THE ROLE OF OXYTOCIN IN BOVINE SATELLITE CELL PROLIFERATION AND DIFFERENTIATION

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

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Sept. 20th, 2017

Blacksburg, VA

Keywords: Satellite cell, Steroid hormone, Tamoxifen, Oxytocin, Proliferation, Differentiation

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ABSTRACT

Steroid hormones are reported to increase oxytocin (OXT) expression in skeletal muscle. It is possible that OXT may play essential roles in satellite cell (SC) proliferation and differentiation, which further contribute to skeletal muscle development and growth. In this dissertation, we tested this hypothesis with in-vivo and in-vitro studies in intrauterine growth restriction (IUGR) sheep, caloric restricted (CR) calves, tamoxifen (TAM) treated heifers, and bovine satellite cells (BSCs), respectively. In the in-vivo studies, we collected (1) longissimus dorsi muscle (LM) from IUGR sheep; (2) infraspinatus muscle (INF), LM, and semitendinosus muscle (ST) from CR calves; (3) LM muscle from TAM heifers. In all samples, mRNA abundance of targeted genes, such as OXT, were measured. Muscle fiber size and BSC number were also determined in skeletal muscle from TAM treated heifers. For the in-vitro studies, different treatments including 17β-estradiol (E2), trenbolone (TBA), TAM, OXT, and atosiban were applied to wild-type BSC and OXT knockout BSC (CRISPR-OXT) separately to investigate OXT’s functions on BSC activity. For in-vivo studies, OXT expression significantly decreased (\(P<0.05\)) in IUGR LM muscle. Caloric restriction decreased OXT expression (\(P<0.05\)) in INF, LD, and ST muscle. Expression of OXT in LM from TAM animals decreased 50% (\(P<0.05\)). Moreover, TAM caused a small statistical reduction (\(P<0.05\)) in cross-sectional area (CSA). In in-vitro studies, OXT alone increased (\(P<0.05\)) fusion index but not proliferation in the wild-type BSC, whereas both proliferation and differentiation were stimulated (\(P<0.05\)) by OXT treatment in the CRISPR-OXT cell. By contrast, E2 and TBA
increased ($P<0.05$) both proliferation rate and fusion index in wild-type BSC. However, E2 and TBA only stimulated proliferation rate ($P<0.05$) but not fusion index for CRISPR-OXT cells. Atosiban treatment resulted in lower proliferation and differentiation ($P<0.05$) in both wild-type BSC and CRISPR-OXT cell compared with OXT and E2 treatment groups. Together, our in-vivo studies indicate that OXT may play important roles in skeletal muscle development and growth. Our in-vitro studies demonstrate that OXT plays important roles in BSC proliferation and differentiation, and it is involved in steroid hormone stimulated BSC activity.

**Key words:** Differentiation, Oxytocin, Proliferation, Satellite cell, Steroid hormone, Tamoxifen
Oxytocin (OXT) is a mammalian neurohypophysial hormone. It has been demonstrated that OXT is highly expressed in skeletal muscle and satellite cell (SC) by steroid hormone administration. However, the role of OXT in SC proliferation and differentiation is not elucidated. In this dissertation, the in-vivo and in-vitro studies are combined and used to investigate if OXT is involved in bovine SC (BSC) activity. In the in-vivo studies, we collected muscle samples from intrauterine growth restriction (IUGR) sheep, caloric restricted (CR) calves, and tamoxifen (TAM) treated heifers. In all samples, mRNA abundance of OXT was measured. For the in-vitro studies, wild-type BSC and OXT knockout BSC (CRISPR-OXT) were treated with different factors including 17β-estradiol (E2), trenbolone (TBA), TAM, OXT, and atosiban separately to investigate OXT’s functions on BSC activity. For in-vivo studies, OXT expression significantly decreased \( (P<0.05) \) in IUGR, CR, and TAM muscle. In in-vitro studies, OXT alone increased \( (P<0.05) \) fusion index but not proliferation in the wild-type BSC, whereas both proliferation and differentiation were stimulated \( (P<0.05) \) by OXT treatment in the CRISPR-OXT cell. By contrast, E2 and TBA, which can stimulate OXT expression in cultured BSC, increased \( (P<0.05) \) both proliferation rate and fusion index in wild-type BSC. However, E2 and TBA only stimulated proliferation rate \( (P<0.05) \) but not fusion index for CRISPR-OXT cells. Atosiban treatment resulted in lower proliferation and differentiation \( (P<0.05) \) in both wild-type BSC and CRISPR-OXT cell compared with OXT and E2 treatment groups. Together, our studies indicate that OXT
plays important roles in BSC proliferation and differentiation, and it is involved in steroid hormone stimulated BSC activity. Studies to investigate specific biological mechanisms of steroid hormone stimulated OXT expression in SC are needed in the future.
ACKNOWLEDGEMENTS

I would like to acknowledge my advisor, Dr. Robert P. Rhoads, for his excellent guidance and support throughout my PhD. He provided me an excellent atmosphere for doing research and helped me with developing my research background, teaching me techniques and correcting my writing. I would never be able to finish my dissertation without his guidance. Besides my advisor, I owe sincere thankfulness to rest of my committee members: Dr. Sally E. Johnson, Dr. Samer W. El-Kadi, Dr. Matthew W. Hulver for their insightful comments.

Special thanks go to Patricia M Williams, who is always willing to help. He helped me to solve different kinds of problems and order most of materials for my study, so that my research can go smoothly. I also would like to express my gratitude to my previous and current lab mates: Guohao Xie, Lidan Zhao, Morgan, Kellie for their help and friendship. We worked together and had a lot of fun in the past several years.

Finally, I would like to thank my parents and my wife for their understanding and endless love. Their love is helping me go through hard times. They are always encouraging me and supporting me no matter where I am.
LIST OF ABBREVIATIONS

AR: androgen receptor
ARE: androgenic response element
BSC: bovine satellite cell
cHRE: composite hormone response element
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats
Cas9: CRISPR associated protein 9
CSA: cross-sectional area
DAPI: 4,6-Diamidino-2-phenylindole
DBD: DNA binding domain
DHEA: dehydroepiandrosterone
DHT: dihydrotestosterone
DM: differentiation medium
DMEM: Dulbecco's Modified Eagle's Medium
DSB: double strand breaks
E2: 17β-estradiol
ECL: ECL cell attachment matrix
EGF: Epidermal growth factor
ER: estrogen receptor
ERE: estrogen response elements
ERK: extracellular signal-regulated kinases
FBS: Fetal Bovine Serum
FSH: follicle stimulating hormone
GKR: glycyl-lysyl-arginine
GM: growth medium
GnRH: Gonadotropin-releasing hormone
GPCR: G-protein coupled receptor
gRNA: guide RNA
HBSS: hanks' balanced salt solution
HDR: homology directed repair
HS: horse serum
HSD: hydroxysteroid dehydrogenase
HSP: heat shock protein
IGF: insulin-like growth factor
IL-6: Interleukin 6
Indels: insertion/deletion
IUGR: intrauterine growth restriction
LBD: ligand-binding domain
LH: luteinizing hormone
MAPK: mitogen-activated protein kinases
MRFs: myogenic regulatory factors
MSC: bone marrow-derived mesenchymal stem cell
MYH: myosin heavy chain
MyoD: myogenic differentiation 1
MyoG: myogenin
NHEJ: non homologous end joining
NO: nitric oxide
OXT: oxytocin
OXTR: oxytocin receptor
MyoG: paired box 7
PBS: phosphate buffered saline
qPCR: real-time quantitative reverse transcription-PCR
T: testosterone
TAM: tamoxifen
TBA: trenbolone acetate
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CHAPTER 1. LITERATURE REVIEW

Introduction

The beef industry is one of the major meat suppliers, which contributed to roughly 21.2% of world’s meat products in 2016 (FAO, 2016: http://www.fao.org/fileadmin/templates/est/COMM_MARKETS_MONITORING/Meat/Documents/FO_Meat_June_2016.pdf). Increasing salable meat, thus economic profit, is of great interest to beef producers. Understanding the growth of skeletal muscle, which is one of the most critical components in meat, has become necessary in this process. Feed supplemented with steroid hormones are routinely applied to beef management to improve feed efficiency, reduce behavioral problems, and enhance meat production (Bartelt-Hunt et al., 2012). The mechanism(s) of steroid hormone stimulated muscle growth is not fully elucidated. The mechanism by which sex steroid mediate muscle growth, in broad terms, is expected to be both direct (Sauerwein and Meyer, 1989) and indirect (Kamanga-Sollo et al., 2008). The direct function is by binding to androgen and estrogen receptors, whereas the indirect function is to induce other substances that subsequently stimulate muscle growth. Recently, one study supported a new indirect function of steroid hormones, that is, it may induce expression of oxytocin (OXT) in skeletal muscle during muscle development (De Jager et al., 2011a). However, whether OXT participates in skeletal muscle satellite cell (SC) proliferation and differentiation is not yet fully understood and explored. Therefore, the ultimate goal of the studies reported in this dissertation was to determine the role of OXT in bovine satellite cell (BSC) activity with emphasis on proliferation and differentiation.
Skeletal Muscle

Introduction of Skeletal Muscle

Skeletal muscle is one of the largest tissues in the vertebrate body, and it accounts for approximately 40% of the adult total body mass (Marieb and Hoehn, 2007). It is also a highly adaptive tissue that can respond to environmental and physiological stimuli such as heat stress, disuse, age, or hormone, and it is able to undergo both hypertrophy and atrophy in accordance with the demands placed on it (Lundby et al., 2009; Patel and Matsakas, 2009). Muscle fiber hypertrophy and atrophy relate to the growth of basic muscle structural units named myofibrils. Myofibrils are an insoluble protein assembly consisting of more than 30 different proteins including actin (thin filament), myosin (thick filament), and titin that are organized into a regular pattern to generate repeating units called sarcomeres (Lieber, 2002). The sarcomere is the basic contractile unit of muscle that is composed of one A-band and two half I-bands. Two sarcomere units are separated by Z-lines. The sarcomere can grow in diameter by addition or subtraction of thick and thin filaments and in length by adding filaments at the end of the cell (Goldspink, 1968).

An increase in strength training causes an increase in muscle size and strength (Fiatarone et al., 1990); conversely, muscle disuse results in muscle atrophy (Powers et al., 2007). Furthermore, many coordinately regulated hormones have evolved to stimulate muscle size by controlling the related signaling pathways such as steroid hormones (Florini et al., 1991; Glass, 2005).
Fig. 1.1: Vertebrate skeletal muscle sarcomere organization. The sarcomere consists of one A-band and two half I-band. Two sarcomeres separate by Z-line. Thick filaments lie parallel to each other in clusters and overlap to the thin filaments. The sarcomere can grow in diameter by adding more thick and thin filaments. Sarcomere can also be added at the end of the cell to increase the overall length. (Schiaffino and Reggiani, 2011)

It is generally accepted that muscle fibers can be divided into two broad types: slow twitch (Type I) muscle fibers and fast twitch (Type II) muscle fibers (Brooke and Kaiser, 1970). Fast twitch fibers can be further categorized into Type IIa, Type IIb, and Type IIx fibers (Essen et al., 1975). The slow muscle fibers are thinner, more efficient at using oxygen to generate ATP for sustained contractions over a long time (Schiaffino and Reggiani, 2011). Therefore, slow twitch fibers are great at helping athletes run marathons and bicycle for hours. Type II fibers are thicker, use anaerobic metabolism to create fuel, contain few mitochondria and less Myoglobin. They are better at generating short bursts of near maximal force than slow muscles, but fatigue quickly (Schiaffino and Reggiani, 2011). Both Type IIa and Type IIx fibers use both aerobic and anaerobic metabolism almost equally to create energy, and Type IIb fibers represent fast twitch fibers with limited oxidative capacity and high glycolytic capacity (Klont et al., 1998).
Table 1.1: Muscle fiber type characteristics (Schiaffino and Reggiani, 2011)

<table>
<thead>
<tr>
<th></th>
<th>Type I fibers</th>
<th>Type II a fibers</th>
<th>Type II x fibers</th>
<th>Type II b fibers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contraction time</td>
<td>Slow</td>
<td>Moderately fast</td>
<td>Fast</td>
<td>Very fast</td>
</tr>
<tr>
<td>Fatigue</td>
<td>High</td>
<td>Fairly high</td>
<td>Moderate</td>
<td>Low</td>
</tr>
<tr>
<td>Activity used for</td>
<td>Aerobic</td>
<td>Long term anaerobic</td>
<td>Short term anaerobic</td>
<td>Short term anaerobic</td>
</tr>
<tr>
<td>Maximum duration of use</td>
<td>Hours</td>
<td>&lt; 30 min</td>
<td>&lt; 5 min</td>
<td>&lt; 1 min</td>
</tr>
<tr>
<td>Power produced</td>
<td>Low</td>
<td>Medium</td>
<td>High</td>
<td>Very high</td>
</tr>
<tr>
<td>Mitochondrial density</td>
<td>Very high</td>
<td>High</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>Capillary density</td>
<td>High</td>
<td>Intermediate</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Oxidative capacity</td>
<td>High</td>
<td>High</td>
<td>Moderate</td>
<td>Low</td>
</tr>
<tr>
<td>Glycolytic capacity</td>
<td>Low</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Myosin heavy chain</td>
<td>MYH 7</td>
<td>MYH 2</td>
<td>MYH 1</td>
<td>MYH 4</td>
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</table>

The primary functions of skeletal muscle are to produce skeletal movement, maintain posture and body position, maintain body temperature, and store nutrient reserves (Millward et al., 1975; Bergh and Ekblom, 1979; MacIntosh et al., 2006). However, less well identified is the important role it plays in body metabolism and health. Since skeletal muscle accounts for ~ 75% of glucose utilization after insulin stimulation, it is the most important tissue to maintain glucose homeostasis (Kramer et al., 2006). Therefore, when muscle tissue loses its sensitivity to insulin (insulin resistance), type 2 diabetes commonly occur (DeFronzo and Tripathy, 2009). In addition, during the past decade, skeletal muscle has been identified as an organ with a high secretory capacity to produce several hundred secreted factors, such as myostatin, Interleukin 6 (IL-6), insulin-like growth factor (IGF) 1, and IGF 2 (Pedersen et al., 2007; Pedersen and Febbraio, 2008; Rodgers and Garikipati, 2008). Some of the factors produced by skeletal muscle are released into the blood stream and exert specific endocrine effects on distant organs (Pedersen and Febbraio, 2012). Other proteins produced by skeletal muscle could work via autocrine or paracrine mechanisms to trigger signaling pathways within the muscle itself (Pedersen and Febbraio, 2012).
Therefore, the importance of maintaining muscle mass is also critical for overall quality of life and health.

**Myogenesis**

In vertebrates, skeletal muscle originates from the paraxial mesoderm layer, and specifically from the somite. The dorsal part of the somite contributes to the skeletal muscle of the body and limbs (Grefte et al., 2007). During embryogenesis, the muscle progenitor cells from the somite migrate to the limb bud, where they proliferate, commit to the myogenic lineage, and fuse together to form myotubes which serve as an important structural component of skeletal muscle (Péault et al., 2007).

Myogenesis is tightly regulated by many muscle specific transcriptional factors. These factors have been found to regulate the progression of myogenic lineage coordinately. In the embryonic stage, the myogenic precursors can be characterized by Paired Box Protein 3 and 7 (Pax3 and MyoG) expression (Biressi et al., 2007). Pax3 is essential for embryonic muscle development. It is only detected in high levels in certain anatomical locations of the adult body, such as the diaphragm (Buckingham, 2007; Day et al., 2007). Thus its function in adult muscle is less understood. MyoG regulates the expression of muscle regulatory basic helix-loop-helix transcriptional factors (known as MRFs) and support the proliferation of myoblasts in the somite and the SC in adult skeletal muscle (Relaix et al., 2005; von Maltzahn et al., 2013). Deletion of MyoG in embryos or adults causes a profound muscle regeneration deficit (von Maltzahn et al., 2013).

Factors regulating proliferation and differentiation involve the four identified MRFs, which are myogenic factor 5 (Myf5), myoblast determination protein (MyoD), muscle-specific regulatory
factor 4 (\textit{MRF4}), and myogenin (\textit{MyoG}) (Sambasivan and Tajbakhsh, 2015). Among those four, the earliest transcription factors involved in proliferation is \textit{Myf5}. \textit{Myf5} is expressed in most quiescent SC (Beauchamp et al., 2000) and activated myoblasts, and is thought to regulate proliferation rate and homeostasis (Ustanina et al., 2007). Freshly isolated SC express high levels of \textit{Myf5} transcripts (Kuang et al., 2007). The initiation of myogenesis is delayed by the presence of \textit{Myf5} alone (Kablar et al., 1997). The second MRF expressed during myogenesis is \textit{MyoD}. The expression of \textit{MyoD} may be required for cell cycle entry and SC differentiation. It is often thought of as the master myogenic transcription factor to induce multipotent precursor cells to the muscle lineage (Tapscott, 2005). After an extensive proliferation stage, some myoblasts withdraw from the cell cycle and start to express the late stage MRFs, \textit{MyoG} and \textit{MRF4} (Kassar-Duchossoy et al., 2004). Both \textit{MyoG} and \textit{MRF4} play essential roles in terminal differentiation and fusion of committed myoblast. The \textit{MyoG} and \textit{MRF4} knock-out mice exhibit severe defects in muscle development (Hasty et al., 1993; Olson et al., 1996).

As myogenesis proceeds, the myoblasts fuse to each other to form the primary muscle fibers. Only a small number of primary fibers are formed which then serve as a template for secondary fiber formation (Bailey et al., 2001). Subsequently, the muscle mass grows extensively during the prenatal and postnatal period. During the muscle development process, small populations of active myoblasts can withdraw from the cell cycle and return to the quiescent state. Those cells are located between the basal lamina and muscle fiber membrane, defined as SC (Mauro, 1961). These cells are of obvious importance to skeletal muscle regeneration (Relaix and Zammit, 2012).
**Postnatal Skeletal Muscle Growth**

Since muscle fiber number is fixed before birth, postnatal muscle growth mainly depends on an increase in muscle fiber size (hypertrophy) rather than new myofiber formation. Postnatal muscle growth can be characterized as a period of muscle hypertrophy due to the substantial increase in DNA content and protein deposition (Verdijk et al., 2009). Because myonuclei are post-mitotic, the increase of DNA content is dependent on SC proliferation (Blaauw and Reggiani, 2014). In healthy human adults, SC make up 2-7% of the muscle nuclei (Yin et al., 2013), which is significantly lower than 30% observed in neonatal muscle in mice (Hawke and Garry, 2001). The SC numbers vary by muscle fiber type. The Type I and Type IIa oxidative muscle fibers display higher numbers of SC compared to Type IIb glycolytic muscles fibers (Schultz, 1989). In addition, lower numbers of SC were observed with age related loss of muscle mass, regeneration capacity (Shefer et al., 2006; Collins et al., 2007), and in patients with diseases such as Duchene Muscular Dystrophy (BLAu et al., 1983). In contrast, SC number can increase with exercise training and hormone treatment (Darr and Schultz, 1987; Sinha-Hikim et al., 2003; Cermak et al., 2013).

In most cases, SC are in a quiescent stage that has limited gene expression and protein synthesis. They become activated by injury, disease, or exercise (Charge and Rudnicki, 2004). When activated, SC become known as myoblasts that undergo proliferation and increase in number. After proliferation, in order to generate multinucleated myofibers, myoblasts committed to differentiation undergo cell-to-cell fusion with existing and damaged myofibers, or they participate in de novo formation of myotubes. A small portion of these myoblasts will revert back to SC and return to the quiescent stage (Le Grand and Rudnicki, 2007).
Estrogens and Skeletal Muscle

Estrogen

Estrogens are a group of 18-carbon corticosteroid molecules that are synthesized from cholesterol (Ghayee and Auchus, 2007). Estrogens are secreted primarily by ovaries in females and testes in males. Other tissues such as muscle, fat, liver, and brain synthesize estrogens as well (Cooke and Naaz, 2004). The most potent and dominant estrogen in mammals is 17β-estradiol (E2), but two other estrogens, estriol and estrone, are present at lower levels. When released into the blood stream, estrogens are bound to plasma proteins and transported to target tissues. The steroid hormones are lipophilic and have a low molecular weight that enables them to pass through the cell membrane by passive diffusion. Estrogens are primarily involved in the development and maintenance of normal sexual and reproductive function (Heldring et al., 2007a). They also exert biological effects on cardiovascular, skeletal muscle, immune, and central nervous systems (Katzenellenbogen et al., 1995). Estrogens prevent bone resorption and reduce osteoporosis in postmenopausal women (Rossouw, 2002). In the nervous system, estrogens can control the hypothalamic-pituitary-gonadal axis, and impact learning and memory (Phillips and Sherwin, 1992; Birge, 1996). The incidence of cardiac disease in premenopausal women is lower than men of the same age, and this observation is correlated with estrogen presence (Milne and Noble, 2008).

Estrogen Receptors

Estrogen receptors (ERs) are ligand-activated transcription factors which are found both inside and on the membrane of the cells. Two classes of ER exist in animals: nuclear estrogen receptors (ERα and ERβ), which are members of the nuclear receptor family of intracellular receptors, and membrane estrogen receptor (GPR30), which belongs to G protein-coupled receptor
family (Carmeci et al., 1997; Dahlman-Wright et al., 2006). In the late 1960s, the presence of a receptor molecule that could bind E2 was first reported by Jensen and Jacobsen (Jensen and Jacobson, 1962). This receptor was isolated and cloned in the mid-1980s by Green et al. (Green et al., 1986) and was for a long time was regarded as the only ER until a second ER was reported in 1996 (Kuiper et al., 1996). The two receptors are today known as ERα and ERβ, respectively.

