equal amounts of protein from each sample in tracking dye were electrophoresed on a 7.5% SDS polyacrylamide gel, and then electrophoretically transferred to a nitrocellulose membrane. Once the protein was transferred to the nitrocellulose membrane the membrane was washed twice with TBS and blocked for 1 hour with 5% NFDM+TBST. Specific details for individual proteins' primary anti-bodies, dilution reagent, and anti-body incubation period are provided in Table 2. Following the primary antibody incubation period, the membranes were extensively washed, and incubated with a secondary antibody for 1 hour. Specific details for individual proteins' secondary anti-bodies and dilution reagent are provided in Table 2. Following the secondary anti-body incubation period the membrane was washed extensively and incubated for 5 minutes in WesternSure Premium Chemiluminescent substrate (LI-COR, Lincoln, NE) and visualized with C-Digit Blot Scanner (LI-COR, Lincoln, NE). Blots were stripped with Restore Western blot Stripping Buffer (Thermo Scientific, Rockford, IL) and probed with anti-tubulin as a loading control.

Statistical Analysis

All data was analyzed using the MIXED procedure of SAS (SAS 9.3, Cary, NC). In each experiment, data from at least three separate assays each with triplicate counts were combined and analyzed. Significant interactions were determined at $p<0.05$ and least squares means were separated using Tukey's ($p<0.05$)

Results

Effects of silencing EGFR expression on ER-α and IGFR-1β mRNA expression

Treatment of BSC cultures with EGFR siRNA resulted in significant knocked down of EGFR mRNA expression compared to BSC cultures treated with non-specific siRNA ($p<0.05$) (Figure 1). Additionally, because ER-α and IGFR-1β have been shown to play a role in E2 stimulated satellite cell proliferation, we also looked at the effect of EGFR siRNA treatment on expression of ER-α and IGFR-1β mRNA. Treatment of BSCs with EGFR siRNA had no impact upon ER-α (Figure 2) or IGFR-1β mRNA (Figure 3) expression indicating that the EGFR siRNA knockdown is specific for EGFR.

Effects of silencing EGFR on EGFR, ER-α and IGFR-1β protein levels

To further establish the efficacy of EGFR knockdown, the EGFR protein levels in BSCs treated with EGFR siRNA and non-specific siRNA were examined. In western immunoblots of protein lysates from BSC cultures treated with EGFR siRNA, EGFR protein was undetectable in comparison to the protein level in BSCs treated with non-specific siRNA (Figure 4). Table 3 shows that the ratio of EGFR protein to tubulin was 4 for non-specific siRNA treated cells and 0 for EGFR siRNA treated cells. Treatment of BSCs with EGFR siRNA had little if any impact upon ER-α protein levels (Figure 5). The ratio of ER-α protein to tubulin was 1.64 for non-specific siRNA and 1.35 for EGFR siRNA treated cells (Table 3). Bovine satellite cell cultures treated with EGFR siRNA had reduced levels of IGFR-1β protein in comparison to non-specific treated BSCs (Figure 6). The ratio of IGFR-1β protein to tubulin was 7.57 for non-specific siRNA and

2.25 for EGFR siRNA treated cells (Table 3). This implies that functional EGFR is necessary to maintain IGFR-1β protein levels in BSC.

Effect of silencing EGFR expression on E2 stimulated proliferation in cultured BSCs

To determine the impact of EGFR in E2 stimulated proliferation of bovine satellite cells we have evaluated the effect of silencing EGFR on the ability of E2, LR3-IGF-1, hb-EGF, and 10% FBS to stimulated proliferation. Figure 7 shows that silencing EGFR expression in BSC cultures completely suppressed hb-EGF, IGF-1, and E2 stimulated BSC proliferation ($p<0.05$). EGFR silenced cells respond to FBS indicating they are able to respond to mitogenic stimulation; however their response is less than cells treated with non-specific siRNA (Figure 7). This lesser response may reflect the inability of EGFR silenced cells to respond to EGF, E2, and possibly IGF-1.

Effects of an inhibitor of GPER-1 on E2 stimulated proliferation in cultured BSCs

In a multitude of tissues it has been shown that the G protein-coupled estrogen receptor (GPER)-1 contributes to rapid non-genomic estrogen response. These studies have shown that binding of E2 to GPER-1 results in transactivation of the EGFR. To assess the role of GPER-1 in E2 stimulated proliferation of BSCs, we evaluated the effect of G15 and G36 (specific GPER-1 antagonists) on E2 and LR3-IGF-1 (IGF-1 analog) stimulated proliferation. Figure 8 portrays that both G15 and G36 completely suppressed E2 stimulated proliferation of BSCs ($p<0.05$), indicating that GPER-1 is necessary for E2 stimulated proliferation. However, G15 and G36 had no impact upon LR3-IGF-1 stimulated proliferation (Figure 8). Thus, it appears that GPER-1 is not required for IGF-1-stimulated BSC proliferation.

Effects of an inhibitor of MMP2/9 on E2- and IGF-1-stimulated proliferation in cultured BSCs

Estrogen mediated activation of GPER-1 stimulates the activity of matrix metalloproteinases (MMP) 2/9 which contributes to the transactivation of EGFR in a variety of non-muscle tissues. To examine the potential role of MMP2 and MMP9 in E2 stimulated proliferation of bovine satellite cells, we assessed the effect of a MMP2/9 inhibitor on E2 stimulated proliferation. The MMP2/9 inhibitor completely suppressed E2 stimulated BSC proliferation ($p<0.05$), indicating that MMP2/9 activity is necessary for E2 stimulated proliferation. We also examined the effect of the MMP2/9 inhibitor on LR3-IGF-1 stimulated BSC proliferation. The MMP2/9 inhibitor partially suppressed IGF-1 stimulated proliferation ($p<0.05$) (Figure 9). The proliferation rate was significantly lower than the IGF-1 proliferation rate; however, it was not suppressed to the level of the control BSCs.

Discussion

Estrogen is an anabolic steroid that is commonly used as a beef management tool. Anabolic steroid implantation in steers has been linked to an increase in average daily gain (ADG) of 18-36% and feed efficiency of 8-34% over non-implanted steers (1; 6-9), both of which are important production characteristics contributing to muscle growth. Treatment of steers with estrogenic implants resulted in an increase in protein accretion and increased body weight gain (64). Determining the mechanism of action that estrogen utilizes to increase and support postnatal muscle growth is important to further improve beef production practices. Satellite cells play a crucial role in postnatal muscle growth by providing the additional nuclei necessary to support muscle growth. Skeletal muscle hypertrophy requires both myofiber size increase and nuclei quantity increase to support additional myofiber growth, both of which estrogen plays a role in. Estrogen has been shown to increase proliferative activity in a multitude of cells including bovine satellite cells (24; 25).

