THE ROLE OF OXYTOCIN IN BOVINE SATELLITE CELL PROLIFERATION AND DIFFERENTIATION

ZHENHE ZHANG

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

Animal and Poultry Sciences

Robert P. Rhoads, Chair

Sally E. Johnson

Samer W. El-Kadi

Matthew W. Hulver

Sept. 20th, 2017

Blacksburg, VA

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

THE ROLE OF OXYTOCIN IN BOVINE SATELLITE CELL PROLIFERATION AND DIFFERENTIATION

ZHENHE ZHANG

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

THE ROLE OF OXYTOCIN IN BOVINE SATELLITE CELL PROLIFERATION AND DIFFERENTIATION

ZHENHE ZHANG

GENERAL AUDIENCE 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. 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

Table of Contents

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).

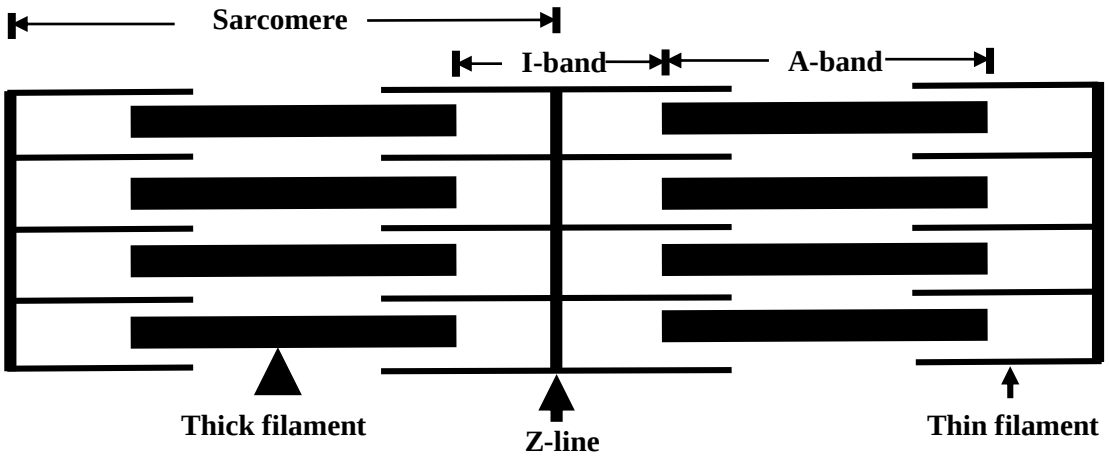


Fig. 1.1: Vertebrate skeletal muscle sarcomere organization. The sarcomere consists of one A-band and two half I-band. Two sarcomeres separate by Z-line. Thick filaments lie parallel to each other in clusters and overlap to the thin filaments. The sarcomere can grow in diameter by adding more thick and 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 *MyoG*lobin. 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)

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

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 (*MRF4*), and myogenin (*MyoG*) (Sambasivan and Tajbakhsh, 2015). Among those four, the earliest transcription factors involved in proliferation is *Myf5*. *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 *Myf5* transcripts (Kuang et al., 2007). The initiation of myogenesis is delayed by the presence of *Myf5* alone (Kablar et al., 1997). The second MRF expressed during myogenesis is *MyoD*. The expression of *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, *MyoG* and *MRF4* (Kassar-Duchossoy et al., 2004). Both *MyoG* and *MRF4* play essential roles in terminal differentiation and fusion of committed myoblast. The *MyoG* and *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.

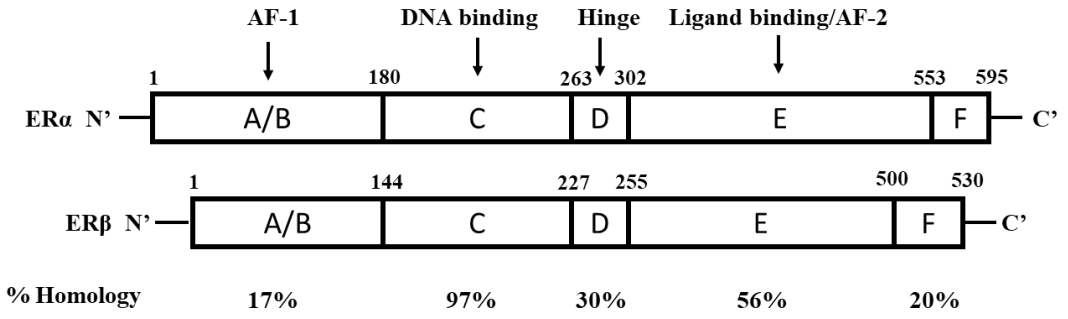


Fig. 1.2: Structure of estrogen receptor. The comparison of human estrogen receptor α (595 AA) and a shorter estrogen receptor β (530 AA) was shown in this picture. These receptors consist of five distinct structural and functional domains: DNA-binding domain (DBD; C domain), hinge domain (D), ligand-binding 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.

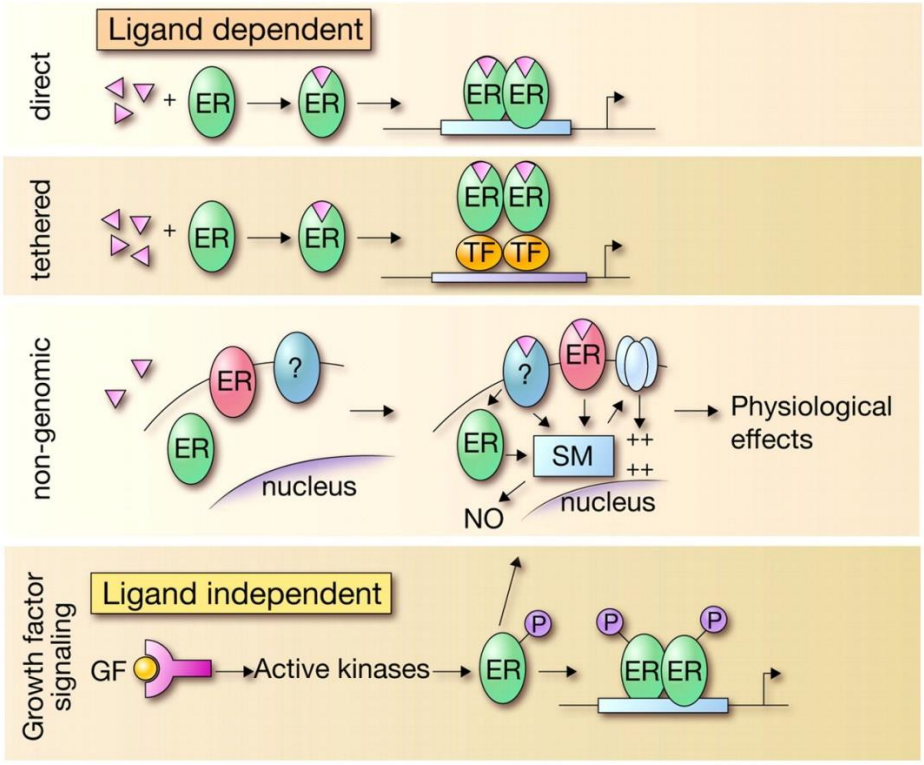


Fig. 1.3: Model representing the mechanistically distinct molecular pathways of estrogen receptor. Four different mechanisms were summarized in this picture: the classical (direct) pathway, the tethered pathway, non-genomic signaling pathway, and the ligand-independent pathway (Heldring et al., 2007b).

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 *MyoD, MyoG, MYF5* positive, whereas quiescence SC are only *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 *in-vitro* (Kahlert et al., 1997) and is also associated with *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 *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 (*MyoG*-positive), activated (*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

et al., 2011). 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 promotor 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).

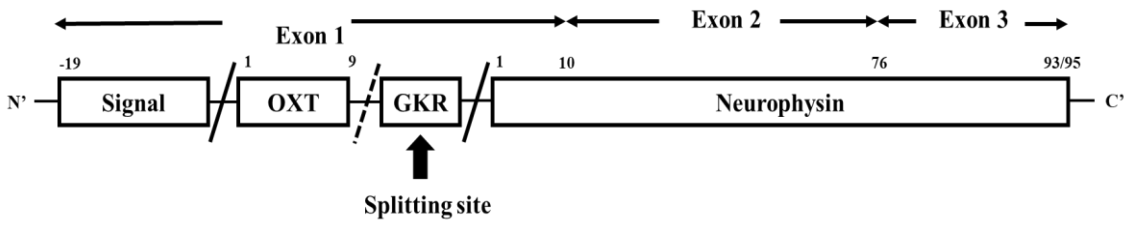


Fig. 1.4 Domain organization of preprooxytocin including the splitting site. The OXT DNA consists of three exons and encodes two separate protein OXT and neurophy. The precursor of OXT is split into the signaling peptide, the OXT-GKR and neurophysin by enzymatic cleavage. OXT-GKR is prooxytocin which 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 hydrolyse 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 (β_2AR) (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+} concertation 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 *in-vivo* or *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 *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 *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.

References

Abmayr, S. M., and G. K. Pavlath. 2012. Myoblast fusion: lessons from flies and mice. Development 139(4):641-656.

Ahtiainen, J. P., J. J. Hulmi, W. J. Kraemer, M. Lehti, K. Nyman, H. Selänne, M. Alen, A. Pakarinen, J. Komulainen, and V. Kovanen. 2011. Heavy resistance exercise training and skeletal muscle androgen receptor expression in younger and older men. Steroids 76(1):183-192.

Al Naib, A., H. Tucker, G. Xie, D. Keisler, F. Bartol, R. Rhoads, R. Akers, and M. Rhoads. 2016. Prepubertal tamoxifen treatment affects development of heifer reproductive tissues and related signaling pathways. Journal of dairy science 99(7):5780-5792.

Andari, E., J.-R. Duhamel, T. Zalla, E. Herbrecht, M. Leboyer, and A. Sirigu. 2010. Promoting social behavior with oxytocin in high-functioning autism spectrum disorders. Proceedings of the National Academy of Sciences 107(9):4389-4394.

Ascenzi, P., A. Bocedi, and M. Marino. 2006. Structure–function relationship of estrogen receptor α and β: impact on human health. Molecular aspects of medicine 27(4):299-402.

Askew, E. B., J. T. Minges, A. T. Hnat, and E. M. Wilson. 2012. Structural features discriminate androgen receptor N/C terminal and coactivator interactions. Molecular and cellular endocrinology 348(2):403-410.

Asp, M. L., J. J. Martindale, and J. M. Metzger. 2013. Direct, differential effects of tamoxifen, 4-hydroxytamoxifen, and raloxifene on cardiac myocyte contractility and calcium handling. PloS one 8(10):e78768.

Bae, G.-U., U. Gaio, Y.-J. Yang, H.-J. Lee, J.-S. Kang, and R. S. Krauss. 2008. Regulation of myoblast motility and fusion by the CXCR4-associated sialomucin, CD164. Journal of Biological Chemistry 283(13):8301-8309.

Bailey, P., T. Holowacz, and A. B. Lassar. 2001. The origin of skeletal muscle stem cells in the embryo and the adult. Current opinion in cell biology 13(6):679-689.

Banerjee, S., K. L. Chambliss, C. Mineo, and P. W. Shaul. 2014. Recent insights into non-nuclear actions of estrogen receptor alpha. Steroids 81:64-69.

Barberis, C., B. Mouillac, and T. Durroux. 1998. Structural bases of vasopressin/oxytocin receptor function. Journal of Endocrinology 156(2):223-229.

Barros, R. P., U. F. Machado, M. Warner, and J.-Å. Gustafsson. 2006. Muscle GLUT4 regulation by estrogen receptors ERβ and ERα. Proceedings of the National Academy of Sciences 103(5):1605-1608.

Bartelt-Hunt, S. L., D. D. Snow, W. L. Kranz, T. L. Mader, C. A. Shapiro, S. J. v. Donk, D. P. Shelton, D. D. Tarkalson, and T. C. Zhang. 2012. Effect of growth promotants on the occurrence of endogenous and synthetic steroid hormones on feedlot soils and in runoff from beef cattle feeding operations. Environmental science & technology 46(3):1352-1360.

Basualto-Alarcón, C., G. Jorquera, F. Altamirano, E. Jaimovich, and M. Estrada. 2013. Testosterone signals through mTOR and androgen receptor to induce muscle hypertrophy. Med Sci Sports Exerc 45(9):1712-1720.

Beauchamp, J. R., L. Heslop, S. David, S. Tajbakhsh, R. G. Kelly, A. Wernig, M. E. Buckingham, T. A. Partridge, and P. S. Zammit. 2000. Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. The Journal of cell biology 151(6):1221-1234.

Beggs, M. L., R. Nagarajan, J. M. Taylor-Jones, G. Nolen, M. MacNicol, and C. A. Peterson. 2004. Alterations in the TGFβ signaling pathway in myogenic progenitors with age. Aging cell 3(6):353-361.

Bennett, H., J. Fleming, J. O'prey, K. Ryan, and H. Leung. 2010. Androgens modulate autophagy and cell death via regulation of the endoplasmic reticulum chaperone glucose-regulated protein 78/BiP in prostate cancer cells. Cell death & disease 1(9):e72.

Bergh, U., and B. Ekblom. 1979. Influence of muscle temperature on maximal muscle strength and power output in human skeletal muscles. Acta physiologica 107(1):33-37.

Berio, E., S. Divari, L. S. Cucuzza, B. Biolatti, and F. T. Cannizzo. 2017. 17β-estradiol upregulates oxytocin and the oxytocin receptor in C2C12 myotubes. PeerJ 5:e3124.

Bhasin, S., T. W. Storer, N. Berman, C. Callegari, B. Clevenger, J. Phillips, T. J. Bunnell, R. Tricker, A. Shirazi, and R. Casaburi. 1996. The effects of supraphysiologic doses of testosterone on muscle size and strength in normal men. N Engl j Med 1996(335):1-7.

Bhasin, S., L. Woodhouse, and T. W. Storer. 2003. Androgen effects on body composition. Growth hormone & IGF research 13:S63-S71.

Biressi, S., M. Molinaro, and G. Cossu. 2007. Cellular heterogeneity during vertebrate skeletal muscle development. Developmental biology 308(2):281-293.

Birge, S. J. 1996. Is there a role for estrogen replacement therapy in the prevention and treatment of dementia? Journal of the American Geriatrics Society 44(7):865-870.

Bjornstrom, L., and M. Sjoberg. 2005. Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. Molecular endocrinology 19(4):833-842.

Blaauw, B., and C. Reggiani. 2014. The role of satellite cells in muscle hypertrophy. Journal of muscle research and cell motility 35(1):3-10.

BLAu, H. M., C. Webster, and G. K. Pavlath. 1983. Defective myoblasts identified in Duchenne muscular dystrophy. Proceedings of the National Academy of Sciences 80(15):4856-4860.

Braga, M., S. Bhasin, R. Jasuja, S. Pervin, and R. Singh. 2012. Testosterone inhibits transforming growth factor-β signaling during myogenic differentiation and proliferation of mouse satellite cells: potential role of follistatin in mediating testosterone action. Molecular and cellular endocrinology 350(1):39-52.

Breton, C., C. Haenggeli, C. Barberis, F. Heitz, C. R. Bader, L. Bernheim, and E. Tribollet. 2002. Presence of functional oxytocin receptors in cultured human myoblasts. The Journal of Clinical Endocrinology & Metabolism 87(3):1415-1418.

Brodsky, I. G., P. Balagopal, and K. S. Nair. 1996. Effects of testosterone replacement on muscle mass and muscle protein synthesis in hypogonadal men--a clinical research center study. The Journal of Clinical Endocrinology & Metabolism 81(10):3469-3475.

Brooke, M. H., and K. K. Kaiser. 1970. Muscle fiber types: how many and what kind? Archives of neurology 23(4):369-379.

Brown, D., A. P. S. Hikim, E. L. Kovacheva, and I. Sinha-Hikim. 2009. Mouse model of testosterone-induced muscle fiber hypertrophy: involvement of p38 mitogen-activated protein kinase-mediated Notch signaling. Journal of Endocrinology 201(1):129-139.

Brownstein, M. J., J. T. Russell, and H. Gainer. 1980. Synthesis, transport, and release of posterior pituitary hormones. Science 207(4429):373-378.

Buckingham, M. 2007. Skeletal muscle progenitor cells and the role of Pax genes. Comptes rendus biologies 330(6):530-533.

Burns, P. D., J. Mendes, José OB, R. S. Yemm, C. M. Clay, S. E. Nelson, S. H. Hayes, and W. J. Silvia. 2001. Cellular Mechanisms by Which Oxytocin Mediates Ovine Endometrial Prostaglandin F2α Synthesis: Role of Gi Proteins and Mitogen-Activated Protein Kinases 1. Biology of reproduction 65(4):1150-1155.

Carmeci, C., D. A. Thompson, H. Z. Ring, U. Francke, and R. J. Weigel. 1997. Identification of a gene (GPR30) with homology to the G-protein-coupled receptor superfamily associated with estrogen receptor expression in breast cancer. Genomics 45(3):607-617.

Cattaneo, M., B. Chini, and L. Vicentini. 2008. Oxytocin stimulates migration and invasion in human endothelial cells. British journal of pharmacology 153(4):728-736.

Cermak, N. M., T. Snijders, B. R. McKay, G. Parise, L. B. Verdijk, M. A. Tarnopolsky, M. J. Gibala, and L. Van Loon. 2013. Eccentric exercise increases satellite cell content in type II muscle fibers. Med Sci Sports Exerc 45(2):230-237.

Charge, S. B., and M. A. Rudnicki. 2004. Cellular and molecular regulation of muscle regeneration. Physiological reviews 84(1):209-238.

Chen, Y., J. D. Zajac, and H. E. MacLean. 2005. Androgen regulation of satellite cell function. Journal of Endocrinology 186(1):21-31.

Chen, Z., I. S. Yuhanna, Z. Galcheva-Gargova, R. H. Karas, M. E. Mendelsohn, and P. W. Shaul. 1999. Estrogen receptor α mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. The Journal of clinical investigation 103(3):401-406.

Cheung, T. H., N. L. Quach, G. W. Charville, L. Liu, L. Park, A. Edalati, B. Yoo, P. Hoang, and T. A. Rando. 2012. Maintenance of muscle stem-cell quiescence by microRNA-489. Nature 482(7386):524-528.

Ciocca, D. R., and L. M. Vargas Roig. 1995. Estrogen receptors in human nontarget tissues: biological and clinical implications. Endocrine Reviews 16(1):35-62.

Collins, C. A., I. Olsen, P. S. Zammit, L. Heslop, A. Petrie, T. A. Partridge, and J. E. Morgan. 2005. Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. Cell 122(2):289-301.

Collins, C. A., P. S. Zammit, A. P. Ruiz, J. E. Morgan, and T. A. Partridge. 2007. A population of myogenic stem cells that survives skeletal muscle aging. Stem cells 25(4):885-894.

Conerly, M. L., Z. Yao, J. W. Zhong, M. Groudine, and S. J. Tapscott. 2016. Distinct activities of Myf5 and MyoD indicate separate roles in skeletal muscle lineage specification and differentiation. Developmental cell 36(4):375-385.

Cooke, P. S., and A. Naaz. 2004. Role of estrogens in adipocyte development and function. Experimental biology and medicine 229(11):1127-1135.

Cranwell, C., J. Unruh, J. Brethour, D. Simms, and R. Campbell. 1996. Influence of steroid implants and concentrate feeding on performance and carcass composition of cull beef cows. Journal of animal science 74(8):1770-1776.

Cuzick, J., I. Sestak, S. Cawthorn, H. Hamed, K. Holli, A. Howell, J. F. Forbes, and I.-I. Investigators. 2015. Tamoxifen for prevention of breast cancer: extended long-term follow-up of the IBIS-I breast cancer prevention trial. The lancet oncology 16(1):67-75.

Dahlberg, E. 1982. Characterization of the cytosolic estrogen receptor in rat skeletal muscle. Biochimica et Biophysica Acta (BBA)-General Subjects 717(1):65-75.

Dahlman-Wright, K., V. Cavailles, S. A. Fuqua, V. C. Jordan, J. A. Katzenellenbogen, K. S. Korach, A. Maggi, M. Muramatsu, M. G. Parker, and J.-Å. Gustafsson. 2006. International union of pharmacology. LXIV. Estrogen receptors. Pharmacological reviews 58(4):773-781.

Dale, H. H. 1906. On some physiological actions of ergot. The Journal of physiology 34(3):163-206.

Darr, K. C., and E. Schultz. 1987. Exercise-induced satellite cell activation in growing and mature skeletal muscle. Journal of Applied Physiology 63(5):1816-1821.

Day, K., G. Shefer, J. B. Richardson, G. Enikolopov, and Z. Yablonka-Reuveni. 2007. Nestin-GFP reporter expression defines the quiescent state of skeletal muscle satellite cells. Developmental biology 304(1):246-259.

De Jager, N., N. J. Hudson, A. Reverter, Y.-H. Wang, S. H. Nagaraj, L. M. Cafe, P. L. Greenwood, R. T. Barnard, K. P. Kongsuwan, and B. P. Dalrymple. 2011. Chronic exposure to anabolic steroids induces the muscle expression of oxytocin and a more than fifty fold increase in circulating oxytocin in cattle. Physiological genomics

DeFronzo, R. A., and D. Tripathy. 2009. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. Diabetes care 32(suppl 2):S157-S163.

Delaunay, F., K. Pettersson, M. Tujague, and J.-Å. Gustafsson. 2000. Functional differences between the amino-terminal domains of estrogen receptors α and β. Molecular pharmacology 58(3):584-590.

Desler, M., S. Jones, C. Smith, and T. Woods. 1996. Effects of dexamethasone and anabolic agents on proliferation and protein synthesis and degradation in C2C12 myogenic cells. Journal of animal science 74(6):1265-1273.

Devost, D., P. Wrzal, and H. H. Zingg. 2008. Oxytocin receptor signalling. Progress in brain research 170:167-176.

Diel, P., D. Baadners, K. Schlüpmann, M. Velders, and J. Schwarz. 2008. C2C12 myoblastoma cell differentiation and proliferation is stimulated by androgens and associated with a modulation of myostatin and MyoG expression. Journal of Molecular Endocrinology 40(5):231-241.