ERα and ERβ show a high degree of similarity when compared at the amino acid level and share a common structure including five distinct domains (Ascenzi et al., 2006). They are named A/B, C, D, E, and F domains. Transcriptional activation of ERα is mediated by two distinct activation factors: AF-1, located in the N-terminal A/B domain, and AF-2, located in the E domain of the receptors (Tora et al., 1989). They are believed to function by binding co-activators and initial the transcription of receptors. ERβ has a weaker corresponding AF-1 function and thus the AF-2 site is the major regulatory site for ERβ transcription (Delaunay et al., 2000). The C domain is the DNA-binding domain which is involved in specific DNA binding and receptor dimerization. The sequence of ERα and ERβ are approximately 97% homologous in this domain which indicates that target genes are the same for two receptors. D domain links the DNA-binding domain and the E domain. The E domain is the ligand-binding domain which exhibits approximately 56% similarity between two receptors. It is critical for ligand binding, receptor dimerization, and transcriptional activation. F domain has less than 20% amino acid identity between the two receptors and the function remains poorly understood.
**Fig. 1.2: Structure of estrogen receptor.** The comparison of human estrogen receptor α (595 AA) and a shorter estrogen receptor β (530 AA) was shown in this picture. These receptors consist of five distinct structural and functional domains: DNA-binding domain (DBD; C domain), hinge domain (D), ligand-binding domain (LBD; E/F domain), and two transcriptional activation function domains AF-1 (in A/B domain) and AF-2 (in F domain). (Gimpl and Fahrenholz, 2001)

ERα and ERβ can be detected in different tissues. The classical estrogen targets are the uterus, mammary gland, placenta, central nervous system, cardiovascular system and bone. In some tissues, both receptors are expressed at similar levels, whereas in others, they are different (Ciocca and Vargas Roig, 1995; Taylor and Al-Azzawi, 2000). This observation suggested that two receptors have distinct biological functions. ERα and ERβ mRNA and protein are detected in skeletal muscle of various animals (Dahlberg, 1982; Saartok, 1984; Wiik et al., 2003). Pfaffl et al. (2001) indicated for the first time the expression of ERβ mRNA in skeletal muscle from the heifers. Both ERα and ERβ protein are found in mouse and pig skeletal muscle and SC (Barros et al., 2006; Kalbe et al., 2007). Blocking ERs with ER antagonist ICI 182,870 completely abolished both exercise- and estrogen-mediated increases in SC numbers (Enns and Tiidus, 2008). This demonstrated that ERs play significant roles in influencing muscle repair processes through augmentation of SC activation and proliferation (Thomas et al., 2010).
**Estrogen Receptor Signaling**

Estrogen action is exerted in target tissues via binding to ERα, ERβ, or G-protein coupled receptor (GPR30). Like other steroid hormone receptors, ERα and ERβ act as dimers to regulate transcriptional activation of target genes (Kushner et al., 2000). However, evidence suggested that there are several distinct pathways besides the classical ligand-dependent activation mechanism.

*Classical Ligand-Dependent Pathway*

In the absence of ligand, estrogen receptors are mainly located in the cytosol binding with the protective heat shock proteins (Gasc et al., 1990). Estrogen binding to the receptor triggers translocation of the receptor from the cytosol into the nucleus and dimerize of the receptor. Ligand-bound ER dimers can bind directly to estrogen response elements (ERE) which are located in the promoter region of target genes. The ERE sequence is a 13-base pair sequence consisting of two half-sites (aGGTCAnnnTGACCt) separated by a three-nucleotide spacer. The binding of ER to ERE recruits other proteins such as nuclear receptor coactivators that are responsible for the transcription of downstream DNA into mRNA and finally protein that results in a change in cell function (Nilsson and Gustafsson, 2002).

*Non-ERE Dependent Pathway*

In addition to binding to the ERE, the estrogen and ER complex can interact with other DNA-bound transcription factors to regulate the transcription of target genes. In this mechanism, ER dimers do not bind promoter DNA themselves. Instead, the complex tethered by protein-protein interaction with other transcription factors that contact the DNA. Fos/Jun (AP-1 responsive elements) and SP-1 (GC-rich SP-1 motifs) are well characterized that could regulate estrogen signaling when no consensus ERE has been found (Bjornstrom and Sjoberg, 2005).
Non-Genomic Signaling Pathway

In addition, estrogen may elicit effects through non-genomic mechanisms. Estrogen binds to the GPR30 in the membrane of target cells (Razandi et al., 2004). This effect occurs within seconds or minutes after addition of estrogen and cannot be blocked by transcription inhibitors. It has been suggested that these effects may be the result of estrogen activation of mitogen-activated protein kinases (MAPKs) and extracellular signal–regulated kinases (ERKs) signaling (Pedram et al., 2006) or release of intracellular calcium (Mermelstein et al., 1996). The MAPK signaling pathway is rapidly activated by E2 in several cell types, including breast cancer (Migliaccio et al., 1996), endothelial (Chen et al., 1999), bone (Jessop et al., 2001), and neuroblastoma (Watters et al., 1997) cells.

Ligand-Independent Activation Pathway

Growth factor signaling leads to activation of kinases which may phosphorylate the well-conserved serine residues in the AF-1 site of ER and thus activate ERs or associated coregulators in the absence of ligand. Phosphorylated ERs were activated and dimerized and thereby bind DNA and regulate transcription of target genes (Kato et al., 1995). ERα can be activated by IGF1 and epidermal growth factor (EGF). The activated IGF1 and EGF receptors result in the intracellular activation of MAPK signal transduction cascade that impacts the transcriptional activity of ERs by phosphorylation of serine residues (Thomas et al., 2008). ERs may be activated by cAMP induced signaling pathway as well. The AF-2 site of ERs contributes to this effect. It appears to be dependent on protein kinase A that is activated by cAMP (El-Tanani and Green, 1997). Peptide hormones are specifically involved in this signaling pathway.
Fig. 1.3: Model representing the mechanistically distinct molecular pathways of estrogen receptor. Four different mechanisms were summarized in this picture: the classical (direct) pathway, the tethered pathway, non-genomic signaling pathway, and the ligand-independent pathway (Heldring et al., 2007b).

Effect of Estrogen on Skeletal Muscle

In the United States beef industry, the administration of exogenous estrogens is given to improve production efficiency while reducing production costs (Boles et al., 2009). Specifically, estrogen implants influence growth rates and body composition (Kahl et al., 1978). The most common estrogenic hormones utilized in beef industry are E2, zeranol and estradiol benzoate (Reinhardt, 2007). Exogenous estrogen has been well characterized to improve muscle composition (Duckett et al., 1999). In feedlot heifers, the implant of estrogen positively increased longissimus muscle area (Schneider et al., 2007). Sauerwein and Meyer (1989) found increased
bovine skeletal muscle mass occurs when animals are treated with E2. Moreover, it was reported that E2 treatment in cull cows and feedlot steers increased LM area and carcass fat free lean (Cranwell et al., 1996; Gonzalez et al., 2007). Gonzalez et al. (2007) also found E2 treated cull cows exhibited larger myofibers (especially Type I muscle fiber) cross-sectional area (CSA) and diameter. It was reported that circulating IGF-1 levels are increased in steers treated with E2 (Pampusch et al., 2003). Additionally, LM IGF-1 mRNA levels in implanted steers were increased after E2 implantation relative to control (Dayton and White, 2014). Hannon et al. (1991) found IGF-1 increased SC numbers and efficiency of muscle growth in implanted steers. Consequently, it appears that direct effects of E2 on skeletal muscle growth is due to increased IGF-1 mRNA expression (Dayton and White, 2014). In rodents, most studies reported that female and estrogen supplemented animals exhibit less myofiber injury following exercise induced muscle injury (Tidball, 2005). In addition, It was reported that estrogens impact muscle contractile properties in the mouse (Moran et al., 2007). In animals, estrogen affects muscle fatigue as well as twitch characteristics including peak tension and half-relaxation time (Hatae, 2001; Schneider et al., 2004). Studies in mice indicated that estrogen administration altered the expression patterns of myosin heavy chain (MHC) proteins in both fast- and slow-twitch muscles (Kadi et al., 2002). In addition, it was reported that the isometric twitch tension of the fast-twitch fibers (extensor digitorum longus) was lower in estrogen supplemented ovary-intact rats compared to ovariectomized rats, with no effect on isometric twitch tension in the slow-twitch fibers (soleus) (Suzuki and Yamamuro, 1985; Kitajima and Ono, 2016).

Although SC only account for approximately 2-7% of total muscle nuclei, proliferation and activation of muscle SC may be involved in steroid-induced muscle growth. Fewer than ten SC are capable of generating over 100 new myotubes (Collins et al., 2005). Maintenance of the
quiescent state was recently shown to be regulated by microRNA-489, which was involved in the proliferative expansion of SC (Cheung et al., 2012). SC are activated in response to myotrauma and begin to proliferate (Zammit et al., 2002). After one round of proliferation, one of the progeny return to quiescence with the second cell withdrawing from the cycle to differentiate. Differentiating SC are \textit{MyoD}, \textit{MyoG}, \textit{MYF5} positive, whereas quiescence SC are only \textit{MyoG} (Tedesco et al., 2010). The differentiating SC can fuse with existing fibers or align to form new myofibers to contribute to muscle growth and regeneration. SC activation and proliferation are regulated by a number of factors released from skeletal muscle or leukocytes (Vierck et al., 2000; Machida and Booth, 2004). Many of these factors are impacted by estrogens. Thus, estrogen may play important roles in SC proliferation, and hence muscle regeneration.

Much less is known about the potential for estrogen to stimulate muscle regenerative processes such as SC activation and proliferation. It was reported that regeneration of rat skeletal muscle following a period of muscle atrophy was dependent upon estrogen status (McClung et al., 2006). Estrogen may influence growth of myoblast cells \textit{in-vitro} (Kahlert et al., 1997) and is also associated with \textit{in-vivo} development of muscle size in female mice (Sciote et al., 2001). Kamanga-Sollo et al. (2008) found treatment with 10 nM E2 stimulated proliferation of cultured bovine SC. The proliferation rate of SC from sheep was also increased with estrogen administration (Johnson et al., 1996b). A E2 administration of E2 increased protein synthesis and inhibited protein degradation, which leaded to larger diameter of myotubes (Kamanga-Sollo et al., 2010). Estrogen treatments increase SC proliferation and differentiation but not the expression of \textit{IGF-1} in cattle, which is known to stimulate bovine SC (BSC) proliferation and differentiation (Kamanga-Sollo et al., 2013). This evidence demonstrated the positive role of estrogen in SC activation and proliferation. In rat, skeletal muscles of estrogen-supplemented animals had increased numbers of
total (\textit{MyoG}-positive), activated (\textit{MyoD}-positive) and proliferating (BrdU-incorporated) SC after 72 hours of downhill running (Tiidus et al., 2005; Enns and Tiidus, 2008).

**Androgens and Skeletal Muscle**

**Androgen**

Androgens are male sex hormones of the steroid family which play crucial physiological roles in establishing and maintaining the male phenotype. Male sex steroids are essential for the differentiation and growth of the male reproductive organs, regulation of spermatogenesis, and control of secondary sexual characteristics. In addition, androgens also have metabolic effects on protein, carbohydrate, and fat metabolism and hence contribute to the increase in muscle mass and strength, and anabolic function on bone and fat as well (Yin et al., 2003; Köhn, 2006). The major biologically active male sex steroid in skeletal muscle is testosterone (T), which can be further metabolized into several biologically active hormones including dihydrotestosterone (DHT) and E2 via 5α-reductase (Bhasin et al., 2003). Testosterone is a 0.288 kDa C19 steroid hormone produced from cholesterol (Vingren et al., 2010). Cholesterol is utilized in the formation of T by a series of enzymatic reactions in the Leydig cells of the testes. The testes account for more than 95% of T production. The production of T is regulated through the hypothalamic-pituitary-gonadal-axis (Vingren et al., 2010). The signal originates in the hypothalamus by the innervations of the Central Nervous System (CNS) which results in secretion of Gonadotropin-releasing hormone (GnRH). GnRH is then transported from hypothalamus to pituitary and stimulates the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the gonadotropes. LH is responsible for T production in the Leydig cells in men and the theca cells in
women. Circulating T exerts a negative feedback in hypothalamus and pituitary by inhibiting the release of GnRH and LH.

The synthesis of T is mediated through two major classes of enzymes named cytochrome p450 (CYP) and hydroxysteroid dehydrogenase (HSD). In gonads and adrenal gland, cholesterol side chain is catalyzed by CYP11A to generate the pregnenolone. Pregnenolone can be catalyzed into progesterone by 3βHSD. Pregnenolone and progesterone are further catalyzed by CYP17 to generate 17α-hydroxypregnenolone and 17α-hydroxyprogesterone. The sex steroid precursor, dehydroepiandrosterone (DHEA) is synthesized by additional cleavage. In gonads, DHEA is catalyzed into active T by 17HSD3 (Miller and Auchus, 2010). Skeletal muscle, as a secretory organ, can produce and secrete androgen from DHEA and DHEA sulfate (Sato et al., 2008; Vandenput and Ohlsson, 2010). The same as in gonads, DHEA and DHEA sulfate are transformed to T via steroidogenic enzymes in skeletal muscle. T can be converted to DHT by 5α-reductase. Both T and DHT can bind to androgen receptor within the cells. However, the binding affinity of DHT is higher compared to T (Bennett et al., 2010). In skeletal muscle, DHT levels are considerably low due to the low amounts of 5α-reductase (Chen et al., 2005). Thus, T is the predominant androgen in skeletal muscle.

**Androgen Receptor**

Androgens are ligands of the androgen receptor (AR), which is a member of the ligand activated nuclear hormone receptor super family (Robinson-Rechavi et al., 2003). Androgens bind to the AR to form complexes which can bind to the promoter or enhancer elements of target genes to regulate their transcription. The AR contains an N-terminal domain which regulates transcription activity, a highly conserved DNA binding domain (DBD), variable hinge region, a
ligand-binding domain (LBD), and two transcriptional activation domains AF-1 and AF-2 (Askew et al., 2012).

The AR is sequestered by specific chaperone proteins such as heat shock protein 90 (HSP90), HSP70, and HSP56 in the cells. Once androgens translocate inside the cell, the hormone will bind to the AR which results in a transformation of the receptor and hence dissociates with HSPs allowing the androgens-AR bound complex to be active (Gelmann, 2002). The active dimerized complex will translocate from the cytosol into the nuclei where it will recruit androgenic response element (ARE) and subsequently bind to the promoter or activation region of the target genes and regulate their expression. This process is considered to be the classic genomic effect of androgen action. However, androgens can also bind to a trans-membrane G-protein coupled receptors and result in a more rapid non-genomic effect. These effects may be the result of intracellular calcium increase or activation of signaling cascades such as MAPK and PI3K (Michels and Hoppe, 2008).

**Effect of androgen on skeletal muscle**

Androgens are important determinants of body composition in male mammals (Venken et al., 2006). In the beef industry, the administration of exogenous androgens influence growth rates and body composition of growing and mature cattle (Kahl et al., 1978). Trenbolone acetate (TBA) and T are common androgenic compounds given to cattle to increase muscle mass (Bartelt-Hunt et al., 2012). Brandstetter et al. (2000) found that the presence of testicular hormones is related to greater muscle growth capacity in steers. Katznelson et al. (1998) indicated that androgen deficiency is associated with decreased muscle mass and increased fat mass. Conversely, T supplementation increases fat-free mass (Snyder et al., 2000) and decreases fat mass (Wang et al., 2000) in cattle (Bartelt-Hunt et al., 2012). Ahtiainen et al. (2011) found androgens have an anabolic
effect on skeletal muscle via increases in protein synthesis and decreases in protein degradation. Treatment of cultured BSC with TBA and dexamethasone separately resulted in a dose-dependent increase in protein synthesis and a decrease in protein degradation (Kamanga-Sollo et al., 2010). Johnson et al. (1996a) reported that implantation with TBA resulted in approximately 10% to 12% more carcass protein than carcasses from nonimplanted steers. In weather lambs, Lobley et al. (1987) demonstrated that breakdown of protein was reduced with T administration. Several studies have also determined the effects of androgen on the skeletal muscle in hypogonadal men. The AR and T levels in plasma are highlighted by gender differences, as males produce more than ten times T and have an increased AR protein than in females (Vingren et al., 2010). The CSA of arm and leg muscle increased significantly following administration of T enanthate at a dose of 25-600mg (Bhasin et al., 1996). Another study implied that muscle mass increased by 20% when hypogonadal men were treated with T enanthate (Brodsky et al., 1996).

Besides hypertrophy, another form of skeletal muscle growth is increasing nuclei number in the muscle fiber (Verdijk et al., 2014). Satellite cells can undergo proliferation and fuse to the existing fibers to increase fiber DNA content or repair damage from injury (Hawke and Garry, 2001). Skeletal muscle fiber number is set at birth, and the change in size of skeletal muscle occurs as a result of SC proliferation and differentiation (McCarthy et al., 2011). Skeletal muscle from steers implanted with 120mg TBA contained 50% more SC than muscle from non-implanted steers (Johnson et al., 1998). The proliferation rate of BSC was increased by TBA, anabolic-androgenic steroid (Kamanga-Sollo et al., 2008). Johnson et al. (1998) reported that SC isolated from TBA implanted steers contained 50% more myotube nuclei than that from non-implanted steers, which indicate that T plays important roles in BSC differentiation. In men, it was reported that T-induced muscle hypertrophy is associated with an increase in SC number (Sinha-Hikim et al., 2003).
Moreover, the SC from men exhibited upregulated AR expression following T treatment. Thus, SC are considered as a direct androgen target in skeletal muscle. The steroid also upregulated SC proliferation rates in rat and pig models (Joubert and Tobin, 1995; Dodson et al., 1996). Dexamethasone and T can induce proliferation of C2C12 myoblasts (Desler et al., 1996). Studies in C2C12 cells demonstrated that cell differentiation was increased by administration of T (Diel et al., 2008).

**Indirect Regulation of SC by Androgens**

IGF-1 is well-characterized and regarded as an important regulator of muscle mass (Adams, 2002). Mice with overexpression of IGF-1 in skeletal muscle exhibit a higher muscle mass compared to controls. Recent studies have suggested that anabolic effects of androgens in skeletal muscle may be mediated by local IGF-1 (Lewis et al., 2002). In humans, T stimulated IGF-1 protein expression in skeletal muscle (Ferrando et al., 2002). The administration of nandrolone generated higher levels of muscle IGF-1 expression in skeletal muscle compared with the rats without nandrolone treatment (Lewis et al., 2002). In BSC, TBA treatment increased IGF-1 mRNA abundance (Kamanga - Sollo et al., 2004). Thus, androgens can stimulate muscle growth by increasing IGF-1 expression in skeletal muscle.

IGF-1 regulates several biochemical pathways and is involved in protein synthesis and myogenesis. The downstream pathways of PI3K/Akt/mTOR play essential roles in myotube hypertrophy. IGF-1 acts through binding to its receptor, and subsequent activation of PI3K. The PI3K can phosphorylate and activate Akt, which subsequently mediates the phosphorylation of mTOR. Protein synthesis is promoted through 4E-BP1 and the phosphorylation of p70S6K. It has Testosterone mediates its androgenic effects through IGF-1 and its downstream signaling (Serra
Dihydrotestosterone induced phosphorylation of p70S6k in skeletal muscle of rats in a dose-dependent manner (Xu et al., 2004). It was reported that acute increases in ERK1/2, Akt, and p70S6K occur with T treatment (Basualto-Alarcón et al., 2013). Besides activation of mTOR, IGF-1 can inhibit the FoxO nuclear translocation and hence suppress the transcription of several genes such as *atrogin-1*, *MuRF-1*, and *cathepsin L*, which are involved in the ubiquitin signaling pathway (Sacheck et al., 2004). These findings highlight the crosstalk between IGF-1 and androgens.

Myostatin/GDF8 is a negative regulator of skeletal muscle mass (McPherron and Lee, 1997). Myostatin belongs to the transforming growth factor-β (TGF-β) superfamily. The TGF-β signaling pathway is responsible for the inhibition of myogenic differentiation (Beggs et al., 2004). TGFβ superfamily ligands bind to a type II receptor, which recruits and phosphorylates a type I receptor. The type I receptor subsequently phosphorylates receptor-regulated SMADs (R-SMADs) which can bind coSMAD. The R-SMAD/coSMAD complexes translocate from cytosol to nuclei where they act as transcription factors and mediate target gene expression such as *p21* which is an inhibitor of cyclin-dependant kinase (CDK). Myostatin can up-regulate *p21* expression, and subsequently withdraw cell cycle in G1 and hence inhibit cell proliferation (Kovacheva et al., 2010). Testosterone supplementation decreased *myostatin* expression (Kovacheva et al., 2010). Recently, T administration to mice activated the TGF-β signaling pathway and inhibited growth and differentiation of SC (Braga et al., 2012). The Myostatin gene promoter contains a putative ARE, identified by silico analysis, although no functional analysis has been reported (Ma et al., 2001). Thus, myogenic androgen action could, at least in part, be mediated through repression of both *myostatin* expression and activity (Mendler et al., 2007).
Notch signaling is responsible for SC activation, proliferation, and myogenic progression (Sinha-Hikim et al., 2006). Thus, androgens may increase SC number via the activation of this pathway. Testosterone increased Delta expression, which is a Notch receptor ligand (Brown et al., 2009). The impact of reduced Notch signaling resulted in decreased expression of Notch 1 and 2 as well as MyoG. Since MyoG plays important roles in SC differentiation and muscle hypertrophy, T supplementation may stimulate muscle growth, by, in part, by altered Notch signaling (Brown et al., 2009). In addition, a study exploring androgen effects on aged muscle indicated that Notch signaling can be restored by T treatment. The activated Notch signaling decreased the expression of p21 and enhanced the regenerative capacity of aged mice (Kovacheva et al., 2010).

