Myogenic Transcription Factors and Ornithine decarboxylase mRNA are Influenced by Polyamine Supplementation *in vitro.*

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Authorization to Submit Thesis

This thesis of Avani Gouru, submitted for the degree of Master of Science with a major in Animal Science and titled "Myogenic Transcription Factors and Ornithine decarboxylase mRNA are Influenced by Polyamine Supplementation *in vitro*", has been reviewed in final form. Permission, as indicated by the signature and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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Abstract

The polyamines putrescine and spermine are important nutrients that support cell proliferation, and growth. Studies on the influence of polyamines in skeletal muscle growth are limited. This research is driven towards understanding nutritional means for optimizing muscle growth in cattle. Using commercial cell lines, c2c12 and sol8 cells, the effects of physiological doses of polyamines in undifferentiated and differentiated cells was assessed in the context of mRNA analyses of key myogenic transcription factors and the mRNA that is essential for the rate limiting step for *de novo* synthesis of polyamines; ornithine decarboxylase is also measured. Myogenic transcription factors (MyoD, Myf5, myogenin) and ornithine decarboxylase (ODC) mRNA were measured at 8 and 16 h time points after treatment with polyamines (putrescine- 5 mM, spermine- 0.5 mM or methionine (amino acid control) or no polyamine supplementation ("control"). Exogenous supplementation with putrescine in undifferentiated c2c12 cells significantly increases myogenic transcription factors (Myf5 8 h- $P = 0.040$, Myf5 16 h- $P = 0.030$, MyoD 16 h- $P = 0.040$, Myogenin 16 h- $P = 0.003$) and ODC mRNA (8 h- $P = 0.010$, 16 h- P = 0.020). However, polyamine treatment of differentiated c2c12 (spermine -myogenin 8 h- $P = 0.020$, ODC 8 h- $P = 0.030$) and sol8 cells (putrescine-myogenin 8 h- $P = 0.050$, ODC 8 h- $P = 0.020$) resulted in a significant decrease in myogenic transcription factors and ODC mRNA. Methionine (10 mM) treatment of undifferentiated c2c12 and sol8 cells did not show significant difference in their myogenic transcription factor mRNA relative to control, however ODC mRNA at 16 h ($P = 0.010$) increases in c2c12 undifferentiated cells. These results indicate that putrescine may induce a promyogenic state in undifferentiated myoblasts (c2c12 and sol8) at 8 h and 16 h as compared to controls. Further evaluation of more time points as well as further studies in differentiated myoblasts is critical for a clearer understanding of the complex role of polyamines in skeletal muscle growth.

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Dedication

I wish to dedicate this thesis to "*Lord Venkateswara Swamy*"

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Abbreviations

µl	Microliter
µM	Micromolar
18S	Eukaryotic ribosomal RNA subunit with sedimentation rate 18
AdoMet	S- adenosylmethionine
AdoMetDC	S-adenosylmethionine decarboxylase
AMPK	AMP activated protein kinase
ATCC	American type culture collection
AZ	Antizyme
bHLH	Basic helix loop helix
C	Celsius
c2c12	Immortalized mouse myoblast cell line
CDNA	Copy deoxyribonucleic acid
Conc/kg	Concentration per kilogram
CT	Threshold cycle
d NTR	Deoxyribonucleotide triphosphate
DcAdoMet	Decarboxylated adenosylmethionine
DFMO	Difluoromethylornithine
DMEM	Dulbeccos modified eagle medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
eIF4E	Eukaryotic initiation factor 4E
Elf5a	Eukaryotic translation initiation factor
FBS	Fetal bovine serum
G	Grams
GH	Growth hormone
H	Hours
H2O2	Hydrogen peroxide
Hcl	Hydrochloric acid
HEPA	High efficiency particulate air

HT29	Human colon adeno carcinoma cells
IPEC	Intestinal epithelial cells
Kg	Kilogram
MAPK	Mitogen-activated protein kinase
MHC	Myosin heavy chain
ML	Milli liters
mM	Milli molar
MMP	Matrix metalloproteases
MRFs	Myogenic transcription factors
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
Myf5	Myogenic factor 5
MyoD	Myogenic determination factor
Ng	Nanogram
NO	Nitric oxide
ODC	Ornithine decarboxylase
PAO	Polyamine oxidase
Pax 3	Paired homeobox 3
Pax 7	Paired homeobox 7
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real time – polymerized chain reaction
RNA	Ribonucleic acid
Shh	Hedgehog signaling pathway
SMO	Spermine oxidase
Sol8	Immortalized mouse myoblast cell line derived from soleus muscle
ΔCt	Difference in cycle threshold fluorescence of gene of interest relative to endogenous reference