Our present study utilized the knockdown of EGFR to further elucidate the interaction of EGFR in the E2 mechanism of action within bovine satellite cells. Treatment of BSC cultures with EGFR siRNA resulted in significant knocked down of EGFR mRNA expression compared to BSC cultures treated with non-specific siRNA (Figure 1). To further establish the efficacy of EGFR knockdown, western immunoblots of protein lysates from BSC cultures treated with EGFR siRNA were examined. EGFR protein was eliminated in comparison to the protein level in BSCs treated with non-specific siRNA (Figure 4). Additionally, Table 3 shows that the ratio of EGFR protein to tubulin was 4 for non-specific siRNA treated cells and 0 for EGFR siRNA treated cells. Our present

study indicates that when EGFR is silenced hb-EGF stimulated proliferation is suppressed in bovine satellite cells, as expected. Additionally, neither E2 nor IGF-1 are able to stimulate proliferation in EGFR silenced BSCs. EGFR silenced cells respond to 10% FBS indicating they are able to respond to mitogenic stimulation; however their response is less than cells treated with non-specific siRNA. This lesser response is not surprising due to the fact that the cells are non-responsive to E2, IGF-1, and hb-EGF. The essential role of EGFR in E2 and IGF-1 stimulated proliferation is supported by studies in non-muscle cells where EGFR was rendered nonfunctional either by using AG1478 (EGFR tyrosine kinase inhibitor) or by knocking down EGFR with a siRNA. These studies show that EGFR inhibition results in inhibition of proliferation induced by E2, IGF-1, and EGF (34; 36-38) in MCF-7 and COS-7 cancer cells. Additionally, AG1478 has been shown to suppress E2 and hb-EGF stimulated proliferation in bovine satellite cells (26). This accumulation of data supports our current findings that suppression of EGFR also suppresses activity of ER-α and IGFR-1. This supports the model that EGFR is the downstream converging point for ER-α and IGFR-1 action.

Due to the fact that E2 and IGF-1 stimulated proliferation is suppressed in EGFR silenced bovine satellite cells, we also looked at the effect of EGFR siRNA treatment on expression of ER-α and IGFR-1 mRNA. Treatment of BSCs with EGFR siRNA had no impact upon ER-α (Figure 2) or IGFR mRNA (Figure 3) expression indicating that the EGFR siRNA knockdown is specific for EGFR. Treatment of BSCs with EGFR siRNA had little if any impact upon ER-α protein levels (Figure 5). The ratio of ER-α protein to tubulin was 1.64 for non-specific siRNA and 1.35 for EGFR siRNA treated cells (Table 3). Bovine satellite cell cultures treated with EGFR siRNA had reduced levels of IGFR-

1β protein in comparison to non-specific siRNA treated BSCs (Figure 6). The ratio of IGFR-1β protein to tubulin was 7.57 for non-specific siRNA and 2.25 for EGFR siRNA treated cells (Table 3). Since IGFR-1β mRNA expression is not reduced in EGFR silenced cells, reduction of IGFR-1β is not the result of non-specific effects of the EGFR siRNA. Instead, it appears that silencing of EGFR results in a post-transcriptional reduction in IGFR-1β protein levels. This is supported by a study done in breast cancer cells where EGFR was knocked down, resulting in an decrease in IGFR-1β protein levels while the IGFR-1β mRNA levels were not impacted (65). Since previous studies have shown that E2 stimulated satellite cell proliferation is suppressed in BSC in which IGFR-1β is silenced (51), the reduction in IGFR-1β protein level in EGFR-silenced cells complicates interpretation of the role of EGFR in E2 stimulated BSC proliferation. Although it is clear that functional EGFR is necessary for E2 stimulated proliferation of BSC, it is not clear to what extent. The study done in breast cancer indicated that EGFR protects IGFR-1β from degradation and impacts IGFR-1β protein stability (65). EGFR may function to maintain the level of IGFR-1β which is necessary for E2 stimulated proliferation or the transactivation of EGFR may directly stimulate proliferation. Additionally, it is possible that the role of EGFR in E2 stimulated BSC proliferation may involve both of these mechanisms. Further research is required to elucidate the roles of EGFR and IGFR-1 in E2-stimulated BSC proliferation.

Numerous studies in non-muscle cells have shown that E2 may stimulate proliferation by binding to GPER-1 which results in transactivation of EGFR (28; 33; 34; 40; 42; 43). Consequently, to further clarify the role of EGFR transactivation in E2 stimulated BSC proliferation; we have examined the effect of inhibiting GPER-1 on E2-stimulated BSC

proliferation. Our study indicates that when GPER-1 is suppressed E2 stimulated proliferation is inhibited indicating that E2 binding to GPER-1 is involved in E2 stimulated bovine satellite cell proliferation. This was also seen in uterine epithelial cells were G15 reduced E2 stimulated proliferation by 50% (26). However, treatment with G1 (GPER-1 agonist) did not stimulate BSC proliferation indicating that activation of GPER-1 alone was not sufficient to stimulate BSC proliferation (57; 60). Additionally, treatment of cultured BSCs with ICI 182,780 (ER antagonist) resulted in suppressed E2 stimulated proliferation (47) indicating that ER-α or ER-β activity was required for E2 stimulated BSC proliferation In BSC cultures in which ER-α was silenced E2 stimulation of proliferation was completely suppressed (51), indicating that ER-α and not ER-β is required for E2 stimulated BSC proliferation. These results establish that E2 functions by activating both GPER-1 and ER to stimulate BSC proliferation. Additionally, our results show that GPER-1 is not involved in IGF-1 stimulated BSC proliferation because inhibition of GPER-1 has no impact on IGF-1 stimulated BSC proliferation.