**Oxytocin and Skeletal Muscle**

**Oxytocin**

Oxytocin (OXT) is a mammalian neurohypophysial hormone which was the first to be chemically synthesized. OXT is synthesized in the hypothalamus and secreted by the posterior pituitary gland, which is considered as the primary source of OXT in the blood (Gimpl and Fahrenholz, 2001). The hormone also is produced in the heart, bone, and skeletal muscle (Jankowski et al., 1998; Tamma et al., 2009; De Jager et al., 2011a). OXT is classically considered to have a fundamental role in human labor (Theobald et al., 1948). It was reported for the first time in 1906 that the posterior pituitary extract can drive uterine contractions (Dale, 1906). Subsequently, OXT was identified as a potent activator of contractions in the pregnant uterus. OXT is responsible for the milk-ejecting activity of the mammary gland (Wakerley and Lincoln, 1973). The biochemical structure of OXT was determined in 1953 (du Vigneaud et al., 1953), and the OXT gene was cloned in 1984 (Ivell and Richter, 1984).
OXT is a nonapeptide hormone (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH$_2$) with a disulfide bridge between Cys residues 1 and 6 (du Vigneaud et al., 1953). The presence of the disulfide bridge results in a six-amino acid cyclic part and a COOH-terminal α-amidated 3 residue tail. This structure is quite similar to other nonapeptide neurohypophysial hormones such as vasopressin and mesotocin. OXT peptide family contains a neutral amino acid at position 8, which can separate to the vasopressin (contains a basic amino acid at position 8) (Gimpl and Fahrenholz, 2001). The difference in these amino acids at position 8 can generate differences in the polarity of the peptides, enabling them to interact with their respective receptors (Barberis et al., 1998).

The OXT cDNA consists of three exons (Ruppert et al., 1984). The first exon encodes a translocator signal, the OXT hormone, glycyl-lysyl-arginine (GKR) tripeptide processing signal and the first nine amino acids of neurophysin. The second exon encodes the majority of neurophysin. The last exon encodes the COOH-terminal region of neurophysin (Gimpl and Fahrenholz, 2001). Neurophysin, a small disulfide-rich protein, serves as the carrier of OXT and contributes to the storage and release of OXT (Breslow and Burman, 1993). The prepropeptide is cleaved into the OXT- GKR and neurophysin by magnolysin (Ando et al., 1987). Subsequent processing produces OXT molecules by the amidase at the position 9 (Gly) to generate C-amidated nonapeptide (Green et al., 2001). At this time, the OXT activated and bond to neurophysin. The activated OXT and its carrier neurophysin are stored in the posterior pituitary until stimuli elicit their release (Brownstein et al., 1980).
**Fig. 1.4 Domain organization of preprooxytocin including the splitting site.** The OXT DNA consists of three exons and encodes two separate protein OXT and neurophy. The precursor of OXT is split into the signaling peptide, the OXT-GKR and neurophysin by enzymatic cleavage.

OXT-GKR is prooxytocin which is subsequently split and generate a glycine residue at the COOH-terminus. Peptidylglycine α-amidating monooxygenase (PAM) converts the glycine residue into a peptidyl-α-hydroxyglycine intermediate, which is subsequently converted to the α-amidated mature OXT (Lossie et al., 1994).

**Oxytocin Receptors**

Oxytocin receptors (OXTR) are found in kidney, ovary, testis, pituitary, heart, vascular endothelium, adipocytes, myoblast, pancreatic islet cells, osteoblast, and several types of cancer cells (Zingg and Laporte, 2003). The OXTR consists of a 7-trans-membrane domain and belongs to the class I G protein-coupled receptor (GPCR). The gene spans 17kb and contains 4 exons and 3 introns. The exon 1 and 2 encode the 5’-prime noncoding region. Exon 3 and 4 correspond to the OXT receptor. Exon 4 contains the COOH terminus and 3’-noncoding region (Gimpl and Fahrenholz, 2001).

The 5’ promoter region contains SP-1, AP-1, AP-2, c-Myb, and GATA-a motif cis elements which may regulate OXTR expression. The OXTR promoter also exhibits strong binding of nuclear orphan receptors, such as steroidogenic factor-1 (SF-1) and COUP-TF which suggested that steroid hormone can stimulate OXTR expression in the body tissues (Koohi et al., 2005). The transcriptional activity of the bovine OXT promoter is dependent on the presence of ER and E2 or tamoxifen (TAM). Site-directed mutation of COUP-TF with the OXTR promoter resulted in a significant reduction of transcriptional activity (Koohi et al., 2005). The stimulation of OXT expression is affected by the binding of ligands to ER but independent of ER binding (Koohi et al., 2005). Thus, OXT expression does not rely on the classical mechanism of ER action. Instead, OXT
expression is regulated by binding at a composite hormone response element (cHRE) which located at -164 from the transcription start site (TSS) of human OXT promoter region (Hiroi et al., 2013). SP1 mutated cells show unchanged expression of OXT after E2 treatment, suggesting that besides SP1, a regulatory site within the region of the promoter between TSS -406 and -224 is responsible for E2-induced OXT expression (Hiroi et al., 2013). Thus, the unknown transcription factors or interactions with other DNA sites may contribute to the actions of E2 on OXT promoter.

The OXTR belongs to G protein-coupled receptor, an integral membrane protein of the Gq/11α class (Gimpl and Fahrenholz, 2001). The general mechanism of this signaling pathway is well described (Rosenbaum et al., 2009). The α-subunits of Gq proteins transduce the signal and stimulate the activity of phospholipase C-β isoforms (PLCβ). PLCβ is a phospholipase which can hydrolyze phosphatidylinositol 4,5-biphosphate (PIP2) to produce inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 triggers Ca2+ release from intracellular stores and DAG stimulates and activates protein kinase C (Zhong et al., 2003). However, the precise mechanisms by which OXT exerts its multiple biological actions are not fully described.

OXTR can stimulate the ERK1/2 pathway during myometrial contractions. OXTR-mediated ERK1/2 activation is prolonged compared to β2-adrenergic receptor (β2AR) (Devost et al., 2008). The ERK1/2 signaling pathway mediates cell proliferation by inducing c-fos synthesis and mediates contractions via β-arrestin (Devost et al., 2008). OXT stimulates a rapid increase in ovine endometrial prostaglandin (PG) F2α synthesis via ERK1/2 signaling pathway (Burns et al., 2001). Activation of OXTR stimulates PGF2α expression and release, which, in turn, not only enhances uterine contractions but also promotes cervical opening and luteolysis (Zingg and Laporte, 2003). OXT induces eEF2 dephosphorylation in a dose dependent manner. Because eEF2 dephosphorylation is associated with an increased peptide chain elongation and protein synthesis
was increased by OXT treatment in myometrial cells, it is accepted that OXT stimulates protein synthesis via eEF2 dephosphorylation. Inhibition of PKC indicated that the kinase was involved in this effect (Devost et al., 2008). OXT can induce phosphorylation of ERK5 (Devost et al., 2008). OXTR is involved in the regulation of MEK5-ERK5 signaling and subsequently activated Myocyte Enhancer Factor 2C (MEF2C) transcription factor. MEF2C can stimulate the transcription of c-Jun and then regulate or promotes target gene expression such as myosin light chain gene (Naya and Olson, 1999; Devost et al., 2008). The activation of Gq also causes the membrane depolarization, which, in turn, increases cytocolic Ca$^{2+}$. High Ca$^{2+}$ concentration in plasma binding to calmodulin and then stimulates Ca2+/calmodulin-dependent protein kinase (CaMK). Activated CaMK mediates various cellular responses, such as smooth muscle contractions, or stimulates different enzymes (Viero et al., 2010).

**Effect of OXT On Skeletal Muscle**

Recently, it was demonstrated that cultured human myoblasts express OXTR (Breton et al., 2002). Treatment of human myoblasts with OXT increased the rate of fusion and myotube formation (Zingg and Laporte, 2003). This result suggested that OXT may be involved in the differentiation of skeletal muscle growth in humans, and possibly regeneration following injury (Breton et al., 2002). In cattle, chronic exposure to anabolic steroids led to a more than 97-fold increase in OXT expression in skeletal muscle and a 50-fold higher level of circulating OXT (De Jager et al., 2011a). Expression of OXT significantly increased during the 3rd trimester, coincident with myofiber hypertrophy (De Jager et al., 2011a). Kongsuwan et al. (2012b) demonstrated that OXT expression was upregulated by E2 and TBA administration. Divari et al. (2013) examined the changes in gene expression of OXT in skeletal muscle of cattle after E2 or dexamethasone treatment. In comparison to the placebo treated animals, a significant over-expression of the OXT
gene was detected in E2 or DEX treated cattle (Divari et al., 2013). A significant increase in myogenic regulated factor gene (MRFs) in skeletal muscle was identified as well (Divari et al., 2013). Estradiol can also upregulate OXT and the OXTR in C2C12 myotubes (Berio et al., 2017). Together, these studies indicate that steroid hormones can stimulate OXT expression in skeletal muscle. Since steroid hormones are widely used in meat industry to increase muscle mass, OXT may play important roles in muscle development and growth.

Circulating OXT levels decreased after ovariectomy which mimics hormone aging (Elabd et al., 2014). Expression of OXT and OXTR in skeletal muscle dramatically decreased with age (Elabd et al., 2014). Administration of OXT improved muscle healing in old mice but did not affect young mice after injury (Elabd et al., 2014). Inhibition of OXT signaling in young animals reduced muscle regeneration, whereas systemic OXT administration rapidly improved muscle regeneration by enhancing aged muscle stem cell activation/proliferation via MAPK/ERK pathway (Elabd et al., 2014). OXT knockout mice exhibits premature aging and sarcopenia (Elabd et al., 2014). Taken together, OXT plays important roles in the age-specific regulation of myogenesis.
Objectives and Hypotheses

To our knowledge, there are no existing studies determining the effect of OXT on bovine skeletal muscle growth \textit{in-vivo} or \textit{in-vitro}. Hence, the overall objective of this dissertation was to investigate and highlight the potential roles of OXT in BSC activity, with a focus on BSC proliferation and differentiation. We have designed and conducted different studies to address those issues. Each study and its corresponding hypotheses or specific aims are presented below:

a): To determine the role of OXT in BSC proliferation and differentiation

In this study, first we wanted to determine if: 1) OXT plays essential roles in BSC proliferation and differentiation; 2) E2 and TBA stimulate the expression of \textit{OXT}, which subsequently regulates BSC proliferation and differentiation. To address these questions, the mRNA expression levels of target genes were detected by qPCR. We also detected the proliferation rate, fusion index, cell death, and migration in primary BSC under different treatments.

b): CRISPR/Cas9-mediated heterozygous knockout of OXT and characterization of its role in BSC proliferation and differentiation

For this specific study, we hypothesized that: 1) OXT may play essential roles in BSC proliferation and differentiation; 2): Furthermore, E2 and TBA stimulate the expression of \textit{OXT} which subsequently regulates BSC proliferation and differentiation. In order to test those hypotheses, CRISPR-OXT and CRISPR-CON cell were treated with various medium including OXT, E2, and TBA during proliferation and differentiation. Proliferation rate and fusion index, as well as expression of target genes, were measured.
c): *OXT* expression is altered in skeletal muscle by intrauterine growth restriction and caloric restriction: in vivo study

In this chapter, we hypothesized that *OXT* expression in skeletal muscle may be impacted by intrauterine growth restriction (IUGR) and caloric restriction (CR). To investigate that, the *OXT* expression pattern in two different animal models was examined: (1) longissimus muscle (LM) was collected from fetal control (FC, n=12), fetal IUGR (FI, n=10), lamb control (LC, n=6), and lamb IUGR (LI, n=6) and gene expression was determined; (2) infraspinatus muscle (INF), LM, and semitendinosus muscle (ST) was collected after a control (CON) or CR diet was treated for 8 wk and target genes expression were measured.

d): *OXT* expression is altered in skeletal muscle by TAM administration: in vivo study

In this chapter, we hypothesized that 1) *OXT* expression in skeletal muscle may be impacted by TAM treatment; 2) TAM may affect bovine skeletal muscle fiber size and BSC number. To investigate that, sixteen Holstein calves were randomly assigned to one of two treatment groups: TAM-injected (TAM) or control (CON), and gene expression were studied. We also investigated the muscle fiber size and BSC number in skeletal muscle of TAM treated heifers.

In the next three chapters, we will present each of above four studies in one chapter followed by conclusion and future research interests. The details of experiment design, result, and discussion are presented.
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CHAPTER 2. OXYTOCIN IS INVOLVED IN STEROID HORMONE STIMULATED BOVINE SATELLITE CELL PROLIFERATION AND DIFFERENTIATION

Abstract

Steroid hormones have been widely used in the meat industry due to their ability to regulate muscle development and hypertrophy. However, the biological mechanisms underlying their action are not fully elucidated. Recent reports demonstrate that steroid hormones increase oxytocin (OXT) expression in skeletal muscle, indicating that OXT may play an essential role in satellite cell proliferation and differentiation, which further contribute to muscle growth. We tested this hypothesis using primary bovine satellite cell (BSC). Steroid hormones (E2: 17β-estradiol; TBA: trenbolone), tamoxifen (TAM), OXT, and atosiban (A: OXT receptor inhibitor) were applied to BSC to investigate OXT regulation of BSC activity. We found that OXT alone increased fusion index ($P<0.05$) but not proliferation in the BSC. Oxytocin also reduced ($P<0.05$) apoptotic cell number and stimulated migration rate ($P<0.05$). On the other hand, E2 and TBA increased ($P<0.05$) BSC proliferation rate, fusion index, and migration, and decreased ($P<0.05$) percentage of apoptotic nuclei compared to control (CON). In comparison with E2 or TBA treatment alone groups, E2 or TBA supplemented with A group had lower ($P<0.05$) BSC proliferation rate, fusion index, and migration and higher ($P<0.05$) percentage of apoptotic nuclei. Furthermore, we found that OXT expression increased ($P<0.05$) in E2 or TBA treated BSC during proliferation. OXT, E2, and TBA increased ($P<0.05$) MyoD and MyoG expression during BSC proliferation. During BSC differentiation, OXT expression increased ($P<0.05$) with E2 or TBA treatments. MyoG expression significantly increased ($P<0.05$) in OXT, E2, and TBA treatment groups compared to CON.
However, A, OXT+A, TAM, TAM+OXT, E2+TAM, E2+A, and TBA+A treatments decreased $(P<0.05)$ MyoG expression during BSC differentiation. These results indicate that OXT is involved in steroid hormone-stimulated BSC proliferation and differentiation.

**Key words:** Differentiation, Oxytocin, Proliferation, Satellite cell, Steroid hormone, Tamoxifen
Introduction

Skeletal muscle is a highly specialized tissue predominantly composed of muscle fibers, whose growth in the neonatal stage is dependent on the activity of a subset of muscle fiber–associated mononuclear muscle stem cells termed satellite cells (Mauro, 1961; Rhoads et al., 2009; Jang et al., 2011). Satellite cells (SC) locate between plasma membrane and basal lamina and play an important role in skeletal muscle growth which is characterized as a period of muscle hypertrophy due to a substantial increase in DNA content and protein deposition (Verdijk et al., 2009). Satellite cell proliferation and differentiation can be regulated or stimulated with various factors including steroid hormone administration (Kahlert et al., 1997; Chen et al., 2005) and they are also associated with in-vivo development of animal muscle size (Sciote et al., 2001; Sinha-Hikim et al., 2003). Kamanga-Sollo et al. reported increased proliferation ($P<0.05$) in bovine SC (BSC) treated with 17β-estradiol (E2) and trenbolone acetate (TBA) (Kamanga-Sollo et al., 2013). Other treatments, such as testosterone or dexamethasone, can also induce proliferation and differentiation ($P<0.05$) in C2C12 myoblasts (Desler et al., 1996; Diel et al., 2008; Vingren et al., 2010). Altogether, steroid hormones play an important role in SC proliferation and differentiation. However, the mechanisms behind steroid hormone stimulated muscle growth are not fully understood.

Recently, several studies support a new indirect function of steroid hormones, that is, they may induce expression of oxytocin (OXT) in skeletal muscle during muscle development (De Jager et al., 2011a; Divari et al., 2013). An in-vitro study found that OXT treatment increased ($P<0.05$) the rate of human myoblast fusion and myotube formation (Breton et al., 2002). This suggests that OXT may involve skeletal muscle differentiation, and possibly its regeneration following injury. In cattle, chronic exposure to anabolic steroids leads to a more than 97-fold increase ($P<0.05$) in
OXT expression in skeletal muscle and a 50-fold higher level of circulating OXT \((P<0.05)\) (De Jager et al., 2011a). Examination of cattle longissimus dorsi muscle (LM) samples indicated that OXT expression significantly increased \((P<0.05)\) in the 3rd trimester until birth, coincident with myofiber hypertrophy (De Jager et al., 2011a). Similarly, a study demonstrated that sheep OXT expression was upregulated \((P<0.05)\) by E2 and TBA administration (Kongsuwan et al., 2012b). Moreover, Divari et al. (2013) observed a significant over-expression of the OXT gene \((P<0.05)\) in E2 or dexamethasone treated cattle in comparison to the placebo treated cattle. In a recent in-vitro study, E2 was found to upregulate OXT and the OXT receptor (OXTR) \((P<0.05)\) in C2C12 myotubes (Berio et al., 2017). Together, these studies indicate that steroid hormones regulate OXT expression in skeletal muscle. However, the role of OXT on skeletal muscle development and specifically, SC activity is unknown. The objective of this study is to investigate the role of OXT during BSC proliferation and differentiation. We hypothesize that steroid hormones stimulate the expression of OXT which subsequently further regulates BSC proliferation and differentiation.

Materials and Methods

BSC isolation

Satellite cells were isolated according to Flann et al. (2014). Briefly, the forelimb skeletal muscle from 6 Angus or Angus crossbred steers were collected, washed with cold PBS containing 1% antibiotic–antimycotic (AbAm; Gibco Life Technologies, Grand Island, NY, USA), and then hand-minced with scissors. Cells were released from minced skeletal muscle and isolated via serial centrifugation as previously described with some modifications (Yates et al., 2014). Minced tissues were divided into 50 ml tubes (~10 g/tube), washed in cold PBS with 1% AbAm, and centrifuged \((1500 \times g, 5 \text{ min})\). The pelleted tissues were re-suspended in PBS with Protease type XIV from Strept. griseus \((1.25 \text{ mg/ml}; \text{Sigma-Aldrich, St Louis, MO, USA})\), digested at 37°C for 1 h (shake
tubes every 15 min), and then centrifuged (1500 × g, 5 min). Digested pellets were re-suspended in PBS with 1% AbAm and centrifuged 3 times (500 × g, 10, 8, and 1 min) to isolate BSC. After each centrifugation, the cell-containing supernatant was collected and centrifuged (1500 × g, 5 min). The pellets were re-suspended in DMEM (GlutaMAX; Gibco) supplemented with 20% fetal bovine serum (FBS, Atlas Biologicals, Ft Collins, CO, USA), 1% AbAm, and 0.5% of gentamicin (Gibco Life Technologies) and incubated for 1 h in 10 cm dish (37°C; 5% CO₂) to remove fibroblasts. Non-adhered BSC were collected and grown in growth media (GM; DMEM + 10% FBS + 1% AbAm + 0.5% of gentamicin) on ECL-coated tissue culture dishes overnight. The BSC were then removed from dishes with 0.25% trypsin (Gibco), centrifuged (1500 × g, 5 min), and frozen in growth media supplemented with 10% dimethyl sulfoxide (Sigma-Aldrich).

**Immunocytofluorescence**

BSC were fixed in 4% paraformaldehyde for 20 min at room temperature (RT) and then incubated with blocking buffer consisting of 2% bovine serum albumin (BSA) and 0.2% Triton X-100 in phosphate buffered saline (PBS) for 1 h at RT to eliminate non-specific antigen sites. Cells were further incubated overnight at 4°C with the oxytocin receptor (OXTR) (1:200; LSBio) antibody diluted in blocking solution. Following incubation, the cells were washed 3 times for 5 min with PBS and incubated for 1 h with goat anti-mouse H&L Alexa-Flour 594 (1:1000; Invitrogen, Carlsbad, CA) diluted in blocking solution. DAPI (4’,6-Diamidino-2-Phenylindole, Dihydrochloride; Invitrogen, Carlsbad, CA) was used for the detection of nuclei. After washing with PBS, the immune complexes were visualized by epifluorescence (Nikon Eclipse Ti-U; Nikon Instruments Inc., Melville, NY). Representative images were captured at 200-fold magnification and digitized with a charge-coupled camera (DS-QiMC, Nikon), and NIS Elements software (Nikon).
**Proliferation**

Cells were plated in 96-well plates which were coated with ECL for at least 1 h, then incubated in growth media overnight to allow cells to adhere. The cells plated at 4,000 cells/well were cultured in low serum medium (DMEM + 2%FBS + 1×ITS + 1%AbAm+0.5%gentamicin) with different treatment: gradient concentration of TAM (100 nM, 1 μM, 10 μM, 100 μM, and 200 μM); gradient concentration of OXT (31.25 nM, 62.5 nM, 125 nM, and 250nM); E2 (10 nM); TBA (1 μM); TAM (1 μM); OXT (125 nM); atosiban (A: 5 μM); OXT (125 nM) supplemented with A (5 μM); TAM (1 μM) + A (5 μM); E2 (10 nM) + A (5 μM); TBA (1 μM) + A (5 μM); E2 (10 nM) + TAM (1 μM); fibroblast growth factor 2 (FGF2; 10 ng/ml; as a positive control); and control (CON; without any treatments). All the cell cultures received fresh media with different treatments daily. Using the CyQUANT® NF Cell Proliferation Assay Kit, proliferation rate was measured every 24 hours during culture. CyQUANT® NF reagent in 1× HBSS buffer was added to each well after moving out the culture medium. After 1h incubation at 37°C, fluorescence intensities of BSC were measured with a fluorescence microplate reader (Infinite M200 PRO, TECAN).