Chapter 1: Literature Review

1.1 Introduction to skeletal muscle:

Skeletal muscle is essential for locomotion, vocalization, maintaining structure/posture of the organism and respiration (Roberts 2002; Merrell and Kardon., 2013). It is the primary tissue that yields the commercially marketed and consumable product in beef livestock meat (Bown et al., 2016). Alterations in muscle because of ageing, disease or injury can impact homeostasis, decrease lifespan and productivity of the organism (Demontis et al., 2013). Fortunately, continual repair and even growth can and does occur in healthy and well-nourished mammals, though in diminished capacity with post-natal age (Goldspink and Harridgeet., 2004). The formation of new muscle fibers (myofibers) during development, growth and in regeneration phases, is most generally referred to as; myogenesis (Rudnicki et al., 1995). For the intent and purpose of this review, this chapter will focus on growth, remodeling and maintenance of muscle tissue after birth, or during the postnatal phase of life.

All healthy mammals, including humans, are predominantly comprised of skeletal muscle tissue (Close, 1972). Therefore, improved understanding of "skeletal muscle" is essential to fully promote growth and regenerative processes in this tissue. In beef cattle, the growth of skeletal muscle yields the primary marketable product, meat, and as such impacts both yield and quality (Ten Broek et al., 2010; Guo et al., 2017). To produce cost effective meat with higher feed conversion ratios in a short amount of time, we need to optimize provision of all required nutrients to reach the maximum growth potential. Polyamines are widely distributed in nature and play a vital role in cells of living creatures (Minois et al., 2011). Polyamine concentration in living cells alters with rate of proliferation with highest levels being observed in rapidly developing cells (Pegg et al., 2016). These are critical for essential cell activities like growth, apoptosis and development in animals (Pegg et al., 2016). Supplementation of polyamines significantly accelerates the growth of cells and is vital for regulation of macromolecular biosynthesis (Marton and Pegg, 1995; Moinard et al., 2005). In the conventional pathway of polyamine synthesis, arginine is converted to ornithine by the enzyme arginase (Lee et al., 2011). The ornithine is in turn decarboxylated by ornithine decarboxylase (ODC) a primary and rate limiting enzyme to putrescine (Lee et al., 2011).

Ornithine decarboxylase is a primary, and rate-limiting, enzyme in the biological synthesis pathway of polyamines (Pegg et al., 2016). This enzyme is essential for maintaining optimum intracellular levels of polyamine (Pegg et al., 2016). Putrescine can be later be converted to spermidine and spermine (Lee et al., 2011). Inclusion of these polyamines in the feed of animals may facilitate growth, health and productivity (Moinard et al., 2005; Murdoch et al., 2006). Polyamine intracellular quantities are strictly controlled by their biosynthesis, transport or catabolism (Minois et al., 2011). They play key roles in stimulation of cell proliferation and differentiation, regulation of programmed cell death (apoptosis), angiogenesis, myogenesis, cell-to-cell communication and synthesis of protein (Pegg et al., 2016). Though the exact mechanism of action of polyamines on regulation of muscle growth is unknown (Soda et al., 2011), polyamines have multiple functions that may directly and/or indirectly contribute to myogenesis (Pegg et al., 2016). Increased expenses in feed and maintenance of livestock production had decreased the profitability in cattle production (Moore et al., 2009). Harvesting the maximum meat through sustainable production in a short amount of time requires effective use of feed (Herd et al., 2003). To optimize livestock production and yield efficient muscle growth, it is important to understand all the physiological substances that can increase the muscle accretion (Webb et al., 2010). By increasing the efficiency of muscle production, we also aim to understand parameters that could affect the meat quality. The end goal of the study is to optimize growth in cattle by minimizing the time on feed through supplementing polyamines or polyamine rich foods while maintaining or improving the meat quality.