Divari, S., P. Pregel, F. Cannizzo, L. S. Cucuzza, N. Brina, and B. Biolatti. 2013. Oxytocin precursor gene expression in bovine skeletal muscle is regulated by 17β-oestradiol and dexamethasone. Food chemistry 141(4):4358-4366.

du Vigneaud, V., C. Ressler, and S. Trippett. 1953. The sequence of amino acids in oxytocin, with a proposal for the structure of oxytocin. Journal of Biological Chemistry 205(2):949-957.

El-Tanani, M. K., and C. D. Green. 1997. Two separate mechanisms for ligand-independent activation of the estrogen receptor. Molecular Endocrinology 11(7):928-937.

Elabd, C., W. Cousin, P. Upadhyayula, R. Y. Chen, M. S. Chooljian, J. Li, S. Kung, K. P. Jiang, and I. M. Conboy. 2014. Oxytocin is an age-specific circulating hormone that is necessary for muscle maintenance and regeneration. Nature communications 5

Enns, D. L., and P. M. Tiidus. 2008. Estrogen influences satellite cell activation and proliferation following downhill running in rats. Journal of applied physiology 104(2):347-353.

Enns, D. L., and P. M. Tiidus. 2010. The influence of estrogen on skeletal muscle. Sports Medicine 40(1):41-58.

Essen, B., E. Jansson, J. Henriksson, A. Taylor, and B. Saltin. 1975. Metabolic characteristics of fibre types in human skeletal muscle. Acta Physiologica 95(2):153-165.

Faghihi, M., A. M. Alizadeh, V. Khori, M. Latifpour, and S. Khodayari. 2012. The role of nitric oxide, reactive oxygen species, and protein kinase C in oxytocin-induced cardioprotection in ischemic rat heart. Peptides 37(2):314-319.

Ferrando, A. A., M. Sheffield-Moore, C. W. Yeckel, C. Gilkison, J. Jiang, A. Achacosa, S. A. Lieberman, K. Tipton, R. R. Wolfe, and R. J. Urban. 2002. Testosterone administration to older men improves muscle function: molecular and physiological mechanisms. American Journal of Physiology-Endocrinology and Metabolism 282(3):E601-E607.

Fiatarone, M. A., E. C. Marks, N. D. Ryan, C. N. Meredith, L. A. Lipsitz, and W. J. Evans. 1990. High-intensity strength training in nonagenarians: effects on skeletal muscle. Jama 263(22):3029-3034.

Florini, J. R., D. Z. Ewton, and K. A. Magri. 1991. Hormones, growth factors, and myogenic differentiation. Annual review of physiology 53(1):201-216.

Fuchs, A.-R., F. Fuchs, P. Husslein, M. S. Soloff, and M. J. Fernstrom. 1982. Oxytocin receptors and human parturition: a dual role for oxytocin in the initiation of labor. Obstetrical & Gynecological Survey 37(9):567-568.

Fuchs, A.-R., R. Romero, D. Keefe, M. Parra, E. Oyarzun, and E. Behnke. 1991. Oxytocin secretion and human parturition: pulse frequency and duration increase during spontaneous labor in women. American journal of obstetrics and gynecology 165(5):1515-1523.

Gasc, J.-M., J.-M. Renoir, L. E. Faber, F. Delahaye, and E.-E. Baulieu. 1990. Nuclear localization of two steroid receptor-associated proteins, hsp90 and p59. Experimental cell research 186(2):362-367.

Gelmann, E. P. 2002. Molecular biology of the androgen receptor. Journal of Clinical Oncology 20(13):3001-3015.

Ghayee, H. K., and R. J. Auchus. 2007. Basic concepts and recent developments in human steroid hormone biosynthesis. Reviews in Endocrine and Metabolic Disorders 8(4):289-300.

Gimpl, G., and F. Fahrenholz. 2001. The oxytocin receptor system: structure, function, and regulation. Physiological reviews 81(2):629-683.

Glass, D. J. 2005. Skeletal muscle hypertrophy and atrophy signaling pathways. The international journal of biochemistry & cell biology 37(10):1974-1984.

Goldspink, G. 1968. Sarcomere length during post-natal growth of mammalian muscle fibres. Journal of cell science 3(4):539-548.

Gonzalez, J., J. Carter, D. Johnson, S. Ouellette, and S. Johnson. 2007. Effect of ractopamine-hydrochloride and trenbolone acetate on longissimus muscle fiber area, diameter, and satellite cell numbers in cull beef cows. Journal of animal science 85(8):1893-1901.

Green, L., D. Fein, C. Modahl, C. Feinstein, L. Waterhouse, and M. Morris. 2001. Oxytocin and autistic disorder: alterations in peptide forms. Biological psychiatry 50(8):609-613.

Green, S., P. Walter, V. Kumar, A. Krust, J.-M. Bornert, P. Argos, and P. Chambon. 1986. Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. Nature 320(6058):134-139.

Grefte, S., A. M. Kuijpers-Jagtman, R. Torensma, and J. W. Von den Hoff. 2007. Skeletal muscle development and regeneration. Stem cells and development 16(5):857-868.

Hasty, P., A. Bradley, J. H. Morris, D. G. Edmondson, J. M. Venuti, E. N. Olson, and W. H. Klein. 1993. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. Nature 364(6437):501.

Hatae, J. 2001. Effects of 17β-Estradiol on tension responses and fatigue in the skeletal twitch muscle fibers of frog. The Japanese journal of physiology 51(6):753-759.

Hawke, T. J., and D. J. Garry. 2001. myogenic satellite cells: physiology to molecular biology. Journal of applied physiology 91(2):534-551.

Heldring, N., A. Pike, S. Andersson, J. Matthews, G. Cheng, J. Hartman, M. Tujague, A. Ström, E. Treuter, and M. Warner. 2007a. Estrogen receptors: how do they signal and what are their targets. Physiological reviews 87(3):905-931.

Heldring, N., A. Pike, S. Andersson, J. Matthews, G. Cheng, J. Hartman, M. Tujague, A. Ström, E. Treuter, M. Warner, and J.-Å. Gustafsson. 2007b. Estrogen Receptors: How Do They Signal and What Are Their Targets. Physiological Reviews 87(3):905-931. doi: 10.1152/physrev.00026.2006

Hiroi, R., A. F. Lacagnina, L. R. Hinds, D. G. Carbone, R. M. Uht, and R. J. Handa. 2013. The androgen metabolite, 5α-androstane-3β, 17β-diol (3β-diol), activates the oxytocin promoter through an estrogen receptor-β pathway. Endocrinology 154(5):1802-1812.

Hughes, S. M., and H. M. Blau. 1990. Migration of myoblasts across basal lamina during skeletal muscle development. Nature 345(6273):350.

Ivell, R., and S. Hartung. 2003. The molecular basis of cryptorchidism*. Molecular human reproduction 9(4):175-181.

Ivell, R., and D. Richter. 1984. The gene for the hypothalamic peptide hormone oxytocin is highly expressed in the bovine corpus luteum: biosynthesis, structure and sequence analysis. The EMBO journal 3(10):2351.

Jang, Y., M. Sinha, M. Cerletti, C. Dall'Osso, and A. Wagers. 2011. Skeletal muscle stem cells: effects of aging and metabolism on muscle regenerative function. In: Cold Spring Harbor symposia on quantitative biology. p 101-111.

Jankowski, M., B. Danalache, D. Wang, P. Bhat, F. Hajjar, M. Marcinkiewicz, J. Paquin, S. M. McCann, and J. Gutkowska. 2004. Oxytocin in cardiac ontogeny. Proceedings of the National Academy of Sciences of the United States of America 101(35):13074-13079.

Jankowski, M., F. Hajjar, S. Al Kawas, S. Mukaddam-Daher, G. Hoffman, S. M. McCann, and J. Gutkowska. 1998. Rat heart: a site of oxytocin production and action. Proceedings of the National Academy of Sciences 95(24):14558-14563.

Jensen, E. V., and H. I. Jacobson. 1962. Basic guides to the mechanism of estrogen action. Recent Prog Horm Res 18(4):387.

Jessop, H., M. Sjöberg, M. Z. Cheng, G. Zaman, C. Wheeler-Jones, and L. E. Lanyon. 2001. Mechanical strain and estrogen activate estrogen receptor α in bone cells. Journal of Bone and Mineral Research 16(6):1045-1055.

Johnson, B., N. Halstead, M. White, M. Hathaway, A. DiCostanzo, and W. Dayton. 1998. Activation state of muscle satellite cells isolated from steers implanted with a combined trenbolone acetate and estradiol implant. Journal of animal science 76(11):2779-2786.

Joulia, D., H. Bernardi, V. Garandel, F. Rabenoelina, B. Vernus, and G. Cabello. 2003. Mechanisms involved in the inhibition of myoblast proliferation and differentiation by myostatin. Experimental cell research 286(2):263-275.

Kablar, B., K. Krastel, C. Ying, A. Asakura, S. J. Tapscott, and M. A. Rudnicki. 1997. MyoD and Myf-5 differentially regulate the development of limb versus trunk skeletal muscle. Development 124(23):4729-4738.

Kadi, F., C. Karlsson, B. Larsson, J. Eriksson, M. Larval, H. Billig, and I. H. Jonsdottir. 2002. The effects of physical activity and estrogen treatment on rat fast and slow skeletal muscles following ovariectomy. Journal of Muscle Research & Cell Motility 23(4):335.

Kahlert, S., C. Grohé, R. H. Karas, K. Löbbert, L. Neyses, and H. Vetter. 1997. Effects of estrogen on skeletal myoblast growth. Biochemical and biophysical research communications 232(2):373-378.

Kalbe, C., M. Mau, K. Wollenhaupt, and C. Rehfeldt. 2007. Evidence for estrogen receptor α and β expression in skeletal muscle of pigs. Histochemistry and cell biology 127(1):95-107.

Kamanga-Sollo, E., M. White, M. Hathaway, K. Chung, B. Johnson, and W. Dayton. 2008. Roles of IGF-I and the estrogen, androgen and IGF-I receptors in estradiol-17β-and trenbolone acetate-stimulated proliferation of cultured bovine satellite cells. Domestic animal endocrinology 35(1):88-97.

Kamanga-Sollo, E., M. White, M. Hathaway, W. Weber, and W. Dayton. 2010. Effect of Estradiol-17β on protein synthesis and degradation rates in fused bovine satellite cell cultures. Domestic animal endocrinology 39(1):54-62.

Kamanga-Sollo, E., M. White, W. Weber, and W. Dayton. 2013. Role of estrogen receptor-α (ESR1) and the type 1 insulin-like growth factor receptor (IGFR1) in estradiol-stimulated proliferation of cultured bovine satellite cells. Domestic animal endocrinology 44(1):36-45.

Kamanga-Sollo, E., M. Pampusch, G. Xi, M. White, M. Hathaway, and W. Dayton. 2004. IGF-I mRNA levels in bovine satellite cell cultures: Effects of fusion and anabolic steroid treatment. Journal of cellular physiology 201(2):181-189.

Kassar-Duchossoy, L., B. Gayraud-Morel, D. Gomès, D. Rocancourt, M. Buckingham, V. Shinin, and S. Tajbakhsh. 2004. MRF4 determines skeletal muscle identity in Myf5: MyoD double-mutant mice. Nature 431(7007):466-471.

Kato, S., H. Endoh, Y. Masuhiro, and T. Kitamoto. 1995. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 270(5241):1491.

Katzenellenbogen, B. S., M. M. Montano, P. Le Goff, D. J. Schodin, W. L. Kraus, B. Bhardwaj, and N. Fujimoto. 1995. Antiestrogens: mechanisms and actions in target cells. The Journal of steroid biochemistry and molecular biology 53(1):387-393.

Kitajima, Y., and Y. Ono. 2016. Estrogens maintain skeletal muscle and satellite cell functions. Journal of Endocrinology 229(3):267-275.

Kiyama, R., and Y. Wada-Kiyama. 2015. Estrogenic endocrine disruptors: molecular mechanisms of action. Environment international 83:11-40.

Klont, R., L. Brocks, and G. Eikelenboom. 1998. Muscle fibre type and meat quality. Meat science 49:S219-S229.

Köhn, F. M. 2006. Testosterone and body functions. The aging male 9(4):183-188.

Kongsuwan, K., M. Knox, P. Allingham, R. Pearson, and B. Dalrymple. 2012a. The effect of combination treatment with trenbolone acetate and estradiol-17β on skeletal muscle expression and plasma concentrations of oxytocin in sheep. Domestic animal endocrinology 43(1):67-73.

Kongsuwan, K., M. R. Knox, P. G. Allingham, R. Pearson, and B. P. Dalrymple. 2012b. The effect of combination treatment with trenbolone acetate and estradiol-17β on skeletal muscle expression and plasma concentrations of oxytocin in sheep. Domestic animal endocrinology 43(1):67-73.

Koohi, M., R. Ivell, and N. Walther. 2005. Transcriptional Activation of the Oxytocin Promoter by Oestrogens Uses a Novel Non-Classical Mechanism of Oestrogen Receptor Action. Journal of neuroendocrinology 17(4):197-207.

Kovacheva, E. L., A. P. Sinha Hikim, R. Shen, I. Sinha, and I. Sinha-Hikim. 2010. Testosterone supplementation reverses sarcopenia in aging through regulation of myostatin, c-Jun NH2-terminal kinase, Notch, and Akt signaling pathways. Endocrinology 151(2):628-638.

Kramer, H. F., C. A. Witczak, E. B. Taylor, N. Fujii, M. F. Hirshman, and L. J. Goodyear. 2006. AS160 regulates insulin-and contraction-stimulated glucose uptake in mouse skeletal muscle. Journal of Biological Chemistry 281(42):31478-31485.

Kuang, S., K. Kuroda, F. Le Grand, and M. A. Rudnicki. 2007. Asymmetric self-renewal and commitment of satellite stem cells in muscle. Cell 129(5):999-1010.

Kuiper, G., E. Enmark, M. Pelto-Huikko, S. Nilsson, and J.-A. Gustafsson. 1996. Cloning of a novel receptor expressed in rat prostate and ovary. Proceedings of the National Academy of Sciences 93(12):5925-5930.

Kushner, P. J., D. A. Agard, G. L. Greene, T. S. Scanlan, A. K. Shiau, R. M. Uht, and P. Webb. 2000. Estrogen receptor pathways to AP-1. The Journal of steroid biochemistry and molecular biology 74(5):311-317.

Le Grand, F., and M. A. Rudnicki. 2007. Skeletal muscle satellite cells and adult myogenesis. Current opinion in cell biology 19(6):628-633.

Lee, H.-J., H. K. Caldwell, A. H. Macbeth, S. G. Tolu, and W. S. Young 3rd. 2008. A conditional knockout mouse line of the oxytocin receptor. Endocrinology 149(7):3256-3263.

Lewis, M. I., G. D. Horvitz, D. R. Clemmons, and M. Fournier. 2002. Role of IGF-I and IGF-binding proteins within diaphragm muscle in modulating the effects of nandrolone. American Journal of Physiology-Endocrinology and Metabolism 282(2):E483-E490.

Lieber, R. L. 2002. Skeletal muscle structure, function, and plasticity. Lippincott Williams & Wilkins.

Lossie, A. C., B. Eipper, T. Hand, and S. Camper. 1994. Localization of the peptidylglycine α-amidating monooxygenase gene (Pam) introduces a region of homology between human Chromosome 5q and mouse Chromosome 1. Mammalian Genome 5(11):738-739.

Loureiro, J., P. Sandoval, G. del Peso, G. Gónzalez-Mateo, V. Fernández-Millara, B. Santamaria, M. A. Bajo, J. A. Sánchez-Tomero, G. Guerra-Azcona, and R. Selgas. 2013. Tamoxifen ameliorates peritoneal membrane damage by blocking mesothelial to mesenchymal transition in peritoneal dialysis. PloS one 8(4):e61165.

Lundby, C., J. A. Calbet, and P. Robach. 2009. The response of human skeletal muscle tissue to hypoxia. Cellular and molecular life sciences 66(22):3615-3623.

Ma, K., C. Mallidis, J. Artaza, W. Taylor, N. Gonzalez-Cadavid, and S. Bhasin. 2001. Characterization of 5′-regulatory region of human myostatin gene: regulation by dexamethasone in-vitro. American Journal of Physiology-Endocrinology And Metabolism 281(6):E1128-E1136.

Machida, S., and F. W. Booth. 2004. Insulin-like growth factor 1 and muscle growth: implication for satellite cell proliferation. Proceedings of the Nutrition Society 63(02):337-340.

MacIntosh, B. R., P. F. Gardiner, and A. J. McComas. 2006. Skeletal muscle: form and function. Human Kinetics.

Magon, N., and S. Kalra. 2011. The orgasmic history of oxytocin: Love, lust, and labor. Indian journal of endocrinology and metabolism 15(7):156.

Mandlekar, S., and A.-N. Kong. 2001. Mechanisms of tamoxifen-induced apoptosis. Apoptosis 6(6):469-477.

Marieb, E. N., and K. Hoehn. 2007. Human anatomy & physiology. Pearson Education.

Matsumine, H., K. Hirato, T. Yanaihara, T. TAMADA, and M. YOSHIDA. 1986. Aromatization by skeletal muscle. The Journal of Clinical Endocrinology & Metabolism 63(3):717-720.

Mauro, A. 1961. Satellite cell of skeletal muscle fibers. The Journal of biophysical and biochemical cytology 9(2):493.

McClung, J., J. Davis, M. Wilson, E. Goldsmith, and J. Carson. 2006. Estrogen status and skeletal muscle recovery from disuse atrophy. Journal of Applied Physiology 100(6):2012-2023.

McPherron, A. C., and S.-J. Lee. 1997. Double muscling in cattle due to mutations in the myostatin gene. Proceedings of the National Academy of Sciences 94(23):12457-12461.

Mermelstein, P. G., J. B. Becker, and D. J. Surmeier. 1996. Estradiol reduces calcium currents in rat neostriatal neurons via a membrane receptor. Journal of Neuroscience 16(2):595-604.

Michels, G., and U. C. Hoppe. 2008. Rapid actions of androgens. Frontiers in neuroendocrinology 29(2):182-198.

Migliaccio, A., M. Di Domenico, G. Castoria, A. de Falco, P. Bontempo, E. Nola, and F. Auricchio. 1996. Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. The EMBO journal 15(6):1292.

Miller, W. L., and R. J. Auchus. 2010. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. Endocrine reviews 32(1):81-151.

Millward, D. J., P. J. Garlick, R. J. Stewart, D. Nnanyelugo, and J. Waterlow. 1975. Skeletal-muscle growth and protein turnover. Biochemical Journal 150(2):235-243.

Milne, K. J., and E. G. Noble. 2008. Response of the myocardium to exercise: sex-specific regulation of hsp70. Medicine and science in sports and exercise 40(4):655-663.

Montarras, D., J. Chelly, E. Bober, H. Arnold, M. Ott, F. Gros, and C. Pinset. 1991. Developmental patterns in the expression of Myf5, MyoD, myogenin, and MRF4 during myogenesis. The New biologist 3(6):592-600.

Moran, A. L., S. A. Nelson, R. M. Landisch, G. L. Warren, and D. A. Lowe. 2007. Estradiol replacement reverses ovariectomy-induced muscle contractile and myosin dysfunction in mature female mice. Journal of applied physiology 102(4):1387-1393.

Moretti, I., S. Ciciliot, K. A. Dyar, R. Abraham, M. Murgia, L. Agatea, T. Akimoto, S. Bicciato, M. Forcato, and P. Pierre. 2016. MRF4 negatively regulates adult skeletal muscle growth by repressing MEF2 activity. Nature Communications 7

Naya, F. J., and E. Olson. 1999. MEF2: a transcriptional target for signaling pathways controlling skeletal muscle growth and differentiation. Current opinion in cell biology 11(6):683-688.

Nilsson, S., and J.-Å. Gustafsson. 2002. Biological role of estrogen and estrogen receptors. Critical reviews in biochemistry and molecular biology 37(1):1-28.

Noiseux, N., M. Borie, A. Desnoyers, A. Menaouar, L. M. Stevens, S. Mansour, B. A. Danalache, D.-C. Roy, M. Jankowski, and J. Gutkowska. 2012. Preconditioning of stem cells by oxytocin to improve their therapeutic potential. Endocrinology 153(11):5361-5372.

Olson, E., H.-H. Arnold, P. Rigby, and B. Wold. 1996. Know your neighbors: three phenotypes in null mutants of the myogenic bHLH gene MRF4. Cell 85(1):1-4.

Olson, E. N. 1992. Interplay between proliferation and differentiation within the myogenic lineage. Developmental biology 154(2):261-272.

Patel, K., and A. Matsakas. 2009. Skeletal muscle fibre plasticity in response to selected environmental and physiological stimuli. Histology and histopathology

Payne, W. G., F. Ko, S. Anspaugh, C. K. Wheeler, T. E. Wright, and M. C. Robson. 2006. Down-regulating causes of fibrosis with tamoxifen: a possible cellular/molecular approach to treat rhinophyma. Annals of plastic surgery 56(3):301-305.

Péault, B., M. Rudnicki, Y. Torrente, G. Cossu, J. P. Tremblay, T. Partridge, E. Gussoni, L. M. Kunkel, and J. Huard. 2007. Stem and progenitor cells in skeletal muscle development, maintenance, and therapy. Molecular Therapy 15(5):867-877.

Pedersen, B. K., T. C. Åkerström, A. R. Nielsen, and C. P. Fischer. 2007. Role of myokines in exercise and metabolism. Journal of applied physiology 103(3):1093-1098.

Pedersen, B. K., and M. A. Febbraio. 2008. Muscle as an endocrine organ: focus on muscle-derived interleukin-6. Physiological reviews 88(4):1379-1406.

Pedersen, B. K., and M. A. Febbraio. 2012. Muscles, exercise and obesity: skeletal muscle as a secretory organ. Nature Reviews Endocrinology 8(8):457-465.

Pedram, A., M. Razandi, and E. R. Levin. 2006. Nature of functional estrogen receptors at the plasma membrane. Molecular endocrinology 20(9):1996-2009.

Phillips, S. M., and B. B. Sherwin. 1992. Effects of estrogen on memory function in surgically menopausal women. Psychoneuroendocrinology 17(5):485-495.

Powers, S. K., A. N. Kavazis, and J. M. McClung. 2007. Oxidative stress and disuse muscle atrophy. Journal of applied physiology 102(6):2389-2397.