**Differentiation**

Cells were plated at the density of 5×10^4 cells/well in 24-well plates coated with ECL. At 75%-85% confluency, the medium was changed to differentiation medium (DM, DMEM+2%FBS+1%AbAm+0.5%gentamicin) with gradient concentration of OXT (31.25 nM, 62.5 nM, 125 nM, and 250nM), E2 (10 nM); TBA (1 μM), TAM (1 μM); A (5 μM), OXT (125 nM) supplemented with A (5 μM), TAM (1 μM) + OXT (125 nM), E2 (10 nM) + A (5 μM), TBA (1 μM) + A (5 μM), E2 (10 nM) + TAM (1 μM), Insulin-like growth factor 2 (IGF2; 10 ng/ml; as a positive control); and control (CON; without any treatments), respectively, to induce
differentiation. All the cell cultures received fresh media with different treatments daily. Myotubes were stained at 48 h and 96 h after inducing differentiation as previously described with some modifications (Joulia et al., 2003). Myotubes were washed three times with cold PBS and then fixed in 4% paraformaldehyde at RT for 20 min. After washing with PBS three times, non-specific binding was blocked with blocking buffer (2% BSA+ 0.2% Triton-X-100 in PBS) for 1h at RT. Primary myosin heavy Chain antibody (MF20; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) diluted in blocking buffer (1:10) was applied overnight at 4°C and revealed using secondary Alexa Flour 488 (96 h) or Alexa Flour 594 (48 h) goat anti-mouse antibodies (1:1000; Invitrogen, Carlsbad, CA, USA) for 1 h. Nuclei were stained with DAPI for 5 min at RT. The fusion index was calculated as a ratio between the number of nuclei within myotubes and total nuclei (at least 600 nuclei were counted under each condition).

**Apoptosis**

Cells were preconditioned with E2 (10 nM); TBA (1 μM), TAM (1 μM), OXT (125 nM), A (5 μM), E2 (10 nM) + A (5 μM), TBA (1 μM) + A (5 μM), TAM (1 μM) + A (5 μM), and OXT (125 nM) + A (5 μM). After 48h treatment, cells were treated with serum free medium and 200μM of hydrogen peroxide (H2O2) for another 48h. Following incubation, cells were washed 3 times for 5min with PBS. Cell number was assessed by CyQUANT NF Proliferation Assay Kit. Apoptotic cells were detected by In Situ Cell death detection Kit. A total of 6 pictures of randomly selected fields were taken under 200-fold magnification fluorescent microscope (Nikon), and digital pictures were analyzed by NIS Elements software (Nikon). Results were expressed as percentage of apoptotic nuclei.

**Migration**
Cells were preconditioned with E2 (10 nM); TBA (1 μM), OXT (125 nM), A (5 μM), E2 (10 nM) + A (5 μM), TBA (1 μM) + A (5 μM), TAM (1 μM) + OXT (125 nM), and OXT (125 nM) + A (5 μM) and were plated in the top of modified Boyden chamber (Corning Costar, Cambridge, MA). Cells were allowed to migrate for 16h in the presence 10% FBS in the bottom chamber. Migrated cells were fixed in 4% paraformaldehyde, and nuclei were stained with DAPI. A total of 6 pictures of randomly selected fields were taken under 100-fold magnification fluorescent microscope (Nikon), and digital pictures were analyzed by NIS Elements software (Nikon). Results were expressed as a migration index (percentages), which was defined as: mean number of cells for test conditions/mean number of cells for controls (Noiseux et al., 2012).

**Real-time quantitative reverse transcription-PCR**

Real-time quantitative reverse transcription-PCR (qPCR) was used to measure the abundance of target genes in cultured BSC with different treatments as described in Rhoads et al. (2013).

The cells were seeded in 6-well plates (1.5×10^5 cell/well). To determine the effect of E2, TBA, and OXT on gene expression during cell proliferation, the cells were cultured in GM with 10nM E2, 1μM TBA, 1μM TAM, 5 μM A, 125nM OXT, OXT (125nM) + A (5 μM), TAM (1μM) + OXT (125nM), E2 (10nM) + TAM (1μM), E2 (10nM) + A (5 μM), and TBA (1μM) + A (5 μM) treatments at 37°C with 5% CO2 for 48 h. The medium was refreshed every 24 h. Total RNA was extracted at 0 h (the first day of treatment), 12 h, 24 h, and 48 h of culture. To investigate effects of E2, TBA, A, and OXT on gene expression during cell differentiation, cells were cultured in GM until 75%-85% confluency and then induced differentiation by replacing GM to DM. After 24 h of differentiation, 10nM E2, 1μM TBA, 1μM TAM, 5 μM A, 125nM OXT, OXT (125nM) + A (5 μM), TAM (1μM) + OXT (125nM), E2 (10nM) + TAM (1μM), E2 (10nM) + A (5 μM), and TBA
(1µM) + A (5 µM) treatments were added into the DM. The medium with treatment(s) was refreshed daily until 96 hours of differentiation. Total RNA was extracted at 0 h (the first day of treatment), 24 h, 48 h, and 72 h of culture.

Total RNA was extracted using Trizol reagent (Invitrogen, US) from cultured cells according to the manufacturer’s protocol, and then RNA samples were cleaned up by RNeasy® Mini Kit (Qiagen, USA). The cDNA was generated from 1µg purified RNA using iscript™ cDNA Synthesis Kit (Bio-Rad, USA). Finally, the cDNA was diluted 1:10. Specific TaqMan probes of OXT and cyclophilin α (PPIA) was used in OXT mRNA expression study. The qPCR reactions were performed in the same well using CFX96 Detection System (BioRad) and in a 20µl final volume containing 10µl of PCR mix, 1µl of each primer (10µM), 0.5µl of each probe (10µM), and 5µl of cDNA (25ng). PCR cycling conditions consisted of 10min incubation at 95°C followed by 40 cycles of 95°C for 15s and 60°C for 1min. For other genes, qPCR was performed on 5 μL of diluted cDNA in a final volume of 20 μL using the SsoAdvanced Universal SYBR Green Supermix (BioRad) in CFX96 Detection System. The parameters used were as follows: 5 min at 95°C, followed by 40 cycles of repeated incubations at 95°C for 10 s and 60°C for 30 s. The fold change for all the samples measured by qPCR was calculated by 2\(^{-\Delta\Delta Cq}\) method. All the primers and probes are given in Table 1.

**Statistical analyses**

The results were analyzed in JMP® (SAS Institute) using two-way analysis of variance (ANOVA). Pairwise comparisons between the least square means of the factor levels were conducted using Tukey's HSD test. Values are expressed as means ± SEM.
Results

Expression of OXTR in BSC

OXTR immunofluorescence was detected in approximately 100% of BSC and fused myotubes stained with OXTR antibody (Fig. 1). The presence of OXTR transcripts in BSC during proliferation and differentiation were detected by qPCR (Fig. 5&6). We found that BSC expressed OXTR during proliferation and differentiation, but there was no difference detected between CON cells and cells with E2, TBA, or OXT treatments. In contrast, OXTR expression considerably decreased \((P<0.05)\) after adding TAM or A into medium during BSC proliferation and differentiation.

Proliferation in BSC

There was no difference in BSC proliferation with lower concentrations of TAM (100nM, 1µM, and 10µM), but greater concentration (100µM and 200µM) of TAM caused cell death (Fig. 2A). In addition, OXT did not affect BSC proliferation (Fig. 2B) at any concentration. However, proliferation rate significantly increased in both E2 and TBA treated BSC compared to CON \((P<0.05)\) (Fig. 2C). There was no difference in BSC proliferation observed with A alone, TAM+OXT, E2+A, TBA+A, or OXT+A treatments at 48h and 72h.

Apoptosis in BSC

 Cultures of BSC failed to proliferate in response to OXT, however, atosiban, an OTXR inhibitor, suppressed E2 and TBA stimulated BSC proliferation. Therefore, we hypothesized that OXT does not directly increase BSC proliferation, but may protect BSC from apoptosis. After preconditioning with OXT, E2, TBA, and TAM treatments, BSC number increased \((P<0.05)\) after serum starvation and oxidative stress for 48h (Fig. 3). However, A dramatically decreased \((P<0.05)\)
cell number compared to OXT, E2, TBA, and TAM treatments. The percentage of apoptotic nuclei, a measurement of apoptosis, was decreased ($P<0.05$) by OXT, E2, TBA, and TAM compared to CON, and was then back to normal (CON) after adding A into the medium. Atosiban increased ($P<0.05$) the percentage of apoptotic nuclei compared to OXT, E2, TBA, and TAM treatments.

**Differentiation in BSC**

The effect of OXT on BSC differentiation was examined following immunohistochemical staining for myosin heavy chain (MyHC) and calculation of a fusion index (Fig. 2.4A). As expected, treatment of BSC with 10 ng/mL IGF2 increased ($P<0.05$) the fusion index at 72 and 96 hr. Treatment with OXT at concentrations ranging from 62-250 nM increased ($P<0.05$) fusion percentage at 48 h to levels comparable to IGF2. No differences in the fusion index were evident for any concentration of OXT at 96 h suggesting that the modest increase at 48 h was attributed to accelerated differentiation.

The effect of OXTR inhibition on basal and steroid-induced BSC differentiation was examined further. As expected, treatment of BSC for 48 h with OXT, E2 or TBA accelerated myofiber formation in an OXTR-dependent manner (Fig 2.4B). Blocking OXTR activity did not affect early fusion percentage but inhibited fusion at 96 h to a level comparable to TAM-mediated inhibition of fusion ($P<0.05$). Treatment with E2 or TBA increased fusion of BSC at 96 h that was prevented by inclusion of A in the media ($P<0.05$). No effect of OXT was observed after 96 h of treatment.

Migration of SC contributes to the terminal differentiation and fusion process. The effect of OXT on SC migration was examined using transwell filter inserts. Results demonstrate that BSC migration was increased by treatment with OXT, E2, and TBA ($P<0.05$; Fig. 4C). Inclusion
of A in the media prevented migration in response to OXT, E2, and TBA ($P<0.05$). The effects of OXT does not require E2 activity as TAM did not alter OXT driven BSC migration.

**Target genes expression during BSC proliferation and differentiation**

Expression of OXT, OXTR, MyoG, MyoD, and MyoG expression with different treatments during proliferation and differentiation for BSC are presented in Fig. 5. Oxytocin expression increased ($P<0.05$) in E2, E2+TAM, or TBA treated BSC at 12h, 24h, and 48h of culture in growth media. OXTR expression significantly decreased ($P<0.05$) after adding A, OXT+A, E2+A, or TBA+A treatments into the growth medium. Furthermore, OXT, E2, and TBA increased MyoD and MyoG expression ($P<0.05$) during BSC proliferation. There was no difference in MyoD and MyoG expression with TAM treatment. However, A significantly decreased MyoG expression during proliferation ($P<0.05$).

During differentiation, OXT expression increased with E2, E2+TAM, or TBA treatments ($P<0.05$; Fig. 6), although the increase caused by E2+TAM was less than E2 and TBA treatments ($P<0.05$). Atosiban decreased OXT expression during BSC differentiation ($P<0.05$). However, A, OXT+A, E2+A, or TAB+A treatments increased OXTR expression ($P<0.05$). Moreover, there was no difference in MyoG expression with different treatments. Only TAM treatment decreased MyoD expression ($P<0.05$). Furthermore, MyoG expression significantly increased ($P<0.05$) in OXT, E2, and TBA treatment groups compared to CON. However, A, OXT+A, TAM, TAM+OXT, E2+TAM, E2+A, and TBA+A treatments dramatically decreased MyoG expression ($P<0.05$) during BSC differentiation.
Discussion

Recent studies indicate that OXT, a nonapeptide hormone, is expressed in skeletal muscle, SC, and myotubes, and regulated by steroid hormones in different species and cell types (De Jager et al., 2011a; Berio et al., 2017). Given that steroid hormone administration regulates SC activity, it is plausible that OXT may stimulate SC proliferation and differentiation. However, the role of OXT in SC activity has not been thoroughly investigated. The current study was undertaken to investigate the hypothesis that steroid hormones stimulate OXT expression which further regulates SC proliferation and differentiation.

In the present study, both E2 and TBA increased OXT expression considerably during BSC proliferation and differentiation, which are consistent with observations from previous studies. Divari et al. (2013) and Kongsuwan et al. (2012b) reported that OXT expression in skeletal muscle is modulated by steroid hormone treatment in cattle and sheep, respectively. Berio et al. (2017) reported that E2 treatment increased OXT expression in C2C12 myoblast. Estrogen regulates OXT expression by ER binding to the estrogen response element (ERE) in the OXT promoter (Richard and Zingg, 1990). However, the binding affinities of ER to the OXT promoter excluded this classical mechanism (Stedronsky et al., 2002). It is also recognized that OXT expression relies on the presence of ER and estrogen orphan receptor in the cells (Koohi et al., 2005). These results may present an indirect mechanism of estrogen in skeletal muscle growth, that is, estrogen may induce OXT expression which further contributes to muscle growth.

In this study, both proliferation rate and fusion index increased in E2 and TBA treated BSC compared to CON BSC. In addition, greater MyoG expression was observed in E2 and TBA treated BSC compared to the CON group, and this finding also confirms that proliferation was increased by those two treatments. Thus, we concluded that steroid hormone can stimulate proliferation,
which is similar to what has been found in studies conducted on cattle and other species. For example, in human, mouse, and cattle, steroid hormones stimulate muscle stem cells proliferation and differentiation (Kamanga-Sollo et al., 2004; Sinha-Hikim et al., 2004; Chen et al., 2005; Enns and Tiidus, 2008). We further noticed that there was no significant difference in proliferation rate with different concentrations of OXT. Both E2 and TBA increased BSC proliferation. After addition of A to GM, the proliferation rate significantly decreased compared to that in E2 or TBA single treatment groups. However, A itself has no impact on BSC proliferation. Together, this data indicates that OXT alone cannot increase BSC proliferation and thus it is more likely that OXT may be involved in E2- and TBA-stimulated BSC proliferation.

We measured MyoD and MyoG expression to determine if OXT is involved in steroid hormone stimulated BSC differentiation. MyoD is an important regulator required for the differentiation potential of skeletal myoblasts (Rudnicki et al., 1993; Conerly et al., 2016). Downregulation of MyoD expression in proliferating myoblasts lead to a return to quiescence (Kuang et al., 2007), and reduced SC differentiation. As SC committed to myogenic lineage, most cells will begin to express MyoG which also plays an important role in SC differentiation (Wright et al., 1989; Montarras et al., 1991; Kassar-Duchossoy et al., 2004; Moretti et al., 2016). Therefore, reducing both MyoD and MyoG expression will result in small myofiber size and accumulation of undifferentiated myoblasts (Wang and Rudnicki, 2012). Herein, OXT, E2, and TBA significantly increased MyoG and MyoD expression in BSC compared to CON during the proliferative state. During differentiation, MyoG expression in BSC dramatically increased with OXT, E2, and TBA treatments as well. Thus, OXT may be involved in E2- and TBA-stimulated BSC differentiation. The proliferation and fusion index both decreased after addition of A to the E2 and TBA treatments. Consequently, the ability of A to suppress BSC proliferation rate and fusion index provides support
that OXT plays an important role in BSC proliferation and differentiation. Similar conclusions can be drawn from the results of mouse studies as well. Lee et al. (2008) reported that OXT and OXTR may participate in mouse skeletal muscle development. In addition, OXT appears to be required for proper muscle tissue regeneration and homeostasis in mouse (Elabd et al., 2014).

During differentiation, TAM significantly decreased *MyoD* and *MyoG* expression. It is possible that, by reducing *MyoD* and *MyoG* expression, TAM impairs SC differentiation, which was confirmed by decreased fusion index by TAM during differentiation. Further, TAM decreased *OXT* expression during BSC differentiation. TAM+OXT treatment increased fusion index compared to TAM treatment, and A can reduce BSC differentiation. Thus, these results suggest that TAM, acting as an estrogen antagonist, attenuated BSC differentiation, and OXT was involved in this process. Although the mechanism underlying TAM regulation of *OXT* expression has not been elucidated, it has been reported that estrogen regulates *OXT* expression by estrogen receptors directly binding with OXT promoter via estrogen response element (ERE) (Richard and Zingg, 1990). Competing with estrogen receptors may contribute to the decrease of *OXT* expression with TAM treatment.

In the current study, we found that the proliferation rate was not affected by OXT treatment in BSC. In contrast, E2 and TBA supplemented with A decreased BSC proliferation compared to E2 or TBA treatment. Since cell apoptosis also directly contributes to cell number and proliferation (Joulia et al., 2003), it is likely that OXT may be involved in BSC apoptosis. By assessing the apoptosis in serum free and oxidative stress conditions, we found that BSC number significantly increased with OXT, E2, TBA, and TAM treatments compared to CON, but this effect diminished after adding A to those treatments. A lower percentage of apoptotic nuclei was found in OXT, E2, TBA, and TAM treatment groups compared to the CON group. OXT+A, E2+A, TBA+A, and
TAM+A treated cells exhibited more apoptotic cells compared to OXT, E2, TBA, or TAM groups. These results indicate that OXT may protect SC from serum starvation and oxidative stress in cattle. Although the protective role of OXT in primary SC and muscle cell line has not been described previously, OXT has been reported to protect other types of stem cells from apoptosis. Noiseux et al. (2012) found that OXT treatment of myocardial infarction in rats significantly reduced cardiomyocytes apoptosis and rat bone marrow-derived mesenchymal stem cell (MSC) survival under serum starvation and hypoxic stress. Since the PI3K/Akt and ERK1/2 pathways are key transducers of anti-apoptotic signals in various cell types, future studies are needed to determine which OXT signaling pathways are involved in the reduction of BSC apoptosis.

The process of myoblast fusion consists of cell migration, adhesion and signaling transduction pathways leading up to the actual fusion event (Abmayr and Pavlath, 2012). Several studies support the importance of migration to myoblast fusion (Hughes and Blau, 1990; Bae et al., 2008). Migration of myoblast across basal lamina is essential for fusion of myoblasts during skeletal muscle development in mouse (Hughes and Blau, 1990). Cell migration must occur at early stage of differentiation to bring C2C12 myoblasts in close enough proximity so that they can fuse (Bae et al., 2008). Thus, it is very likely that OXT regulates cell differentiation by stimulating BSC migration. Subsequent investigation on migration in the present study demonstrated that OXT, E2, and TBA significantly increased BSC migration compared to control. And this increase disappeared after adding A. Thus, these findings confirmed that OXT is a potential regulator of myoblast motility and this property contributes to its ability to promote myoblast fusion into myotubes. To our knowledge, there is no existing study examining the role of OXT in BSC migration. However, a study in MSC demonstrated that OXT stimulates the migration of MSC (Noiseux et al., 2012). Further, OXT stimulates activation of PI3K and endothelial nitric oxide
(NO) synthase which were required for the pro-migratory effect of OXT on human umbilical vein endothelial cells (Cattaneo et al., 2008) and in ischemic rat heart (Faghihi et al., 2012). Thus, it is possible that OXT stimulates BSC migration via PI3K/Akt and NO signaling pathway. In addition, OXT increased fusion index at 48h after inducing differentiation but not at 96h. This finding is similar to a report by Elabd et al. (2014) where fusion index was increased by OXT in mouse C2C12 cells compared to control cells and fewer myotubes were generated by OXT antagonist treatment after 48h differentiation induced by low serum medium. Berio et al. (2017) reported no significant difference in fusion index between control cells and OXT treated cells at day 7 in their C2C12 cell line study. Altogether, this may indicate that OXT is only involved in early myoblast fusion, but not at a later stage. Studies to investigate specific biological mechanisms of OXT on SC migration are needed in the future.

**Conclusion**

In conclusion, the data in our study indicates that OXT stimulates BSC differentiation and migration. E2 and TBA increased BSC proliferation, fusion index, and migration, and OXT was involved in these processes. Moreover, both steroid hormones and OXT protected BSC from serum starvation and oxidative stress conditions. Together, the results presented support the hypothesis that OXT plays essential roles in BSC proliferation and differentiation and involves functions of the steroid hormones on BSC activity. Future studies investigating the signaling pathways related to OXT stimulated BSC proliferation and differentiation and steroid hormones stimulated OXT expression may help us better understand the mechanisms of steroid hormones and OXT stimulated SC activity.
References


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<td>MyoG (NM_001111325)</td>
<td>Cattle</td>
<td>F: GTGCCCACTGAATGCAGCTC R: GTCTGTAGGGTCGCCTGGGA</td>
<td>110</td>
</tr>
<tr>
<td>MyoG (XM_002685738)</td>
<td>Cattle</td>
<td>F: GAGTTTGATTAGCCCGAGTGC R: ATGCTGCTTGGCTTTCTT</td>
<td>105</td>
</tr>
</tbody>
</table>

\(^1\)Note: F, forward primer; R, reverse primer; P, TaqMan probe
**Fig. 2.1. Immunocytofluorescence of OXTR.** OXTR antibody was used to detect the expression of OXTR in BSC during proliferation and differentiation. Both BSC and myotubes expressed OXTR. Magnification bar = 100 μm.
**Fig. 2.2. Proliferation rate in BSC.** CyQUANT® NF Cell Proliferation Assay Kit was used to determine the proliferation rate in low serum medium (DMEM + 2%FBS + 1×ITS) with (A) different concentrations of TAM in BSC; (B) different concentrations of OXT in BSC; (C) with E2 (10 nM), TAM (1 µM), TBA (1 µM), OXT (125 nM), atosiban (5 µM), OXT (125 nM) supplemented with atosiban (5 µM), TAM (1 µM) + OXT (125 nM), E2 (10 nM) + atosiban (5 µM), E2 (10 nM) + TAM (1 µM), TBA (1 µM) + atosiban (5 µM), fibroblast growth factor 2 (FGF2; 10 ng/ml; as a positive control), and control (CON; without any treatments). Values are means and SEMs. Groups with different letters have significant difference with $P<0.05$. 
Fig. 2.3. Cell death in BSC.