Approach of the study:

We hypothesized that not only carbohydrates (Figueiredo et al., 2013), lipids (Muro et al., 2014), and proteins (Kung et al., 1996) are required for growth, but other micro-nutrients derived from amino acids called “polyamines” are also important for optimizing muscle growth potential (Murdoch et al., 2006; Lee et al., 2011). In this experimental study we focused on treatment with physiological doses of polyamines, to ascertain their role in different stages of myogenesis (8 h = determination, 16 h = differentiation). We hypothesize that polyamines increase the myogenic transcription factors (Myf5, MyoD and myogenin) mRNA and ornithine decarboxylase (ODC) gene expression (rate limiting enzyme for *de novo*

polyamine synthesis pathway) thereby increasing myogenesis. This may be relevant to the muscle production in cattle as we know that muscle growth is supported by increasing the myogenic transcription factors transcription. Through quantitative real-time PCR, we analyzed the mRNA abundance of different myogenic transcription factors along with ODC mRNA in polyamine treated myoblasts and compared this expression to that of untreated control cells. Overall, this study will contribute to a better understanding of postnatal muscle growth in the presence of a specific nutrient class (polyamines). As this is a preliminary study, we chose c2c12 and sol8 myoblast cell lines to test our hypothesis *in vitro* prior to more complex and expensive large animal trials.

1.2 Postnatal myogenesis:

In adult skeletal muscles, growth and regeneration is critically dependent upon satellite cells or muscle progenitor cells residing within the stromal vascular tissue of muscle (Le Grand et al., 2007; Murphy et al., 2011). Satellite cells are special cells located between the basal lamina and sarcolemma along each muscle fiber, first identified by Mauro and colleagues (1961). These satellite cells, which are predominately quiescent, will proliferate upon activation and physiological signaling within the muscle (Charge and Rudnicki, 2004). In this instance quiescence does not indicate the complete inactive basal state, instead it is a physiological state under which the cells are under active transcriptional control (Charge and Rudnicki, 2004). Common stimulatory signals include trauma, injury, mechanical stretch and in pathological myo-degenerative conditions (Yin et al., 2013). The mechanism (activation trigger), that causes the cell to enter the proliferation phase in myogenesis is not completely understood. There are extrinsic and intrinsic pathways that will predictably promote satellite cells to enter the myogenesis process or maturational cascade (Rudnicki, 2001; Collins et al., 2005)

1.3 Signal activation of Satellite cells:

Satellite cell activation occurs by coordination of both intrinsic and extrinsic signaling pathways; inhibition of either one of these signals will seriously affect its activation, proliferation and differentiation rates (Dumont et al., 2015). One of the earliest intrinsic signals to activate satellite cell is presence of sphingomyelin (Danieli-Betto et al., 2010)

Sphingosine-1-phosphate, produced from sphingomyelin (situated in the plasma membrane), is released after injury (Nagata et al., 2014). This Sphingosine-1-phosphate along with caveolin-1 activates satellite cell and triggers the cells to enter the cell cycle phase (Charge and Rudnicki, 2004; Ten Broek et al., 2010; Dumont et al., 2015). There are some canonical growth pathways called "Notch" and "Fox head box protein" signaling pathways that are known to be involved in a regulatory feedback loop of activating satellite cells and self-renewing the satellite cell pool (Yin et al., 2013; Dumont et al., 2015). Growth factors, expressed at the time of regeneration activate the Akt/mTOR pathway (involved in protein synthesis), inducing satellite cells to undergo determination, and encourage their progress through differentiation stages of myogenesis (Schiaffino et al., 2011). After the regeneration process ends, the notch signal is again activated, and this inhibits the cells from entering determination phase and encourages them to instead proceed through a process of self-renewal (Yin et al., 2013; Dumont et al., 2015). Others signaling pathways such as wnt, shh, and myostatin along with pax7 and pax3 are constitutively expressed in the quiescent satellite cells; upon an appropriate "activation trigger" they downregulate (Bernardi et al.2012). Subsequent upregulation of MyoD and Myf5 directs these cells to proceed through the ordered phases of myogenesis (Dumont et al., 2015). Extrinsic satellite cell activation mainly depends upon its microenvironment created by local cells, microvasculature, neural stimulation through the neuromuscular junction and immune cells (Carlson et al., 2007). The appropriate combination and sequence of extrinsic stimuli will trigger the satellite cells not only to activate, proliferate and differentiate to myocytes but also help in the critical self-renewal process (Ten Broek et al., 2010). Injury to the muscle causes an increase in FGF and HGF signaling. These growth factors bind to their respective receptors on the satellite cells, thereby activating the cell to exit from G0 phase and to enter the cell cycle phase (Brack et al., 2012; Ornitz et al., 2015). FGF is also involved in activating the MAPK signaling pathway thereby triggering the satellite cell activation (Le Grand and Rudnicki, 2007; Bentzinger et al., 2010). Anabolic growth factors such as insulin-like growth factor, secreted by fibroblasts and myofibers, upon growth stimulus can increase the Akt/mTOR pathway, which in turn increases the protein synthesis (McCarthy et al., 2010).