Various studies in non-muscle tissue indicate that MMP2/9 play an important role in GPER-1 mediated proliferation. Ligand binding to GPER-1 results in activation of MMP2/9 and subsequent proteolytic release of membrane bound hb-EGF which binds to EGFR resulting in transactivation of the EGFR. In breast cancer cells when the MMPs are inhibited they are unable to cleave the hb-EGF, reducing the available free hb-EGF. This indicates that MMPs are necessary for E2/GPER-1 mediated release of hb-EGF, a crucial aspect of EGFR transactivation (42; 49; 50). The reduction in hb-EGF resulted in the EGFR phosphorylation being suppressed (42; 49; 50). Similarly, our study showed that when MMP2/9 activity is inhibited in BSC cultures, E2 stimulated proliferation is

also suppressed. This finding supports the hypothesis that GPER-1 stimulated transactivation of EGFR is involved in E2 stimulated proliferation of BSC.

Our studies also show that IGF-1 stimulated proliferation of cultured BSC is suppressed when MMP2/9 are inhibited. These data suggest that there is an interaction between IGFR-1 and MMP2/9 but further studies are needed to clarify this relationship.

The compilation of results from this study contributes to the theory that binding of E2 to GPER-1 results in transactivation of the Epidermal Growth Factor Receptor (EGFR) (Figure 10) (28; 33; 34; 40; 42; 43). Estrogen binds to GPER-1 with high affinity and that binding activates GPER-1 releasing the Gβγ protein complex (44). These subunits of the GPER-1 stimulate the activity of matrix metalloproteinases (MMP) 2/9. MMPs enable the proteolytic cleavage and release of heparin-binding Epidermal Growth Factor (hb-EGF) from the cell surface. The cleaved hb-EGF is able to bind to Epidermal Growth Factor Receptor (EGFR) resulting in EGFR transactivation which mediates downstream pathways (28; 33; 34; 40; 42; 43) which regulates proliferation. GPER-1 transactivation of EGFR is crucial for the rapid, non-genomic response to E2 in cells. Additionally, it appears that IGFR-1 also is involved in E2 stimulated BSC proliferation and that functional EGFR is required in order to maintain IGFR-1 level. Further studies are required to elucidate the relationship and specific roles of IGFR-1 and EGFR in the mechanism of E2 stimulated BSC proliferation.

Table 1. Nucleotide sequences for bovine quantitative real-time PCR primers

	Primer	GenBank accession no.
IGFR-1β primers		NM_001244612
Forward	5'-GCA GAT GGC ATG GCA TAC CT-3'	
Reverse	5'-TCT TCG GCC ACC ATG CA-3'	
ESR1 primers		NM_001001443
Forward	5'-ACG ATT GAT AAA AAC AGG AGG AAG A-3'	
Reverse	5'-GCC TTT CAT CAT GCC CAC TT-3'	
EGFR primers		XM_002696890.2
Forward	5'- CCC CAG GCC ATG AAC GT -3'	
Reverse	5'- GCA GTG AGG GCC ATC AAT GT -3'	
Cyclophilin		NM_178320
Forward	5'-GGT CCT GGC ATC TTG TCC AT-3'	
Reverse	5'-TGG CAG TGC AAA TGA AAA ACT G-3'	

Table 2. Antibodies for western immunoblots and their designated protein, source, catalog number, dilution rate, dilution reagent, and incubation period.

Designated Protein	Specific Antibody	Antibody Source	Catalog Number	Antibody Dilution Rate	Antibody Dilution Reagent	Antibody Incubation Period
EGFR Primary	anti-EGF-Rec	Cell Signaling	2646	1:1000	5% BSA +TBST	Overnight
IGFR-1β Primary	anti-IGF-1 Rec β	Cell signaling	3027	1:3000	5% BSA +TBST	Overnight
ER-α Primary	anti-ESR1	Santa Cruz Biotechnology Inc	sc-71064	1:200	5% NFDM +TBST	1 hour
Tubulin Primary	anti-β Tubulin	Santa Cruz Biotechnology Inc	sc-51712	1:1000	5% NFDM +TBST	1 hour
EGFR Secondary	HRP-conjugated goat anti-rabbit IgG	Cell Signaling	7074	1:2000	5% NFDM +TBST	1 hour
IGFR-1β Secondary	HRP-conjugated goat anti-rabbit IgG	Cell Signaling	7074	1:2000	5% NFDM +TBST	1 hour
ER-α Secondary	goat anti-mouse IgG-HRP	Santa Cruz	sc-2005	1:2000	5% NFDM +TBST	1 hour
Tubulin Secondary	HRP-conjugated goat anti-mouse IgM	Santa Cruz	Sc-2064	1:10,000	5% NFDM +TBST	1 hour

Table 3. Ratio of EGFR, IGFR-1 and ER-α protein levels to tubulin levels in Western immunoblots of protein lysates obtained from BSC cultures treated with EGFR siRNA or Non-specific siRNA.