Cells were preconditioned with E2 (10 nM), TBA (1 µM), OXT (125 nM), E2 (10 nM)+A (5 µM), TBA (1 µM)+A (5 µM), and OXT (125 nM)+A (5 µM). After 48h treatments, cells were treated with serum free medium and 200µM of hydrogen peroxide (H2O2) for another 48h. Following incubation, cells were washed 3 times for 5min with PBS. Cell number was assessed by CyQUANT NF Proliferation Assay Kit. Apoptotic cell was detected by In Situ Cell death detection Kit. Groups with different letters have significant difference with $P<0.05$. Means and SEMs are shown.
Fig. 2.4. Fusion index and migration in BSC. For fusion index study, cells were plated at the density of 5×10^4 cells/well in 24-well plates coated with ECL. At 75%-85% confluency, the medium was changed to differentiation medium (DM, DMEM+2%FBS+1%AbAm+0.5% of gentamicin) with (A) gradient concentration of OXT (31.25 nM, 62.5 nM, 125 nM, and 250nM) in BSC; (B) with E2 (10 nM), TAM (1 µM), TBA (1 µM), OXT (125 nM), atosiban (5 µM), OXT (125 nM) supplemented with atosiban (5 µM), TAM (1 µM) + OXT (125 nM), E2 (10 nM) + atosiban (5 µM), E2 (10 nM) + TAM (1 µM), TBA (1 µM) + atosiban (5 µM), Insulin-like growth factor 2 (IGF2; 10 ng/ml; as a positive control), and control (CON; without any treatments). All SC cultures received fresh media with different treatments daily. Myotubes were stained by MF20, myonuclei were stained by DAPI. The fusion index was calculated as the number of nuclei within myotubes divided by total nuclei at 48 h and 96 h after inducing differentiation. For migration study, (C) BSC were preconditioned with E2 (10 nM), TBA (1 µM), OXT (125 nM), TAM (1 µM), E2 (10 nM) + A (5 µM), TBA (1 µM) + A (5 µM), TAM (1 µM) + A (5 µM) and OXT + A (5 µM). Cells were plated in the top of modified Boyden chamber (Corning Costar, Cambridge, MA). Cells were allowed to migrate for 16h in the presence 10% FBS in the bottom chamber. Migrated cells were fixed in 4% paraformaldehyde, and nuclei were stained with DAPI. Results were expressed as a migration index (in percentage) defined as a ratio between mean number of cells for test conditions and mean number of cells for controls. Groups with different letters have significant difference with $P<0.05$. Means and SEMs are shown.
(A) Proliferation

(B) OXTR Expression

(C) Proliferation

(D) MyoD Expression

(E) Proliferation
**Fig. 2.5. Gene expression during BSC proliferation.** To determine the effect of OXT, E2, and TBA on gene expression during BSC proliferation, the cells were cultured in GM with or without different treatments for 48 h. The medium was refreshed every 24 h. Total RNA was extracted at 0 h (the first day of treatment), 12 h, 24 h, and 48 h of culture. CON and treatment myoblast lysates during proliferation were analyzed for gene mRNA expression by qPCR. Groups with different letters have significant difference with $P<0.05$. Means and SEMs are shown.
Fig. 2.6. Gene expression during BSC differentiation. To determine the effect of OXT, E2, TBA on gene expression during SC differentiation, the BSC were cultured in DM with or without different treatment for 72 h. The medium was refreshed every 24 h. Total RNA was extracted at 0 h (the first day of treatment), 24 h, 48 h, and 72 h of culture. CON and treated myoblast lysates during differentiation were analyzed for gene mRNA expression by qPCR. Groups with different letters have significant difference with $P<0.05$. Means and SEMs are shown.
CHAPTER 3. CRISPR/CAS9-MEDIATED HETEROZYGOUS KNOCKOUT OF OXYTOCIN AND CHARACTERIZATION OF ITS ROLE IN BOVINE SATELLITE CELL PROLIFERATION AND DIFFERENTIATION

Abstract

Oxytocin (OXT) is a mammalian neurohypophysial hormone. It has been demonstrated that OXT is highly expressed in skeletal muscle and satellite cell (SC) by steroid hormone administration. Using OXT knockout bovine SC (CRISPR-OXT) generated by the CRISPR/Cas9 system, the current studies investigated the hypothesis that OXT plays essential roles in bovine satellite cell (BSC) proliferation and differentiation. Different treatments including steroid hormones (E2: 17β-estradiol; TBA: trenbolone), and OXT were applied to CRISPR-OXT cells. Using CRISPR/Cas9, we detected 11 types of insertion and deletion (indels), which resulted in a nonfunctional protein or prevented protein expression. Gene editing efficiency was greater than 76%. Next, we determined the knockout efficiency by qPCR. The OXT expression dramatically decreased in CRISPR-OXT cell compared to wild-type BSC by more than 97%, though OXT expression declined in CRISPR-CON cell by 39.6% compared to wild-type BSC. Thus, we conducted experiments using both CRISPR-OXT and CRISPR-CON cells. There was no difference in proliferation with OXT treatment between CRISPR-CON and CRISPR-OXT cell. E2 and TBA decreased \( P < 0.05 \) proliferation in CRISPR-OXT cell compared to CRISPR-OXT cells. OXT reduced \( P < 0.05 \) apoptosis in both CRISPR-CON and CRISPR-OXT cells. E2 and TBA reduced \( P < 0.05 \) apoptosis in CRISPR-CON cell but not CRISPR-OXT cells. Migration increased with OXT treatment in both CRISPR-CON and CRISPR-OXT cells. E2 and TBA
increased migration in CRISPR-CON cell but not CRISPR-OXT cells. OXTR, MyoG, MyoD, and MyoG expression decreased in both CRISPR-CON and CRISPR-OXT cells compared to the wild-type BSC. These results indicate that myogenic processes were reduced in both CRISPR-CON and CRISPR-OXT cell. The CRISPR experiment altered BSC phenotype and cannot be used to clearly define the role of OXT in BSC proliferation and differentiation.

**Key words:** CRISPR/Cas9, Differentiation, Oxytocin, Proliferation, Satellite cell
Introduction

Oxytocin (OXT) is a mammalian neurohypophysial hormone which is synthesized in the hypothalamus and secreted by the posterior pituitary gland. It is considered as the primary source of OXT in the blood (Gimpl and Fahrenholz, 2001). This hormone is also produced in other parts of the body such as heart, bone, and skeletal muscle (Jankowski et al., 1998; Tamma et al., 2009; De Jager et al., 2011a). OXT is classically considered to have a fundamental roles in human labor and milk-ejecting activity of the mammary gland (Theobald et al., 1948; Wakerley and Lincoln, 1973). Recently, it has been demonstrated that cultured human myoblasts express oxytocin receptor (OXTR) (Breton et al., 2002). In an in-vitro study, OXT treatment increased the rate of myoblast fusion and myotube formation in humans (Zingg and Laporte, 2003). This result indicated that OXT and OXTR system may be involved in the differentiation of skeletal muscle growth in human, and possibly its regeneration following injury (Breton et al., 2002). In cattle, chronic exposure to anabolic steroids led to a more than 97-fold increase in OXT expression in skeletal muscle and a 50-fold higher level of circulating OXT (De Jager et al., 2011a). A re-investigation of cattle longissimus dorsi muscle (LM) samples indicate that OXT expression significantly increased in the 3rd trimester until birth, coincident with myofiber hypertrophy (De Jager et al., 2011a). Taken together, OXT may play important roles in muscle development and growth. Although OXT gene was essential in the process of muscle development and growth, the molecular mechanisms of OXT in BSC activity has not been fully elucidated.

Gene editing is an important method to study the function of genes (Urnov et al., 2010). In recent years, a novel gene editing system, named as CRISPR/Cas9 system, has been developed (Hsu et al., 2014). The word “CRISPR/Cas9” is abbreviated for Clustered, repetitive interspaced, short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9). CRISPR/Cas9
technology was widely used to generate the stable insertion, deletion, and substitution of a specific genome sequence, based on a single-RNA guided nuclease (Ran et al., 2013). In order to edit target genes, cells are transfected with a plasmid carrying the Cas9 gene and a specifically targeted RNA guides (Gaj et al., 2013). After cleavage by Cas9, cellular DNA repair pathways are activated to complete the editing process (Sander and Joung, 2014). There are two general repair pathways: non homologous end joining (NHEJ) and homology directed repair (HDR) (Lieber, 2010). HDR allows an exogenous 'donor' repair template to be inserted into the genome for the repair of double strand breaks (DSB), causing specific changes in sequence (Lieber, 2010). NHEJ is the default DNA repair mechanism of cell (Mahaney et al., 2009).

Based on the recent usage of CRISPR/Cas9 technology, the purpose of this study was to investigate the role of OXT in bovine satellite cell (BSC). BSC were transfected by both CRISPR/Cas9 mediated OXT-knockout lentivirus plasmid and CRISPR/Cas9 mediated control lentivirus plasmid separately. The proliferation, apoptosis, migration, and target gene expression of CRISPR cells were measured.
Materials and Methods

BSC isolation

The forelimb skeletal muscle from 6 Angus or Angus crossbred steers were collected, washed with cold PBS containing 1% antibiotic–antimycotic (AbAm; Gibco Life Technologies, Grand Island, NY, USA), and then hand-minced with scissors. BSC were released from minced skeletal muscle and isolated via serial centrifugation as previously described with some modifications (Yates et al., 2014). Minced tissues were divided into 50 ml tubes (~10 g/tube), washed in cold PBS with 1% AbAm, and centrifuged (1500 × g, 5 min). The pelleted tissues were re-suspended in PBS with Protease type XIV from Strept. griseus (1.25 mg/ml; Sigma-Aldrich, St Louis, MO, USA), digested at 37°C for 1 h (shake tubes every 15 min), and then centrifuged (1500 × g, 5 min). Digested pellets were re-suspended in PBS with 1% AbAm and centrifuged 3 times (500 × g, 10, 8, and 1 min) to isolate BSC. After each centrifugation, the cell-containing supernatant was collected and centrifuged (1500 × g, 5 min). The pellets were re-suspended in DMEM (GlutaMAX; Gibco) supplemented with 20% fetal bovine serum (FBS, Atlas Biologicals, Ft Collins, CO, USA), 1% AbAm, and 0.5% of gentamicin (Gibco Life Technologies) and incubated for 1 h in 10 cm dish (37°C; 5% CO₂) to remove fibroblasts. Non-adhered BSC were collected and grown in growth media (GM; DMEM + 10% FBS + 1% AbAm+0.5% of gentamicin) on ECL cell attachment matrix (ECL)-coated tissue culture dishes overnight. BSC were then removed from dishes with 0.25% trypsin (Gibco), centrifuged (1500 × g, 5 min), and frozen in GM supplemented with 10% dimethyl sulfoxide (Sigma-Aldrich).

OXT- knockout BSC generation

Lentiviral vector digestion, oligo annealing, and cloning into digested vector
In order to investigate the role of OXT in myogenic, we sought to knockout (KO) OXT in primary BSC using the CRISPR-Cas9 technique. We used CHOPCHOP (http://chopchop.cbu.uib.no/) to choose guide RNA (gRNA) targets for bovine OXT (Montague et al., 2014; Labun et al., 2016). Two oligos of gRNA were annealed to form double-stranded duplexes using 10×T4 Ligation Buffer and T4 PNK (NEB). Annealed oligos were diluted at a 1:200 dilutions by Eluting Buffer (EB). The plasmid containing gRNA were generated using the cloning protocol implemented in Zhang’s lab. In brief, the LentiCRISPR V2 plasmids (5µg) were digested and dephosphorylated with BsmBI for 30 min at 37°C. If BsmBI digested, a ~2kb filler piece and a larger band should be presented on the gel. Only the larger band was purified by QIAquick Gel Extraction Kit (QIAGEN) and eluted in EB. Digested plasmids and double – stranded duplexes were ligated by T4 ligase and then transformed into StbI3 bacteria. 6 to 10 colonies were picked into 5ml LB-ampicillin culture each and cultured overnight. DNA purification was performed using a mini-prep kit from Qiagen. Using hU6F and Oligo 2 primer, we identified the positive clones by PCR. The positive clones were then sequenced by hU6F primer to confirm insert.

We designed gRNA that recognized OXT hormone sequence in exon 1, because exon 1 encodes OXT hormone and GKR tripeptide processing signal which is a splicing site (Gimpl and Fahrenholz, 2001). Gimpl and Fahrenholz (2001) reported that OXT gene transcript the prepropeptide which consists of signal peptide, OXT, GKR, and neurophysin. The prepropeptide is enzymatically cleaved into the OXT- GKR and neurophysin by magnolysin (Ando et al., 1987). Subsequent processing produces active OXT by the amidase at the position 9 (Gly) to generate C-amidated nonapeptide (Green et al., 2001). Thus, C-terminus of OXT sequence and GKR splicing
site play an essential role in OXT activation. Indels mutations on OXT sequence and OXT-GKR splitting cite may impact OXT expression and activation (Wang et al., 2016).

**Generation of pseudovirus**

The pseudovirus was generated in HEK293FT cells. HEK293FT cells (2.5×10⁶ cells) were plated in a 10cm dish and cultured at 37°C. After overnight culture, 5µg of lenti plasmids and 6µg of pCas-Guide plasmid (ORIGene) were added into the medium. MegaTran transfection reagent was used for increasing virus transduction efficiency. Medium was changed 12-18 h after transduction, then collected viral supernatant at 24 and 48 h. The supernatant was filtered through a 0.45-micron filter to remove cellular debris and stored in -80°C before use.

**Generation of OXT knockout BSC**

BSC were seeded into a 15cm dish coated with ECL cell attachment matrix for at least 1h. After 4 days culture, cells were suspended by 0.25% Trypsin and replated in five 10cm dishes (2×10⁶ cell/dish) with different culture medium. Among five dishes, two of them contained 3ml lentiCRISPRv2-OXT virus and 3ml GM (CRISPR-OXT) in each, another two contained 3ml empty lentiCRISPR V2 and 3ml GM for control (CRISPR-CON) in each, and the last one had 3ml PLJM1-EGFP virus (GFP; as transduction control) and 3ml GM. Cells were incubated at 37°C with 5% CO2 for 24h. After 24h transduction, medium was replaced by 10ml of fresh GM and cells were incubated for another 24 h. Puromycin (1µg/ml) was added into the culture medium. After puromycin selection for 48h, the remaining cells were suspended by 0.25% Trypsin and transferred to a new 10cm dish. When the cell confluency reached 70%, cells were suspended by and 0.25% Trypsin and virus transduction and puromycin selection performed a second time. Cells
were passaged and parts of them were used for DNA extraction, PCR amplification, and sequencing, rest of them storage in liquid nitrogen for downstream experiments.

**Proliferation**

The cells were plated in 96-well plates which were coated with ECL for at least 1 h, then incubated in GM overnight to allow cells to adhere. BSC plated at 4,000 cells/well were cultured in low serum medium (DMEM + 2%FBS + 1xITS) with different treatment: 17β-estradiol (E2: 10 nM); OXT (125 nM); trenbolone acetate (TBA: 1 µM); fibroblast growth factor 2 (FGF2; 10 ng/ml; as a positive control); or control (CON; without any treatments). All cell cultures received fresh media with different treatments daily. Using the CyQUANT® NF Cell Proliferation Assay Kit, proliferation rate was measured every 24 hours during culture. CyQUANT® NF reagent in 1× HBSS buffer was added to each well after moving out the culture medium. After 1h incubation at 37°C, fluorescence intensities of BSC were measured with a fluorescence microplate reader (Infinite M200 PRO, TECAN).

**Apoptosis**

Cells were preconditioned with E2 (10 nM), OXT (125 nM), and TBA (1 µM). After 48h treatment, cells were treated with serum free medium and 200µM of hydrogen peroxide (H2O2) for another 48h. Following incubation, cells were washed 3 times for 5min with PBS. Cell number was assessed by CyQUANT NF Proliferation Assay Kit. Cell death was detected by In Situ Cell death detection Kit. A total of 6 pictures of randomly selected fields were taken under 200-fold magnification fluorescent microscope (Nikon), and digital pictures were analyzed by NIS Elements software (Nikon). Results were expressed as percentage of apoptotic nuclei.

**Differentiation**
The cells were plated at the density of 5×10^4 cells/well in 24-well plates coated with ECL. At 75%-85% confluency, the medium was changed to differentiation medium (DM, DMEM+2%FBS+1%AbAm+0.5 of gentamicin) with E2 (10 nM), OXT (125 nM), TBA (1 µM), or control (CON; without any treatments), respectively, to induce differentiation. All cell cultures received fresh media with different treatments daily. Myotubes were stained at 72 h after inducing differentiation as previously described with some modifications (Joulia et al., 2003). Myotubes were washed three times with cold PBS, and then fixed in 4% paraformaldehyde at RT for 20 min. After washing with PBS three times, non-specific bindings were blocked with blocking buffer (2% BSA+ 0.2% Triton-X-100 in PBS) for 1h at RT. Primary myosin heavy chain antibody (MF20; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) diluted in blocking buffer (1:10) was applied overnight at 4°C and revealed using secondary Alexa Flour 488 (96 h) goat anti-mouse antibodies (1:1000; Invitrogen, Carlsbad, CA, USA) for 1 h. Nuclei were stained with DAPI for 5 min at RT. Since CRISPR-CON and CRISPR-OXT exhibited poor differentiation, and fusion index with different treatment were very low (all lower than 3%), we count myotube number in the whole well as the measurement of BSC differentiation.

**Migration**

Cells were preconditioned with E2 (10 nM), OXT (125 nM), and TBA (1 µM) for 48h. Then the treated CRISPR-CON and CRISPR-OXT cell were plated in the top of modified Boyden chamber (Corning Costar, Cambridge, MA). Cells were allowed to migrate for 16h in the presence 10% FBS in the bottom chamber. Migrated cells were fixed in 4% paraformaldehyde, and nuclei were stained with DAPI. A total of 6 pictures of randomly selected fields were taken under 100-fold magnification fluorescent microscope (Nikon), and digital pictures were analyzed by NIS Elements software (Nikon). Results were expressed as a migration index (percentages), which was
defined as: mean number of cells for test conditions/mean number of cells for controls (Noiseux et al., 2012).

**Real-Time quantitative reverse transcription-PCR**

Real-time quantitative reverse transcription-PCR (qPCR) was used to measure the abundance of target genes in cultured CRISPR-CON and CRISPR-OXT cell. The cells were seeded in 6-well plates (1.5×10^5 cell/well). The cells were cultured in GM with 10nM E2, 1 µM TBA, or 125nM OXT treatment at 37°C with 5% CO2 for 48 h. The medium was refreshed every 24 h. Total RNA was extracted at 48 h of culture.

During cell differentiation, effects of OXT on gene expression were investigated. The cells were cultured in GM until 75% - 85% confluency and then induced differentiation by replacing GM to DM. After 24 h of differentiation, 10nM E2, 1 µM TBA, or 125nM OXT were added into the DM. The medium with treatment was refreshed daily until 96 hours of differentiation. Total RNA was extracted at 72h (48h treatment) of culture.

Total RNA was extracted using Trizol reagent (Invitrogen, US) from cultured cells according to the manufacturer’s protocol, and then RNA samples were cleaned up by RNeasy® Mini Kit (Qiagen, USA). The cDNA was generated from 1µg purified RNA using iscript™ cDNA Synthesis Kit (Bio-Rad, USA). Finally, the cDNA was diluted 1:10. Specific TaqMan probes of OXT and cyclophilin α (PPIA) was used in OXT mRNA expression study. The qPCR reactions were performed in the same well using CFX96 Detection System (BioRad) and in a 20µl final volume containing 10µl of PCR mix, 1µl of each primer (10µM), 0.5µl of each probe (10µM), and 5µl of cDNA (25ng). PCR cycling conditions consisted of 10min incubation at 95°C followed by 40 cycles of 95°C for 15s and 60°C for 1min. For other genes, qPCR was performed on 5 µL of
diluted cDNA in a final volume of 20 μL using the SsoAdvanced Universal SYBR Green Supermix (BioRad) in CFX96 Detection System. The parameters used were as follows: 5 min at 95°C, followed by 40 cycles of repeated incubations at 95°C for 10 s and 60°C for 30 s. The fold change for all the samples measured by qPCR was calculated by \(2^{-\Delta\Delta C_q}\) method. All the primers and probes are given in Table 1.

**Statistical analyses**

The results were analyzed in JMP® (SAS Institute) using two-way analysis of variance (ANOVA). Pairwise comparisons between the least square means of the factor levels were conducted using Tukey’s HSD test. Values are expressed as means ± SEM.

**Results**

**OXT-knockout BSC generation**

As a template for mutagenesis, we designed a gRNA that recognizes exon 1 of OXT to induce a double-strand break (see Figure 1B). After 48h puromycin selection, more than 95% cells were GFP positive (Figure 1D). Since insertion/deletion (indels) mutations will be caused through NHEJ during genomic region repair, we generated a cell pool which including wild-type BSC and CRISPR-OXT cells. There are three genotypes in the cell pool including double-strands modified, single-strand modified, and wild type. The exon 1 PCR products were cloned into TOPO vector, and then 47 TOPO clones were sequenced to identify mutations in both alleles. 25 clones contained deletions in both alleles, 11 clones introduced 1- insertion in both alleles, and 11 clones exhibited unmodified DNA (Figure 1C). Gene editing efficiency was more than 76%. All the indels generated the in-frame shift causing the absence of 1-5 amino acids at the COOH-terminal end of OXT and a glycyl-lysyl-arginine (GKR) sequence which is a cleavage site involved in activation
and maturation of OXT. Thus, in-frame deletions and insertions in the OXT KO BSC either created a nonfunctional protein or prevented protein expression. Next, we determined the knockout efficiency by qPCR (Figure 2). The OXT expression dramatically decreased in CRISPR-OXT cell compared to wild-type BSC by more than 97%, however OXT expression declined in CRISPR-CON cell by 39.6% compared to wild-type BSC.

**proliferation**

Proliferation rate for wild-type BSC, CRISPR-CON cell and CRISPR-OXT cell under different treatments is presented in Figure 3. There was no difference in proliferation with OXT treatment between CRISPR-CON and CRISPR-OXT cell. Proliferation decreased ($P<0.05$) with E2 and TBA treatments between CRISPR-CON and CRISPR-OXT cell.