Powles, T. J., S. Ashley, A. Tidy, I. E. Smith, and M. Dowsett. 2007. Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. Journal of the National Cancer Institute 99(4):283-290.

Ramaswamy, B., Y. Lu, K.-y. Teng, G. Nuovo, X. Li, C. L. Shapiro, and S. Majumder. 2012. Hedgehog signaling is a novel therapeutic target in tamoxifen-resistant breast cancer aberrantly activated by PI3K/AKT pathway. Cancer research 72(19):5048-5059.

Razandi, M., A. Pedram, I. Merchenthaler, G. L. Greene, and E. R. Levin. 2004. Plasma membrane estrogen receptors exist and functions as dimers. Molecular Endocrinology 18(12):2854-2865.

Relaix, F., D. Rocancourt, A. Mansouri, and M. Buckingham. 2005. A Pax3/MyoG-dependent population of skeletal muscle progenitor cells. Nature 435(7044):948-953.

Relaix, F., and P. S. Zammit. 2012. Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage. Development 139(16):2845-2856.

Rhoads, R. P., R. M. Johnson, C. R. Rathbone, X. Liu, C. Temm-Grove, S. M. Sheehan, J. B. Hoying, and R. E. Allen. 2009. Satellite cell-mediated angiogenesis in-vitro coincides with a functional hypoxia-inducible factor pathway. American Journal of Physiology-Cell Physiology 296(6):C1321-C1328.

Richard, S. p., and H. Zingg. 1990. The human oxytocin gene promoter is regulated by estrogens. Journal of Biological Chemistry 265(11):6098-6103.

Robinson-Rechavi, M., H. E. Garcia, and V. Laudet. 2003. The nuclear receptor superfamily. Journal of cell science 116(4):585-586.

Rodgers, B. D., and D. K. Garikipati. 2008. Clinical, agricultural, and evolutionary biology of myostatin: a comparative review. Endocrine reviews 29(5):513-534.

Rossouw, J. E. 2002. Writing Group for the Women's Health Initiative Investigators. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. Jama 288:321-333.

Rudnicki, M. A., P. N. Schnegelsberg, R. H. Stead, T. Braun, H.-H. Arnold, and R. Jaenisch. 1993. MyoD or Myf-5 is required for the formation of skeletal muscle. Cell 75(7):1351-1359.

Saartok, T. 1984. Steroid receptors in two types of rabbit skeletal muscle. International journal of sports medicine 5(03):130-136.

Sacheck, J. M., A. Ohtsuka, S. C. McLary, and A. L. Goldberg. 2004. IGF-I stimulates muscle growth by suppressing protein breakdown and expression of atrophy-related ubiquitin ligases, atrogin-1 and MuRF1. American Journal of Physiology-Endocrinology and Metabolism 287(4):E591-E601.

Sambasivan, R., and S. Tajbakhsh. 2015. Adult skeletal muscle stem cells, Vertebrate myogenesis. Springer. p. 191-213.

Sato, K., M. Iemitsu, K. Aizawa, and R. Ajisaka. 2008. Testosterone and DHEA activate the glucose metabolism-related signaling pathway in skeletal muscle. American Journal of Physiology-Endocrinology and Metabolism 294(5):E961-E968.

Sauerwein, H., and H. Meyer. 1989. Androgen and estrogen receptors in bovine skeletal muscle: relation to steroid-induced allometric muscle growth. Journal of animal science 67(1):206-212.

Schiaffino, S., and C. Reggiani. 2011. Fiber types in mammalian skeletal muscles. Physiological reviews 91(4):1447-1531.

Schiff, R., P. Reddy, M. Ahotupa, E. Coronado-Heinsohn, M. Grim, S. G. Hilsenbeck, R. Lawrence, S. Deneke, R. Herrera, and G. C. Chamness. 2000. Oxidative stress and AP-1 activity in tamoxifen-resistant breast tumors in-vivo. Journal of the National Cancer Institute 92(23):1926-1934.

Schneider, B. S. P., J. P. Fine, T. Nadolski, and P. M. Tiidus. 2004. The effects of estradiol and progesterone on plantarflexor muscle fatigue in ovariectomized mice. Biological research for nursing 5(4):265-275.

Schulster, M., A. M. Bernie, and R. Ramasamy. 2016. The role of estradiol in male reproductive function. Asian journal of andrology 18(3):435.

Schultz, E. 1989. Satellite cell behavior during skeletal muscle growth and regeneration. Medicine and science in sports and exercise 21(5 Suppl):S181-186.

Sciote, J., M. Horton, Y. Zyman, and G. Pascoe. 2001. Differential effects of diminished oestrogen and androgen levels on development of skeletal muscle fibres in hypogonadal mice. Acta physiologica scandinavica 172(3):179-187.

Serra, C., S. Bhasin, F. Tangherlini, E. R. Barton, M. Ganno, A. Zhang, J. Shansky, H. H. Vandenburgh, T. G. Travison, and R. Jasuja. 2011. The role of GH and IGF-I in mediating anabolic effects of testosterone on androgen-responsive muscle. Endocrinology 152(1):193-206.

Shang, Y. 2006. Molecular mechanisms of oestrogen and SERMs in endometrial carcinogenesis. Nature Reviews Cancer 6(5):360-368.

Shang, Y., and M. Brown. 2002. Molecular determinants for the tissue specificity of SERMs. Science 295(5564):2465-2468.

Sharples, A. P., D. C. Hughes, C. S. Deane, A. Saini, C. Selman, and C. E. Stewart. 2015. Longevity and skeletal muscle mass: the role of IGF signalling, the sirtuins, dietary restriction and protein intake. Aging cell 14(4):511-523.

Shefer, G., D. P. Van de Mark, J. B. Richardson, and Z. Yablonka-Reuveni. 2006. Satellite-cell pool size does matter: defining the myogenic potency of aging skeletal muscle. Developmental biology 294(1):50-66.

Shupnik, M. A. 2004. Crosstalk between steroid receptors and the c-Src-receptor tyrosine kinase pathways: implications for cell proliferation. Oncogene 23(48):7979-7989.

Sinha-Hikim, I., M. Cornford, H. Gaytan, M. L. Lee, and S. Bhasin. 2006. Effects of testosterone supplementation on skeletal muscle fiber hypertrophy and satellite cells in community-dwelling older men. The Journal of Clinical Endocrinology & Metabolism 91(8):3024-3033.

Sinha-Hikim, I., S. M. Roth, M. I. Lee, and S. Bhasin. 2003. Testosterone-induced muscle hypertrophy is associated with an increase in satellite cell number in healthy, young men. American Journal of Physiology-Endocrinology and Metabolism 285(1):E197-E205.

Sinha-Hikim, I., W. E. Taylor, N. F. Gonzalez-Cadavid, W. Zheng, and S. Bhasin. 2004. Androgen receptor in human skeletal muscle and cultured muscle satellite cells: up-regulation by androgen treatment. The Journal of Clinical Endocrinology & Metabolism 89(10):5245-5255.

Soloff, M. S., M. Alexandrova, and M. J. Fernstrom. 1979. Oxytocin receptors: triggers for parturition and lactation? Science 204(4399):1313-1315.

Stedronsky, K., R. Telgmann, G. Tillmann, N. Walther, and R. Ivell. 2002. The affinity and activity of the multiple hormone response element in the proximal promoter of the human oxytocin gene. Journal of neuroendocrinology 14(6):472-485.

Suzuki, S., and T. Yamamuro. 1985. Long-term effects of estrogen on rat skeletal muscle. Experimental neurology 87(2):291-299.

Tamma, R., G. Colaianni, L.-l. Zhu, A. DiBenedetto, G. Greco, G. Montemurro, N. Patano, M. Strippoli, R. Vergari, and L. Mancini. 2009. Oxytocin is an anabolic bone hormone. Proceedings of the National Academy of Sciences 106(17):7149-7154.

Tapscott, S. J. 2005. The circuitry of a master switch: MyoD and the regulation of skeletal muscle gene transcription. Development 132(12):2685-2695.

Taylor, A., and F. Al-Azzawi. 2000. Immunolocalisation of oestrogen receptor beta in human tissues. Journal of molecular endocrinology 24(1):145-155.

Tedesco, F. S., A. Dellavalle, J. Diaz-Manera, G. Messina, and G. Cossu. 2010. Repairing skeletal muscle: regenerative potential of skeletal muscle stem cells. The Journal of clinical investigation 120(1):11-19.

Theobald, G., A. Graham, J. Campbell, P. Gange, and W. Driscoll. 1948. Use of post-pituitary extract in obstetrics. British medical journal 2(4567):123.

Thomas, A., K. Bunyan, and P. Tiidus. 2010. Oestrogen receptor-alpha activation augments post-exercise myoblast proliferation. Acta physiologica 198(1):81-89.

Thomas, R. S., N. Sarwar, F. Phoenix, R. C. Coombes, and S. Ali. 2008. Phosphorylation at serines 104 and 106 by Erk1/2 MAPK is important for estrogen receptor-α activity. Journal of molecular endocrinology 40(4):173-184.

Tidball, J. G. 2005. Inflammatory processes in muscle injury and repair. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 288(2):R345-R353.

Tiidus, P., M. Deller, and X. Liu. 2005. Oestrogen influence on myogenic satellite cells following downhill running in male rats: a preliminary study. Acta physiologica scandinavica 184(1):67-72.

Tiidus, P. M., D. A. Lowe, and M. Brown. 2013. Estrogen replacement and skeletal muscle: mechanisms and population health. Journal of Applied Physiology 115(5):569-578.

Tora, L., J. White, C. Brou, D. Tasset, N. Webster, E. Scheer, and P. Chambon. 1989. The human estrogen receptor has two independent nonacidic transcriptional activation functions. Cell 59(3):477-487.

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.

Ustanina, S., J. Carvajal, P. Rigby, and T. Braun. 2007. The myogenic factor Myf5 supports efficient skeletal muscle regeneration by enabling transient myoblast amplification. Stem Cells 25(8):2006-2016.

Vandenput, L., and C. Ohlsson. 2010. Sex steroid metabolism in the regulation of bone health in men. The Journal of steroid biochemistry and molecular biology 121(3):582-588.

Verdijk, L. B., B. G. Gleeson, R. A. Jonkers, K. Meijer, H. H. Savelberg, P. Dendale, and L. J. van Loon. 2009. Skeletal muscle hypertrophy following resistance training is accompanied by a fiber type–specific increase in satellite cell content in elderly men. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences:gln050.

Vierck, J., B. O'Reilly, K. Hossner, J. Antonio, K. Byrne, L. Bucci, and M. Dodson. 2000. Satellite cell regulation following myotrauma caused by resistance exercise. Cell biology international 24(5):263-272.

Viero, C., I. Shibuya, N. Kitamura, A. Verkhratsky, H. Fujihara, A. Katoh, Y. Ueta, H. H. Zingg, A. Chvatal, and E. Sykova. 2010. REVIEW: Oxytocin: Crossing the bridge between basic science and pharmacotherapy. CNS neuroscience & therapeutics 16(5):e138-e156.

Vingren, J. L., W. J. Kraemer, N. A. Ratamess, J. M. Anderson, J. S. Volek, and C. M. Maresh. 2010. Testosterone physiology in resistance exercise and training. Sports medicine 40(12):1037-1053.

von Maltzahn, J., A. E. Jones, R. J. Parks, and M. A. Rudnicki. 2013. MyoG is critical for the normal function of satellite cells in adult skeletal muscle. Proceedings of the National Academy of Sciences 110(41):16474-16479.

Wakerley, J., and D. Lincoln. 1973. The milk-ejection reflex of the rat: a 20-to 40-fold acceleration in the firing of paraventricular neurones during oxytocin release. Journal of Endocrinology 57(3):477-493.

Wang, Y. X., and M. A. Rudnicki. 2012. Satellite cells, the engines of muscle repair. Nature reviews Molecular cell biology 13(2):127-133.

Watters, J. J., J. S. Campbell, M. J. Cunningham, E. G. Krebs, and D. M. Dorsa. 1997. Rapid membrane effects of steroids in neuroblastoma cells: effects of estrogen on mitogen activated protein kinase signalling cascade and c-fos immediate early gene transcription. Endocrinology 138(9):4030-4033.

Wiik, A., B. Glenmark, M. Ekman, M. Esbjörnsson-Liljedahl, O. Johansson, K. Bodin, E. Enmark, and E. Jansson. 2003. Oestrogen receptor β is expressed in adult human skeletal muscle both at the mRNA and protein level. Acta Physiologica Scandinavica 179(4):381-387.

Wright, W. E., D. A. Sassoon, and V. K. Lin. 1989. myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. Cell 56(4):607-617.

Xu, T., Y. Shen, H. Pink, J. Triantafillou, S. A. Stimpson, P. Turnbull, and B. Han. 2004. Phosphorylation of p70 s6 kinase is implicated in androgen-induced levator ani muscle anabolism in castrated rats. The Journal of steroid biochemistry and molecular biology 92(5):447-454.

Yates, D. T., D. S. Clarke, A. R. Macko, M. J. Anderson, L. A. Shelton, M. Nearing, R. E. Allen, R. P. Rhoads, and S. W. Limesand. 2014. Myoblasts from intrauterine growth-restricted sheep fetuses exhibit intrinsic deficiencies in proliferation that contribute to smaller semitendinosus myofibres. The Journal of physiology 592(14):3113-3125.

Yin, D., W. Gao, J. D. Kearbey, H. Xu, K. Chung, Y. He, C. A. Marhefka, K. A. Veverka, D. D. Miller, and J. T. Dalton. 2003. Pharmacodynamics of selective androgen receptor modulators. Journal of Pharmacology and Experimental Therapeutics 304(3):1334-1340.

Yin, H., F. Price, and M. A. Rudnicki. 2013. Satellite cells and the muscle stem cell niche. Physiological reviews 93(1):23-67.

Zammit, P. S., L. Heslop, V. Hudon, J. D. Rosenblatt, S. Tajbakhsh, M. E. Buckingham, J. R. Beauchamp, and T. A. Partridge. 2002. Kinetics of myoblast proliferation show that resident satellite cells are competent to fully regenerate skeletal muscle fibers. Experimental cell research 281(1):39-49.

Zhong, H., S. M. Wade, P. J. Woolf, J. J. Linderman, J. R. Traynor, and R. R. Neubig. 2003. A Spatial Focusing Model for G Protein Signals REGULATOR OF G PROTEIN SIGNALING (RGS) PROTEIN-MEDIATED KINETIC SCAFFOLDING. Journal of Biological Chemistry 278(9):7278-7284.

Zingg, H. H., and S. A. Laporte. 2003. The oxytocin receptor. Trends in Endocrinology & Metabolism 14(5):222-227.

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 × 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% CO_2) 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 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. 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 5min 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 Instuments 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 (H_2O_2) 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 (1.5×10^5cell/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^{TM} 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 concerntration (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

Al Naib, A., H. Tucker, G. Xie, D. Keisler, F. Bartol, R. Rhoads, R. Akers, and M. Rhoads. 2016. Prepubertal tamoxifen treatment affects development of heifer reproductive tissues and related signaling pathways. Journal of dairy science 99(7):5780-5792.

Andari, E., J.-R. Duhamel, T. Zalla, E. Herbrecht, M. Leboyer, and A. Sirigu. 2010. Promoting social behavior with oxytocin in high-functioning autism spectrum disorders. Proceedings of the National Academy of Sciences 107(9):4389-4394.

Asp, M. L., J. J. Martindale, and J. M. Metzger. 2013. Direct, differential effects of tamoxifen, 4-hydroxytamoxifen, and raloxifene on cardiac myocyte contractility and calcium handling. PloS one 8(10):e78768.

Banerjee, S., K. L. Chambliss, C. Mineo, and P. W. Shaul. 2014. Recent insights into non-nuclear actions of estrogen receptor alpha. Steroids 81:64-69.

Collins, C. A., I. Olsen, P. S. Zammit, L. Heslop, A. Petrie, T. A. Partridge, and J. E. Morgan. 2005. Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. Cell 122(2):289-301.

Conerly, M. L., Z. Yao, J. W. Zhong, M. Groudine, and S. J. Tapscott. 2016. Distinct activities of Myf5 and MyoD indicate separate roles in skeletal muscle lineage specification and differentiation. Developmental cell 36(4):375-385.

Cranwell, C., J. Unruh, J. Brethour, D. Simms, and R. Campbell. 1996. Influence of steroid implants and concentrate feeding on performance and carcass composition of cull beef cows. Journal of animal science 74(8):1770-1776.

Cuzick, J., I. Sestak, S. Cawthorn, H. Hamed, K. Holli, A. Howell, J. F. Forbes, and I.-I. Investigators. 2015. Tamoxifen for prevention of breast cancer: extended long-term follow-up of the IBIS-I breast cancer prevention trial. The lancet oncology 16(1):67-75.

De Jager, N., N. J. Hudson, A. Reverter, Y.-H. Wang, S. H. Nagaraj, L. M. Cafe, P. L. Greenwood, R. T. Barnard, K. P. Kongsuwan, and B. P. Dalrymple. 2011. Chronic exposure to anabolic steroids induces the muscle expression of oxytocin and a more than fifty fold increase in circulating oxytocin in cattle. Physiological genomics

Divari, S., P. Pregel, F. Cannizzo, L. S. Cucuzza, N. Brina, and B. Biolatti. 2013. Oxytocin precursor gene expression in bovine skeletal muscle is regulated by 17β-oestradiol and dexamethasone. Food chemistry 141(4):4358-4366.

Elabd, C., W. Cousin, P. Upadhyayula, R. Y. Chen, M. S. Chooljian, J. Li, S. Kung, K. P. Jiang, and I. M. Conboy. 2014. Oxytocin is an age-specific circulating hormone that is necessary for muscle maintenance and regeneration. Nature communications 5

Enns, D. L., and P. M. Tiidus. 2008. Estrogen influences satellite cell activation and proliferation following downhill running in rats. Journal of applied physiology 104(2):347-353.

Enns, D. L., and P. M. Tiidus. 2010. The influence of estrogen on skeletal muscle. Sports Medicine 40(1):41-58.

Fuchs, A.-R., F. Fuchs, P. Husslein, M. S. Soloff, and M. J. Fernstrom. 1982. Oxytocin receptors and human parturition: a dual role for oxytocin in the initiation of labor. Obstetrical & Gynecological Survey 37(9):567-568.

Gimpl, G., and F. Fahrenholz. 2001. The oxytocin receptor system: structure, function, and regulation. Physiological reviews 81(2):629-683.

Gonzalez, J., J. Carter, D. Johnson, S. Ouellette, and S. Johnson. 2007. Effect of ractopamine-hydrochloride and trenbolone acetate on longissimus muscle fiber area, diameter, and satellite cell numbers in cull beef cows. Journal of animal science 85(8):1893-1901.

Hawke, T. J., and D. J. Garry. 2001. myogenic satellite cells: physiology to molecular biology. Journal of applied physiology 91(2):534-551.

Ivell, R., and S. Hartung. 2003. The molecular basis of cryptorchidism*. Molecular human reproduction 9(4):175-181.

Jang, Y., M. Sinha, M. Cerletti, C. Dall'Osso, and A. Wagers. 2011. Skeletal muscle stem cells: effects of aging and metabolism on muscle regenerative function. In: Cold Spring Harbor symposia on quantitative biology. p 101-111.

Jankowski, M., B. Danalache, D. Wang, P. Bhat, F. Hajjar, M. Marcinkiewicz, J. Paquin, S. M. McCann, and J. Gutkowska. 2004. Oxytocin in cardiac ontogeny. Proceedings of the National Academy of Sciences of the United States of America 101(35):13074-13079.

Joulia, D., H. Bernardi, V. Garandel, F. Rabenoelina, B. Vernus, and G. Cabello. 2003. Mechanisms involved in the inhibition of myoblast proliferation and differentiation by myostatin. Experimental cell research 286(2):263-275.

Kassar-Duchossoy, L., B. Gayraud-Morel, D. Gomès, D. Rocancourt, M. Buckingham, V. Shinin, and S. Tajbakhsh. 2004. MRF4 determines skeletal muscle identity in Myf5: MyoD double-mutant mice. Nature 431(7007):466-471.

Kitajima, Y., and Y. Ono. 2016. Estrogens maintain skeletal muscle and satellite cell functions. Journal of Endocrinology 229(3):267-275.

Kiyama, R., and Y. Wada-Kiyama. 2015. Estrogenic endocrine disruptors: molecular mechanisms of action. Environment international 83:11-40.

Kongsuwan, K., M. Knox, P. Allingham, R. Pearson, and B. Dalrymple. 2012. The effect of combination treatment with trenbolone acetate and estradiol-17β on skeletal muscle

expression and plasma concentrations of oxytocin in sheep. Domestic animal endocrinology 43(1):67-73.

Koohi, M., R. Ivell, and N. Walther. 2005. Transcriptional Activation of the Oxytocin Promoter by Oestrogens Uses a Novel Non-Classical Mechanism of Oestrogen Receptor Action. Journal of neuroendocrinology 17(4):197-207.

Kuang, S., K. Kuroda, F. Le Grand, and M. A. Rudnicki. 2007. Asymmetric self-renewal and commitment of satellite stem cells in muscle. Cell 129(5):999-1010.

Loureiro, J., P. Sandoval, G. del Peso, G. Gónzalez-Mateo, V. Fernández-Millara, B. Santamaria, M. A. Bajo, J. A. Sánchez-Tomero, G. Guerra-Azcona, and R. Selgas. 2013. Tamoxifen ameliorates peritoneal membrane damage by blocking mesothelial to mesenchymal transition in peritoneal dialysis. PloS one 8(4):e61165.

Mandlekar, S., and A.-N. Kong. 2001. Mechanisms of tamoxifen-induced apoptosis. Apoptosis 6(6):469-477.

Matsumine, H., K. Hirato, T. Yanaihara, T. TAMADA, and M. YOSHIDA. 1986. Aromatization by skeletal muscle. The Journal of Clinical Endocrinology & Metabolism 63(3):717-720.

Mauro, A. 1961. Satellite cell of skeletal muscle fibers. The Journal of biophysical and biochemical cytology 9(2):493.

Montarras, D., J. Chelly, E. Bober, H. Arnold, M. Ott, F. Gros, and C. Pinset. 1991. Developmental patterns in the expression of Myf5, MyoD, myogenin, and MRF4 during myogenesis. The New biologist 3(6):592-600.