The vast majority of satellite cells (68-82%) reside in close proximity (5μm) to the microvasculature. Several growth factors in blood such as platelet derived growth factor,

epidermal growth factor, vascular endothelial growth factor, brain derived neurotrophic factor and interleukin 6, present in the blood contribute to satellite cell activation (Kuang et al., 2008; Bentzinger et al., 2010; Ten Broek et al., 2010). Nitric oxide (NO) and tumor necrosis factor-alpha (TNF- α) are also released following muscle injury, whereas NO stimulates expression of matrix metalloproteases (MMP) and other growth factors which combine to stimulate satellite cell activation and proliferation (Le Grand and Rudnicki, 2007; Bentzinger et al., 2010). Further, "microRNAs", which are recognized as non-coding molecules, can bind to mRNAs of muscle related genes soon after the transcription and regulate translation levels (Horak et al., 2016). It has been reported that there are twenty-two microRNAs that are upregulated in quiescent satellite cells and downregulated in activated satellite cells, suggesting a critical role of these molecules in the regulation of satellite cell state (Kuang et al., 2008; Yin et al., 2013). In fact, satellite cell quiescence and activation can be influenced by many factors including mechanical (stretch and injury; Hill et al., 2003), electrical (neuronal action potential; Guo et al., 2012) and chemical (neuro transmitters) (Kuang et al., 2008; Ten Broek et al., 2010) stimulus regulated by multiple pathways (intrinsic and extrinsic) at multiple steps (transcription, translation) in a highly intricate manner.

1.4 Pathways influencing satellite cell division and differentiation:

Once satellite cells are activated to proceed out of the quiescent state, they can either undertake stochastic differentiation or asymmetric division (Almeida et al., 2012). In stochastic differentiation, one stem cell divides in a planar division (symmetrical division) to form two identical daughter cells that can enter the determination phase (Knoblich, 2008). Another stem cell divides similarly (symmetrical division) giving rise to two identical stem cells that can replenish the satellite cell pool (Bentzinger et al., 2012). In asymmetrical division, stem cells divide and form two different daughter cells (Knoblich, 2008). One of the daughter cells forms a new satellite cell (self- renewal) and the other cell further divides symmetrically and enters the determination stage of myogenesis as a myoblast (Charge and Rudnicki, 2004; Collins et al., 2006). Though the satellite cells participate in several cycles of regeneration, in the optimal state they retain their regenerative capacity and maintain the satellite pool size at a constant (Brack et al., 2012). For example, it is observed that one myofiber with 7-22 satellite cells when grafted to irradiated muscles of immunodeficient dystrophic mice, could potentially

support 100 new myofibers (Yin et al., 2013). The newly formed myofibers comprised of 25,000– 30,000 myonuclei, while maintaining the same pool size, emphasizing that the satellite cells have high regenerative capacity (Collin et al., 2005). Upon injury to muscle cells, satellite cells not only activate at the damaged site but are activated across an entire satellite cell niche along the myofiber (Jockusch et al., 2003). The distantly activated satellite cells are motile and migrate to the areas of regeneration by crossing the myofibers and even muscles during phases of muscle growth and regeneration (Hughes et al., 1990; Jockusch et al., 2003). Determined myoblasts actively proliferate in this stage, which is initiated by many signaling cascades through the direct action of multiple growth factors cytokines and prostaglandins (Jeffrey et al., 2005). The satellite cell pool does not remain constant throughout the adult stage and declines during old age, this in part explains the poor regenerative capacity of the aged muscle (Hwang et al., 2018). In conclusion, once satellite cells are stimulated, they act as a critical resource that undergoes the process of myogenesis giving rise to new myofibers, thus helping build muscles through growth and regenerative processes.

1.5 Stages in myogenesis:

Myogenesis, the active maturation and addition of new muscle cells to multinucleated myofibers, consists of three stages 1) commitment or determination phase, 2) differentiation phase and 3) maturation phase with some molecular and physiological overlap between all of these stages (Sabourin et al., 2000; Figure 1.1.)