**Apoptosis**

The cell number gradually decreased ($P<0.05$) from wild-type BSC to CRISPR-OXT cell (Figure 3.4A). OXT increased ($P<0.05$) cell number in both CRISPR-CON and CRISPR-OXT cell (Figure 3.4A). E2 and TBA increased ($P<0.05$) cell number in CRISPR-CON cell but not CRISPR-OXT cell (Figure 3.4A). The percentage of apoptotic nuclei was reduced ($P<0.05$) in CRISPR-OXT cell compared to CRISPR-CON cell and wild-type BSC (Figure 3.4B). OXT decreased ($P<0.05$) percentage of apoptotic nuclei in both CRISPR-CON and CRISPR-OXT cell (Figure 3.4B). E2 and TBA decreased ($P<0.05$) percentage of apoptotic nuclei in CRISPR-CON cell but not CRISPR-OXT cell (Figure 3.4B).

**Differentiation**

Effect of OXT on CRISPR-OXT cell and CRISPR-CON cell differentiation based on immunohistochemical staining were shown. The number of myotubes decreased ($P<0.05$) in
CRISPR-OXT cell compared to CRISPR-CON cell (Figure 3.5). OXT increased \( P<0.05 \) the number of myotube in both CRISPR-CON and CRISPR-OXT cell (Figure 3.5). E2 and TBA increased \( P<0.05 \) number of myotube in CRISPR-CON cell but not CRISPR-OXT cell (Figure 3.5).

**Migration**

Because migration contributes to the fusion of BSC, we also investigated if OXT altered BSC migration as well (Figure 3.6). Migration decreased \( P<0.05 \) in CRISPR-OXT cell compared to CRISPR-CON and wild-type BSC cell. OXT increased migration in both CRISPR-CON and CRISPR-OXT cell (Figure 3.6). E2 and TBA increased migration in CRISPR-CON cell but not CRISPR-OXT cell (Figure 3.6).

**Effect of OXT on gene expression during CRISPR cell proliferation and differentiation**

Since we found a significant increase in \( MyoD \) and \( MyoG \) expression with E2 and TBA treatments at 48h for wild-type BSC during proliferation and differentiation stages in a previous study, we elected to detect target genes expression in CRISPR-OXT cell at 48h as well. The expression of OXTR, \( MyoG, MyoD, \) and \( MyoG \) decreased \( P<0.05 \) in both CRISPR-CON and CRISPR-OXT cell compared to wild-type BSC during proliferation (Figure 3.7) and differentiation (Figure 3.8). OXT, E2, and TBA cannot stimulate OXTR and \( MyoG \) expression in both CRISPR-CON and CRISPR-OXT cell during proliferation (Figure 3.7 A&B) and differentiation (Figure 3.8 A&B). OXT increased \( P<0.05 \) \( MyoD \) and \( MyoG \) expression in CRISPR-CON cell but not CRISPR-OXT cell during proliferation (Figure 3.7 C&D). During differentiation, OXT, E2, and TBA cannot stimulate \( MyoD \) expression in both CRISPR-CON and
CRISPR-OXT cell (Figure 3.8 C). The expression of MyoG increased ($P<0.05$) in CRISPR-OXT cell but not CRISPR-CON cell during proliferation (Figure 3.8 D).

**Discussion**

Our study with CRISPR-OXT cells has demonstrated that OXT, a nonapeptide hormone, expressed in skeletal muscle and SC, and upregulated by estrogen in BSC, similar to what has been reported in other studies (De Jager et al., 2011a; Berio et al., 2017). Since estrogen administration regulates SC activity, it is likely that OXT may stimulate SC proliferation and differentiation. However, the role of OXT in SC activity is poorly documented. The current study was performed to generate OXT knockout BSC model using CRISPR/Cas9 technology. Then using this model, we further determined and confirmed the role of OXT in BSC proliferation and differentiation.

The expression of $OXT$ declined in CRISPR-CON cell by 39.6% compared to wild-type BSC. This unexpected result indicates that control lentiviral transduction also impacts $OXT$ expression in BSC. However, $OXT$ expression further decreased in CRISPR-OXT cells by 97% compared to wild-type BSC. This decrease was more dramatic than that in CRISPR-CON cell. Thus, we evaluated the effect of OXT on proliferation and differentiation in both CRISPR-CON and CRISPR-OXT cell. A similar observation was also reported by Liu and Conboy (2017), who evaluated the effect of control lentiviral transduction on $OXTR$ expression. They found that lentiviral control vectors considerably downregulate $OXTR$ expression at mRNA and protein levels and diminish ERK signaling, downstream effectors of $OXTR$, in mouse and human skeletal muscle cells (Liu and Conboy, 2017). These results suggest that experimental transduced with control lentiviruses may not behave the same as un-transduced skeletal muscle cells, at least that control viral vectors significantly change the intensity of OXT/OXTR system. Our results further demonstrate that lentiviral transduction significantly decreases BSC proliferation and
differentiation, which suggests that viral infections, in general, may play a role in decreasing muscle health and regeneration, as these rely on effective OXTR signaling (Elabd et al., 2014).

Since cell apoptosis also directly contributes to the proliferation (Joulia et al., 2003), it is likely that OXT may be involved in BSC apoptosis. By assessing the cell in serum free and oxidative stress conditions, we confirmed that cell number increased with OXT in both CRISPR-CON and CRISPR-OXT cell. E2 and TBA treatments increased cell number in CRISPR-CON cell but not CRISPR-OXT cell. These results indicate that OXT plays protective roles in BSC, and it may contribute to the protective function of E2 and TBA in BSC. This founding was later again confirmed by the results that apoptotic cell number significantly decreased with OXT treatment compared to CON in both CRISPR-CON and CRISPR-OXT cell. So far, there is no publication disclosed the protective role of OXT in primary BSC or muscle cell line before. However, Noiseux et al. (2012) found that OXT treatment of myocardial infarct in rats significantly reduced cardiomyocytes apoptosis and rat MSC survival under serum starvation and hypoxic stress. OXT-OXTR system positively regulates muscle stem cell responses through the MAPK/ERK pathway which is also a key transducer of anti-apoptotic signals in various cell types (Kornasio et al., 2009; Elabd et al., 2014). Future studies focused on determining the molecular mechanisms underlying reduction of BSC apoptosis by OXT are necessary.

The process of myoblast fusion consists of cell migration, adhesion and signaling transduction pathways leading up to the actual fusion event (Abmayr and Pavlath, 2012). Several studies support the importance of migration to myoblast fusion (Hughes and Blau, 1990; Bae et al., 2008). In a migration study for both CRISPR-CON and CRISPR-OXT cells, we confirmed that OXT dramatically increased migration rate compared to CON. E2 and TBA increased CRISPR-CON cell migration but not CRISPR-OXT cell. Thus, these findings confirmed that OXT is a
potential regulator of myoblast motility and this property contributes to E2’s and TBA’s ability to promote myoblast fusion into myotubes. To our knowledge, there is no existing OXT study examining BSC migration. Noiseux et al. (2012) demonstrated that OXT stimulates the migration of bone marrow-derived mesenchymal stem cell (MSC). OXT stimulates activation of PI3K and endothelial nitric oxide (NO) synthase which were required for the pro-migratory effect of OXT on human umbilical vein endothelial cells (Cattaneo et al., 2008) and in ischemic rat heart (Faghihi et al., 2012). Thus, it is possible that OXT stimulates BSC migration via PI3K/Akt and NO signaling pathway. Studies to investigate the specific biological mechanisms of OXT on SC migration are needed in the future.

For detection of target genes mRNA expression in CRISPR-OXT cell, MyoD and MyoG expression significantly increased with OXT treatment in both CRISPR-CON and CRISPR-OXT cell during proliferation. E2 and TBA increased MyoD and MyoG expression in CRISPR-CON cell but not CRISPR-OXT cell during proliferation. During differentiation, OXT significantly increased MyoG expression in both CRISPR-CON and CRISPR-OXT cell, although there was no difference in MyoD expression with different treatments. E2 and TBA increased MyoG expression in CRISPR-CON cell but not CRISPR-OXT cell. These results demonstrate that OXT regulates BSC differentiation by regulating MyoD and MyoG expression. MyoD has been reported to be an important regulator required for the differentiation potential of skeletal myoblasts (Rudnicki et al., 1993; Conerly et al., 2016). Kuang et al. (2007) found downregulation of MyoD expression in proliferating myoblast lead to return to quiescence, and resist of SC differentiation. As the SC commits to the myogenic lineage, most cells will begin to express MyoG which also plays important roles in SC differentiation (Wright et al., 1989; Montarras et al., 1991; Kassar-Duchossoy et al., 2004; Moretti et al., 2016). So, reduction of both MyoD and MyoG expression
will result in small myofiber size and accumulation of undifferentiated myoblasts (Wang and Rudnicki, 2012). Our previous study indicated that E2 and TBA increased MyoD and MyoG expression during wild-type BSC differentiation, but E2 and TBA did not increase MyoD and MyoG expression in CRISPR-OXT cell. Thus, OXT, at least partially, contributes to E2 and TBA stimulated BSC differentiation by regulating MyoG expression. These results indicate a new indirect function of steroid hormones, that is, they induce expression of OXT which further stimulates BSC differentiation by regulating MyoD and MyoG expression.

**Conclusion**

In conclusion, these results indicate that OXT stimulated CRISPR-OXT cell proliferation, differentiation, and migration. E2 or TBA increased BSC proliferation, differentiation, and migration, and OXT is involved in those processes. OXT stimulates BSC differentiation by regulating MyoD and MyoG expression. Moreover, OXT protected CRISPR-OXT cell from serum starvation and oxidative stress conditions. Together, the results support the hypothesis that OXT plays essential roles in BSC proliferation and differentiation and it is involved in the functions of E2 and TBA on BSC activity.

**References**


<table>
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<tr>
<th>Gene (RefSeq ID)</th>
<th>Animal</th>
<th>Primer (5'-3')</th>
<th>Amplicon size(bp)</th>
</tr>
</thead>
</table>
| OXT (NM_176855) | Cattle | F: CTGCCAAGAGGAGAACTACC  
R: CCTGGGGATGATTACAGAGG  
P: FAM-CTTCTCCAGCACTGAGACC-BHQ1 | 218 |
| PPIA (NM_178320) | Cattle | F: GCCCCAACACAAATGGTT  
R: CCCTCTTTCACCTTGCCAAAG  
P: HEX-TGCTTGCCATCCAACCACCTCAGTC-BHQ1 | 96 |
| OXTR (NM_174134) | Cattle | F: CAAGGAAGCCTCACCTTTCA  
R: TGCACAAAGTCTCTGGAAGAGG | 111 |
| MyoD (NM_001040478) | Cattle | F: CGACTCGGACGCTTCCAGT  
R: GATGCTGGACAGGCACTCGA | 180 |
| MyoG (NM_001111325) | Cattle | F: GTGCCCATGAATGCAGCAGCTC  
R: GTCTGTAGGTCCGCTGGGA | 110 |
| MyoG (XM_002685738) | Cattle | F: GAGTTCGATTAGCCGAGTGC  
R: ATGCTGTGCTTGGCTTTCTT | 105 |

Note: F, forward primer; R, reverse primer; P, TaqMan probe
Fig. 3.1. OXT disruption in BSC using CRISPR/Cas9 genome editing. (A) Domain organization of preprooxytocin including the splitting site. (B) sgRNA sequence was designed to bind sequence in the exon 1 region of the OXT gene, such that gene editing could disrupt OXT expression in BSC. PAM: protospacer-adjacent motif. (C) Transduction efficiency was determined by pLJM1-EGFP transfection. After 48h puromycin selecting, more than 95% BSC were GFP positive. (D) The exon1 was PCR amplified from BSC treated with LentiCRISPR V2 and gRNA expression cassettes. Sequence of individual clone was determined by Sanger sequencing. The number of clones for each sequence is shown in parentheses. Δ means deletion or insertion. The top sequence is the unmodified. In the right peptide sequence, the signaling peptide, OXT (nonapeptide), and splicing site were highlighted in orange, red, and blue, respectively.
Fig. 3.2. Knockout efficiency after CRISPR/Cas9 genome editing. OXT mRNA expression was detected in wild-type BSC, CRISPR-CON cell, and CRISPR-OXT cell by qPCR. Groups with different letters have significant difference with $P < 0.05$. Means and SEMs are shown.
Fig. 3.3. Proliferation rate in CRISPR-CON and CRISPR-OXT cell. CyQUANT® NF Cell Proliferation Assay Kit was used to determine the proliferation rate in low serum medium (DMEM + 2%FBS + 1×ITS) with different treatments in CRISPR-CON cell and CRISPR-OXT cell. Values are means and SEMs. Groups with different letters have significant difference with $P < 0.05$. 
Fig. 3.4. Effect of OXT on cell death in CRISPR-CON and CRISPR-OXT cell. CRISPR-CON and CRISPR-OXT cell were preconditioned with E2 (10 nM), OXT (125 nM) and TBA (1 µM). After 48h treatments, cells were treated with serum free medium and 200µM of hydrogen peroxide (H2O2) for another 48h. Following incubation, cells were washed 3 times for 5min with PBS. Cell number was assessed by CyQUANT NF Proliferation Assay Kit. Apoptotic nuclei were detected by In Situ Cell death detection Kit. Groups with different letters have significant difference with $P < 0.05$. Means and SEMs are shown.
Fig. 3.5. Effect of OXT on differentiation in CRISPR-CON and CRISPR-OXT cell. CRISPR-CON and CRISPR-OXT cell were plated at the density of $5 \times 10^4$ cells/well in 24-well plates coated with ECL. At 75%-85% confluency, the medium was changed to differentiation medium (DM, DMEM+2%FBS+1%AbAm+0.5% of gentamicin) with different treatments. All the cells cultures received fresh media with different treatments daily. Myotubes were stained by MF20, myonuclei were stained by DAPI. We count myotube number in entire well as the measurement of differentiation. Means and SEMs are shown. Groups with different letters have significant difference with $P < 0.05$. 
Fig. 3.6. Effect of OXT on migration in CRISPR-CON and CRISPR-OXT cell. CRISPR-CON and CRISPR-OXT cell were preconditioned with E2 (10 nM), OXT (125 nM), and TBA (1 µM). Then, the cells were plated in the top of modified Boyden chamber (Corning Costar, Cambridge, MA) and were allowed to migrate for 16h in the presence 10% FBS in the bottom chamber. Migrated cells were fixed in 4% paraformaldehyde, and nuclei were stained with DAPI. Results were expressed as a migration index (in percentage) defined as a ratio between mean number of cells for test conditions and mean number of cells for controls. Groups with different letters have significant difference with $P < 0.05$. Means and SEMs are shown.
Fig. 3.7. Gene expression during CRISPR cell proliferation. The cells were cultured in DM with or without different treatments for 48 h. The medium was refreshed every 24 h. Total RNA was extracted at 48 h of culture. CON and treated myoblast lysates during proliferation were analyzed for gene mRNA expression by qPCR. (A) OXTR, (B) MyoG, (C) MyoD, and (D) MyoG expression were determined by qPCR. Groups with different letters have significant difference with $P < 0.05$. Means and SEMs are shown.
**Fig. 3.8. Gene expression during CRISPR cell differentiation.** The cells were cultured in DM with or without different treatments for 48 h. The medium was refreshed every 24 h. Total RNA was extracted at 48 h of culture. CON and treated myoblast lysates during differentiation were analyzed for gene mRNA expression by qPCR. (A) OXTR, (B) MyoG, (C) MyoD, and (D) MyoG expression were determined by qPCR. Groups with different letters have significant difference with $P < 0.05$. Means and SEMs are shown.
CHAPTER 4. OXYTOCIN EXPRESSION IS ALTERED IN SKELETAL MUSCLE BY INTRAUTERINE GROWTH RESTRICTION AND CALORIC RESTRICTION

Abstract

Intrauterine growth restriction (IUGR) and caloric restriction (CR) reduce skeletal muscle mass in mammals, principally because of a reduction in fiber number and/or size. Skeletal muscle has been reported to express oxytocin (OXT). Thus, in this study, the OXT expression pattern was examined in IUGR sheep and CR calves. We collected (1) longissimus dorsi muscle (LM) from fetal control (FC, n=12), fetal IUGR (FI, n=10), lamb control (LC, n=6), and lamb IUGR (LI, n=6); (2) infraspinatus muscle (INF), LM, and semitendinosus muscle (ST) from newborn calves after a control (CON) or caloric restricted (CR) diet for eight weeks. In all samples, mRNA abundance of target genes, including OXT, OXT receptor (OXTR), and myogenic regulating factors (MRFs), were measured. Compared to the FC, OXT expression decreased (P<0.05) in FI LM muscle, whereas there was no significant difference in OXT expression between LI and LC groups. However, OXTR expression decreased (P<0.05) in FI group compared to FC group. Caloric restriction decreased OXT expression (P<0.05) in INF, LD, and ST muscle. Moreover, CR decreased (P<0.05) MHC4 expression but increased (P<0.05) MHC2 and MHC7 expression in skeletal muscle. These findings support that OXT may play important roles in skeletal muscle development and growth.

Key words: Caloric restriction, IUGR, Oxytocin, Skeletal muscle
Introduction

Permanent changes in individual organs will develop in organisms during intrauterine life and neonatal stages (Valsamakis et al., 2006). Thus, environmental challenges (malnutrition, thermal stress, etc.) during fetal development – especially in the second trimester – can result in insufficient development of the placenta, which may further affect the transportation of nutrients, oxygen, and other factors and cause placental insufficiency intrauterine growth restriction (PI-IUGR) in fetuses (Chen et al., 2010). Fetal skeletal muscle growth in the IUGR fetus is compromised under conditions of placental insufficiency (Tchirikov et al. 1998, Yajnik 2004). However, there is no effective therapy to reduce the risk of diseases for IUGR offspring. Nutrition plays a central role in preventing diseases and promoting health. Caloric restriction (CR), defined as a diet low in calories without under-nutrition, reduces skeletal muscle mass in mammals (Lu et al., 2017). However, the molecular mechanism underlying CR stimulated skeletal muscle loss is not fully understood.

Oxytocin (OXT) is a hormone best known for its role in lactation (Soloff et al., 1979), parturition (Fuchs et al., 1982), and social behaviors (Andari et al., 2010). The main source of OXT is the hypothalamus, from where OXT is transported to posterior pituitary, and then released into the bloodstream (Gimpl and Fahrenholz, 2001). However, OXT can also be produced by other tissues, such as uterus (Ivell and Hartung, 2003), placenta (Gimpl and Fahrenholz, 2001), and heart (Jankowski et al., 2004). Researchers recently discovered that OXT is greatly expressed in skeletal muscle by steroid hormone treatment in cattle (De Jager et al., 2011a). OXT is also required for proper muscle tissue regeneration and homeostasis in mouse as well (Elabd et al., 2014).

The overall objective of present study is to determine target genes expression pattern in IUGR sheep and CR calves, with a focus on OXT expression. To achieve this goal, we performed
TaqMan qPCR assay to measure the OXT mRNA expression level in developing skeletal muscle of IUGR fetuses in sheep and CR calves. Expression of other important genes, such as OXT receptor (OXTR), paired box 7 (MyoG), myogenic differentiation 1 (MyoD), myogenin (MyoG), myogenic factor 5 (MYF5), muscle regulatory factors (MRF4), myostatin (MSTN), proliferating cell nuclear antigen (PCNA), and myosin heavy chain gene (e.g. MHC 1, 2, 4, and 7) were also determined by qPCR.

**Materials and Methods**

**Animals**

This study was approved by the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee. Two sets of experiments were provided previously (Tucker et al., 2016; Lu et al., 2017).

Briefly, the ovine model of the PI-IUGR system generated by exposing pregnant ewes to hyperthermia (40°C × 12h/d, 35°C ×12h/d; 30-40% RH) from 40 to 95 days of gestational age (dGA). Control ewes were pair fed, dGA-matched, and maintained at 25°C. Fetal skeletal muscle was collected from treatment groups (PI-IUGR and control) generated above. Longissimus dorsi muscle (LM) were collected (snap frozen in liquid nitrogen) from fetuses at 140 dGA (0.97 of gestation) and from lambs at 21 days of age. In total, four groups were generated: Fetal control (FC, n=12); Fetal IUGR (FI, n=10); Lamb control (LC, n=6); Lamb IUGR (LI, n=6). Each sample was immediately frozen in liquid nitrogen and stored at -80 °C before use.

New born Holstein bull calf (N=11) were divided into two groups: Group 1 (N=5) was served with low plane of nutrition (CR; 20% CP, 20% fat; 441 g DM/d); Group 2 (N=6) was served with normal plane of nutrition (CON; 27% CP, 10% fat; 882 g DM/d during wk 1 and 1131 g
DM/d thereafter). At eight weeks of age, calves were slaughtered and infraspinatus muscle (INF), LM, and semitendinosus muscle (ST) were harvested from each animal. Each sample was immediately frozen in liquid nitrogen and stored at -80 °C before use.

**Isolation of RNA and production of cDNA**

Total RNA from muscle was isolated using Trizol reagent (Invitrogen, US) according to the manufacturer’s protocol, and then RNA samples were cleaned up by RNeasy® Mini Kit (Qiagen, USA). Total RNA was assessed by agarose gel and Nanophotometer (IMPLEN, USA). The presence of two clear 18S and 28S bands and a ratio of ~2.0 in 260/280 confirmed the integrity of the RNA. Total RNA was stored at -80°C before transcription. cDNA was synthesized from 1µg purified RNA using iscript™ cDNA Synthesis Kit (Bio-Rad, USA). To this, in 20ul total volume per reaction, 4µl 5×iScript reaction mix, 1µl iScript reverse transcriptase, 1µg total RNA, and H₂O mix well in PCR tube. This mixture was incubated 5min at 25°C, 30min at 42°C, 5min at 85 °C, and hold at 4°C. Finally, the cDNA was diluted 1:10.