Moretti, I., S. Ciciliot, K. A. Dyar, R. Abraham, M. Murgia, L. Agatea, T. Akimoto, S. Bicciato, M. Forcato, and P. Pierre. 2016. MRF4 negatively regulates adult skeletal muscle growth by repressing MEF2 activity. Nature Communications 7

Olson, E. N. 1992. Interplay between proliferation and differentiation within the myogenic lineage. Developmental biology 154(2):261-272.

Payne, W. G., F. Ko, S. Anspaugh, C. K. Wheeler, T. E. Wright, and M. C. Robson. 2006. Down-regulating causes of fibrosis with tamoxifen: a possible cellular/molecular approach to treat rhinophyma. Annals of plastic surgery 56(3):301-305.

Powles, T. J., S. Ashley, A. Tidy, I. E. Smith, and M. Dowsett. 2007. Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. Journal of the National Cancer Institute 99(4):283-290.

Ramaswamy, B., Y. Lu, K.-y. Teng, G. Nuovo, X. Li, C. L. Shapiro, and S. Majumder. 2012. Hedgehog signaling is a novel therapeutic target in tamoxifen-resistant breast cancer aberrantly activated by PI3K/AKT pathway. Cancer research 72(19):5048-5059.

Rhoads, R. P., R. M. Johnson, C. R. Rathbone, X. Liu, C. Temm-Grove, S. M. Sheehan, J. B. Hoying, and R. E. Allen. 2009. Satellite cell-mediated angiogenesis in-vitro coincides with a functional hypoxia-inducible factor pathway. American Journal of Physiology-Cell Physiology 296(6):C1321-C1328.

Richard, S. p., and H. Zingg. 1990. The human oxytocin gene promoter is regulated by estrogens. Journal of Biological Chemistry 265(11):6098-6103.

Rudnicki, M. A., P. N. Schnegelsberg, R. H. Stead, T. Braun, H.-H. Arnold, and R. Jaenisch. 1993. MyoD or Myf-5 is required for the formation of skeletal muscle. Cell 75(7):1351-1359.

Schiff, R., P. Reddy, M. Ahotupa, E. Coronado-Heinsohn, M. Grim, S. G. Hilsenbeck, R. Lawrence, S. Deneke, R. Herrera, and G. C. Chamness. 2000. Oxidative stress and AP-1 activity in tamoxifen-resistant breast tumors in-vivo. Journal of the National Cancer Institute 92(23):1926-1934.

Schulster, M., A. M. Bernie, and R. Ramasamy. 2016. The role of estradiol in male reproductive function. Asian journal of andrology 18(3):435.

Shang, Y. 2006. Molecular mechanisms of oestrogen and SERMs in endometrial carcinogenesis. Nature Reviews Cancer 6(5):360-368.

Shang, Y., and M. Brown. 2002. Molecular determinants for the tissue specificity of SERMs. Science 295(5564):2465-2468.

Sharples, A. P., D. C. Hughes, C. S. Deane, A. Saini, C. Selman, and C. E. Stewart. 2015. Longevity and skeletal muscle mass: the role of IGF signalling, the sirtuins, dietary restriction and protein intake. Aging cell 14(4):511-523.

Shupnik, M. A. 2004. Crosstalk between steroid receptors and the c-Src-receptor tyrosine kinase pathways: implications for cell proliferation. Oncogene 23(48):7979-7989.

Soloff, M. S., M. Alexandrova, and M. J. Fernstrom. 1979. Oxytocin receptors: triggers for parturition and lactation? Science 204(4399):1313-1315.

Stedronsky, K., R. Telgmann, G. Tillmann, N. Walther, and R. Ivell. 2002. The affinity and activity of the multiple hormone response element in the proximal promoter of the human oxytocin gene. Journal of neuroendocrinology 14(6):472-485.

Tiidus, P. M., D. A. Lowe, and M. Brown. 2013. Estrogen replacement and skeletal muscle: mechanisms and population health. Journal of Applied Physiology 115(5):569-578.

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.

Wang, Y. X., and M. A. Rudnicki. 2012. Satellite cells, the engines of muscle repair. Nature reviews Molecular cell biology 13(2):127-133.

Wright, W. E., D. A. Sassoon, and V. K. Lin. 1989. myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. Cell 56(4):607-617.

Yates, D. T., D. S. Clarke, A. R. Macko, M. J. Anderson, L. A. Shelton, M. Nearing, R. E. Allen, R. P. Rhoads, and S. W. Limesand. 2014. Myoblasts from intrauterine growth-restricted sheep fetuses exhibit intrinsic deficiencies in proliferation that contribute to smaller semitendinosus myofibres. The Journal of physiology 592(14):3113-3125.

Yin, H., F. Price, and M. A. Rudnicki. 2013. Satellite cells and the muscle stem cell niche. Physiological reviews 93(1):23-67.

Table 2.1

Primer sequences and TaqMan probes for RT-PCR

Gene (RefSeq ID)	Animal	Primer (5'-3')	Amplicon size(bp)
OXT (NM_176855)	Cattle	F[1]: CTGCCAAGAGGAGAACTACC R[1]: CCTGGGGATGATTACAGAGG P[1]: FAM-CTTCTCCCAGCACTGAGACC-BHQ1	218
PPIA (NM_178320)	Cattle	F: GCCCCAACACAAATGGTT R: CCCTCTTTCACCTTGCCAAAG P: HEX-TGCTTGCCATCCAACCACTCAGTC-BHQ1	96
OXTR (NM_174134)	Cattle	F: CAAGGAAGCCTCACCTTTCA R: TGCACAAGTTCTTGGAAGAGG	111
MyoD (NM_001040478)	Cattle	F: CGACTCGGACGCTTCCAGT R: GATGCTGGACAGGCAGTCGA	180
MyoG (NM_001111325)	Cattle	F: GTGCCCAGTGAATGCAGCTC R: GTCTGTAGGGTCCGCTGGGA	110
MyoG (XM_002685738)	Cattle	F: GAGTTCGATTAGCCGAGTGC R: ATGCTGTGCTTGGCTTTCTT	105

[1]Note: F, forward primer; R, reverse primer; P, TaqMan probe

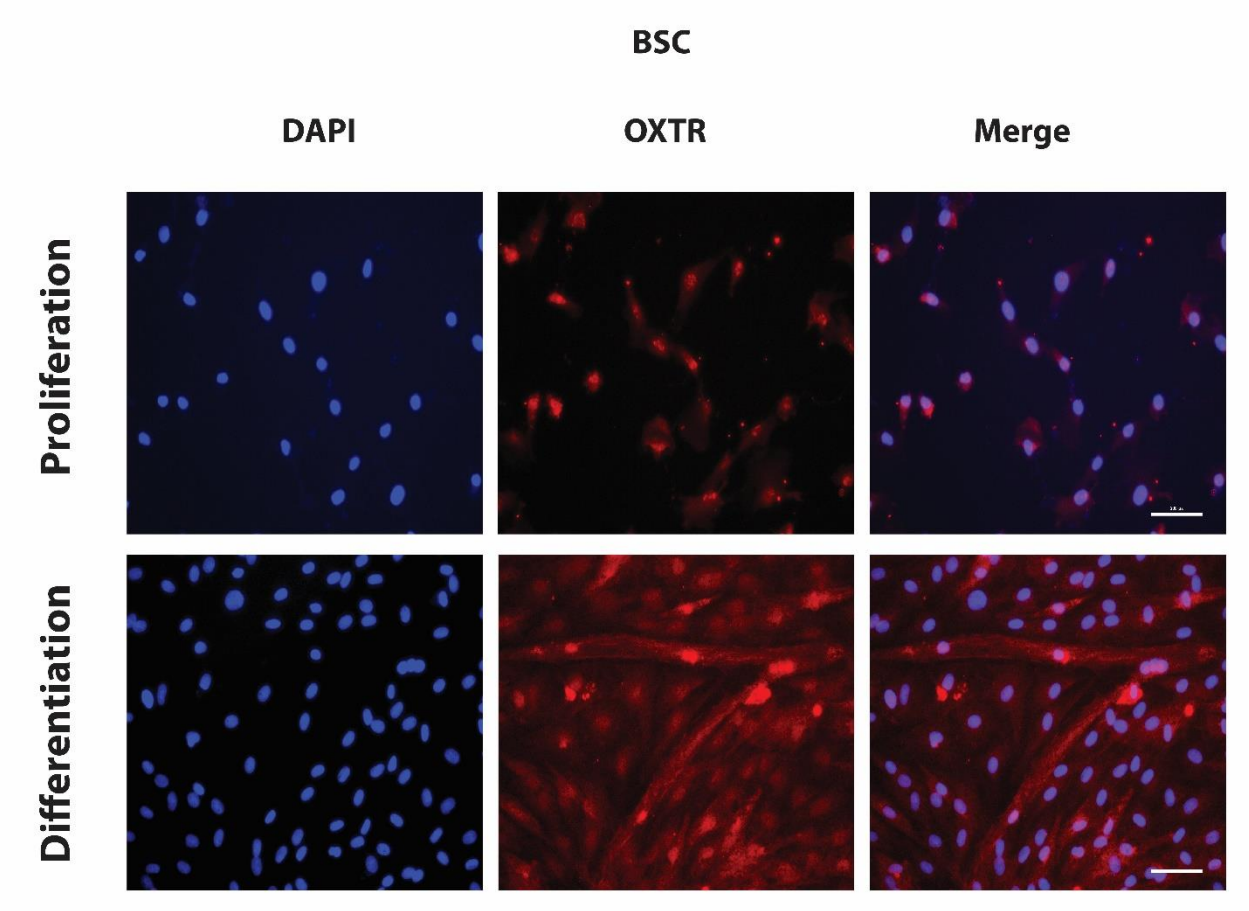
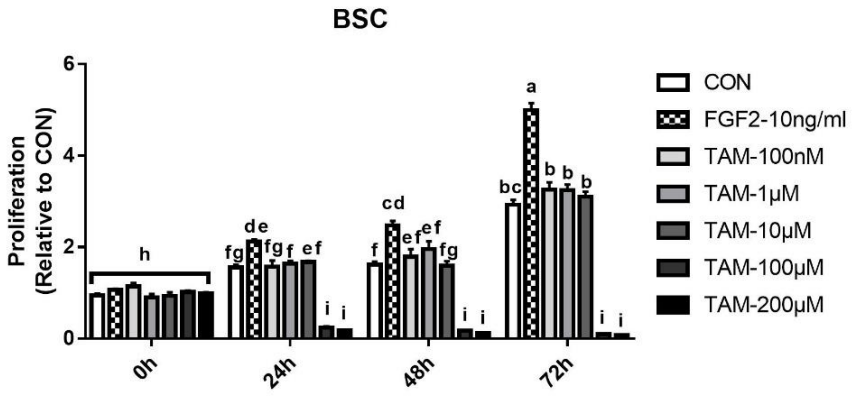
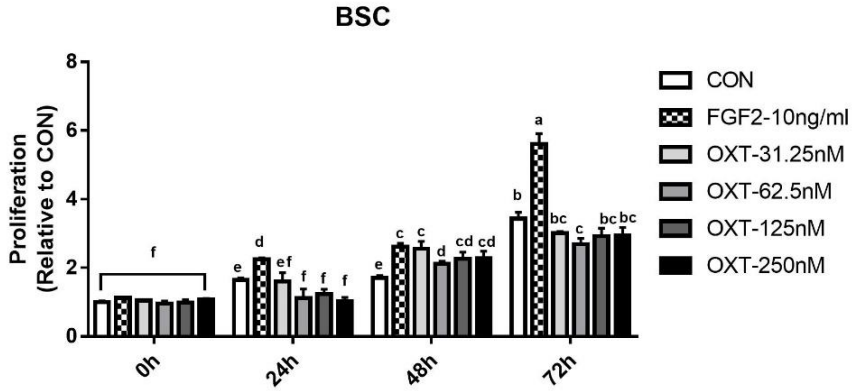


Fig. 2.1. Immunocytofluorescence of OXTR. OXTR antibody was used to detect the expression of OXTR in BSC during proliferation and differentiation. Both BSC and myotubes expressed OXTR. Magnification bar = 100 μm.

(A)


BSC
Proliferation (Relative to CON)
0h
24h
48h
72h
CON
FGF2-10ng/ml
TAM-100nM
TAM-1μM
TAM-10μM
TAM-100μM
TAM-200μM

(B)


BSC
Proliferation (Relative to CON)
0h
24h
48h
72h
CON
FGF2-10ng/ml
OXT-31.25nM
OXT-62.5nM
OXT-125nM
OXT-250nM

(C)

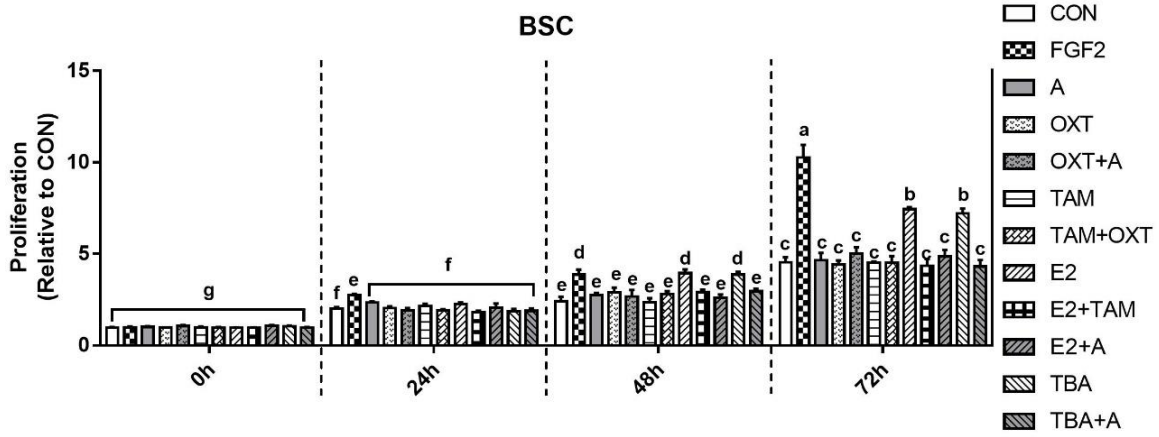
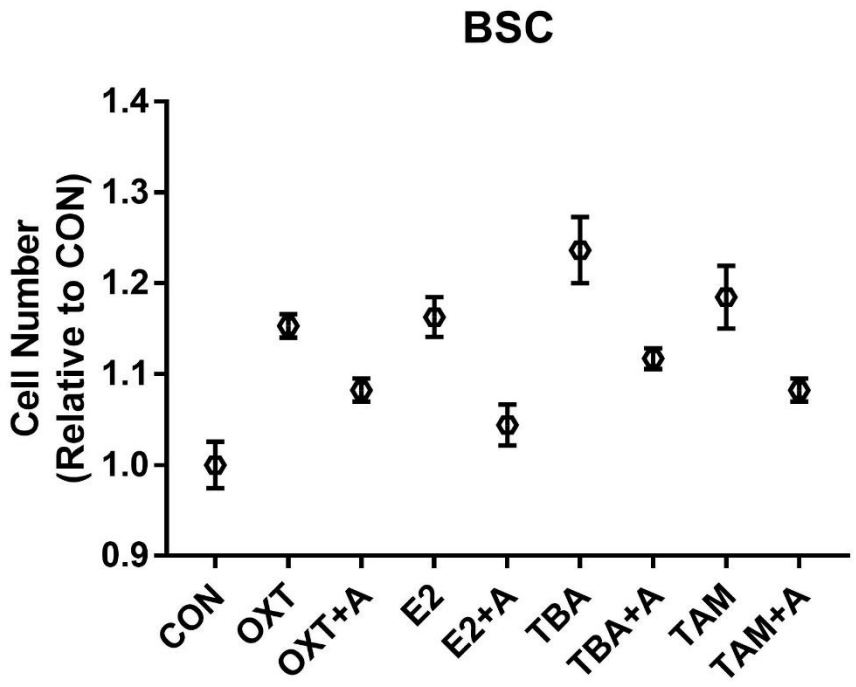

BSC
Proliferation (Relative to CON)
0h
24h
48h
72h
CON
FGF2
A
OXT
OXT+A
TAM
TAM+OXT
E2
E2+TAM
E2+A
TBA
TBA+A

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 $P<0.05$.

(A)


BSC
Cell Number (Relative to CON)
1.4
1.3
1.2
1.1
1.0
0.9
CON
OXT
OXT+A
E2
E2+A
TBA
TBA+A
TAM
TAM+A

(B)

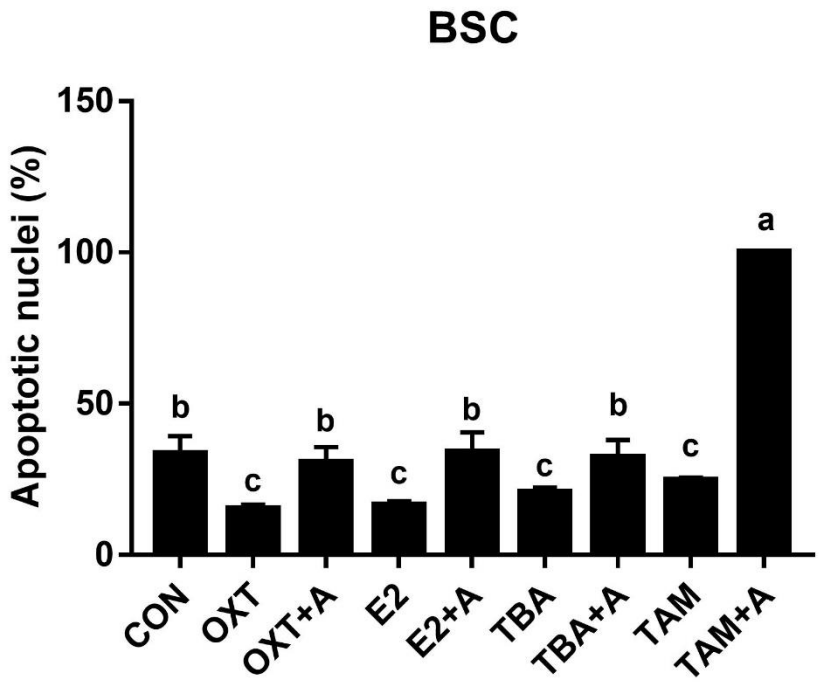
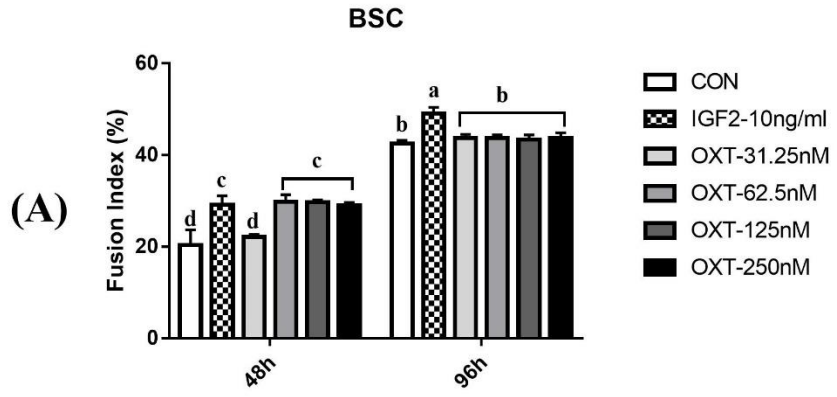
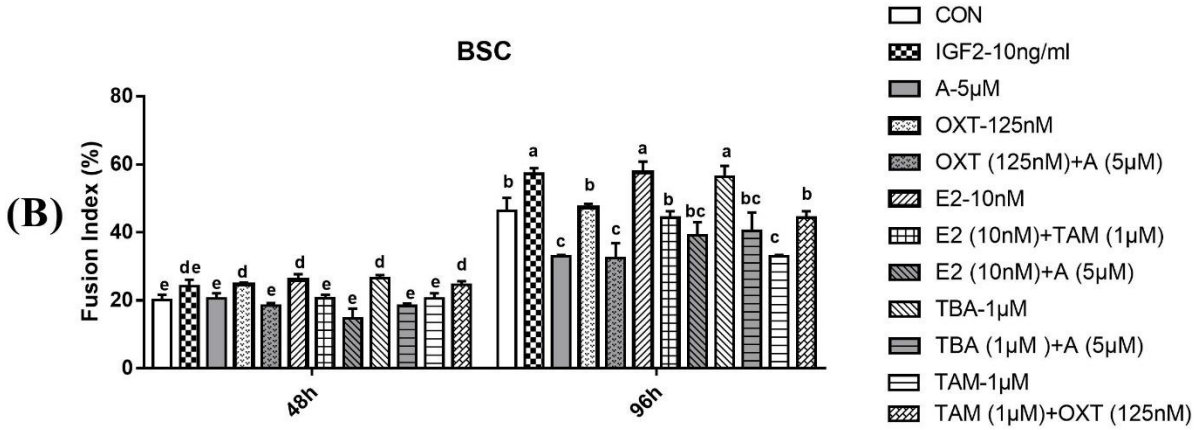

BSC
Apoptotic nuclei (%)
150
100
50
0
b
c
b
c
b
c
b
c
a
CON
OXT
OXT+A
E2
E2+A
TBA
TBA+A
TAM
TAM+A

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.


BSC
(A)
Fusion Index (%)
60
40
20
0
48h
96h
CON
IGF2-10ng/ml
OXT-31.25nM
OXT-62.5nM
OXT-125nM
OXT-250nM


BSC
(B)
Fusion Index (%)
80
60
40
20
0
48h
96h
CON
IGF2-10ng/ml
A-5μM
OXT-125nM
OXT (125nM)+A (5μM)
E2-10nM
E2 (10nM)+TAM (1μM)
E2 (10nM)+A (5μM)
TBA-1μM
TBA (1μM)+A (5μM)
TAM-1μM
TAM (1μM)+OXT (125nM)

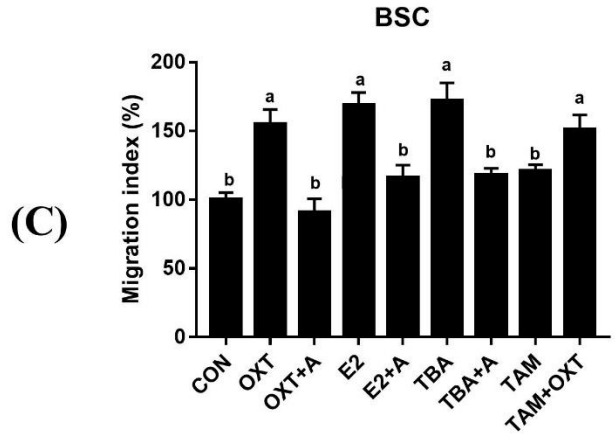

BSC
(C)
Migration index (%)
200
150
100
50
0
CON
OXT
OXT+A
E2
E2+A
TBA
TBA+A
TAM
TAM+OXT

Fig. 2.4. Fusion index and migration in BSC. For fusion index study, cells were plated at the density of 5×104 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.