Protein	Non-specific siRNA	EGFR siRNA
EGFR	4	0
IGFR-1β	7.57	2.25
ER-α	1.64	1.35

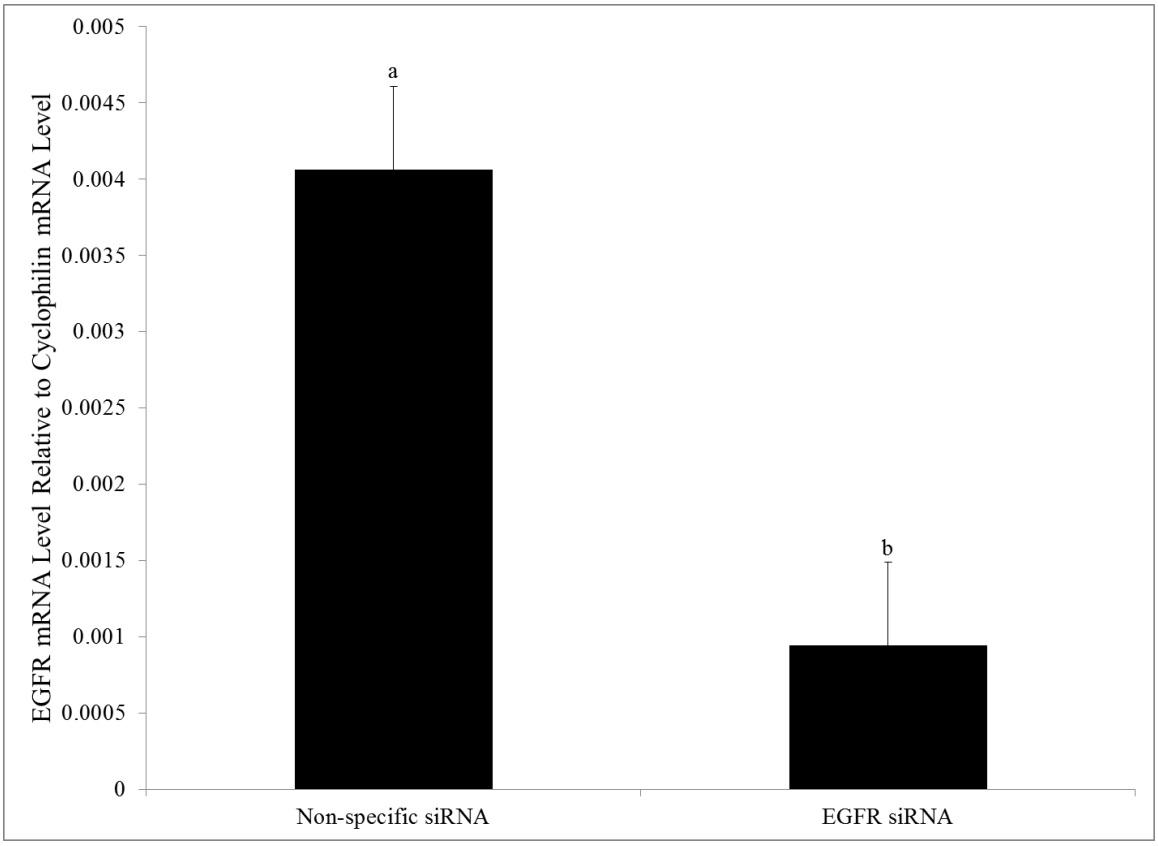

Figure 1. Relative EGFR mRNA level in bovine satellite cell (BSC) cultures treated with non-specific siRNA and in BSC cultures treated with EGFR siRNA. Cells were plated as detailed in Materials and Methods, and during the first 72 hours were grown in Dulbecco Modified Eagle Medium (DMEM) containing 10% FBS. At 72 hours cells were fed with 1mL 10%FBS+DMEM without any antibiotics and the transfection complex containing 200 µl Opti-MEM I Reduced Serum Media, 1.5 µl Lipofectamine RNAiMAX, and either control(non-specific) or EGFR siRNA (2nM). This antibiotic-free media was incubated on the cells for 6 hours. After 6 hours media was removed and fresh 10%FBS+DMEM with antibiotics was added and cells were incubated for an additional 18 hours. At 96 hours total RNA was isolated and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays were utilized to determine EGFR mRNA levels relative to the level of cyclophilin mRNA in cultures treated with non-specific siRNA or EGFR siRNA. Relative EGFR mRNA levels are significantly reduced ($p < 0.05$) in EGFR siRNA-treated cultures. Data are the pooled mean ± SEM from 8 separate assays each containing triplicate determinations and using cells isolated from two different animals. Bars with different letters are significantly different ($p<0.05$).