**Quantitative real-time PCR (qPCR)**

The cDNA was subjected to qPCR using specific TaqMan probe of OXT and housekeeping genes by 2×IQ Multiplex Powermix (BioRad). The β-actin (sheep) and cyclophilin α (cattle; PPIA) gene were used as housekeeping gene control as previously reported (Kongsuwan et al., 2012a; Divari et al., 2013). Primer sequences and the TaqMan probe of OXT and housekeeping genes (Table1) were synthesized by Fisher Scientific. The qPCR reactions were performed in the same well using CFX96 Detection System (BioRad) and in a 20µl final volume containing 10µL of PCR mix, 1µL of each primer (10µM), 0.5µl of each probe (10µM), and 5µl of cDNA (25ng). The PCR cycling conditions consisted of 10min incubation at 95°C followed by 40 cycles of 95°C for 15s
and 60°C for 1min. Each sample was amplified in triplicate. The standard curve method was used to calculate the relative gene expression level between OXT and housekeeping genes. Measurements of other interest genes expression (OXTR, MyoD, MyoG, Myf5, MRF4, MSTN, PCNA, MHCs) were performed using SsoAdvanced Universal SYBR Green Supermix (BioRad). The qPCR reactions were the same as former TaqMan study but no probe added. The fold change for all the samples was calculated by $2^{-\Delta\Delta C_q}$ method.

**Generation of standard curve**

Using TOPO® TA Cloning® Kit (Invitrogen), the PCR products of OXT gene, β-actin, and PPIA were ligated with vector and then transformed into Match1™-T1R Competent Cells (Invitrogen, USA). The cell-vector mixture was incubated on ice for 30min, heat shocked for 30s at 42°C in a water bath, and immediately placed them on ice for 2min. After adding 250µl of S.O.C. medium, the mixture was shaken horizontally at 37°C for 1hour at 225rpm. The cells were then spread onto prewarmed selective LB agar and incubated at 37°C overnight. White recombinant colonies were picked and subjected to colony PCR to confirm the presence of the target fragment. Positive colonies were grown in liquid LB medium overnight, and the plasmids were purified with the QIAprep Spin Miniprep Kit (Qiagen, USA), in compliance with the manufacturer’s instructions.

After purification, each recombinant plasmid DNA (recDNAOXT, recDNAβ-actin, and recDNAPPIA) was sequenced by Virginia Bioinformatics Institute at Virginia Tech (VBI, Virginia Tech) using M13R or M13F primers to confirm its identity. The concentration of each purified recDNA was calculated by Nanophotometer (IMPLEN, USA). Standard curves were generated using serial dilutions of recDNAs from $3 \times 10^6$ to 30 molecules.
Results

Target genes expression in skeletal muscle of IUGR sheep

Compared to the control fetus, *OXT* mRNA expression significantly decreased in LM of IUGR fetus, whereas there was no difference in *OXT* expression between the IUGR and control lambs (Fig. 4.1. A). *OXTR* mRNA expression significantly decreased in IUGR fetuses compared to control fetuses (Fig. 4.1. B). The expression of *MyoG, MyoD, MyoG* significantly decreased as well in LM of IUGR fetuses compared to control fetuses (Fig. 4.1. C, D, &E).

Target genes expression in skeletal muscle of CR calves

The expression of *OXT* decreased in INF, LD, and ST muscle in CR group compared to CON group (Fig. 4.2). However, *OXTR* and *MyoG* expression increased in INF, LD, and ST muscle in CR group compared to CON group (Fig. 4.2). There was no difference on expression of *MyoD, MyoG, Myf5, MRF4*, and *MSTN* in INF muscle (Fig. 4.2. A). But LD and ST muscle exhibited lower expression on those genes in CR groups compared to CON group (Fig. 4.2. B, C). There was no significant difference on *PCNA* expression in INF, LD, and ST muscle of CR group compared to CON group (Fig. 4.2). The expression of *MHC4* significantly decreased with CR treatment compared to CON in INF, LD, and ST muscle (Fig. 4.2). *MHC2* and *MHC7* expression increased with CR treatment in INF, LD, or ST muscle (Fig. 4.2). There was no difference in *MHC1* expression with CR treatment compared to CON in INF, LD, and ST muscle (Fig. 4.2).

Discussion

In the present study, we found that *MyoG, MyoD, and MyoG* expression decreased in IUGR fetuses compared to CON. In CR calves, *MyoD, MyoG, Myf5*, and *MRF4* expression also significantly decreased in LD or ST muscles. *MyoD, MyoG, Myf5*, and *MRF4* belong to the MRF
family (Sambasivan and Tajbakhsh, 2015). Thus, our results demonstrate that both IUGR and CR decrease MRF expression in skeletal muscle. The MRF are involved in myogenic lineage which play important roles in skeletal muscle development and growth (Dodson et al., 2010). Myf5 is expressed in most of the quiescent SC (Beauchamp et al., 2000) and activated myoblasts, and is thought to regulate proliferation rate and homeostasis (Ustanina et al., 2007). MyoD has also been reported to be an important regulator required for the differentiation potential of skeletal myoblasts (Rudnicki et al., 1993; Conerly et al., 2016). Kuang et al. (2007) found downregulation of MyoD expression in proliferating myoblast lead to a return to quiescence, and prevent satellite cell differentiation. Both MyoG and MRF4 play essential roles in terminal differentiation and fusion of committed myoblasts (Le Grand and Rudnicki, 2007). Similarly, Hasty et al. (1993) and Olson et al. (1996) indicated that MyoG and MRF4 knock-out mice exhibit severe defects in muscle development and growth. In addition, we also observed that IUGR fetuses reduced OXT and OXTR expression in skeletal muscle. We have shown that OXT functions in muscle development in Chapter 2 & 3. Thus, it is possible that IUGR and CR impair muscle development and growth by decreasing MRFs expression or regulating OXT. Yates et al. (2012) and Lu et al. (2017) have found that IUGR sheep and CR cattle exhibited smaller skeletal muscle fiber and lower muscle mass, which also imply that IUGR and CR impact skeletal muscle development and growth in sheep and cattle.

Similarly, CR calves exhibited lower OXT expression in INF, LD, and ST muscle, whereas the OXTR transcripts were much more abundant in CR calves. However, in IUGR sheep study, both OXT and OXTR expression significantly decreased in IUGR LM muscle. This different observation may be explained by different species or because of possible complex processing response to IUGR and CR (Lu et al., 2017). Since IUGR and CR decreases skeletal muscle mass
(Yates et al., 2012; Lu et al., 2017), our results indicate that OXT-OXTR system may be involved in this phenomenon. Skeletal muscle growth requires myoblast activation, proliferation, and differentiation (Dodson et al., 2010). Breton et al. (2002) reported that treating human myoblast with OXT for five days increased the percentage of fusion to over 60% compared to control cells. Our in-vitro studies in chapter 2 & 3 also demonstrated that OXT is involved in BSC proliferation and differentiation. Together, these results indirectly indicated that OXT, as an important skeletal muscle developing hormone, may help explain smaller muscle fiber size and less muscle mass in near-term IUGR fetuses and CR calves. Similar results has been reported by De Blasio et al. (2007) and MacGhee et al. (2017) who observed that skeletal muscle mass decreased with IUGR and CR in mammals. We also found OXT expression significantly decreased in LI and LC LM compared to FC LM muscle, which indicate that OXT expression increases near term. De Jager et al. (2011a) reported the similar finding that OXT expression peaked at least three months prior to birth and was sustained neonatally in LM muscle, coincident with myofiber hypertrophy during muscle development. These results again confirm that OXT may play important roles in skeletal muscle development and growth.

Although we did not detect the muscle fiber type by immunohistochemistry in this in-vivo study, the MHC isoforms expression were determined by qPCR. Compared to CON, CR significantly decreased MHC4 expression in LM muscles. CR increased MHC2 expression in INF and LD muscle and MHC7 expression in LD and ST muscle. These results indicate that CR can lead to skeletal muscle fiber switch by switch type IIb fibers into type I fibers or type IIa fibers. De Andrade et al. (2015) demonstrated that an overall conversion of fast fibers into slower fibers was found in CR rats. As a result, the ratio of type I to IIB fibers was more than doubled (over
127% in CR rats compared to control rats (De Andrade et al., 2015). Because CR decreased OXT expression, OXT may be involved in skeletal muscle fiber switch.

**Conclusion**

In conclusion, we performed initial *in-vivo* analysis of target gene expression pattern in cattle and sheep during IUGR and CR conditions. We found that IUGR and CR decreased MRFs expression in skeletal muscle, which may explain why IUGR and CR impair skeletal muscle development and growth. CR significantly decreased MHC4 expression but increased MHC2 and MHC7 expression in skeletal muscle. These results indicate that CR can lead to skeletal muscle fiber switch by switch type IIb fibers into type I fibers or type IIa fibers. Moreover, our work revealed that OXT expression significantly decreased in skeletal muscle of IUGR sheep and CR calves. These findings confirm that OXT may play important roles in skeletal muscle development and growth. Elucidation of the therapeutic role of OXT during IUGR will be an area of future interest.

**References**


Tucker, H., C. Parsons, S. Ellis, M. Rhoads, and R. Akers. 2016. Tamoxifen impairs prepubertal mammary development and alters expression of estrogen receptor α (ESR1) and progesterone receptors (PGR). Domestic animal endocrinology 54:95-105.


Table 1

Primer sequences and TaqMan probes for RT-PCR

<table>
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<tr>
<th>Gene (RefSeq ID)</th>
<th>Animal</th>
<th>Primer (5'-3')</th>
<th>Amplicon size(bp)</th>
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R: CCTGGGGATGATTACAGAGG  
P: FAM-CTTCTCCACGACTGAGACC-BHQ1 | 218 |
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R: CCCTCTTTACACCTGCAAAAG  
P: HEX-TGCTTGCCATCCAACACCACCTGTC-BHQ1 | 96 |
| OXTR (NM_174134) | Cattle | F: CAAGGAAGCCTCACCTTTCA  
R: TGCAACAGTTCTTGGAGAGG | 111 |
| M_{yoD} (NM_001040478) | Cattle | F: CGACTCGGACGCTTCCAGT  
R: GATGCTGGACAGGCAGTCTGA | 180 |
| M_{yoG} (NM_001111325) | Cattle | F: GGTGCCAGTGTAAGTGACCTC  
R: GTCTGTAGGTCCTGCTGGGA | 110 |
| Myf5 (NM_181811) | Cattle | F: ACCAGCCCCACCTCAAGTTG  
R: GCAATCCTCAAGGATAAGGAG | 150 |
| MRF4 (NM_181811) | Cattle | F: GGTTGACCCCTTCAGCTACAG  
R: TGCTTGTCTTCCTTCTCGCGA | 140 |
| MSTN (AB076403)  | Cattle | F: GGCATGATCTTCTGTAACCT  
R: GCATCGAGATTCTTGAGT | 144 |
| M_{yoG} (XM_002685738) | Cattle | F: GAGTTCGATTAGCCGAGTGC  
R: ATGCTGTGTGCTGCTTT | 105 |
| PCNA (NM_001034494) | Cattle | F: GTGAACCTGCAGAGCATGGACTCGT  
R: CGTGTCCGCGTTATCTTCAGCTCTT | 192 |
| MHC 1 (NM_174117.1) | Cattle | F: GACAATCTCCTCGCTTGG  
R: GCTCTGAGCTGGAGGTGAC | 122 |
| MHC 2 (NM_001166227.1) | Cattle | F: TCAAAGGGGAGTCACTCCGTC  
R: TCAAGCAACTCTCCAGCTG | 204 |
| MHC 4 (XM_015468059.1) | Cattle | F: CTTCAACCACCACATGTCCG  
R: GCTTCTGGAAGTTGCTGGAC | 233 |
| MHC 7 (NM_174727.1) | Cattle | F: TGTCATACCGTTCAACCTTA  
R: TGGCTGCAAACACAGCAAAG | 238 |

^1Note: F, forward primer; R, reverse primer; P, TaqMan probe
Figure 4.1. Gene mRNA expression level in IUGR sheep model. Fetal control (FC, n=12), fetal IUGR (FI, n=10), lamb control (LC, n=6), and lamb IUGR (LI, n=6) LM lysates were analyzed for gene mRNA expression. (A) OXT expression; (B) OXTR expression; (C) MyoG expression; (D) MyoD expression; (E) MyoG expression. Different letters above bars indicate a significant difference ($P < 0.05$). Means and SEMs are shown.
**Fig 4.2. Gene mRNA expression level in CR calves.** Infraspinatus muscle (INF), longissimus muscle (LM), and semitendinosus muscle (ST) was collected after a control (CON) or caloric restricted (CR) diet was treated for 8 wk and target genes expression were measured. (A) gene expression in INF; (B) gene expression in LD; (C) gene expression in ST. Different letters above bars indicate a significant difference ($P < 0.05$). Means and SEMs are shown.
CHAPTER 5. OXYTOCIN EXPRESSION IS ALTERED IN SKELETAL MUSCLE BY TAMOXIFEN

Abstract

Improving muscle mass production is one of the most important goals in beef industry, which can be achieved by introducing hormones such as estrogens. Tamoxifen (TAM) has been reported as a selective estrogen receptor modulator (SERM) that either mimics or antagonizes estrogen in a tissue dependent manner. Studies have not identified the effect of TAM on bovine skeletal muscle growth. Our study aimed to understand the effect of TAM on bovine skeletal muscle fiber size and bovine satellite cell (BSC) number in vivo, and also examine target genes expression pattern in skeletal muscle with an emphasis on oxytocin (OXT). In this study, sixteen Holstein calves were randomly assigned to one of two treatment groups: TAM-injected (TAM) or control (CON). Longissimus dorsi muscle (LM) muscle were collected after a CON or TAM treatment for 120 days. Our TAM study indicated that the statistically-significant difference of cross-sectional area (CSA) was small. There was no significant difference in BSC number and myonuclei per fiber in TAM treated animals compared to CON animals. LM muscle in TAM animals exhibited more than 50\% decrease ($P < 0.05$) in OXT expression and about 30\% increase ($P<0.05$) in OXTR and MyoG expression. The expression of myosin heavy chain (MHC) isoforms (e.g. $MHC1$, $MHC2$, $MHC4$, and $MHC7$) were not significantly different between TAM and CON heifers. Together, these results indicate that TAM does not impact skeletal muscle fiber growth, however the OXT-OXTR system may be involved in TAM functions, and long-term TAM treatment does not stimulate fiber type shift in heifers.

Keywords: Bovine, Oxytocin, Skeletal muscle, Tamoxifen
Introduction

Skeletal muscle is a highly specialized tissue predominantly composed of muscle fiber, whose growth in the neonate stage depends on the activity of a subset of muscle fiber–associated mononuclear muscular stem cells called satellite cells (Mauro, 1961; Rhoads et al., 2009; Jang et al., 2011). Satellite cell (SC), which normally remain in a non-proliferative quiescent state, locate between the basement membrane and the sarcolemma of muscle fibers (Collins et al., 2005). Following activation, the SC migrate out of their niche (Myoblast) and begin to proliferate (Yin et al., 2013). The progeny of SC after proliferation will ultimately differentiate and commit to a myogenic lineage (Olson, 1992). Upon differentiation, the myoblasts fuse to preexisting fibers which results in a contribution of additional nuclei to the multinucleated muscle fibers, and this addition can allow for muscle growth (hypertrophy) (Hawke and Garry, 2001).

Modification of skeletal muscle growth in young can be achieved by using hormones (Tiidus et al., 2013; Sharples et al., 2015). Estrogen is widely used in the meat industry to increase muscle mass, although they are primarily involved in the development and maintenance of normal sexual and reproductive function (Tiidus et al., 2013; Schulster et al., 2016). Skeletal muscles are the major sites of estrogen production in males (Matsumine et al., 1986). With respect to skeletal muscle, estrogen has been shown to influence proliferation and differentiation of SC in-vitro (Enns and Tiidus, 2008) and is also associated with muscle development and regeneration in-vivo (Enns and Tiidus, 2010). Tamoxifen (TAM), a first-generation of selective estrogen receptor modulator (SERM) involving in many anti-estrogenic activity on the mammary gland, has been used to prevent and treat breast cancer for decades (Powles et al., 2007; Cuzick et al., 2015). TAM also has other effects including prevention of oxidative stress (Schiff et al., 2000), protection membrane damage (Loureiro et al., 2013), modulation of calcium handling (Asp et al., 2013), prevention of
mitochondria-mediated cell death (Mandlekar and Kong, 2001), and inhibition of fibrosis (Payne et al., 2006). Al Naib et al. (2016) has demonstrated that TAM treatment impaired the development of heifer reproductive tract. However, little attention has been focused on the effect of TAM on skeletal muscle growth during the market growth period of postnatal life. Like estradiol, TAM can signal through direct canonical genomic and indirect nongenomic pathways (Kiyama and Wada-Kiyama, 2015). The direct mechanisms were well investigated in different cell types (Shupnik, 2004; Ramaswamy et al., 2012). The indirect mechanisms were less well elucidated but could contribute to the tissue-specific SERM effects (Shang, 2006; Banerjee et al., 2014).

To date, effects of TAM on bovine skeletal muscle growth in-vivo have not been investigated. Through this study, we determined the effect of TAM on skeletal muscle fiber size and bovine satellite cell (BSC) number in-vivo. Expression of target genes, such as paired box 7 (MyoG), myogenic differentiation 1 (MyoD), myogenin (MyoG), myogenic factor 5 (MYF5), muscle regulatory factors (MRF4), myostatin (MSTN), proliferating cell nuclear antigen (PCNA), and myosin heavy chain gene (e.g., MHC 1, 2, 4, and 7) were also determined. In recent years, several studies seemed to indicate a new indirect function of estrogen, that is, estrogen may induce expression of oxytocin (OXT) in skeletal muscle during muscle development. Thus, OXT may play an important role in skeletal muscle growth. Next, we also monitored the OXT and OXT receptor (OXTR) mRNA expression level in TAM treated longissimus dorsi muscle (LM) muscle tissue.

Materials and Methods

Animals
This study was approved by the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee. All experimental procedures involving animals were described before (Tucker et al., 2016). Holstein heifer calves (n = 16) were purchased from a commercial dairy farm at two weeks of age and housed individually in calf hutches until weaning at eight weeks of age and then group housed through the completion of the study. The calves were randomly assigned to control (CON; n = 8) or treatment (n = 8) groups (TAM; tamoxifen). All calves were fed milk replacer twice daily. After weaning, they received only pelleted grain and hay. Calves were weighed and measured hip height every two weeks.

**Injection and sampling**

The heifers were enrolled into the experiment when they reached 28 days of age. They were injected subcutaneously daily for 120 days of age with a weight dependent dosage of each treatment. Heifers in the TAM group were administered of TAM at a rate of 0.3 mg/kg of BW per day. Heifers in the CON group were injected with an equivalent volume of carrier which was composed of 30% ethanol, 30% benzyl benzoate, and 40% corn oil. Four injected sites (left and right shoulder, upper and lower portion of the scapula) were used in this study to minimize the possible site of injection reaction. Because no literature described an optimal dose of TAM administered to calves of this age to suppress estrogen effects on the bovine skeletal muscle. Therefore, the dose administered was based on the amount given to human breast cancer patients.

The heifers were euthanized using a commercial phenobarbital solution administered intravenously (Euthasol, 10 mg/kg BW) at 121±1 days of age. Two pieces of longissimus dorsi muscle (LM) were collected for muscle morphometry and gene expression. The samples for gene expression froze in liquid nitrogen immediately, whereas samples for immunohistology were
embedded in optimal cutting temperature medium (Tissue-Tek OCT, VWR Scientific, Radnor, PA) and freeze in liquid nitrogen. All the samples were stored at -80°C before use.

**Immunohistology**

Subsamples of the LM were embedded in O.C.T. and 10µm cryosections were collected onto glass slides (Superfrost, ThermoFisher Scientific, WilLDington, MA). Cryosections were fixed in 4% paraformaldehyde for 20min at room temperature (RT) and then incubated with blocking buffer consisting of 2% bovine serum albumin (BSA) and 0.2% Triton X-100 in phosphate buffered saline (PBS) for 1h at RT to eliminate non-specific antigen sites. Tissue cryosections were further incubated overnight at 4°C with the MyoG (1:200, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) antibodies diluted in blocking solution. Following incubation, cryosections were washed 3 times for 5min with PBS and incubated for 1h with the following substrates diluted in blocking solution; Wheat Germ Agglutinin (WGA) Alexa Fluor 488 conjugate (5µg/ml, Invitrogen, Carlsbad, CA), goat anti-mouse H&L Alexa-Flour 594 (1:1000; Invitrogen, Carlsbad, CA). DAPI (4’,6-Diamidino-2-Phenylindole, Dihydrochloride; Invitrogen, Carlsbad, CA) was used for the detection of nuclei. After washing with PBS, the slides were coverslipped and immune complexes were visualized by epifluorescence (Nikon Eclipse Ti-U; Nikon Instruments Inc., Melville, NY). Representative images were captured at 200-fold magnification and digitized with a charge- coupled camera (DS-QiMC, Nikon), and NIS Elements software (Nikon). The region constrained by WGA was measured for cross-sectional area (CSA). Both DAPI (blue) and MyoG (red) positive cells (pink) were classified as SC. Minimum of 500 muscle fibers for each individual were analyzed for CSA and BSC number.

**Isolation of RNA and production of cDNA**
Total RNA from muscle was isolated using Trizol reagent (Invitrogen, US) according to the manufacturer’s protocol, and then RNA samples were cleaned up by RNaseasy® Mini Kit (Qiagen, USA). Total RNA was assessed by agarose gel and Nanophotometer (IMPLEN, USA). The presence of two clear 18S and 28S bands and a ratio of ~2.0 in 260/280 confirmed the integrity of the RNA. Total RNA was stored at −80°C before transcription. cDNA was synthesized from 1µg purified RNA using iscript™ cDNA Synthesis Kit (Bio-Rad, USA). To this, in 20µl total volume per reaction, 4µl 5×iScript reaction mix, 1µl iScript reverse transcriptase, 1µg total RNA, and H2O mix well in PCR tube. This mixture was incubated 5min at 25°C, 30min at 42°C, 5min at 85°C, and hold at 4°C. Finally, the cDNA was diluted 1:10.