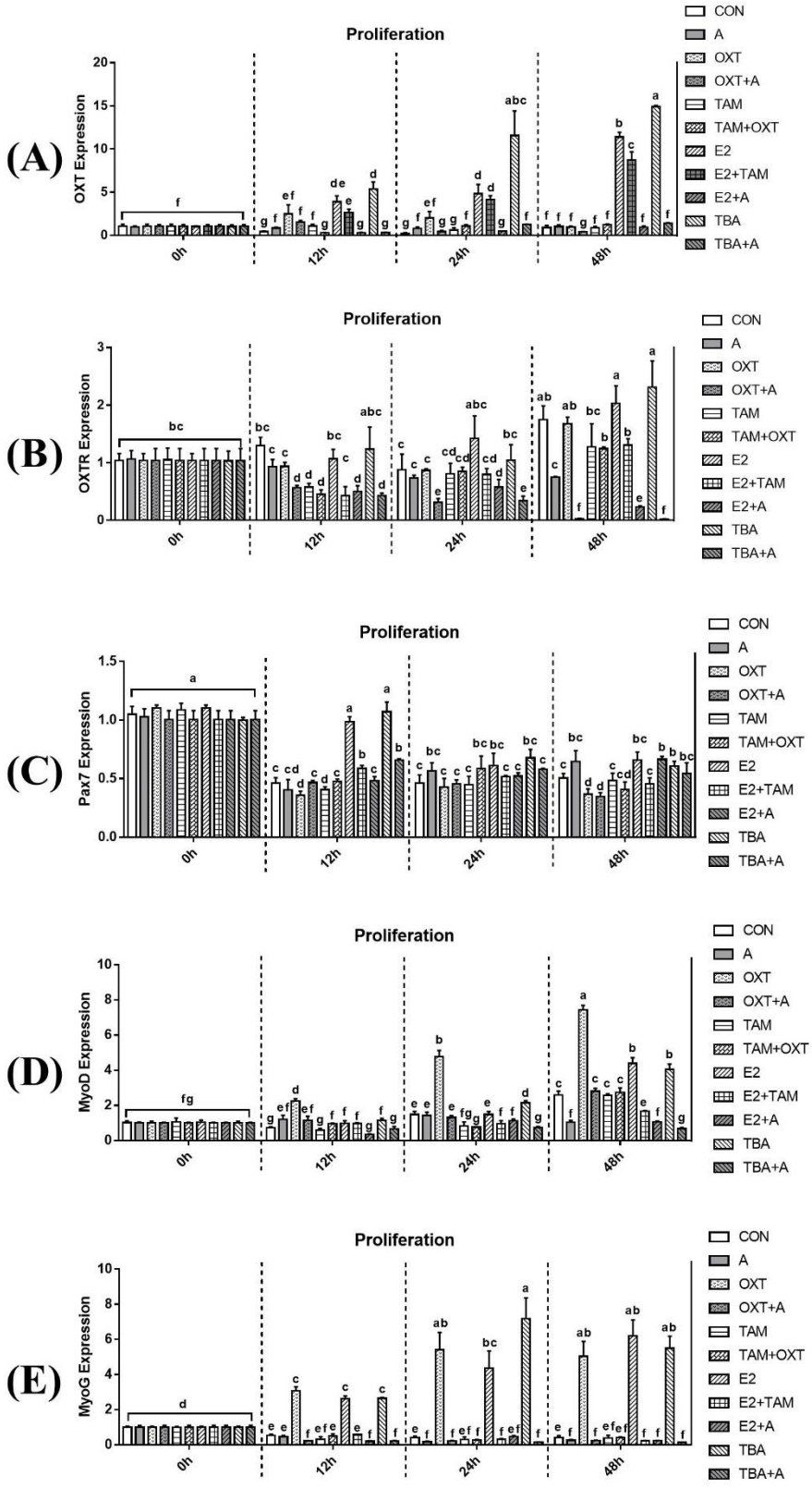

(A)
Proliferation
OXT Expression
0h
12h
24h
48h
CON
A
OXT
OXT+A
TAM
TAM+OXT
E2
E2+TAM
E2+A
TBA
TBA+A
(B)
Proliferation
OXTR Expression
(C)
Proliferation
Pax7 Expression
(D)
Proliferation
MyoD Expression
(E)
Proliferation
MyoG Expression

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.

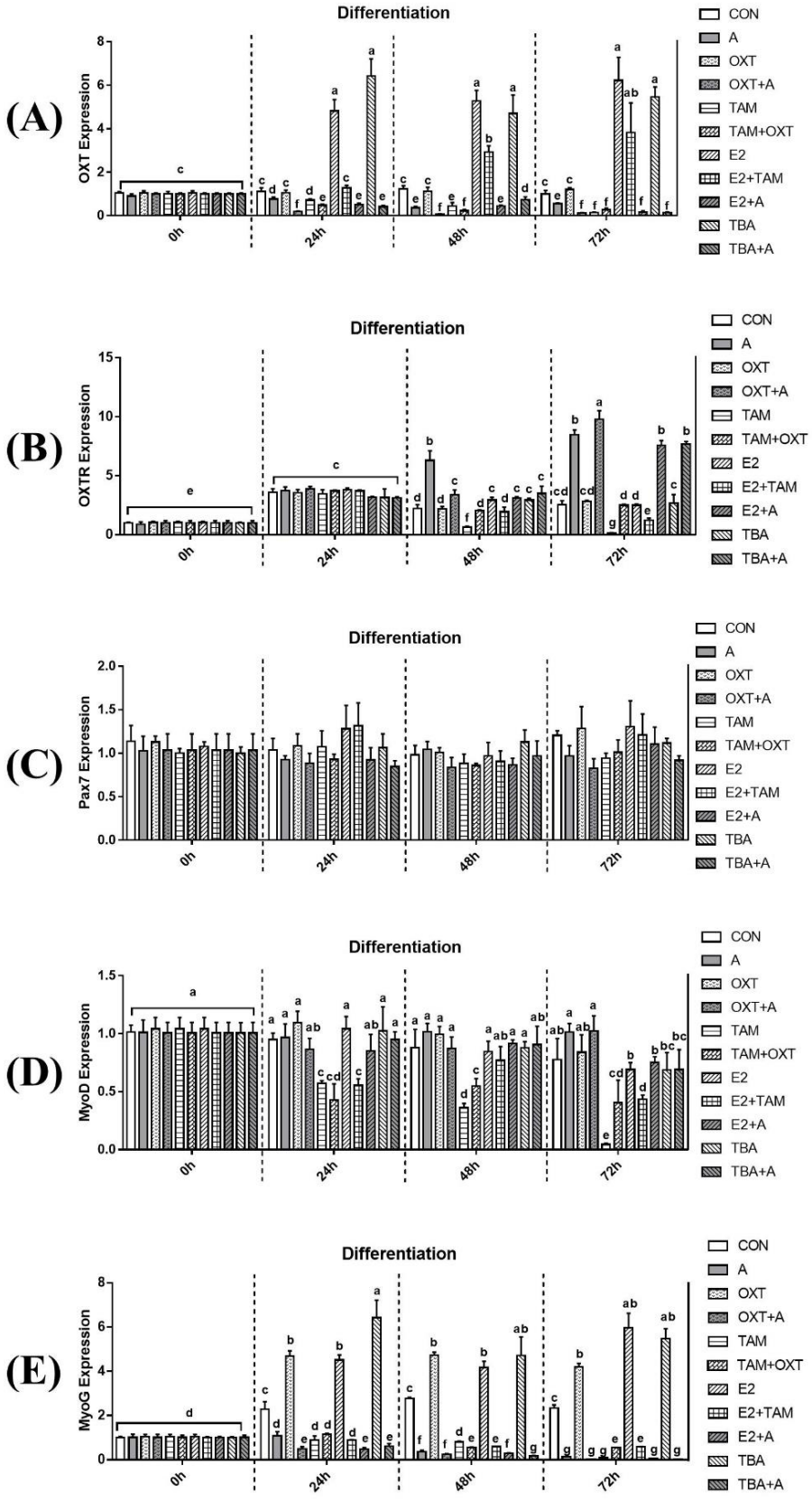

(A)
OXT Expression
Differentiation
0h
24h
48h
72h
CON
A
OXT
OXT+A
TAM
TAM+OXT
E2
E2+TAM
E2+A
TBA
TBA+A
(B)
OXTR Expression
(C)
Pax7 Expression
(D)
MyoD Expression
(E)
MyoG Expression

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 CRISRP-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% CO_2) 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℃. 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 transcribts 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 (2.5×10^6 cells) were plated in a 10cm dish and cultured at 37℃. 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℃ 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 (2×10^6 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℃ 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 + 1×ITS) 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 (1.5×10^5cell/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 $2^{-\Delta\Delta Cq}$ 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

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 CRIPSR-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 CRIPSR-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

Abmayr, S. M., and G. K. Pavlath. 2012. Myoblast fusion: lessons from flies and mice. Development 139(4):641-656.

Bae, G.-U., U. Gaio, Y.-J. Yang, H.-J. Lee, J.-S. Kang, and R. S. Krauss. 2008. Regulation of myoblast motility and fusion by the CXCR4-associated sialomucin, CD164. Journal of Biological Chemistry 283(13):8301-8309.

Berio, E., S. Divari, L. S. Cucuzza, B. Biolatti, and F. T. Cannizzo. 2017. 17β-estradiol upregulates oxytocin and the oxytocin receptor in C2C12 myotubes. PeerJ 5:e3124.

Blaauw, B., and C. Reggiani. 2014. The role of satellite cells in muscle hypertrophy. Journal of muscle research and cell motility 35(1):3-10.

Breton, C., C. Haenggeli, C. Barberis, F. Heitz, C. R. Bader, L. Bernheim, and E. Tribollet. 2002. Presence of functional oxytocin receptors in cultured human myoblasts. The Journal of Clinical Endocrinology & Metabolism 87(3):1415-1418.

Brownstein, M. J., J. T. Russell, and H. Gainer. 1980. Synthesis, transport, and release of posterior pituitary hormones. Science 207(4429):373-378.

Cattaneo, M., B. Chini, and L. Vicentini. 2008. Oxytocin stimulates migration and invasion in human endothelial cells. British journal of pharmacology 153(4):728-736.

Charge, S. B., and M. A. Rudnicki. 2004. Cellular and molecular regulation of muscle regeneration. Physiological reviews 84(1):209-238.

Chen, Y., J. D. Zajac, and H. E. MacLean. 2005. Androgen regulation of satellite cell function. Journal of Endocrinology 186(1):21-31.

De Jager, N., N. J. Hudson, A. Reverter, Y.-H. Wang, S. H. Nagaraj, L. M. Cafe, P. L. Greenwood, R. T. Barnard, K. P. Kongsuwan, and B. P. Dalrymple. 2011. Chronic exposure to anabolic steroids induces the muscle expression of oxytocin and a more than fifty fold increase in circulating oxytocin in cattle. Physiological genomics

Divari, S., P. Pregel, F. Cannizzo, L. S. Cucuzza, N. Brina, and B. Biolatti. 2013. Oxytocin precursor gene expression in bovine skeletal muscle is regulated by 17β-oestradiol and dexamethasone. Food chemistry 141(4):4358-4366.

Elabd, C., W. Cousin, P. Upadhyayula, R. Y. Chen, M. S. Chooljian, J. Li, S. Kung, K. P. Jiang, and I. M. Conboy. 2014. Oxytocin is an age-specific circulating hormone that is necessary for muscle maintenance and regeneration. Nature communications 5

Enns, D. L., and P. M. Tiidus. 2008. Estrogen influences satellite cell activation and proliferation following downhill running in rats. Journal of applied physiology 104(2):347-353.

Faghihi, M., A. M. Alizadeh, V. Khori, M. Latifpour, and S. Khodayari. 2012. The role of nitric oxide, reactive oxygen species, and protein kinase C in oxytocin-induced cardioprotection in ischemic rat heart. Peptides 37(2):314-319.

Fuchs, A.-R., R. Romero, D. Keefe, M. Parra, E. Oyarzun, and E. Behnke. 1991. Oxytocin secretion and human parturition: pulse frequency and duration increase during spontaneous labor in women. American journal of obstetrics and gynecology 165(5):1515-1523.

Gimpl, G., and F. Fahrenholz. 2001. The oxytocin receptor system: structure, function, and regulation. Physiological reviews 81(2):629-683.

Green, L., D. Fein, C. Modahl, C. Feinstein, L. Waterhouse, and M. Morris. 2001. Oxytocin and autistic disorder: alterations in peptide forms. Biological psychiatry 50(8):609-613.

Hughes, S. M., and H. M. Blau. 1990. Migration of myoblasts across basal lamina during skeletal muscle development. Nature 345(6273):350.

Jankowski, M., F. Hajjar, S. Al Kawas, S. Mukaddam-Daher, G. Hoffman, S. M. McCann, and J. Gutkowska. 1998. Rat heart: a site of oxytocin production and action. Proceedings of the National Academy of Sciences 95(24):14558-14563.

Joulia, D., H. Bernardi, V. Garandel, F. Rabenoelina, B. Vernus, and G. Cabello. 2003. Mechanisms involved in the inhibition of myoblast proliferation and differentiation by myostatin. Experimental cell research 286(2):263-275.

Kahlert, S., C. Grohé, R. H. Karas, K. Löbbert, L. Neyses, and H. Vetter. 1997. Effects of estrogen on skeletal myoblast growth. Biochemical and biophysical research communications 232(2):373-378.

Kamanga-Sollo, E., M. White, M. Hathaway, K. Chung, B. Johnson, and W. Dayton. 2008. Roles of IGF-I and the estrogen, androgen and IGF-I receptors in estradiol-17β-and trenbolone acetate-stimulated proliferation of cultured bovine satellite cells. Domestic animal endocrinology 35(1):88-97.

Kamanga-Sollo, E., M. White, W. Weber, and W. Dayton. 2013. Role of estrogen receptor-α (ESR1) and the type 1 insulin-like growth factor receptor (IGFR1) in estradiol-stimulated proliferation of cultured bovine satellite cells. Domestic animal endocrinology 44(1):36-45.

Kamanga-Sollo, E., M. Pampusch, G. Xi, M. White, M. Hathaway, and W. Dayton. 2004. IGF-I mRNA levels in bovine satellite cell cultures: Effects of fusion and anabolic steroid treatment. Journal of cellular physiology 201(2):181-189.

Kongsuwan, K., M. R. Knox, P. G. Allingham, R. Pearson, and B. P. Dalrymple. 2012. The effect of combination treatment with trenbolone acetate and estradiol-17β on skeletal muscle expression and plasma concentrations of oxytocin in sheep. Domestic animal endocrinology 43(1):67-73.

Labun, K., T. G. Montague, J. A. Gagnon, S. B. Thyme, and E. Valen. 2016. CHOPCHOP v2: a web tool for the next generation of CRISPR genome engineering. Nucleic acids research:gkw398.

Lee, H.-J., H. K. Caldwell, A. H. Macbeth, S. G. Tolu, and W. S. Young 3rd. 2008. A conditional knockout mouse line of the oxytocin receptor. Endocrinology 149(7):3256-3263.

Magon, N., and S. Kalra. 2011. The orgasmic history of oxytocin: Love, lust, and labor. Indian journal of endocrinology and metabolism 15(7):156.

Montague, T. G., J. M. Cruz, J. A. Gagnon, G. M. Church, and E. Valen. 2014. CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing. Nucleic acids research 42(W1):W401-W407.

Noiseux, N., M. Borie, A. Desnoyers, A. Menaouar, L. M. Stevens, S. Mansour, B. A. Danalache, D.-C. Roy, M. Jankowski, and J. Gutkowska. 2012. Preconditioning of stem cells by oxytocin to improve their therapeutic potential. Endocrinology 153(11):5361-5372.

Sauerwein, H., and H. Meyer. 1989. Androgen and estrogen receptors in bovine skeletal muscle: relation to steroid-induced allometric muscle growth. Journal of animal science 67(1):206-212.

Sciote, J., M. Horton, Y. Zyman, and G. Pascoe. 2001. Differential effects of diminished oestrogen and androgen levels on development of skeletal muscle fibres in hypogonadal mice. Acta physiologica scandinavica 172(3):179-187.

Sinha-Hikim, I., S. M. Roth, M. I. Lee, and S. Bhasin. 2003. Testosterone-induced muscle hypertrophy is associated with an increase in satellite cell number in healthy, young men. American Journal of Physiology-Endocrinology and Metabolism 285(1):E197-E205.

Sinha-Hikim, I., W. E. Taylor, N. F. Gonzalez-Cadavid, W. Zheng, and S. Bhasin. 2004. Androgen receptor in human skeletal muscle and cultured muscle satellite cells: up-regulation by androgen treatment. The Journal of Clinical Endocrinology & Metabolism 89(10):5245-5255.

Tamma, R., G. Colaianni, L.-l. Zhu, A. DiBenedetto, G. Greco, G. Montemurro, N. Patano, M. Strippoli, R. Vergari, and L. Mancini. 2009. Oxytocin is an anabolic bone hormone. Proceedings of the National Academy of Sciences 106(17):7149-7154.

Theobald, G., A. Graham, J. Campbell, P. Gange, and W. Driscoll. 1948. Use of post-pituitary extract in obstetrics. British medical journal 2(4567):123.

Tiidus, P., M. Deller, and X. Liu. 2005. Oestrogen influence on myogenic satellite cells following downhill running in male rats: a preliminary study. Acta physiologica scandinavica 184(1):67-72.

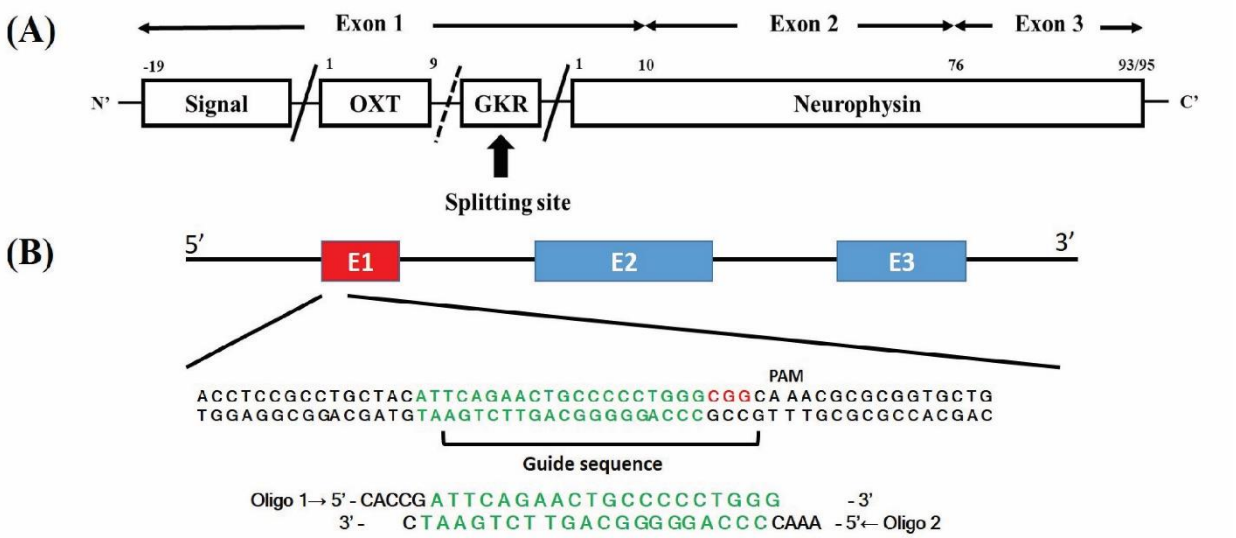
Verdijk, L. B., B. G. Gleeson, R. A. Jonkers, K. Meijer, H. H. Savelberg, P. Dendale, and L. J. van Loon. 2009. Skeletal muscle hypertrophy following resistance training is accompanied by a fiber type–specific increase in satellite cell content in elderly men. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences:gln050.

Yates, D. T., D. S. Clarke, A. R. Macko, M. J. Anderson, L. A. Shelton, M. Nearing, R. E. Allen, R. P. Rhoads, and S. W. Limesand. 2014. Myoblasts from intrauterine growth-restricted sheep fetuses exhibit intrinsic deficiencies in proliferation that contribute to smaller semitendinosus myofibres. The Journal of physiology 592(14):3113-3125.