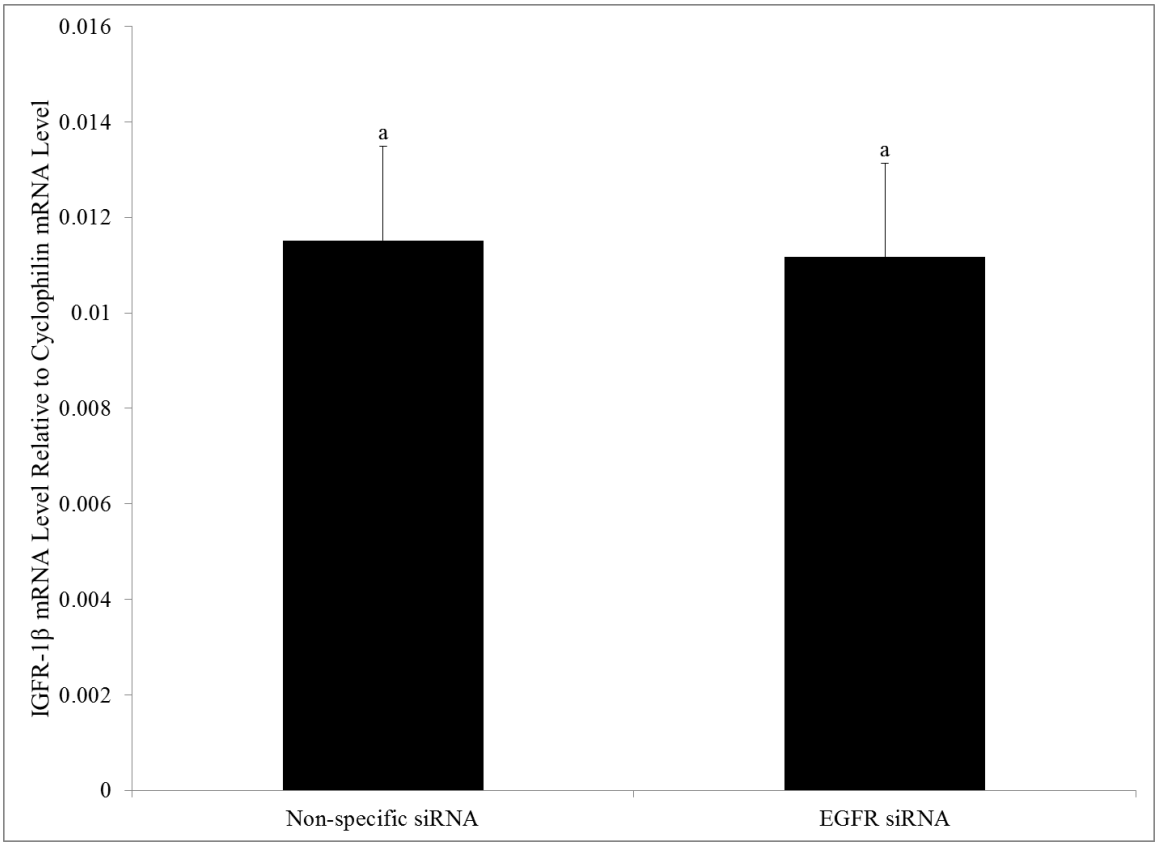

Figure 2. Relative IGFR-1β mRNA level in bovine satellite cell (BSC) cultures treated with non-specific siRNA and in BSC cultures treated with EGFR siRNA. Cells were plated as detailed in Materials and Methods, and during the first 72 hours were grown in Dulbecco Modified Eagle Medium (DMEM) containing 10% FBS. At 72 hours cells were fed with 1mL 10%FBS+DMEM without any antibiotics and the transfection complex containing 200 µl Opti-MEM I Reduced Serum Media, 1.5 µl Lipofectamine RNAiMAX, and either control(non-specific) or EGFR siRNA (2nM). This antibiotic-free media was incubated on the cells for 6 hours. After 6 hours media was removed and fresh 10%FBS+DMEM with antibiotics was added and cells were incubated for an additional 18 hours. At 96 hours total RNA was isolated and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays were utilized to determine IGFR mRNA levels relative to the level of cyclophilin mRNA in cultures treated with non-specific siRNA or EGFR siRNA. Relative IGFR mRNA levels were not significantly reduced ($p < 0.05$) in EGFR siRNA-treated cultures. Data are the pooled mean ± SEM from 8 separate assays each containing triplicate determinations and using cells isolated from two different animals. Bars with different letters are significantly different ($p<0.05$).

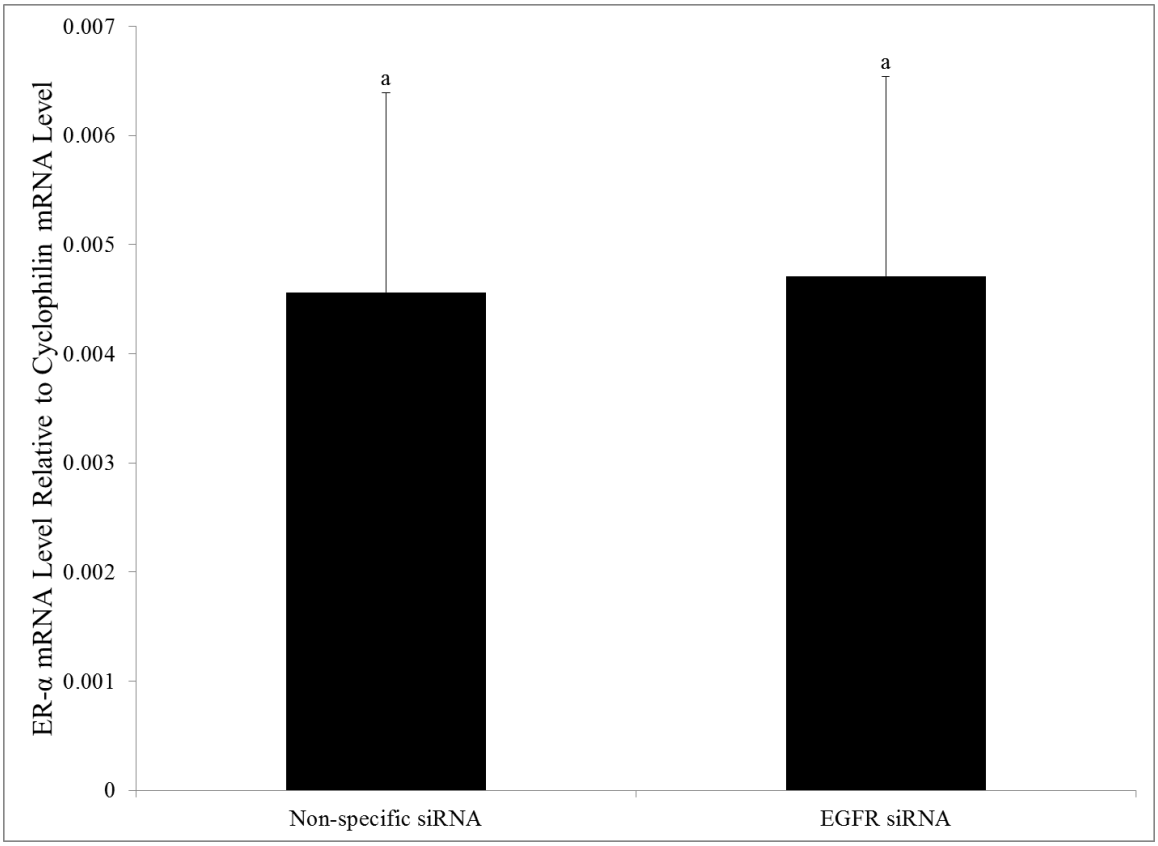

Figure 3. Relative ER-α mRNA level in bovine satellite cell (BSC) cultures treated with non-specific siRNA and in BSC cultures treated with EGFR siRNA. Cells were plated as detailed in Materials and Methods, and during the first 72 hours were grown in Dulbecco Modified Eagle Medium (DMEM) containing 10% FBS. At 72 hours cells were fed with 1mL 10%FBS+DMEM without any antibiotics and the transfection complex containing 200 µl Opti-MEM I Reduced Serum Media, 1.5 µl Lipofectamine RNAiMAX, and either control(non-specific) or EGFR siRNA (2nM). This antibiotic-free media was incubated on the cells for 6 hours. After 6 hours media was removed and fresh 10%FBS+DMEM with antibiotics was added and cells were incubated for an additional 18 hours. At 96 hours total RNA was isolated and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays were utilized to determine ER-α mRNA levels relative to the level of cyclophilin mRNA in cultures treated with non-specific siRNA or EGFR siRNA. Relative ER-α mRNA levels were not significantly reduced ($p < 0.05$) in EGFR siRNA-treated cultures. Data are the pooled mean ± SEM from 8 separate assays each containing triplicate determinations and using cells isolated from two different animals. Bars with different letters are significantly different ($p<0.05$).

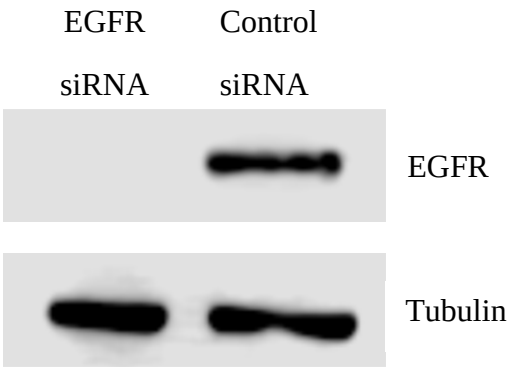

Figure 4. Western immunoblot showing EGFR protein levels in bovine satellite cell (BSC) cultures treated with non-specific siRNA (**Control siRNA**) or EGFR siRNA (**EGFR siRNA**). Cells were lysed in tracking dye (20% SDS, 1M Tris-HCl pH6.8, Glycerol, 0.4% Bromophenol blue, Water, β-Mercaptoethanol) and analyzed using Western immunoblotting. Tubulin was used as a loading control. Cells treated with EGFR siRNA show no detectable EGFR protein.