**Quantitative real-time PCR (qPCR)**

The cDNA was subjected to qPCR using specific TaqMan probe of OXT and housekeeping genes by 2×IQ Multiplex Powermix (BioRad). The β-actin (sheep) and cyclophilin α (cattle; PPIA) gene were used as housekeeping gene control as previously reported (Kongsuwan et al., 2012a; Divari et al., 2013). Primer sequences and the TaqMan probe of OXT and housekeeping genes (Table 5.1) were synthesized by Fisher Scientific. The qPCR reactions were performed in the same well using CFX96 Detection System (BioRad) and in a 20µl final volume containing 10µL of PCR mix, 1µL of each primer (10µM), 0.5µl of each probe (10µM), and 5µl of cDNA (25ng). The PCR cycling conditions consisted of 10min incubation at 95°C followed by 40 cycles of 95°C for 15s and 60°C for 1min. Each sample was amplified in triplicate. The standard curve method was used to calculate the relative gene expression level between OXT and housekeeping genes. Measurements of other interest genes expression (OXTR, MyoD, MyoG, Myf5, MRF4, MSTN, PCNA, MHCs) were performed using SsoAdvanced Universal SYBR Green Supermix (BioRad).
The qPCR reactions were the same as former TaqMan study but no probe added. The fold change for all the samples was calculated by $2^{-\Delta\Delta C_q}$ method.

**Generation of standard curve**

Using TOPO® TA Cloning® Kit (Invitrogen), the PCR products of OXT gene, β-actin, and PPIA were ligated with vector and then transformed into Match1™-T1R Competent Cells (Invitrogen, USA). The cell-vector mixture was incubated on ice for 30min, heat shocked for 30s at 42°C in a water bath, and immediately placed them on ice for 2min. After adding 250μl of S.O.C. medium, the mixture was shaken horizontally at 37°C for 1hour at 225rpm. The cells were then spread onto prewarmed selective LB agar and incubated at 37°C overnight. White recombinant colonies were picked and subjected to colony PCR to confirm the presence of the target fragment. Positive colonies were grown in liquid LB medium overnight, and the plasmids were purified with the QIAprep Spin Miniprep Kit (Qiagen, USA), in compliance with the manufacturer’s instructions.

After purification, each recombinant plasmid DNA (recDNAOXT, recDNAβ-actin, and recDNAPPIA) was sequenced by Virginia Bioinformatics Institute at Virginia Tech (VBI, Virginia Tech) using M13R or M13F primers to confirm its identity. The concentration of each purified recDNA was calculated by Nanophotometer (IMPLEN, USA). Standard curves were generated using serial dilutions of recDNAs from $3\times10^6$ to 30 molecules.

**Statistical analyses**

The results were analyzed in JMP® (SAS institute) using two-way analysis of variance (ANOVA). Pairwise comparisons between the least square means of the factor levels were
conducted using Tukey's HSD test. The fold change for all the samples measured by qPCR was calculated by $2^{-\Delta\Delta C_q}$ method. Values are expressed as means ± SEM.

**Results**

**Effect of long-term TAM treatment on muscle fiber size and BSC number**

Long-term effects of TAM were examined in LM muscle of Holstein heifers. Subsamples of CON and TAM LM muscle were immunostained (Fig. 5.1A) for Wheat Germ Agglutinin (WGA; green), *MyoG* (red), and DAPI (blue). The area constrained by the sarcolemma border was measured by NIS Elements software. The CSA in TAM animals statistically significantly decreased compared to CON (Fig. 5.1B), but the magnitude of difference was very small and likely not biologically meaningful. The frequency of fibers confirmed that the difference of CSA between TAM and CON animals was small (Fig. 5.1C). There was no difference in the number of *MyoG*-positive SC (Fig. 5.1D) and myonuclei per fiber (Fig. 5.1E) between TAM and CON animals.

**Effect of long-term TAM treatment on target genes expression in skeletal muscle**

Expression of the target genes were measured by qPCR (Fig. 5.2). LM in TAM animals exhibited ~30% increase ($P < 0.05$) in *MyoG* mRNA expression compared to that in the CON animals. There was no difference between TAM and CON in terms of the expression of myogenic differentiation 1 (*MyoD*), myogenin (*MyoG*), myogenic factor 5 (*MYF5*), muscle regulatory factors (*MRF4*), myostatin (*MSTN*), proliferating cell nuclear antigen (*PCNA*), and myosin heavy chain gene (*MHC 1, 2, 4, and 7*). *OXT* mRNA expression decreased ($P < 0.05$) in TAM heifers compared to CON heifers, whereas the *OXTR* transcripts were much more abundant in TAM heifers ($P<0.05$).
Discussion

As a SERM, TAM has been intensively studied for its ability to treat breast cancer (Cuzick et al., 2015). TAM also has other effects including prevention of oxidative stress (Schiff et al., 2000), protection membrane damage (Loureiro et al., 2013), modulation of calcium handling (Asp et al., 2013), prevention of mitochondria-mediated cell death (Mandlekar and Kong, 2001), and inhibition of fibrosis (Payne et al., 2006). Many studies have demonstrated that TAM effect in tissue-specific manner (Shang and Brown, 2002). However, to the best of our knowledge, there have been no studies to investigate the effects of TAM on skeletal muscle growth in cattle. Our study generally demonstrated that: i) TAM does not impact bovine skeletal muscle fiber size and BSC number *in vivo*; ii) *OXT* expression decreased in TAM heifers *in vivo*.

First, we determined the effect of TAM on skeletal muscle growth. We found that, although 0.3mg/kg/day TAM treatment statistically decreased LM of heifers, the overall effect was small. The frequency of fiber size confirmed that the difference of CSA between TAM and CON animals was negligible. BSC number and myonuclei per fiber did not change in response to the TAM treatment. Thus, these results indicate that TAM does not impact skeletal muscle fiber growth *in vivo*. However, TAM impaired BSC differentiation *in-vitro* and TAM treated BSC supplemented with OXT increased BSC differentiation compared to TAM alone treatment (see chapter 2). Given that TAM also decreased *OXT* expression in cultured BSC, we believe that OXT is involved in the effect of TAM on BSC differentiation *in-vitro*. Moreover, it has been reported that E2 treatment in cull cows and feedlot steers increased LM area and carcass fat free lean (Cranwell et al., 1996; Gonzalez et al., 2007) and larger myofibers (especially Type I muscle fiber) CSA and diameter were founded in E2 treated cull cows (Gonzalez et al., 2007). In mice, it has also been found that estrogen insufficiency (Ovariectomized: OVX) results in a significant decrease in the CSA of the
tibialis anterior (TA) muscle after 8 and 24 weeks compared to control mice (Kitajima and Ono, 2016). In this study, we found lower concentration of TAM does not impact skeletal muscle fiber size and BSC number in-vivo, though OXT expression significantly decreased in TAM LM muscle. We surmised that the possible reason for the differences between the in-vivo and in-vitro studies and TAM and E2 treatment studies may be the dosage of TAM used in this study. We used 1μM TAM in the cellular study, which likely leads to much higher local concentrations in the extracellular fluid of cultured cells. But the total concentration of TAM on LM muscle of heifers was low, which likely triggered MyoG and OXTR expression in BSC. Gonzalez et al. (2007) administered of E2 in cull cows at a rate of 16 mg/kg of BW per day. This E2 amount was over 50 folds higher than TAM amount we used in this study. Moreover, in chapter 2, we found that the proliferation rate cannot be stimulated at 100nM, 1μM, and 10 μM dosage of TAM, yet cells died when we increased the dosage to 100μM during proliferation and differentiation. Thus, TAM treatment may regulate BSC activity in a dose-dependent manner, which may explain why TAM does not impact skeletal muscle fiber size and BSC number.

In the present study, we found that OXT expression significantly decreased in LM of TAM treated heifers, which suggests that OXT may be involved in TAM effects, such that TAM attenuates BSC differentiation. OXT expression activation relies on the presence of estrogen receptor and estrogen orphan receptor in the cells (Koohi et al., 2005). Competing with estrogen receptors may contribute to the decrease of OXT expression with TAM treatment. In chapter 2, we demonstrated that TAM impaired BSC differentiation in-vitro and TAM treated BSC supplemented with OXT increased BSC differentiation compared to TAM alone treatment. Given that TAM also decreased OXT expression in cultured BSC, we believe that OXT is involved in TAM effects, at least the effect of TAM on BSC differentiation. It is interesting to note that OXTR
expression increased about 30% \((P<0.05)\) in TAM LM compared to CON \textit{in-vivo}, whereas \textit{OXTR} expression level significantly decreased with TAM treatment during BSC differentiation \textit{in-vitro}. In addition, \textit{MyoG} expression considerably increased TAM LM compared to CON, but there was no difference detected in \textit{MyoG}-positive satellite number \textit{in-vivo}. These discordant observations point to the possible complex processing response to chronic TAM treatment \textit{in-vivo}.

Although we did not detect muscle fiber type with TAM treatment in the present study, the \textit{MHC} isoforms expression were determined by qPCR. The expression of all \textit{MHC} isoforms (e.g. \textit{MHC1}, \textit{MHC2}, \textit{MHC4}, and \textit{MHC7}) were not significantly different between TAM and CON heifers. These results indicate that long term TAM treatment cannot stimulate fiber type shift in heifers. Using E2 treated cull cattle, Gonzalez et al. (2007) also found there was no fiber type shift between the different MHC isoforms. Thus, it is possible that TAM, as a SERM, has no impact on fiber type shift.

\textbf{Conclusion}

In conclusion, our TAM study indicated that the statistical difference of CSA was small. There was no significant difference in BSC number and myonuclei per fiber in TAM treated animals compared to CON animals. These results imply that TAM does not impact skeletal muscle fiber growth. LM muscle in TAM animals exhibited more than 50% decrease \((P < 0.05)\) in \textit{OXT} expression and about 30% increase in \textit{OXTR} and \textit{MyoG} expression, which indicate that OXT may be involved in TAM functions in skeletal muscle. The expression of MHC isoforms (e.g. \textit{MHC1}, \textit{MHC2}, \textit{MHC4}, and \textit{MHC7}) were not significantly different between TAM and CON heifers. Those results indicate that long term TAM treatment does not alter fiber types in heifers.
References


Tucker, H., C. Parsons, S. Ellis, M. Rhoads, and R. Akers. 2016. Tamoxifen impairs prepubertal mammary development and alters expression of estrogen receptor α (ESR1) and progesterone receptors (PGR). Domestic animal endocrinology 54:95-105.

Table 5.1. Primer sequences and TaqMan probes for RT-PCR

<table>
<thead>
<tr>
<th>Gene (RefSeq ID)</th>
<th>Animal</th>
<th>Primer (5’-3’)</th>
<th>Amplicon size(bp)</th>
</tr>
</thead>
</table>
| OXT (NM_176855)  | Cattle | F: CTGCCAAGAGGAGAAGTACC  
R: CCTGGGGAATGATTACAGAGG  
P: FAM-CTTCTCCACGTGAGACC-BHQ1 | 218 |
| PPIA (NM_178320) | Cattle | F: GCCCCCAACACAAATGTT  
R: CCCTTTCACCTTGCCAAAG  
P: HEX-TCTTCTCCACGTGAGACC-BHQ1 | 96 |
| OXTR (NM_174134) | Cattle | F: CAAGGAAGCCTCACCCCTTTCA  
R: TGCACAAGCTTCTTGAGAG | 111 |
| MyoD (NM_001040478) | Cattle | F: CGACTCGGAGCTTCCAGT  
R: GATGCTGGACAGGCAGTCGA | 180 |
| MyoG (NM_001111325) | Cattle | F: GTGCCCCAGTGAAAGTCACTC  
R: GTCTGTAGGGTCCGCTGGGA | 110 |
| Myf5 (NM_174116) | Cattle | F: ACCAGCCCCACCTCAAGTTG  
R: GCAATCCAAAGCTTGGAGAG | 150 |
| MRF4 (NM_181811) | Cattle | F: GGTTGGACCCCTTCAGCTCAG  
R: TGCTTGTCCCTCCTTCTTTGG | 140 |
| MSTN (AB076403)  | Cattle | F: GGCCCATGATCTTGCTGAACTC  
R: GCATCGAGATTCTGTGGAAG | 144 |
| MyoG (XM_002685738) | Cattle | F: GAGTTCGATTAGCCGAGTGC  
R: ATGCTGCTTCTTCTTCTTCTTCTT | 105 |
| PCNA (NM_001034494) | Cattle | F: GTGAACCTGCAGAGCATGGACTCGT  
R: CGTGTCCGCGTTATCTTCAGCTCTT | 192 |
| MHC 1 (NM_174117.1) | Cattle | F: GACAACTTCTCCTGCTTTTG  
R: GCCTTCACGTGAAAGTGAC  | 122 |
| MHC 2 (NM_001166227.1) | Cattle | F:TCAAGGGGAGATCAAGACTCC  
R: TCAGCAATCCCTCAAGTGGAC | 204 |
| MHC 4 (XM_015468059.1) | Cattle | F: CTCTAACCCACATGTCTCC  
R: GCTTCTGGAAGTTGCTGGAC | 233 |
| MHC 7 (NM_174727.1) | Cattle | F: TGTGTCACCGTCACCCCTTA  
R: TGGCTGCAATAAACAGCAAAG | 238 |

Note: F, forward primer; R, reverse primer; P, TaqMan probe
Figure 5.1. The effect of TAM on muscle size, number of BSC, number of myonuclei per fiber in Holstein heifers. Cryosections from longissimus of TAM and CON were analyzed by MyoG, DAPI, and wheat germ agglutinin (WGA) histology (A) for the measurement of number of BSC (B), CSA (B), fiber size distribution (B), myonuclei per fiber (B); white arrows point to the BSC; magnification bar = 100 μm. Asterisk denotes significant difference from control (*P < 0.05).
**Figure 5.2. Gene mRNA expression level in LM.** CON and TAM longissimus lysates were analyzed for gene mRNA expression. The TAM treatment led to a marked decrease in OXT expression and a significant increase in OXTR and MyoG expression in LM. Values are means and SEMs. Asterisk denotes significant difference from control (*$P < 0.05$).
CHAPTER 6. CONCLUSION AND FUTURE DIRECTIONS

Conclusion

To our knowledge, there is no existing study that determines the effect of OXT on skeletal muscle growth \textit{in-vivo or in-vitro}. Hence, the overall objective of this thesis is to investigate and highlight the potential roles of OXT in bovine muscle satellite cell (BSC) activity, especially related to proliferation and differentiation. We have designed and conducted two different studies to address such issue.

In the first study, the data indicates that OXT stimulates BSC differentiation and migration. E2 and TBA increased BSC proliferation, fusion index, and migration, and OXT was involved in these processes. Moreover, both steroid hormones and OXT protected BSC from serum starvation and oxidative stress conditions. Together, the results presented support the hypothesis that OXT plays essential roles in BSC proliferation and differentiation and involves functions of the steroid hormones on BSC activity. Future studies investigating the signaling pathways related to OXT stimulated BSC proliferation and differentiation and steroid hormones stimulated OXT expression may help us better understand the mechanisms of steroid hormones and OXT stimulated SC activity.

In the second study, the results indicated that OXT stimulated CRISPR-OXT cell proliferation, differentiation, and migration. E2 or TBA increased BSC proliferation, differentiation, and migration, and OXT is involved in those processes. OXT stimulates BSC differentiation by regulating \textit{MyoG} expression. Moreover, OXT, E2, or TBA protected CRISPR-OXT cell from serum starvation and oxidative stress conditions. Similar to Chapter 2, the results
presented in Chapter 3 support the hypothesis that OXT plays essential roles in BSC proliferation and differentiation and it is involved in the functions of E2 and TBA on BSC activity.

In the third study, we performed initial in-vivo analysis of target genes expression pattern in cattle and sheep during IUGR and CR conditions. We found IUGR and CR decreased MRFs expression in skeletal muscle, which may explain why IUGR and CR impair skeletal muscle development and growth. CR significantly decreased MHC4 expression but increased MHC2 and MHC7 expression in skeletal muscle. These results indicate that CR can lead to skeletal muscle fiber switch by switch type IIb fibers into type I fibers or type IIa fibers. Besides, our work revealed that OXT expression significantly decreased in skeletal muscle of IUGR sheep and CR calves. These findings confirm that OXT may play important roles in skeletal muscle development and growth. Elucidation of the therapeutic role of OXT during IUGR will be an area of future interest.

In the fourth study, our TAM study indicated that the statistically-significant difference of CSA was small. There was no significant difference in BSC number and myonuclei per fiber in TAM treated animals compared to CON animals. These results imply that TAM does not impact skeletal muscle fiber growth. LM muscle in TAM animals exhibited more than 50% decrease ($P < 0.05$) in OXT expression and about 30% increase in OXTR and MyoG expression, which indicate that OXT-OXTR system may be involved in TAM functions in skeletal muscle. The expression of MHC isoforms (e.g. MHC1, MHC2, MHC4, and MHC7) were not significantly different between TAM and CON heifers. Those results indicate that long term TAM treatment cannot affect fiber type shift in heifers.
Future Directions

Mechanisms of OXT underlying cell death and migration

In my dissertation research, we demonstrate that OXT protects BSC from serum starvation and oxidative stress and plays important roles in BSC migration. However, the underlying mechanisms remain unknown. It has been reported that PI3K/Akt and ERK1/2 pathways are key transducers of antiapoptotic signals in various cell types. OXT stimulates activation of PI3K and endothelial nitric oxide (NO) synthase which were required for the pro-migratory effect of OXT on human umbilical vein endothelial cells (Cattaneo et al., 2008) and in ischemic rat heart (Faghihi et al., 2012). It is possible that OXT protects BSC from apoptosis by PI3K/Akt and ERK1/2 pathways, and stimulates BSC migration via PI3K/Akt and NO signaling pathways. Thus, the pathways by which OXT stimulates migration and protects BSC from apoptosis warrant further investigation.

How steroid hormones regulate differentiation in BSC

In the present study, we found that OXT expression both significantly decreased in LM in TAM treated heifers and TAM treated differentiating BSC. Both E2 and TBA increased OXT expression considerably during BSC proliferation and differentiation. However, much less is known about how steroid hormones stimulate OXT expression in BSC. It has been reported that estrogen regulates OXT expression by estrogen receptors directly binding with OXT promoter via estrogen response element (ERE) (Richard and Zingg, 1990). However, the binding affinities of estrogen receptor to the OXT promoter excluded this classical mechanism (Stedronsky et al., 2002). In addition, it is also recognized that OXT expression activation relies on the presence of estrogen receptor and estrogen orphan receptor in the cells (Koohi et al., 2005). Therefore, it is worthwhile
to determine the mechanisms of steroid hormones in regulating OXT expression in BSC. Furthermore, we also found that OXT can upregulate the expression of MyoG both in wild-type BSC and CRISPR-OXT cell during proliferation and differentiation. However, E2 and TBA can stimulate MyoG expression only in wild-type BSC but not in CRISPR-OXT cell. Therefore, it appears that E2 and TBA upregulate MyoG expression by regulating OXT expression, which further stimulates BSC differentiation. Future studies on which signaling pathways involve the steroid hormones stimulated OXT expression and OXT stimulated MyoG expression may help us to better understand the mechanisms of steroid hormone and the role of OXT in BSC.

**Role of OXT in protein deposition**

Since muscle fiber numbers are fixed before birth, postnatal muscle growth mainly depends on an increase in muscle fiber size (hypertrophy) rather than new myofiber formation. Postnatal muscle growth can be characterized as a period of muscle hypertrophy due to a substantial increase in DNA content and protein turnover (Verdijk et al., 2009). Because myonuclei are post-mitotic, the increase of DNA content is dependent on SC proliferation and differentiation (Blaauw and Reggiani, 2014). Protein turnover is the balance between protein synthesis and protein degradation (Hinkson and Elias, 2011). An anabolic state that builds skeletal muscle tissues will happen when the protein synthesis outweighs degradation, a catabolic state that reduces skeletal muscle tissues will occur when the protein synthesis is less than degradation (Munro, 2012). We have already investigated the effect of OXT on BSC proliferation and differentiation in this dissertation study. Thus, it is equally important to determine the role of OXT in protein synthesis and degradation.

**Effect of OXT on skeletal muscle of Intrauterine Growth Retardation (IUGR) sheep**
Environmental challenges (malnutrition, thermal stress, etc.) during fetal development – especially the second trimester – can result in the insufficient development of the placenta, which may further affect the transportation of nutrients, oxygen, and other factors and cause placenta insufficiency intrauterine growth restriction (PI-IUGR) fetus generation (Chen et al., 2010). Professor Barker first introduced an idea called a “Barker hypothesis” that size at birth is related to the risk of developing the disease in later life (Hales and Barker, 1992). It is suggested that the fetus makes physiological adaptations in response to changes in its environment to prepare itself for postnatal life. IUGR fetuses show lower body weights and lower skeletal muscle mass than normal fetuses (Valsamakis et al., 2006; Wang et al., 2008; Yates et al., 2012). Thus, low muscle mass affects adult health and has important implications for quality of life, excess weight gain, and risk of developing insulin resistance and type 2 diabetes.

However, studies have not defined effective treatment(s) that can be used to reduce the effect of IUGR in human and animals. Recently, several studies in cattle and sheep indicated that the expression of OXT peaked at least three months prior to birth and was sustained perinatally (De Jager et al., 2011b; Kongsuwan et al., 2012a; Divari et al., 2013). This timing coincides with secondary myogenesis (increased expression muscle fiber subunit) and subsequent fusion of differentiated myoblasts into myotubes. According to a study using mice, OXT also plays important roles in the regeneration of skeletal muscle (Elabd et al., 2014). Since our results show that ovine skeletal muscle express OXT and OXTR mRNA and OXT expression decreased in IUGR fetuses compared to control fetuses, it is likely that OXT may provide benefit to patients with IUGR. However, the effect of OXT on ovine skeletal muscle development is still unknown. Consequently, it is necessary to investigate the impact of OXT on skeletal muscle development in IUGR sheep.
References