Table 3.1. Primer sequences and TaqMan probes for RT-PCR

Gene (RefSeq ID)	Animal	Primer (5'-3')	Amplicon size(bp)
OXT (NM_176855)	Cattle	F: CTGCCAAGAGGAGAACTACC R: CCTGGGGATGATTACAGAGG P: FAM-CTTCTCCCAGCACTGAGACC-BHQ1	218
PPIA (NM_178320)	Cattle	F: GCCCCAACACAAATGGTT R: CCCTCTTTCACCTTGCCAAAG P: HEX-TGCTTGCCATCCAACCACTCAGTC-BHQ1	96
OXTR (NM_174134)	Cattle	F: CAAGGAAGCCTCACCTTTCA R: TGCACAAGTTCTTGGAAGAGG	111
MyoD (NM_001040478)	Cattle	F: CGACTCGGACGCTTCCAGT R: GATGCTGGACAGGCAGTCGA	180
MyoG (NM_001111325)	Cattle	F: GTGCCCAGTGAATGCAGCTC R: GTCTGTAGGGTCCGCTGGGA	110
MyoG (XM_002685738)	Cattle	F: GAGTTCGATTAGCCGAGTGC R: ATGCTGTGCTTGGCTTTCTT	105

Note: F, forward primer; R, reverse primer; P, TaqMan probe



(C)

Deletions

TGCTACATTCAGAACTGCCCCCTGGGCGGCAAACGCGCGG	(×11)	Length	MAGSSLACCLLGLLALTSACYIQNCPLGGKR	Length
TGCTACATTCAGAACTGCCCCCT------CAAACGCGCGG	(×1)	Δ6	MAGSSLACCLLGLLALTSACYIQNCPL--KR	Δ2
TGCTACATTCAGAACTGC-------GCGGCAAACGCGCGG	(×1)	Δ7	MAGSSLACCLLGLLALTSACYIQNC------	Δ6
TGCTACATTCAGAACTGC------------------GCGG	(×1)	Δ18	MAGSSLACCLLGLLALTSACYIQNC------	Δ6
TGCTACATTCAGAACTGC--------------------GG	(×1)	Δ20	MAGSSLACCLLGLLALTSACYIQNC------	Δ6
TGCTACATTCA--------------------AACGCGCGG	(×2)	Δ20	MAGSSLACCLLGLLALTSACYIQ--------	Δ7
TGCTACATTCAGAACTGCCCC------GGCAAACGCGCGG	(×1)	Δ6	MAGSSLACCLLGLLALTSACYIQNCP--GKR	Δ2
TGCTACATTCAGAACTGCCCCCT--------------CGG	(×1)	Δ14	MAGSSLACCLLGLLALTSACYIQNCPLG---	Δ3
TGCTACATTCAGAACTGCCCC-----CGGCA--CGCGCGG	(×3)	Δ6+2	MAGSSLACCLLGLLALTSACYIQNCP-----	Δ5
TGCTACATTCAGAACTGC---------GGCAAACGCGCGG	(×13)	Δ9	MAGSSLACCLLGLLALTSACYIQNC---GKR	Δ3
TGCTACATTCAGAAC------------------GCGCGG	(×1)	Δ19	MAGSSLACCLLGLLALTSACYIQN-------	Δ7

Insertions

TGCTACATTCAGAACT-GCCCCCTGGGCGGCAAACGCGCGG	(×11)		MAGSSLACCLLGLLALTSACYIQNCPLGGKR	Length
TGCTACATTCAGAACTTGCCCCCTGGGCGGCAAACGCGCGG	(×11)	Δ1	MAGSSLACCLLGLLALTSACYIQNCPLG---	Δ2

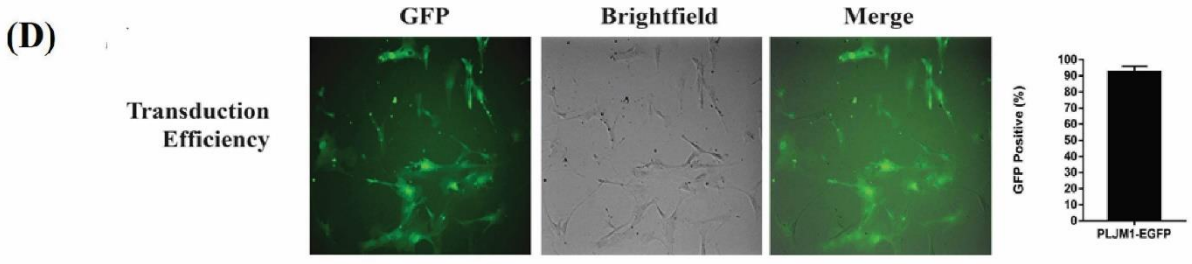


Fig. 3.1. OXT disruption in BSC using CRISPR/Cas9 genome editing. (A) Domain organization of preprooxytocin including the splitting site. (B) sgRNA sequence was designed to bind sequence in the exon 1 region of the OXT gene, such that gene editing could disrupt *OXT* expression in BSC. PAM: protospacer-adjacent 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.

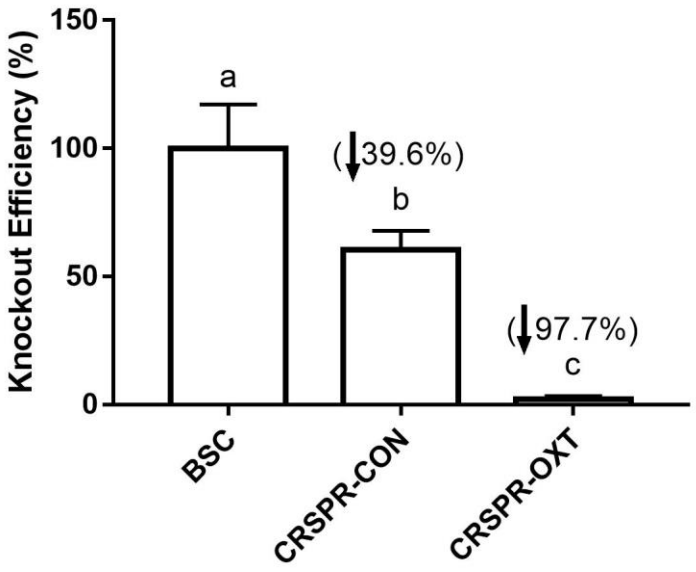


Fig. 3.2. Knockout efficiency after CRISPR/Cas9 genome editing. OXT mRNA expression was detected in wild-type BSC, CRISPR-CON cell, and CRISPR-OXT cell by qPCR. Groups with different letters have significant difference with $P < 0.05$. Means and SEMs are shown.

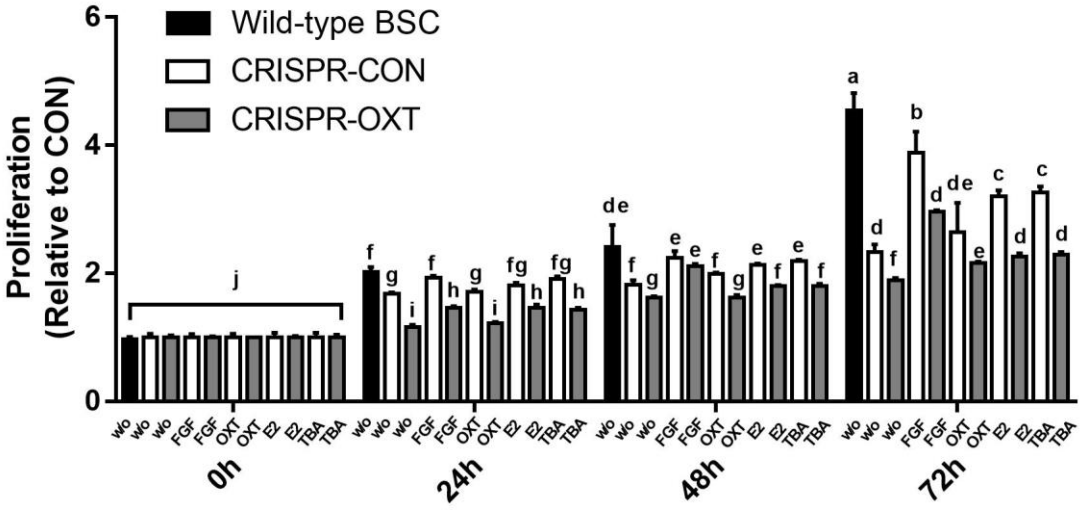


Fig. 3.3. Proliferation rate in CRISPR-CON and CRISPR-OXT cell. CyQUANT® NF Cell Proliferation Assay Kit was used to determine the proliferation rate in low serum medium (DMEM + 2%FBS + 1×ITS) with different treatments in CRISPR-CON cell and CRISPR-OXT cell. Values are means and SEMs. Groups with different letters have significant difference with $P < 0.05$.

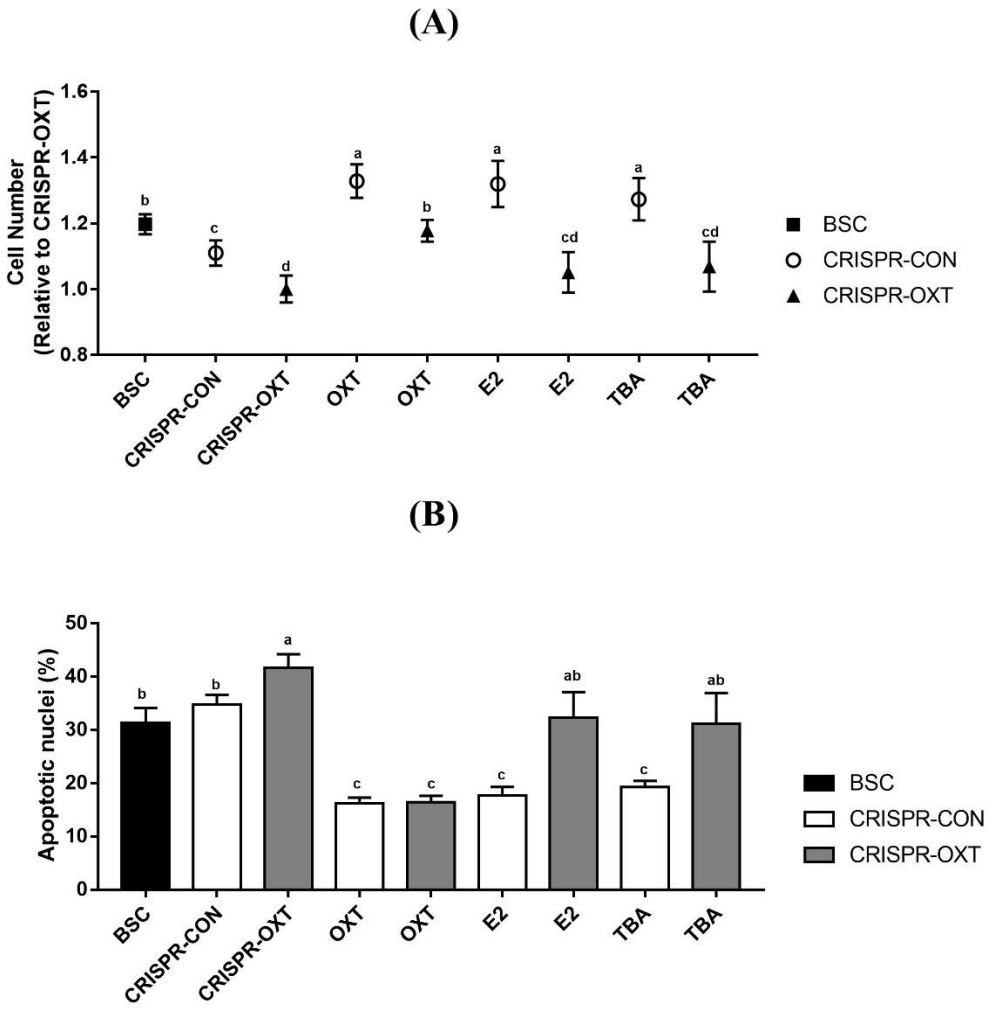


Fig. 3.4. Effect of OXT on cell death in CRISPR-CON and CRISPR-OXT cell. CRISPR-CON and CRISPR-OXT cell were preconditioned with E2 (10 nM), OXT (125 nM) and TBA (1 μM). After 48h treatments, cells were treated with serum free medium and 200μM of hydrogen peroxide (H2O2) for another 48h. Following incubation, 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.

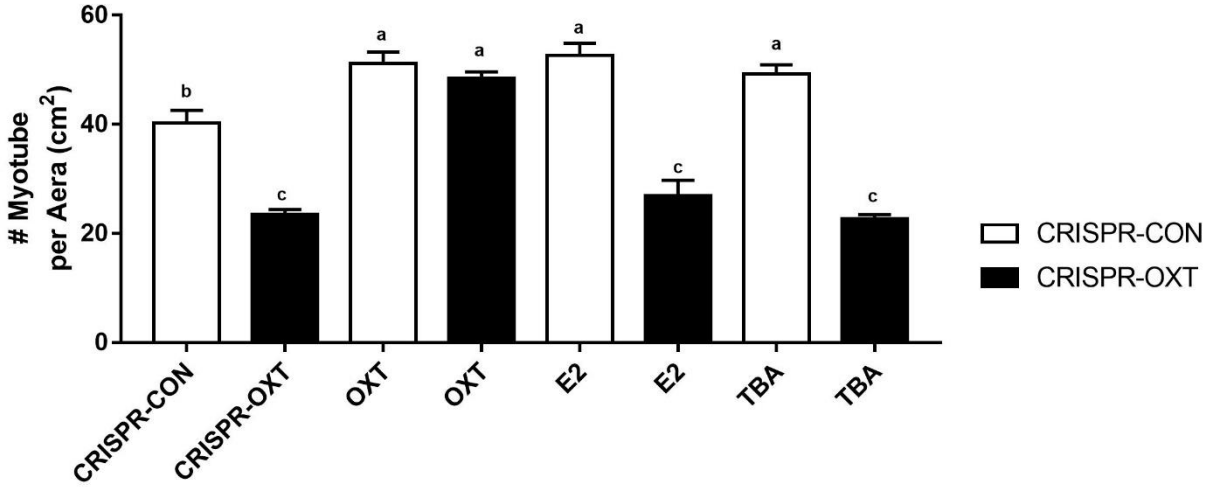


Fig. 3.5. Effect of OXT on differentiation in CRISPR-CON and CRISPR-OXT cell. CRISPR-CON and CRISPR-OXT cell were plated at the density of 5×10^4 cells/well in 24-well plates coated with ECL. At 75%-85% confluency, the medium was changed to differentiation medium (DM, DMEM+2%FBS+1%AbAm+0.5% of gentamicin) 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 $P < 0.05$.

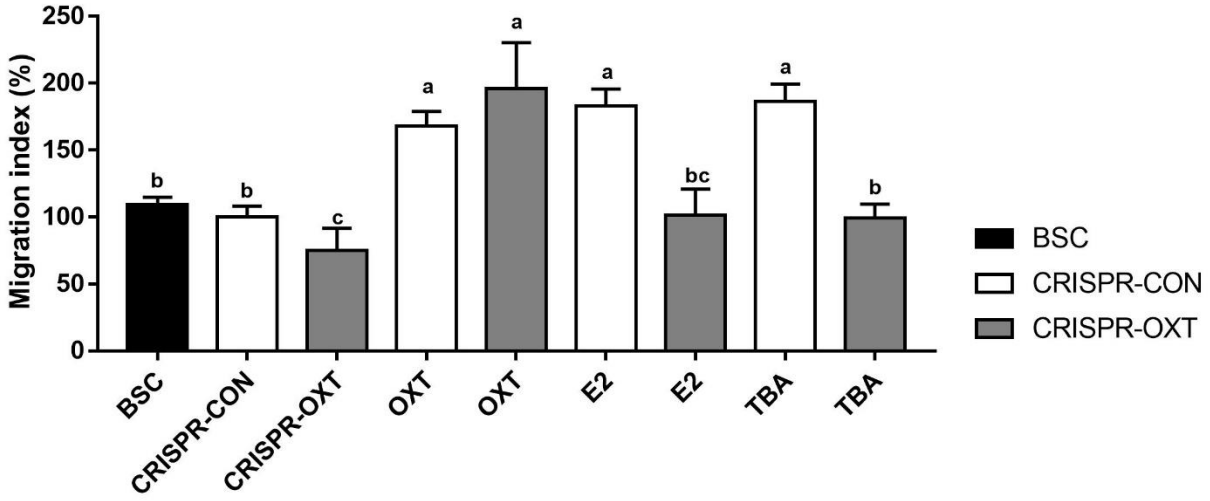


Fig. 3.6. Effect of OXT on migration in CRISPR-CON and CRISPR-OXT cell. CRISPR-CON and CRISPR-OXT cell were preconditioned with E2 (10 nM), OXT (125 nM), and TBA (1 μM). Then, the cells were plated in the top of modified Boyden chamber (Corning Costar, Cambridge, MA) and were allowed to migrate for 16h 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.

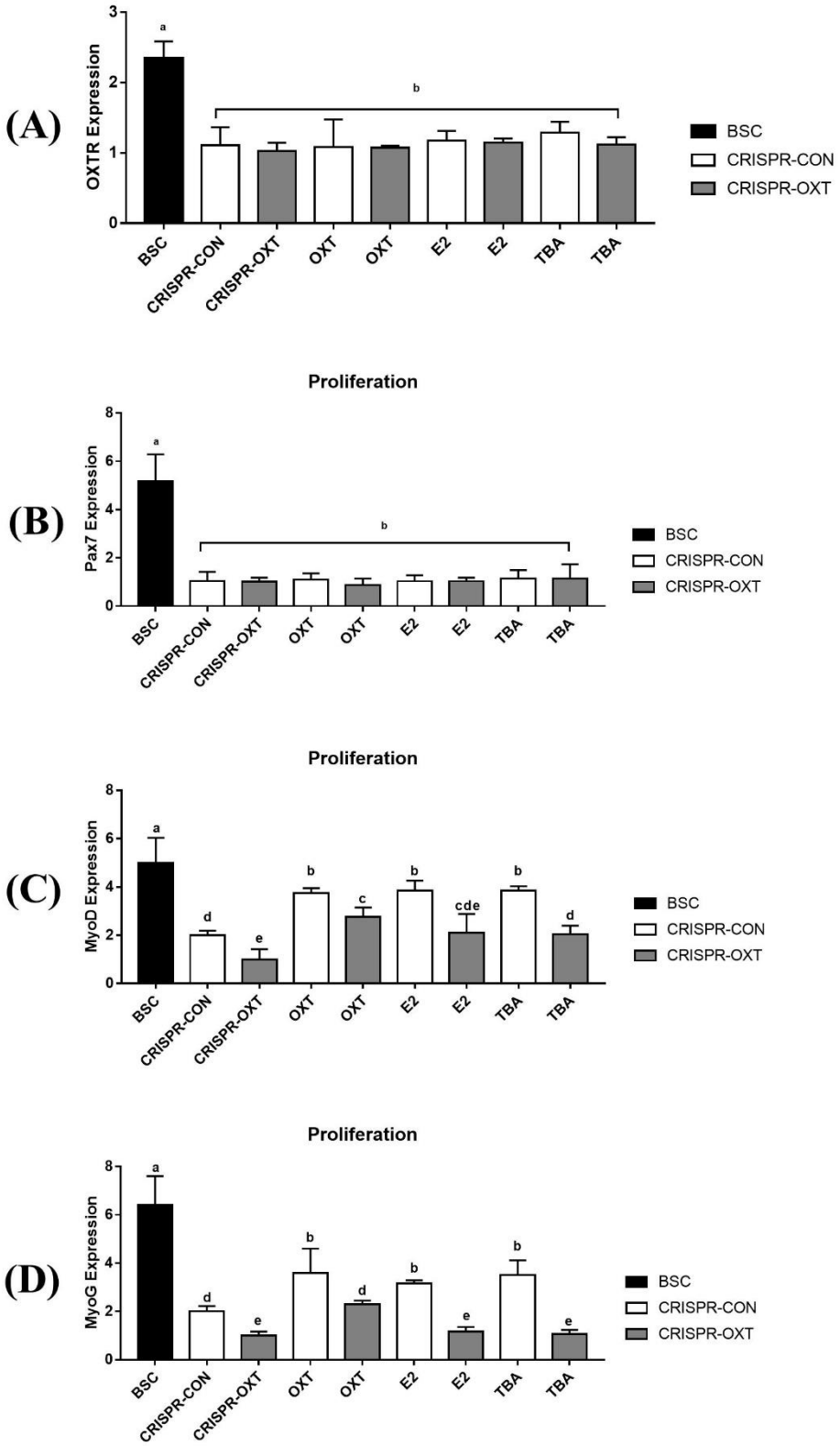
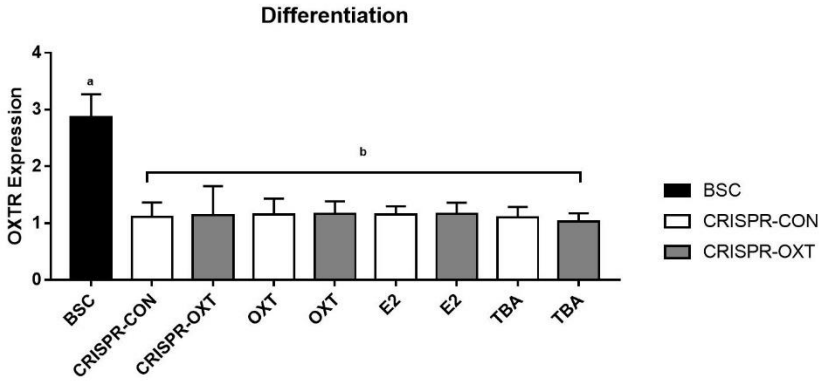
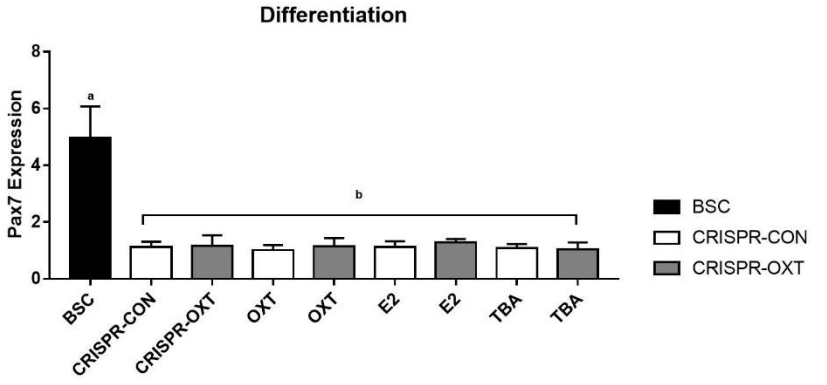

(A)
OXTR Expression
BSC
CRISPR-CON
CRISPR-OXT
BSC
CRISPR-CON
CRISPR-OXT
OXT
OXT
E2
E2
TBA
TBA
Proliferation
(B)
Pax7 Expression
Proliferation
(C)
MyoD Expression
Proliferation
(D)
MyoG Expression

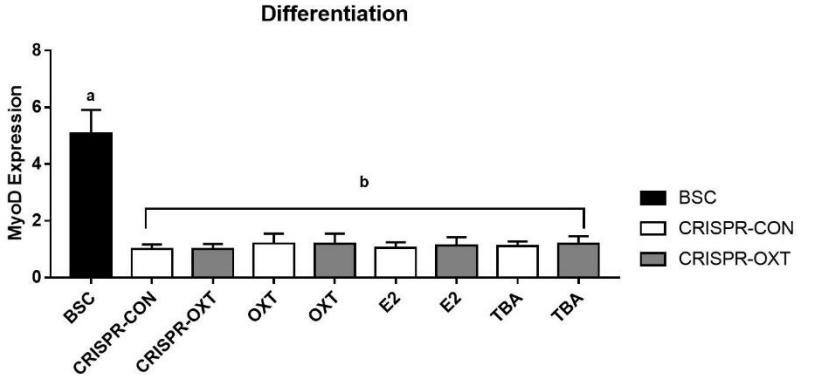
Fig. 3.7. Gene expression during CRISPR cell proliferation. The cells were cultured in DM with or without different treatments for 48 h. The medium was refreshed every 24 h. Total RNA was extracted at 48 h of culture. CON and treated myoblast lysates during proliferation were analyzed for gene mRNA expression 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.