In the determination phase, satellite cells are roused out of quiescence and phenotypically altered to proceed down the developmental and maturational sequence to become myoblasts. (Yablonka-Reuveni, 2011). The myoblasts actively proliferate and subsequently down regulate pax7 and sprouty 1 genes, thereby entering the next transitional stage as a myoblast (immature muscle cell) (Bentzinger et al., 2010). In the differentiation stage, myoblasts are further phenotypically altered and “differentiated” into myocytes and at this stage these cells withdraw from the cell cycle and mature rather than proliferate through myogenesis (Chal et al., 2017). In the maturation phase, myocytes ultimately transition to the muscle phenotype and express muscle specific proteins such as sarcomeric myosin, among others (Braun et al., 2011). Myocytes will finally migrate, adhere and fuse with each other to generate nascent

myotubes and these nascent myotubes fuse with other myotubes or myocytes to produce mature myofibers (Kassar et al., 2004). Alternatively, and more commonly postnatally these myocytes will adhere and fuse with existing mature myofibers in a process referred to as myonuclear addition. (Klimczak et al., 2016). Within the myofibers, sarcomeric myofibrils are generated (myofibrillogenesis) and their assembly gives rise to new or expanded skeletal muscle contractile units referred to as "sarcomeres" (Sanger et al., 2002). Skeletal muscle sarcomeres are comprised of structural proteins such as nebulin, titin, and α-actinin (Labeit et al., 2011). Additionally, contractile myofilaments mainly comprised of f-actin and myosin as well as regulatory proteins such as troponin, and tropomyosin are expressed (Koubassova et al., 2011). All three classes of proteins: structural, regulatory and contractile, are required for the functional contraction and are therefore essential components of the physiological phenotype of skeletal muscles (Glisovic et al., 2008; Abmayr et al., 2012).

1.6 Determination phase:

During the stage of recruitment and activation of the reserve pool of satellite cells the intent is to facilitate protein accretion within existing mature multinucleated myofibers (Shefer et al., 2006). Therefore, as an essential starting point satellite cells must be "determined" to form myoblasts upon reducing pax 7 and sprouty 1 expression and concomitantly increasing the expression of MyoD or Myf5 or both (Buckingham et al., 2015). Whereas satellite cells that express either MyoD, Myf5 or both can enter the determination stages (Figure 1.1.) and become myoblasts, cells that do not express either one of these transcription factors (MyoD or Myf5) are mitogenically active and serve to increase or replenish the satellite reservoir pool (self-renewal; Francetic et al., 2011; Mokalled et al.,2012). Satellite cells expressing both Myf5 and MyoD represent the majority of determined satellite cells (50%), while those exclusively expressing Myf5 and cells that are expressing only MyoD comprise 20% and 30% of the determined pool, respectively (Brack et al., 2012). This indicates that there is some redundancy in the function of MyoD and Myf5 though expression of both yields the most successful path for myogenesis (Wei et al., 2001). MyoD and Myf5 genes are critically important in promoting the successful myogenesis process especially in determination and differentiation stages (Wei et al., 2001). Loss of MyoD and Myf5 expression during this phase causes increased cell apoptosis and result in only very few cells differentiating into myocytes,

leading to poor muscle growth (Yamamoto dt al., 2018). It has been shown that MyoD-/- myoblasts fail to initiate the increased myogenin gene transcription, which is the critical regulator of sarcomeric gene expression thereby affecting both the differentiation process and muscle regeneration process, and phenotypic skeletal muscle development (Dumont et al., 2015).

1.7 Differentiation phase:

In this stage of myogenesis the myoblasts are phenotypically altered further and progress through a time specific and organized process where they are "differentiated" into myocytes (Bentzinger et al., 2010). A key component of this phenotypic transformation is associated with the commencement of myogenin gene expression along with continued Myf5 and MyoD gene expression (Wei et al., 2001) (Figure 1.1.). As mentioned previously, these myoblasts exit the mitogenic proliferative stage and lose this capacity as they mature and transition to form myocytes (Dumont et al., 2015; Almeida et al., 2016). The myogenin expression activates the expression of muscle specific genes including sarcomeric myosin, promoting the maturation of cells into a more muscle specific phenotype (Miller 1990). In proliferative stage, myoblast cells are motile, and they migrate to different locations upon the action of chemokines and other growth promoting substances (hepatocyte growth factor, platelet derived growth factor) whereas in differentiation stage cell motility decreases and myocyte response to chemokine attraction decreases (Griffin et al., 2009; Griffin et al., 2010). This differentiation phase is an irreversible process; once the cells are differentiated to myocytes, they can't undergo self-renewal process and can only proceed forward to maturation (Ruijtenberg et al., 2016). The lack of expression of myogenic transcription factors at any step causes the cells to undergo apoptosis. (Yamamoto et al., 2018). Lack of the expression of myogenin and MyoD genes are a key example of unfavorable conditions and will cause cells to undergo apoptosis (Almedia et al., 2016). Hence, expression of appropriate myogenic transcription at every step of myogenesis is necessary for the cells to be in a promyogenic state and ultimately contribute to postnatal muscle growth.