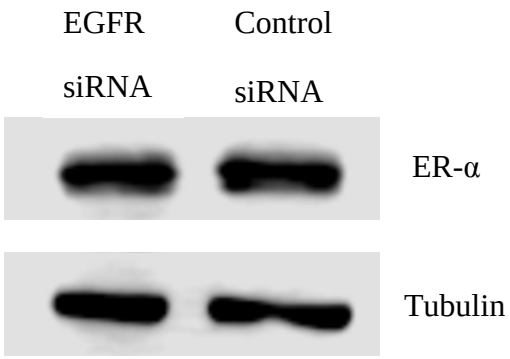

Figure 5. Western immunoblot showing ER-α protein levels in bovine satellite cell (BSC) cultures treated with non-specific siRNA **(Control siRNA)** and EGFR siRNA **(EGFR siRNA)**. Cells were lysed in tracking dye (20% SDS, 1M Tris-HCl pH6.8, Glycerol, 0.4% Bromophenol blue, Water, β-Mercaptoethanol) and analyzed using Western immunoblotting. Tubulin was used as a loading control. Levels of ER-α protein in EGFR siRNA treated were not reduced compared to levels in non-specific siRNA treated BSCs.

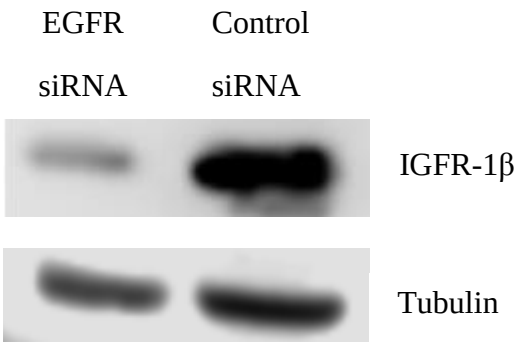

Figure 6. Western immunoblot showing IGFR-1β protein levels in bovine satellite cell (BSC) cultures treated with non-specific siRNA **(Control siRNA)** and EGFR siRNA **(EGFR siRNA)**. Cells were lysed in tracking dye (20% SDS, 1M Tris-HCl pH6.8, Glycerol, 0.4% Bromophenol blue, Water, β-Mercaptoethanol) and analyzed using Western immunoblotting. Tubulin was used as a loading control. Levels of IGFR-1 protein in EGFR siRNA treated BSC are reduced compared to the levels in BSC treated with non-specific siRNA.