(A)


Differentiation
OXTR Expression
4
3
2
1
0
a
b
BSC
CRISPR-CON
CRISPR-OXT
BSC
CRISPR-CON
CRISPR-OXT
OXT
OXT
E2
E2
TBA
TBA


Differentiation
Pax7 Expression
8
6
4
2
0
a
b
BSC
CRISPR-CON
CRISPR-OXT
BSC
CRISPR-CON
CRISPR-OXT
OXT
OXT
E2
E2
TBA
TBA

(C)


Differentiation
MyoD Expression
8
6
4
2
0
a
b
BSC
CRISPR-CON
CRISPR-OXT
BSC
CRISPR-CON
CRISPR-OXT
OXT
OXT
E2
E2
TBA
TBA

(D)

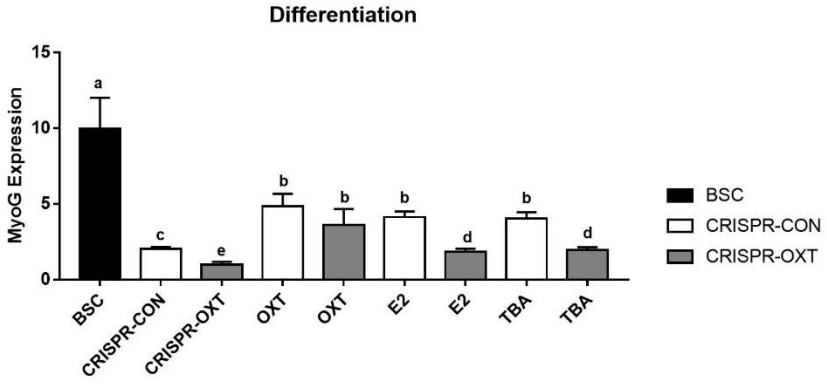

Differentiation
MyoG Expression
15
10
5
0
a
c
e
b
b
b
d
b
d
BSC
CRISPR-CON
CRISPR-OXT
BSC
CRISPR-CON
CRISPR-OXT
OXT
OXT
E2
E2
TBA
TBA

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℃ × 12h/d, 35℃ ×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℃. 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 21days 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 ℃ 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 harvest from each animal. Each sample was immediately frozen in liquid nitrogen and stored at -80 ℃ 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^{TM} 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 H_2O mix well in PCR tube. This mixture was incubated 5min at 25℃, 30min at 42℃, 5min at 85 ℃, and hold at 4℃. 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℃ followed by 40 cycles of 95℃ for 15s

and 60℃ 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 $2^{-\Delta\Delta Cq}$ 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℃ 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℃ for 1hour at 225rpm. The cells were then spread onto prewarmed selective LB agar and incubated at 37℃ 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 3×10^6 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 comparted 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 comparted 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

Andari, E., J.-R. Duhamel, T. Zalla, E. Herbrecht, M. Leboyer, and A. Sirigu. 2010. Promoting social behavior with oxytocin in high-functioning autism spectrum disorders. Proceedings of the National Academy of Sciences 107(9):4389-4394.

Banerjee, S., K. L. Chambliss, C. Mineo, and P. W. Shaul. 2014. Recent insights into non-nuclear actions of estrogen receptor alpha. Steroids 81:64-69.

Berio, E., S. Divari, L. S. Cucuzza, B. Biolatti, and F. T. Cannizzo. 2017. 17β-estradiol upregulates oxytocin and the oxytocin receptor in C2C12 myotubes. PeerJ 5:e3124.

Breton, C., C. Haenggeli, C. Barberis, F. Heitz, C. R. Bader, L. Bernheim, and E. Tribollet. 2002. Presence of functional oxytocin receptors in cultured human myoblasts. The Journal of Clinical Endocrinology & Metabolism 87(3):1415-1418.

Chen, X., A. L. Fahy, A. S. Green, M. J. Anderson, R. P. Rhoads, and S. W. Limesand. 2010. β2-Adrenergic receptor desensitization in perirenal adipose tissue in fetuses and lambs with

placental insufficiency-induced intrauterine growth restriction. The Journal of physiology 588(18):3539-3549.

Cranwell, C., J. Unruh, J. Brethour, D. Simms, and R. Campbell. 1996. Influence of steroid implants and concentrate feeding on performance and carcass composition of cull beef cows. Journal of animal science 74(8):1770-1776.

De Jager, N., N. J. Hudson, A. Reverter, Y.-H. Wang, S. H. Nagaraj, L. M. Cafe, P. L. Greenwood, R. T. Barnard, K. P. Kongsuwan, and B. P. Dalrymple. 2011. Chronic exposure to anabolic steroids induces the muscle expression of oxytocin and a more than fifty fold increase in circulating oxytocin in cattle. Physiological genomics

Deschenes, M. R. 2004. Effects of aging on muscle fibre type and size. Sports Medicine 34(12):809-824.

Divari, S., P. Pregel, F. Cannizzo, L. S. Cucuzza, N. Brina, and B. Biolatti. 2013. Oxytocin precursor gene expression in bovine skeletal muscle is regulated by 17β-oestradiol and dexamethasone. Food chemistry 141(4):4358-4366.

Dodson, M. V., G. J. Hausman, L. Guan, M. Du, T. P. Rasmussen, S. P. Poulos, P. Mir, W. G. Bergen, M. E. Fernyhough, and D. C. McFarland. 2010. Skeletal muscle stem cells from animals I. Basic cell biology. International Journal of Biological Sciences 6(5):465.

Elabd, C., W. Cousin, P. Upadhyayula, R. Y. Chen, M. S. Chooljian, J. Li, S. Kung, K. P. Jiang, and I. M. Conboy. 2014. Oxytocin is an age-specific circulating hormone that is necessary for muscle maintenance and regeneration. Nature communications 5

Enns, D. L., and P. M. Tiidus. 2010. The influence of estrogen on skeletal muscle. Sports Medicine 40(1):41-58.

Fuchs, A.-R., F. Fuchs, P. Husslein, M. S. Soloff, and M. J. Fernstrom. 1982. Oxytocin receptors and human parturition: a dual role for oxytocin in the initiation of labor. Obstetrical & Gynecological Survey 37(9):567-568.

Gimpl, G., and F. Fahrenholz. 2001. The oxytocin receptor system: structure, function, and regulation. Physiological reviews 81(2):629-683.

Gonzalez, J., J. Carter, D. Johnson, S. Ouellette, and S. Johnson. 2007. Effect of ractopamine-hydrochloride and trenbolone acetate on longissimus muscle fiber area, diameter, and satellite cell numbers in cull beef cows. Journal of animal science 85(8):1893-1901.

Hunter, R. 2010. Hormonal growth promotant use in the Australian beef industry. Animal Production Science 50(7):637-659.

Ivell, R., and S. Hartung. 2003. The molecular basis of cryptorchidism*. Molecular human reproduction 9(4):175-181.

Jankowski, M., B. Danalache, D. Wang, P. Bhat, F. Hajjar, M. Marcinkiewicz, J. Paquin, S. M. McCann, and J. Gutkowska. 2004. Oxytocin in cardiac ontogeny. Proceedings of the National Academy of Sciences of the United States of America 101(35):13074-13079.

Kamanga-Sollo, E., M. White, M. Hathaway, K. Chung, B. Johnson, and W. Dayton. 2008. Roles of IGF-I and the estrogen, androgen and IGF-I receptors in estradiol-17β-and trenbolone acetate-stimulated proliferation of cultured bovine satellite cells. Domestic animal endocrinology 35(1):88-97.

Kitajima, Y., and Y. Ono. 2016. Estrogens maintain skeletal muscle and satellite cell functions. Journal of Endocrinology 229(3):267-275.

Kiyama, R., and Y. Wada-Kiyama. 2015. Estrogenic endocrine disruptors: molecular mechanisms of action. Environment international 83:11-40.

Kongsuwan, K., M. Knox, P. Allingham, R. Pearson, and B. Dalrymple. 2012. The effect of combination treatment with trenbolone acetate and estradiol-17β on skeletal muscle expression and plasma concentrations of oxytocin in sheep. Domestic animal endocrinology 43(1):67-73.

Koohi, M., R. Ivell, and N. Walther. 2005. Transcriptional Activation of the Oxytocin Promoter by Oestrogens Uses a Novel Non-Classical Mechanism of Oestrogen Receptor Action. Journal of neuroendocrinology 17(4):197-207.

Lu, Y., J. S. Bradley, S. R. McCoski, J. M. Gonzalez, A. D. Ealy, and S. E. Johnson. 2017. Reduced skeletal muscle fiber size following caloric restriction is associated with calpain-mediated proteolysis and attenuation of IGF-1 signaling. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 312(5):R806-R815.

Powles, T. J., S. Ashley, A. Tidy, I. E. Smith, and M. Dowsett. 2007. Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. Journal of the National Cancer Institute 99(4):283-290.

Ramaswamy, B., Y. Lu, K.-y. Teng, G. Nuovo, X. Li, C. L. Shapiro, and S. Majumder. 2012. Hedgehog signaling is a novel therapeutic target in tamoxifen-resistant breast cancer aberrantly activated by PI3K/AKT pathway. Cancer research 72(19):5048-5059.

Sauerwein, H., and H. Meyer. 1989. Androgen and estrogen receptors in bovine skeletal muscle: relation to steroid-induced allometric muscle growth. Journal of animal science 67(1):206-212.

Shang, Y. 2006. Molecular mechanisms of oestrogen and SERMs in endometrial carcinogenesis. Nature Reviews Cancer 6(5):360-368.

Shupnik, M. A. 2004. Crosstalk between steroid receptors and the c-Src-receptor tyrosine kinase pathways: implications for cell proliferation. Oncogene 23(48):7979-7989.

Soloff, M. S., M. Alexandrova, and M. J. Fernstrom. 1979. Oxytocin receptors: triggers for parturition and lactation? Science 204(4399):1313-1315.

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.

Valsamakis, G., C. KANAKA-GANTENBEIN, A. MALAMITSI-PUCHNER, and G. Mastorakos. 2006. Causes of intrauterine growth restriction and the postnatal development of the metabolic syndrome. Annals of the New York Academy of Sciences 1092(1):138-147.

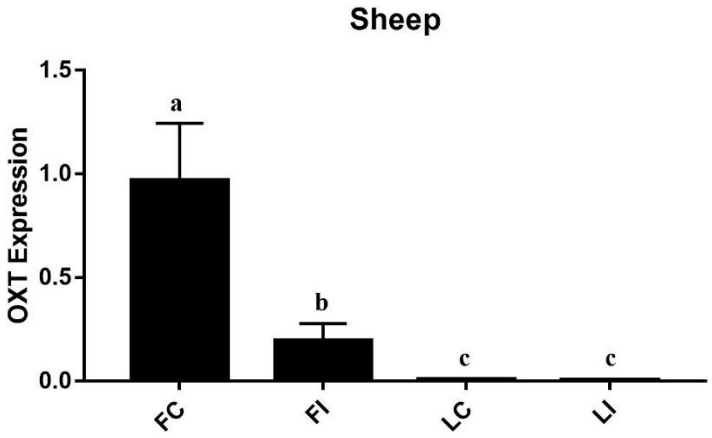
Yates, D. T., C. N. Cadaret, K. A. Beede, H. E. Riley, A. R. Macko, M. J. Anderson, L. E. Camacho, and S. W. Limesand. 2016. Intrauterine growth-restricted sheep fetuses exhibit smaller hindlimb muscle fibers and lower proportions of insulin-sensitive Type I fibers near term. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 310(11):R1020-R1029.

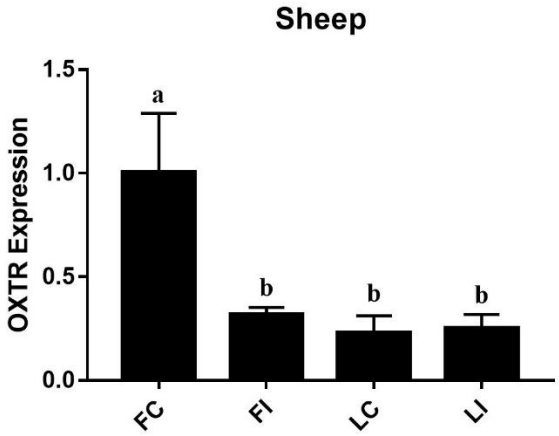
Table 1

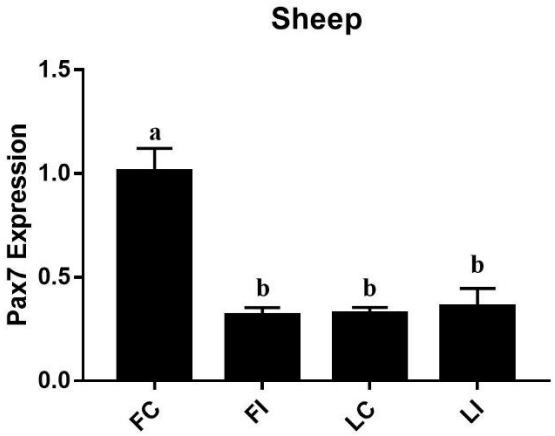
Primer sequences and TaqMan probes for RT-PCR

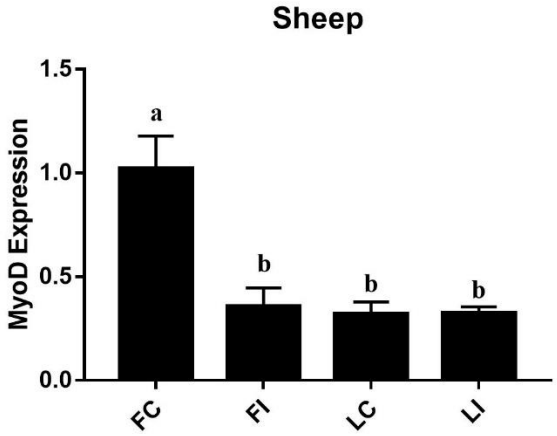
Gene (RefSeq ID)	Animal	Primer (5'-3')	Amplicon size(bp)
OXT (NM_176855)	Cattle	F[1]: CTGCCAAGAGGAGAACTACC R[1]: CCTGGGGATGATTACAGAGG P[1]: FAM-CTTCTCCCAGCACTGAGACC-BHQ1	218
PPIA (NM_178320)	Cattle	F: GCCCCAACACAAATGGTT R: CCCTCTTTCACCTTGCCAAAG P: HEX-TGCTTGCCATCCAACCACTCAGTC-BHQ1	96
OXTR (NM_174134)	Cattle	F: CAAGGAAGCCTCACCTTTCA R: TGCACAAGTTCTTGGAAGAGG	111
MyoD (NM_001040478)	Cattle	F: CGACTCGGACGCTTCCAGT R: GATGCTGGACAGGCAGTCGA	180
MyoG (NM_001111325)	Cattle	F: GTGCCCAGTGAATGCAGCTC R: GTCTGTAGGGTCCGCTGGGA	110
Myf5 (NM_174116)	Cattle	F: ACCAGCCCCACCTCAAGTTG R: GCAATCCAAGCTGGATAAGGAG	150
MRF4 (NM_181811)	Cattle	F: GGTGGACCCCTTCAGCTACAG R: TGCTTGTCCCTCCTTCCTTGG	140
MSTN (AB076403)	Cattle	F: GGCCATGATCTTGCTGTAACCT R: GCATCGAGATTCTGTGGAGTG	144
MyoG (XM_002685738)	Cattle	F: GAGTTCGATTAGCCGAGTGC R: ATGCTGTGCTTGGCTTTCTT	105
PCNA (NM_001034494)	Cattle	F: GTGAACCTGCAGAGCATGGACTCGT R: CGTGTCCGCGTTATCTTCAGCTCTT	192
MHC 1 (NM_174117.1)	Cattle	F:GACAACTCCTCTCGCTTTGG R:GCCTTCAGCTGGAAAGTGAC	122
MHC 2 (NM_001166227.1)	Cattle	F:TCAAGGGGAGATCACAGTCC R:TCAGCAACTTCAGTGCCATC	204
MHC 4 (XM_015468059.1)	Cattle	F:CTTCAACCACCACATGTTCG R:GCTTCTGGAAGTTGCTGGAC	233
MHC 7 (NM_174727.1)	Cattle	F: TGTGTCACCGTCAACCCTTA R: TGGCTGCAATAACAGCAAAG	238

[1]Note: F, forward primer; R, reverse primer; P, TaqMan probe


Sheep
OXT Expression
1.5
1.0
0.5
0.0
a
b
c
c
FC
FI
LC
LI


Sheep
OXTR Expression
1.5
1.0
0.5
0.0
a
b
b
b
FC
FI
LC
LI


Sheep
Pax7 Expression
1.5
1.0
0.5
0.0
a
b
b
b
FC
FI
LC
LI


Sheep
MyoD Expression
1.5
1.0
0.5
0.0
a
b
b
b
FC
FI
LC
LI

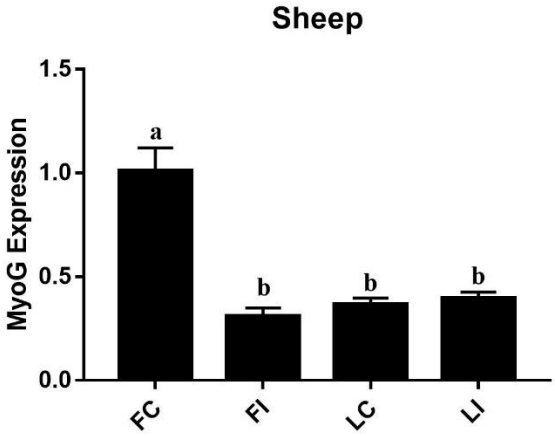

Sheep
MyoG Expression
1.5
1.0
0.5
0.0
a
b
b
b
FC
FI
LC
LI

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.

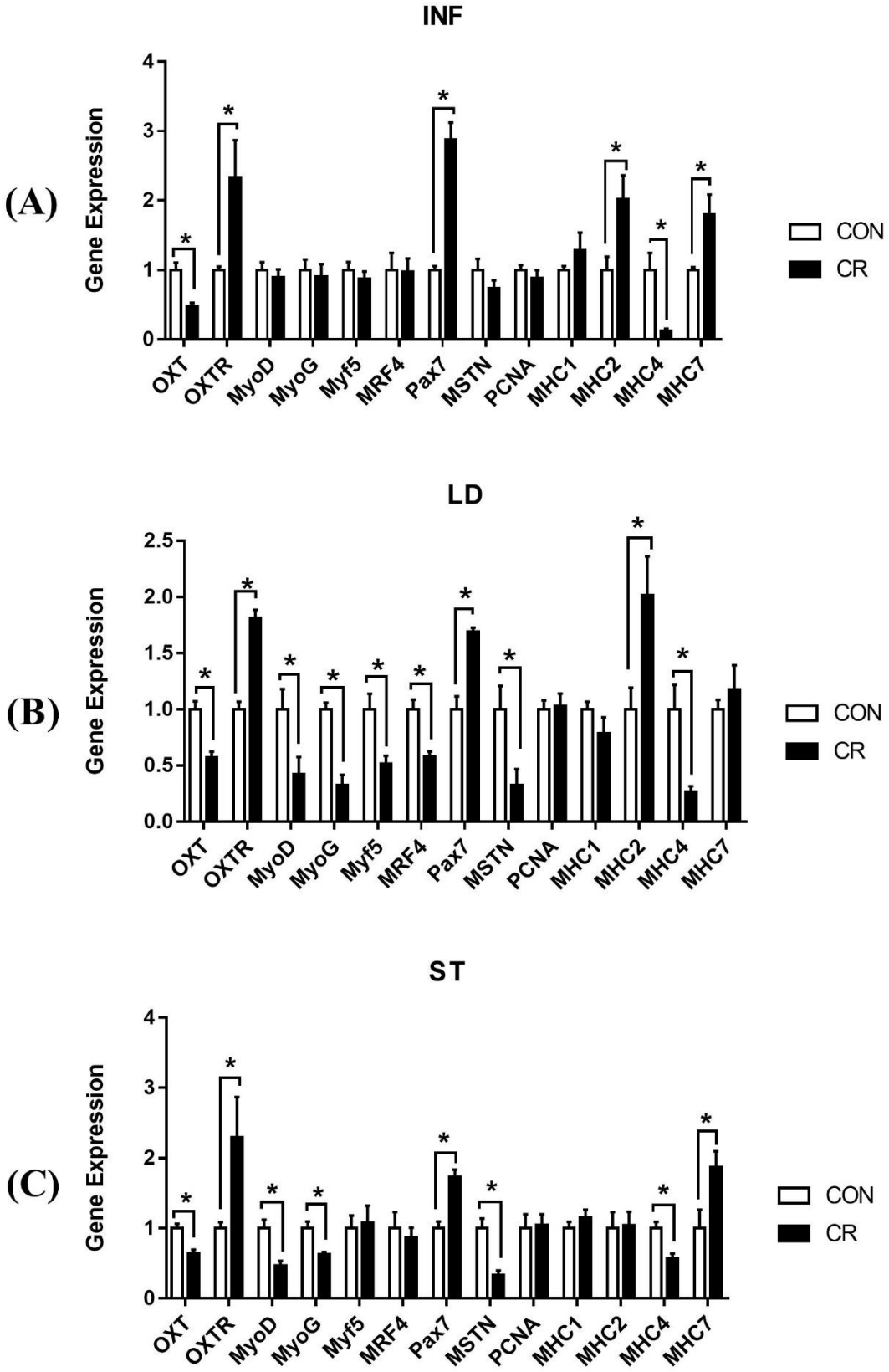

INF
(A)
Gene Expression
CON
CR
OXT
OXTR
MyoD
MyoG
Myf5
MRF4
Pax7
MSTN
PCNA
MHC1
MHC2
MHC4
MHC7
LD
(B)
ST
(C)

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 activitiy 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 Instuments 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 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^{TM} 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 H_2O mix well in PCR tube. This mixture was incubated 5min at 25℃, 30min at 42℃, 5min at 85 ℃, and hold at 4℃. 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℃ followed by 40 cycles of 95℃ for 15s and 60℃ 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 $2^{-\Delta\Delta Cq}$ 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℃ 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℃ for 1hour at 225rpm. The cells were then spread onto prewarmed selective LB agar and incubated at 37℃ 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 3×10^6 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 $2^{-\Delta\Delta Cq}$ 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 activitiy 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 *in-vivo*, whereas *OXTR* expression level significantly decreased with TAM treatment during BSC differentiation *in-vitro*. In addition, *MyoG* expression considerably increased TAM LM compared to CON, but there was no difference detected in *MyoG*-positive satellite number *in-vivo*. These discordant observations point to the possible complex processing response to chronic TAM treatment *in-vivo*.