1.8 Maturation phase:

Myocytes in maturation phase express myogenin and this initiates expression of phenotypically and functionally critical proteins associated with skeletal muscle contractility. (Shima et al., 2008). The cells in this phase appear elongated with flattened appearance compared with myoblasts cells, which are round and small (Abmayr et al., 2012). Early maturation is again divided into early, middle and late fusion stages (Dumont et al., 2015). In the early stage, myocytes start to express myogenin, in the middle stage, myocytes are attracted to one another and myocyte-myocyte contact is initiated (Jiwlawat et al., 2017). In late stage, myocytes fuse with the nascent multinucleated myotubes as they are ready to signal and prepare for fusion with an existing myofiber, resulting in postnatal myonuclear addition (Abmayr et al., 2012). Special proteins like actin binding protein helps in the fusion of these structures (Figure 1.1.; Dumont et al., 2015). In late maturation phase the nascent myotubes undergo a maturation process to become functional myofibers (Hindi et al., 2013). In all maturation phases, expression of myogenic transcription factors such as myogenin, MyoD and Myf5 genes are required for the cell to be in a promyogenic state (Peter et al., 2017). In this fully matured stage, the myocytes express myosin heavy chain proteins, actins, myosin and troponins, which lead to maturation of a cell that reaches its final phenotypic maturity prior to fusing with an existing functional myofiber (Buckingham et al., 2016). Sarcomeric myofibers start to regenerate within the myofiber and complete the process of myogenesis (Hindi et al., 2016). Cao and associates reported that there are 23k MyoD binding sites in myoblasts and 26k MyoD binding sites in myotubes, which regulate the genes and participate in myogenesis (2010). This means that myogenic transcription factors, especially MyoD, are critically important at determination and differentiation, and in the maturation process for the cell to successfully complete the myogenesis process (Yin et al., 2013). With the fusion and myonuclear addition, there is more cellular machinery, which increases the capacity of the recipient myofiber to increase its protein synthesis for intracellular accretionary hypertrophic growth.

1.9 Importance of Satellite cells role in myogenesis:

These are the primary stem cells responsible for postnatal muscle growth, muscle hypertrophy and regeneration (Dhawan et al., 2006). They account for 30% of muscle nuclei at birth and

facilitate prolific growth of the muscle fibers after birth, ultimately giving rise to 50% of mature myofibers nuclei (Pallafacchina et al., 2013). This capacity for myonuclear addition decreases to 6% in mice as they reach adulthood and eventually systemic muscle growth decreases at this phase because satellite cells remain in a predominantly quiescent state (Cardasis et al., 1975). It has been demonstrated that ablation of the satellite cells from mice tissues through gamma irradiation completely inhibits the myonuclear addition and hypertrophic growth. (Grounds et al., 1998). The number of satellite cells and muscle renewing capacity declines with age resulting in a condition called "sarcopenia" and degenerative skeletal muscle loss (Demontis et al., 2013). In humans this represents an average loss of 8% muscle mass per decade after the age of 50 (Grounds et al., 1998). Though it is very well known that satellite cells play a fundamental role in muscle regeneration process, the exact mechanism through which these cells expression of MRFs genes are regulated and function to develop skeletal muscle is unknown (Murphy et al., 2011).