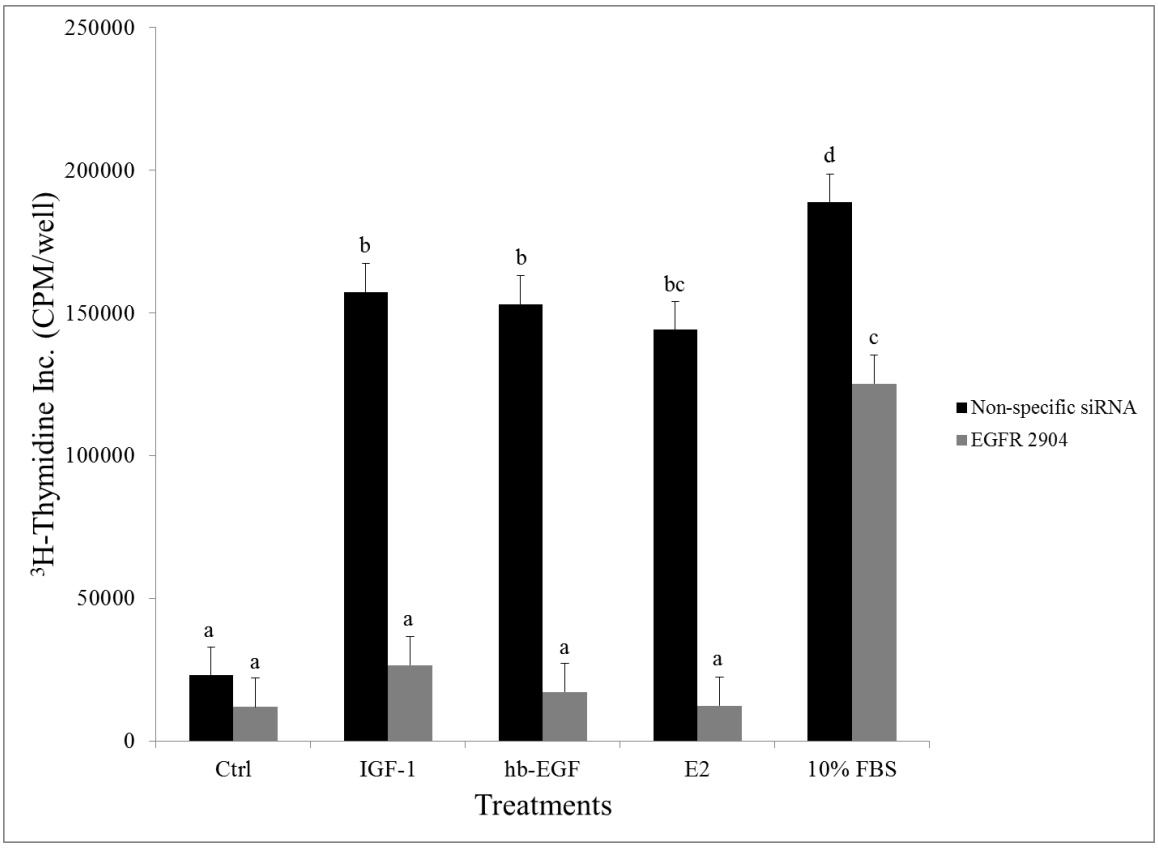

Figure 7. ^{3}H-Thymidine incorporation assay showing the effects of LR3-IGF-1 (IGF-1), estradiol **(**E2), heparin binding-epidermal growth factor (hb-EGF) or fetal bovine serum (FBS) on proliferation in bovine satellite cell (BSC) cultures treated with non-specific small interfering RNA (siRNA) or EGFR siRNA. Cells were plated as detailed in Materials and Methods, and during the first 72 hours were grown in Dulbecco Modified Eagle Medium (DMEM) containing 10% FBS. At 72 hours cells were fed with 0.5mL 10%FBS+DMEM without any antibiotics and the transfection complex containing 100 µl Opti-MEM I Reduced Serum Media, 0.75 µl Lipofectamine RNAiMAX, and either control(non-specific) or EGFR siRNA (2nM). This antibiotic-free media was incubated on the cells for 6 hours. After 6 hours media was removed and fresh 10%FBS+DMEM with antibiotics was added to the cells and incubation was continued for 18 hours. At 96 hours cultures were treated with DMEM/1%IGFBP-3-free swine serum (**Ctrl**) or DMEM/1%IGFBP-3-free swine serum plus 25ng LR3-IGF-1/mL **(IGF-1)**, 5ng hbEGF/mL (**hb-EGF**), 10nM E2 (**E2**) or 10% FBS (**10% FBS**). Bars with different letters are significantly different ($p < 0.05$). Data are the pooled mean ± SEM from 8 separate assays each containing triplicate determinations and using cells isolated from two different animals.

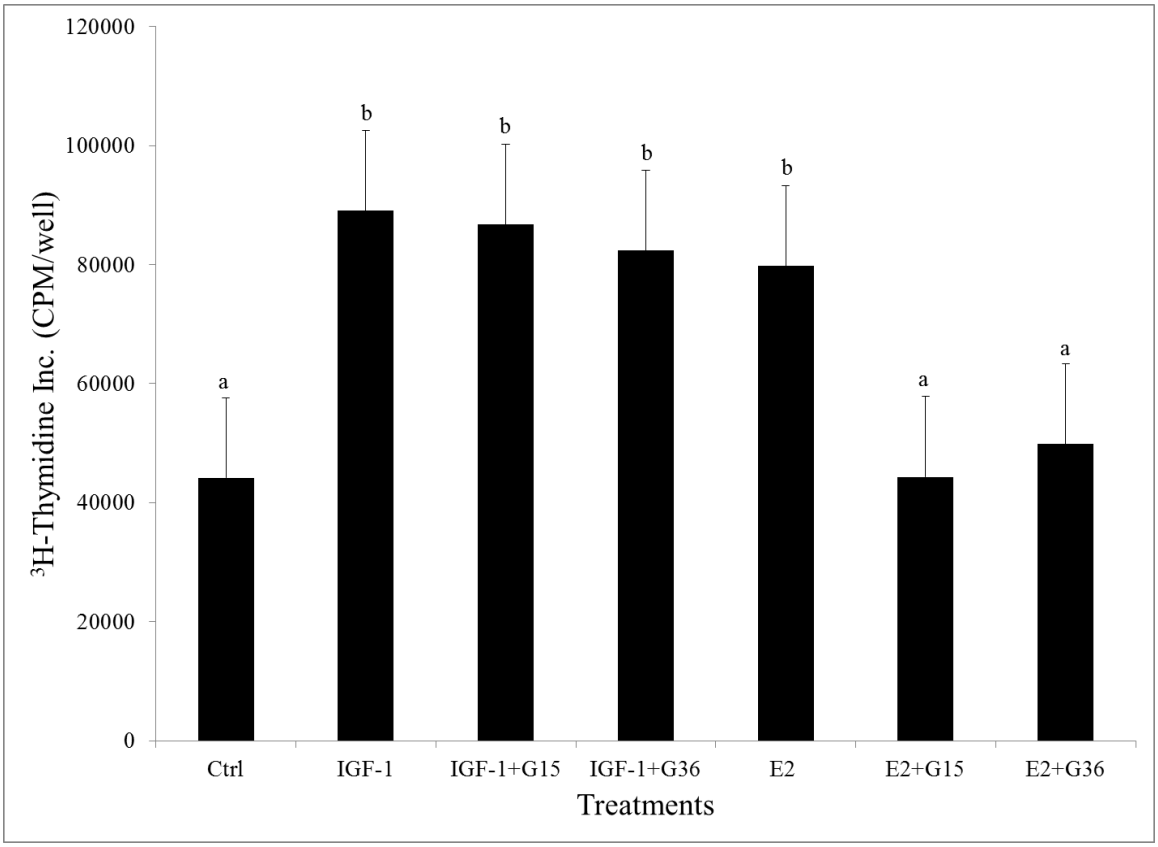

Figure 8. ^{3}H-Thymidine incorporation assay showing the effects of LR3-IGF-1 (IGF-1) or estradiol (E2) on proliferation in bovine satellite cell (BSC) cultures treated with G15 and G36 (specific G protein-coupled estrogen receptor (GPER)-1 antagonists). Cells were plated as detailed in Materials and Methods, and during the first 72 hours were grown in Dulbecco Modified Eagle Medium (DMEM) containing 10% FBS. At 72 hours cells were treated with DMEM/1% IGFBP-3-free swine serum (**Ctrl**) or DMEM/1% IGFBP-3-free swine serum plus 25ng LR3-IGF-1/mL (**IGF-1**) or 10nM E2 (**E2**) +/- 20nM G15 or G36. Bars with different letters are significantly different from each other ($p < 0.05$). Data are the pooled mean ± SEM from 3 separate assays each containing triplicate determinations and using cells isolated from two different animals.