Although we did not detect muscle fiber type with TAM treatment in the present study, the *MHC* isoforms expression were determined by qPCR. The expression of all *MHC* isoforms (e.g. *MHC1*, *MHC2*, *MHC4*, and *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.

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 *OXT* expression and about 30% increase in *OXTR* and *MyoG* expression, which indicate that OXT 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 does not alter fiber types in heifers.

References

Al Naib, A., H. Tucker, G. Xie, D. Keisler, F. Bartol, R. Rhoads, R. Akers, and M. Rhoads. 2016. Prepubertal tamoxifen treatment affects development of heifer reproductive tissues and related signaling pathways. Journal of dairy science 99(7):5780-5792.

Asp, M. L., J. J. Martindale, and J. M. Metzger. 2013. Direct, differential effects of tamoxifen, 4-hydroxytamoxifen, and raloxifene on cardiac myocyte contractility and calcium handling. PloS one 8(10):e78768.

Banerjee, S., K. L. Chambliss, C. Mineo, and P. W. Shaul. 2014. Recent insights into non-nuclear actions of estrogen receptor alpha. Steroids 81:64-69.

Collins, C. A., I. Olsen, P. S. Zammit, L. Heslop, A. Petrie, T. A. Partridge, and J. E. Morgan. 2005. Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. Cell 122(2):289-301.

Cranwell, C., J. Unruh, J. Brethour, D. Simms, and R. Campbell. 1996. Influence of steroid implants and concentrate feeding on performance and carcass composition of cull beef cows. Journal of animal science 74(8):1770-1776.

Cuzick, J., I. Sestak, S. Cawthorn, H. Hamed, K. Holli, A. Howell, J. F. Forbes, and I.-I. Investigators. 2015. Tamoxifen for prevention of breast cancer: extended long-term follow-up of the IBIS-I breast cancer prevention trial. The lancet oncology 16(1):67-75.

De Jager, N., N. J. Hudson, A. Reverter, Y.-H. Wang, S. H. Nagaraj, L. M. Cafe, P. L. Greenwood, R. T. Barnard, K. P. Kongsuwan, and B. P. Dalrymple. 2011. Chronic exposure to anabolic steroids induces the muscle expression of oxytocin and a more than fifty fold increase in circulating oxytocin in cattle. Physiological genomics

Divari, S., P. Pregel, F. Cannizzo, L. S. Cucuzza, N. Brina, and B. Biolatti. 2013. Oxytocin precursor gene expression in bovine skeletal muscle is regulated by 17β-oestradiol and dexamethasone. Food chemistry 141(4):4358-4366.

Enns, D. L., and P. M. Tiidus. 2008. Estrogen influences satellite cell activation and proliferation following downhill running in rats. Journal of applied physiology 104(2):347-353.

Enns, D. L., and P. M. Tiidus. 2010. The influence of estrogen on skeletal muscle. Sports Medicine 40(1):41-58.

Gonzalez, J., J. Carter, D. Johnson, S. Ouellette, and S. Johnson. 2007. Effect of ractopamine-hydrochloride and trenbolone acetate on longissimus muscle fiber area, diameter, and satellite cell numbers in cull beef cows. Journal of animal science 85(8):1893-1901.

Hawke, T. J., and D. J. Garry. 2001. myogenic satellite cells: physiology to molecular biology. Journal of applied physiology 91(2):534-551.

Jang, Y., M. Sinha, M. Cerletti, C. Dall'Osso, and A. Wagers. 2011. Skeletal muscle stem cells: effects of aging and metabolism on muscle regenerative function. In: Cold Spring Harbor symposia on quantitative biology. p 101-111.

Kitajima, Y., and Y. Ono. 2016. Estrogens maintain skeletal muscle and satellite cell functions. Journal of Endocrinology 229(3):267-275.

Kiyama, R., and Y. Wada-Kiyama. 2015. Estrogenic endocrine disruptors: molecular mechanisms of action. Environment international 83:11-40.

Kongsuwan, K., M. Knox, P. Allingham, R. Pearson, and B. Dalrymple. 2012. The effect of combination treatment with trenbolone acetate and estradiol-17β on skeletal muscle expression and plasma concentrations of oxytocin in sheep. Domestic animal endocrinology 43(1):67-73.

Koohi, M., R. Ivell, and N. Walther. 2005. Transcriptional Activation of the Oxytocin Promoter by Oestrogens Uses a Novel Non-Classical Mechanism of Oestrogen Receptor Action. Journal of neuroendocrinology 17(4):197-207.

Loureiro, J., P. Sandoval, G. del Peso, G. Gónzalez-Mateo, V. Fernández-Millara, B. Santamaria, M. A. Bajo, J. A. Sánchez-Tomero, G. Guerra-Azcona, and R. Selgas. 2013. Tamoxifen ameliorates peritoneal membrane damage by blocking mesothelial to mesenchymal transition in peritoneal dialysis. PloS one 8(4):e61165.

Mandlekar, S., and A.-N. Kong. 2001. Mechanisms of tamoxifen-induced apoptosis. Apoptosis 6(6):469-477.

Matsumine, H., K. Hirato, T. Yanaihara, T. TAMADA, and M. YOSHIDA. 1986. Aromatization by skeletal muscle. The Journal of Clinical Endocrinology & Metabolism 63(3):717-720.

Mauro, A. 1961. Satellite cell of skeletal muscle fibers. The Journal of biophysical and biochemical cytology 9(2):493.

Olson, E. N. 1992. Interplay between proliferation and differentiation within the myogenic lineage. Developmental biology 154(2):261-272.

Payne, W. G., F. Ko, S. Anspaugh, C. K. Wheeler, T. E. Wright, and M. C. Robson. 2006. Down-regulating causes of fibrosis with tamoxifen: a possible cellular/molecular approach to treat rhinophyma. Annals of plastic surgery 56(3):301-305.

Powles, T. J., S. Ashley, A. Tidy, I. E. Smith, and M. Dowsett. 2007. Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. Journal of the National Cancer Institute 99(4):283-290.

Ramaswamy, B., Y. Lu, K.-y. Teng, G. Nuovo, X. Li, C. L. Shapiro, and S. Majumder. 2012. Hedgehog signaling is a novel therapeutic target in tamoxifen-resistant breast cancer aberrantly activated by PI3K/AKT pathway. Cancer research 72(19):5048-5059.

Rhoads, R. P., R. M. Johnson, C. R. Rathbone, X. Liu, C. Temm-Grove, S. M. Sheehan, J. B. Hoying, and R. E. Allen. 2009. Satellite cell-mediated angiogenesis in vitro coincides with a functional hypoxia-inducible factor pathway. American Journal of Physiology-Cell Physiology 296(6):C1321-C1328.

Schiff, R., P. Reddy, M. Ahotupa, E. Coronado-Heinsohn, M. Grim, S. G. Hilsenbeck, R. Lawrence, S. Deneke, R. Herrera, and G. C. Chamness. 2000. Oxidative stress and AP-1 activity in tamoxifen-resistant breast tumors in vivo. Journal of the National Cancer Institute 92(23):1926-1934.

Schulster, M., A. M. Bernie, and R. Ramasamy. 2016. The role of estradiol in male reproductive function. Asian journal of andrology 18(3):435.

Shang, Y. 2006. Molecular mechanisms of oestrogen and SERMs in endometrial carcinogenesis. Nature Reviews Cancer 6(5):360-368.

Shang, Y., and M. Brown. 2002. Molecular determinants for the tissue specificity of SERMs. Science 295(5564):2465-2468.

Sharples, A. P., D. C. Hughes, C. S. Deane, A. Saini, C. Selman, and C. E. Stewart. 2015. Longevity and skeletal muscle mass: the role of IGF signalling, the sirtuins, dietary restriction and protein intake. Aging cell 14(4):511-523.

Shupnik, M. A. 2004. Crosstalk between steroid receptors and the c-Src-receptor tyrosine kinase pathways: implications for cell proliferation. Oncogene 23(48):7979-7989.

Tiidus, P. M., D. A. Lowe, and M. Brown. 2013. Estrogen replacement and skeletal muscle: mechanisms and population health. Journal of Applied Physiology 115(5):569-578.

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.

Yin, H., F. Price, and M. A. Rudnicki. 2013. Satellite cells and the muscle stem cell niche. Physiological reviews 93(1):23-67.

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

Gene (RefSeq ID)	Animal	Primer (5'-3')	Amplicon size(bp)
OXT (NM_176855)	Cattle	F: CTGCCAAGAGGAGAACTACC R: CCTGGGGATGATTACAGAGG P: FAM-CTTCTCCCAGCACTGAGACC-BHQ1	218
PPIA (NM_178320)	Cattle	F: GCCCCAACACAAATGGTT R: CCCTCTTTCACCTTGCCAAAG P: HEX-TGCTTGCCATCCAACCACTCAGTC-BHQ1	96
OXTR (NM_174134)	Cattle	F: CAAGGAAGCCTCACCTTTCA R: TGCACAAGTTCTTGGAAGAGG	111
MyoD (NM_001040478)	Cattle	F: CGACTCGGACGCTTCCAGT R: GATGCTGGACAGGCAGTCGA	180
MyoG (NM_001111325)	Cattle	F: GTGCCCAGTGAATGCAGCTC R: GTCTGTAGGGTCCGCTGGGA	110
Myf5 (NM_174116)	Cattle	F: ACCAGCCCCACCTCAAGTTG R: GCAATCCAAGCTGGATAAGGAG	150
MRF4 (NM_181811)	Cattle	F: GGTGGACCCCTTCAGCTACAG R: TGCTTGTCCCTCCTTCCTTGG	140
MSTN (AB076403)	Cattle	F: GGCCATGATCTTGCTGTAACCT R: GCATCGAGATTCTGTGGAGTG	144
MyoG (XM_002685738)	Cattle	F: GAGTTCGATTAGCCGAGTGC R: ATGCTGTGCTTGGCTTTCTT	105
PCNA (NM_001034494)	Cattle	F: GTGAACCTGCAGAGCATGGACTCGT R: CGTGTCCGCGTTATCTTCAGCTCTT	192
MHC 1 (NM_174117.1)	Cattle	F:GACAACTCCTCTCGCTTTGG R:GCCTTCAGCTGGAAAGTGAC	122
MHC 2 (NM_001166227.1)	Cattle	F:TCAAGGGGAGATCACAGTCC R:TCAGCAACTTCAGTGCCATC	204
MHC 4 (XM_015468059.1)	Cattle	F:CTTCAACCACCACATGTTCG R:GCTTCTGGAAGTTGCTGGAC	233
MHC 7 (NM_174727.1)	Cattle	F: TGTGTCACCGTCAACCCTTA R: TGGCTGCAATAACAGCAAAG	238

Note: F, forward primer; R, reverse primer; P, TaqMan probe

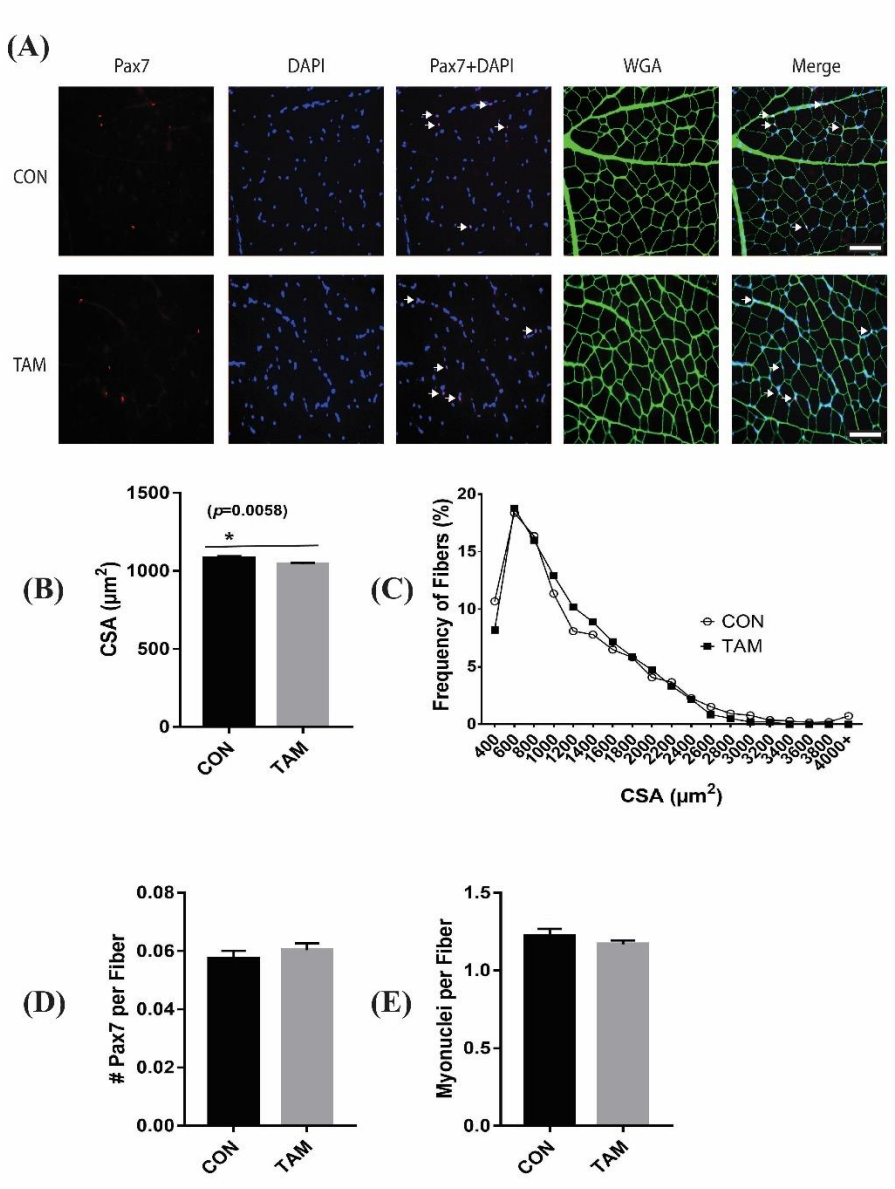


Figure 5.1. The effect of TAM on muscle size, number of BSC, number of myonuclei per fiber in Holstein heifers. Cryosections from longissimus of TAM and CON were analyzed by *MyoG*, DAPI, and wheat germ agglutinin (WGA) histology (A) for the measurement of number of BSC (B), CSA (B), fiber size distribution (B), myonuclei per fiber (B); white arrows point to the BSC; magnification bar = 100 µm. Asterisk denotes significant difference from control (*$P < 0.05$).

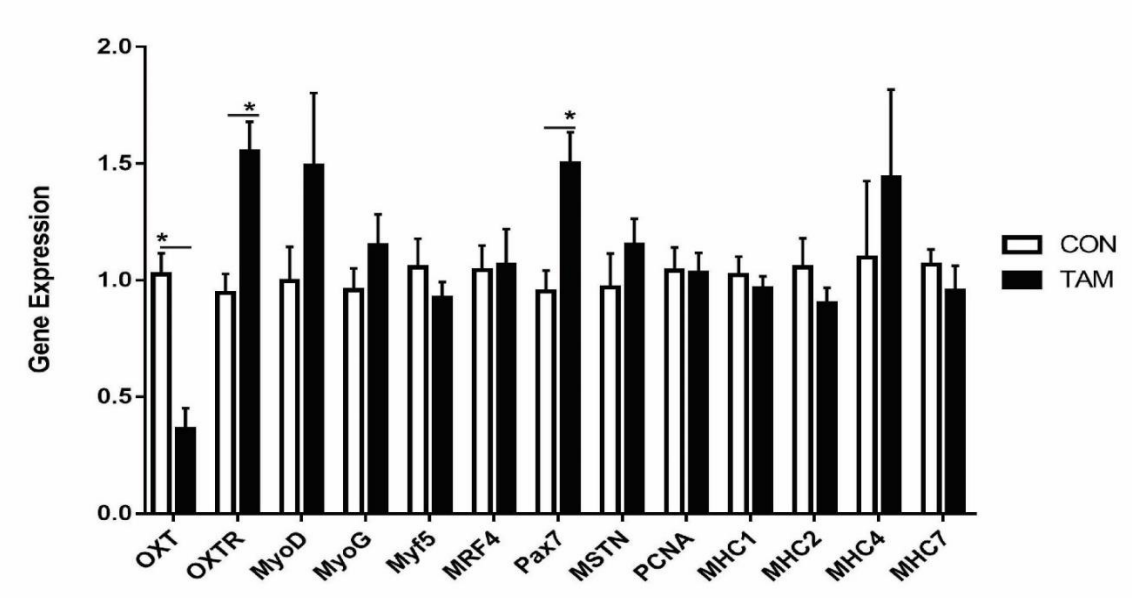


Figure 5.2. Gene mRNA expression level in LM. CON and TAM longissimus lysates were analyzed for gene mRNA expression. The TAM treatment led to a marked decrease in *OXT* expression and a significant increase in OXTR and *MyoG* expression in LM. Values are means and SEMs. Asterisk denotes significant 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 *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 *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

Blaauw, B., and C. Reggiani. 2014. The role of satellite cells in muscle hypertrophy. Journal of muscle research and cell motility 35(1):3-10.

Cattaneo, M., B. Chini, and L. Vicentini. 2008. Oxytocin stimulates migration and invasion in human endothelial cells. British journal of pharmacology 153(4):728-736.

Chen, X., A. L. Fahy, A. S. Green, M. J. Anderson, R. P. Rhoads, and S. W. Limesand. 2010. β2-Adrenergic receptor desensitization in perirenal adipose tissue in fetuses and lambs with placental insufficiency-induced intrauterine growth restriction. The Journal of physiology 588(18):3539-3549.

De Jager, N., N. J. Hudson, A. Reverter, Y.-H. Wang, S. H. Nagaraj, L. M. Cafe, P. L. Greenwood, R. T. Barnard, K. P. Kongsuwan, and B. P. Dalrymple. 2011. Chronic exposure to anabolic steroids induces the muscle expression of oxytocin and a more than fiftyfold increase in circulating oxytocin in cattle. Physiological genomics 43(9):467-478.

Divari, S., P. Pregel, F. Cannizzo, L. S. Cucuzza, N. Brina, and B. Biolatti. 2013. Oxytocin precursor gene expression in bovine skeletal muscle is regulated by 17β-oestradiol and dexamethasone. Food chemistry 141(4):4358-4366.

Elabd, C., W. Cousin, P. Upadhyayula, R. Y. Chen, M. S. Chooljian, J. Li, S. Kung, K. P. Jiang, and I. M. Conboy. 2014. Oxytocin is an age-specific circulating hormone that is necessary for muscle maintenance and regeneration. Nature communications 5

Faghihi, M., A. M. Alizadeh, V. Khori, M. Latifpour, and S. Khodayari. 2012. The role of nitric oxide, reactive oxygen species, and protein kinase C in oxytocin-induced cardioprotection in ischemic rat heart. Peptides 37(2):314-319.

Hales, C. N., and D. J. Barker. 1992. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. Diabetologia 35(7):595-601.

Hinkson, I. V., and J. E. Elias. 2011. The dynamic state of protein turnover: It's about time. Trends in cell biology 21(5):293-303.

Kongsuwan, K., M. Knox, P. Allingham, R. Pearson, and B. Dalrymple. 2012. The effect of combination treatment with trenbolone acetate and estradiol-17β on skeletal muscle expression and plasma concentrations of oxytocin in sheep. Domestic animal endocrinology 43(1):67-73.

Koohi, M., R. Ivell, and N. Walther. 2005. Transcriptional Activation of the Oxytocin Promoter by Oestrogens Uses a Novel Non-Classical Mechanism of Oestrogen Receptor Action. Journal of neuroendocrinology 17(4):197-207.

Munro, H. N. 2012. Mammalian protein metabolism. Elsevier.

Richard, S. p., and H. Zingg. 1990. The human oxytocin gene promoter is regulated by estrogens. Journal of Biological Chemistry 265(11):6098-6103.

Stedronsky, K., R. Telgmann, G. Tillmann, N. Walther, and R. Ivell. 2002. The affinity and activity of the multiple hormone response element in the proximal promoter of the human oxytocin gene. Journal of neuroendocrinology 14(6):472-485.

Valsamakis, G., C. KANAKA-GANTENBEIN, A. MALAMITSI-PUCHNER, and G. Mastorakos. 2006. Causes of intrauterine growth restriction and the postnatal development of the metabolic syndrome. Annals of the New York Academy of Sciences 1092(1):138-147.

Verdijk, L. B., B. G. Gleeson, R. A. Jonkers, K. Meijer, H. H. Savelberg, P. Dendale, and L. J. van Loon. 2009. Skeletal muscle hypertrophy following resistance training is accompanied by a fiber type–specific increase in satellite cell content in elderly men. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences:gln050.

Wang, J., L. Chen, D. Li, Y. Yin, X. Wang, P. Li, L. J. Dangott, W. Hu, and G. Wu. 2008. Intrauterine growth restriction affects the proteomes of the small intestine, liver, and skeletal muscle in newborn pigs. The Journal of nutrition 138(1):60-66.

Yates, D., A. Macko, M. Nearing, X. Chen, R. Rhoads, and S. Limesand. 2012. Developmental programming in response to intrauterine growth restriction impairs myoblast function and skeletal muscle metabolism. Journal of pregnancy 2012