1.10 Role and action of myogenic transcription factors in myogenesis:

Members of the myogenic regulatory factor (MRF) family include Myoblast determination protein (MyoD), Myogenic factor 5 (Myf5), myogenic factor 4 (myf4) and myogenin (MyoG) are basic helix-loop-helix (bHLH) protein transcription factors, which contain a highly conserved bHLH binding domain (Harada et al., 2017). These MRF's heterodimerize with a ubiquitous class of bHLH transcription factors called E proteins (E2 and E47) (Wang et al., 2015). These heterodimers then bind to E box sequences (CANNTG) (Enhancer box nucleotide sequence) present in DNA promoter regions and starts the transcription and hence expression of myogenic genes (Wang et al., 2015). Myogenic transcription factors are essential for the satellite cells to undergo myogenesis and these factors share limited overlapping actions in this process. (Le grand et al., 2007). These factors regulate and turn off the genes in a timely manner. Inhibitory (Id) proteins lack DNA binding domain and thus their transcription is stopped (Wang et al., 2015). These Id proteins are vital in regulating cell to cell development and differentiation by modifying unique cell cycle mechanisms (Wang et al., 2015). Myf5 called as myogenic determination factor expresses earlier in activated satellite cells followed by MyoD, this helps the cells to enter determination and early differentiation stages of specific myogenic lineage (Ustanina et al., 2007). Myogenin, which

is expressed afterwards, helps the cells to enter late differentiation stages of myogenesis (reviewed in Manuel et al., 2017).

1.11 Specific functions of myogenic transcription factors:

Satellite cells present underneath the basal lamina of muscle fibers are divided into Myf5 positive satellite cells (90%) and Myf5 negative satellite cells (10%) (Hernández-Hernández et al., 2017). Satellite cells populations, which do not transcribe Myf5 (10% of total satellite cells), have long periods of self-renewing ability whereas the satellite cells, which transcribe MyoD or Myf5 or both, enters determination phases of myogenesis (Hernández-Hernández et al., 2017). Myf5 is the first myogenic regulatory factor to specify the skeletal muscle lineage (Kosenko et al., 2011). The distinct role of Myf5 in myogenesis includes histone protein acetylation around binding sites, which opens and unfolds DNA for transcription, whereas MyoD functions by binding to the nearly identical sites and directly induces transcription machinery recruitment (Melissa et al., 2016). Though MyoD and Myf5 performs similar functions in chromatin remodeling and activating myogenic specific genes, only MyoD can efficiently recruit DNA polymerase II (DNA Pol II) (polymerase) and robustly activate transcription (Melissa et al., 2016). The expression of these two factors (Myf5 and MyoD) commits the satellite cells to enter determination stage resulting in the muscle satellite cells becoming myoblasts (Peter, 2017). As MyoD induces myogenin expression and down-regulates Myf5 expression the cells exit from the cell cycle and enter the differentiation stage thereby converting myoblasts to myocytes and myotubes (Deato et al., 2008). MyoD and myogenin later express muscle specific genes to form multinucleated myofibers and turn off both the genes as they mature (Du et al., 2012). MyoD which acts as a master switch for muscle differentiation has its highest expression and function during the period between proliferating myoblasts to differentiated myotubes and then gets degraded by ubiquitin-proteasome system after the cell matures (Yasuo et al., 2018). When MRFs are expressed artificially, they are very potent in inducing myogenesis even in non-muscle cells such as fibroblasts, nerve cells and fat cells in-vitro thereby converting them into muscle cells (Kosenko et al., 2011; Klimczak et al., 2016). Therefore, every myogenic factor has a specific function to perform, though MyoD and Myf5 have some redundancy in function, they can't be fully replaced and are thus both required for optimal myogenic activity.