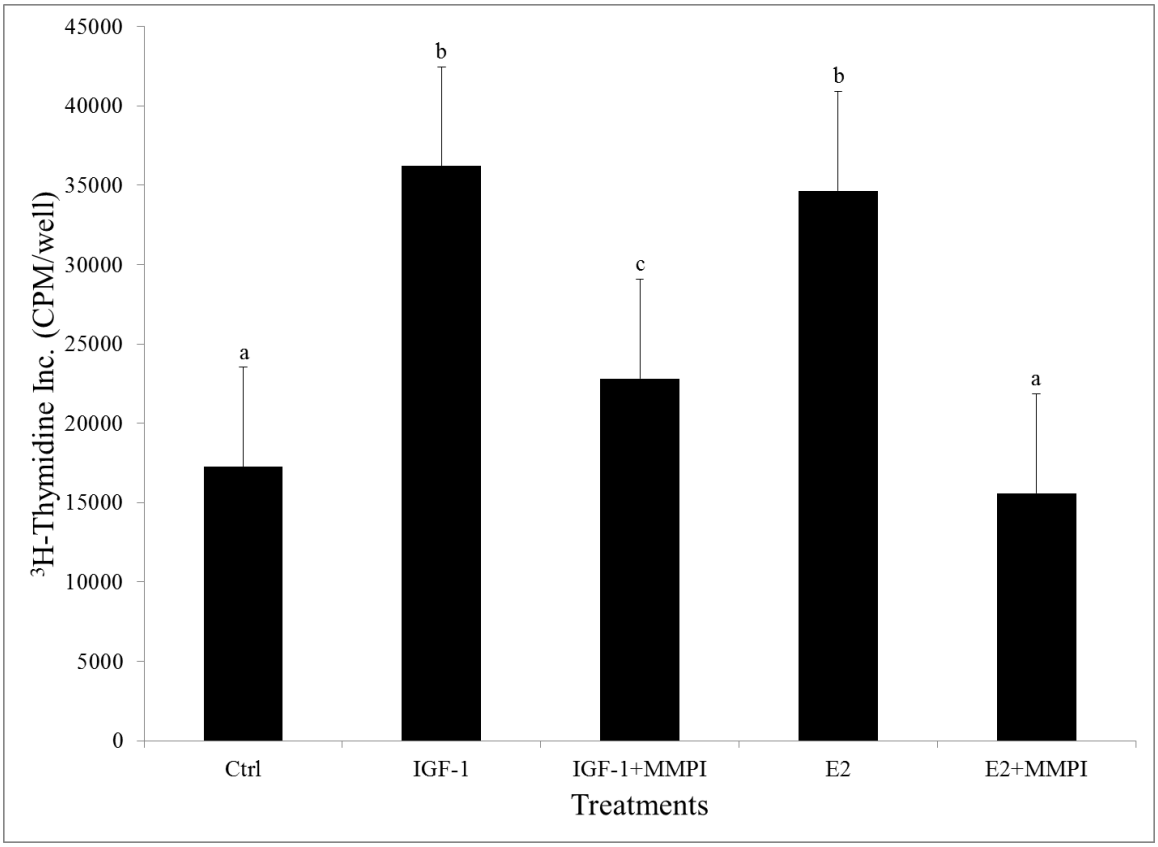

Figure 9. ^{3}H-Thymidine incorporation assay showing the effects of LR3-IGF-1 (IGF-1), estradiol (E2), heparin binding-epidermal growth factor (hb-EGF) or fetal bovine serum (FBS) on proliferation in bovine satellite cell (BSC) cultures treated with a matrix metalloproteinase 2/9 inhibitor. Cells were plated as detailed in Materials and Methods, and during the first 72 hours were grown in Dulbecco Modified Eagle Medium (DMEM) containing 10% FBS. At 72 hours cultures were treated with DMEM/1%IGFBP-3-free swine serum (**Ctrl**) or DMEM/1%IGFBP-3-free swine serum plus 25ng LR3-IGF-1/mL **(IGF-1)**, 10nM E2 (**E2**) ± 10μM MMP2/9 inhibitor (**MMPI**). Bars with different letters are significantly different ($p < 0.05$). Data are the pooled mean ± SEM from 3 separate assays each containing triplicate determinations and using cells isolated from two different animals.

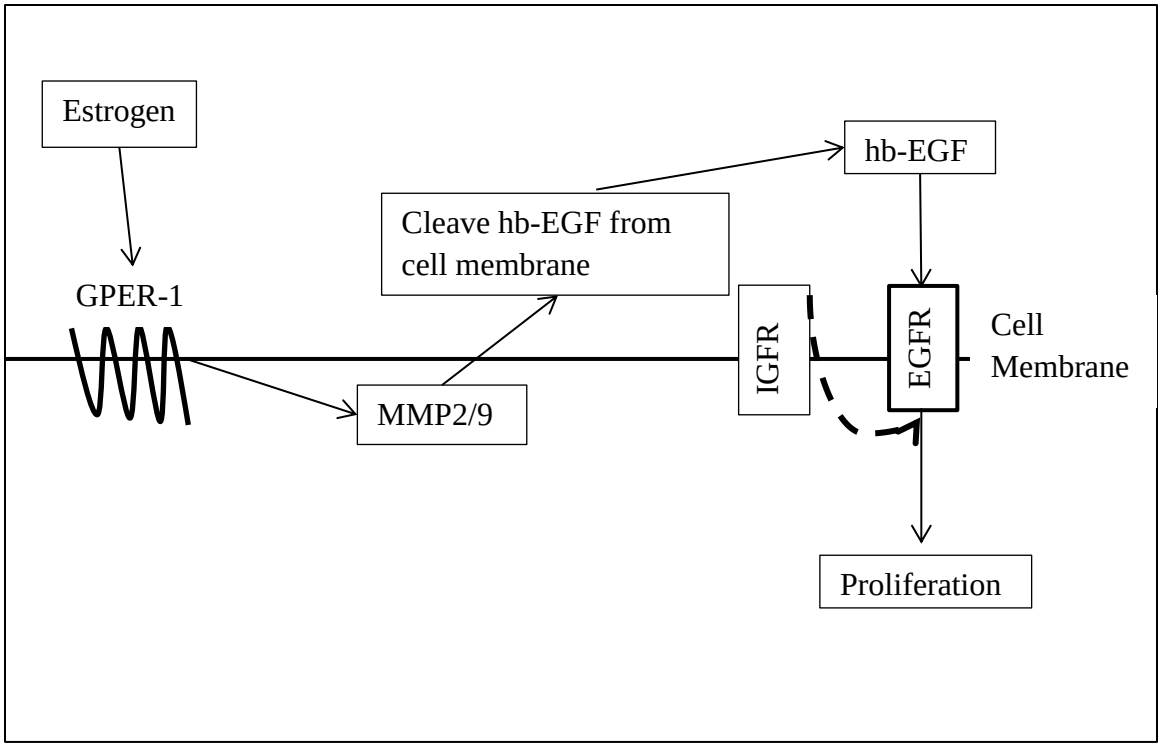

Figure 10. Proposed estrogen mechanism of action schematic as detailed in this paper

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