1.12 Importance of Muscle specific genes as indicated by knock-outs and associated pathologies:

Yamamoto in the year 2018 states that either MyoD or Myf5 is essential for skeletal muscle regeneration and mice with double knock outs (MyoD and myf5) completely failed in regeneration process (Yamamoto et al., 2018). Early studies reported that mice lacking MyoD gene did not exhibit any apparent abnormalities in their muscles but instead increased amounts of Myf5 mRNA were observed (Braun et al., 1992). Similarly, Myf5 null mice did not show visible defects in skeletal muscles formation but died after birth because of rib abnormalities and had reduced muscle mass (Braun et al., 1992). Pups lacking both MyoD and Myf5 had severe abnormalities and lacked muscle, resulting in death within minutes of birth (Rudnicki et al., 1993). Although, during regeneration, either MyoD or Myf5 expression is enough to repair damaged muscle cells, satellite cell defects are more evident in MyoD mutant cells. (Cooper et al., 1999). Satellite cells isolated from double MyoD knock-out mice fail to fuse with myofibers and resulted in multiple skeletal muscle myopathies in postnatal life (Francetic et al., 2011; Mokalled et al., 2012). Satellite cells having single functional MyoD allele have normal regeneration capacity (Melissa et al., 2016). Whereas, mice with single Myf5 allele showed delayed regeneration, decreased fiber diameter at endpoint and increased fatty deposition (Melissa et al., 2016). Skeletal muscle structure in Myf5 null (Myf5loxP/loxP) mice showed decreased muscle mass and reduced myofiber cross sectional area in comparison to normal mice (Peter, 2017). Francetic (2011) also observed a decreased transcription rate in MyHC protein in hind limb muscle. While MyoD null mice (MyoDm1/m1) showed structurally normal skeletal muscles, there were few myofibers (1%) which had morphological anomalies like being forked or having sprouts (Peter et al., 2017). MyoD mainly involves in the differentiation process of skeletal myoblasts whereas myf5 role is to regulate the proliferation rate and to maintain homeostasis within the muscle (Le grand et al., 2007). Myf5 null mice also showed a decreased myoblast amplification while myoD null mice showed an increased self- renewing capacity of satellite cells, rather than undergoing differentiation and entering myogenic differentiation (Ustanina et al., 2007). Removal of myogenin gene before the development of embryonic muscles leads to deficiencies in myofiber formation and the myogenin null mice die at birth (Allen 1979). Deletion of myogenin gene after the formation of embryonic muscles (post-natal), can slightly increases the transcription levels of MyoD,

Mrf4, but an 30% smaller growth rate is observed in comparison to normal wild-type mice (Knapp et al., 2006).

1.13 Factors influencing muscle growth:

Muscle is a dynamic tissue as there is always a constant production of new protein molecules and the breakdown of older protein occurring within muscle tissue; this is collectively known as protein turnover (Goldspink et al., 1991) The anabolic state of the muscle is defined as the state when muscle protein synthesis is greater than the muscle breakdown leading to net protein accretion and inhibition of muscle breakdown or apoptosis could also lead to building up of muscle mass (Robert et al., 2017). For the muscle to be in the anabolic state, there should be adequate supply of all macronutrients (proteins, carbohydrates and lipids), micronutrients (vitamins and minerals) including essential (not in ruminants), nonessential amino acids and their metabolites (polyamines). Muscle growth and anabolism also requires a distinct cell signaling stimulus to grow. Several studies demonstrate that, various factors including blood supply (and vasculature), innervation, hormones, nutrition and degree of tissue injury influence the determination of activated satellite cells to effectively undergo myogenic lineage progression (Charge et al., 2004; Yin et al., 2013). Accordingly, to obtain efficient muscle growth it is essential to consider the overall health of the animal. A proper understanding of all nutrients and their significant role in myogenesis will provide a potentially cost-effective means of optimizing lean muscle growth in mammals.

1.14 Macro and micronutrients role in increasing muscle growth:

Though macronutrients and micronutrients are required for adequate muscle growth, among them proteins play a crucial role in muscle synthesis and turnover rates. Essential amino acids play a significant role in activation of muscle protein synthesis (Hou et al., 2015). Limited supply of essential amino acids could notably decrease the muscle production whereas limiting the non-essential amino acid supply can be compensated through de novo synthesis (Elena et al., 2003). Intracellular signaling pathways, such as mTOR are well associated with muscle production and can be initiated or activated by presence or through diet availability of branched chain amino acids availability (Jewell et al., 2013). In fact, these amino acids are required by most non- ruminant mammals as they can’t be synthesized hence must be obtained

through diet (Cahill et al., 1976). Likewise, all amino acids are the building blocks for proteins, and perhaps even amino acid metabolites such as polyamines may be required for optimal skeletal muscle growth (Pegg et al., 2016).

1.15 Introduction to polyamines:

Polyamines, namely putrescine (diamine), spermidine (triamine) and spermine (tetra-amine), are low molecular weight aliphatic polycations ubiquitously found in all living cells and are considered as vital growth factors (Marton and Pegg, 1995; Moinard et al., 2005). They were first discovered in 1678 by Leeuwenhoek in seminal fluid. Polyamines play a vital role in various cellular activities including cell growth, proliferation, differentiation and migration. They also participate in gene transcription, translation, signal transduction, DNA stabilization and functioning of ion channels (Pegg et al., 2016). These polyamines are present in varied concentrations from high micromolar to low millimolar quantities in different cell types, with the highest levels being found in rapidly growing cells (Soda et al., 2011). There are the two sources through which cells can obtain polyamines. The first source is through exogenous supply through the diet. The second source is through endogenous production or de novo synthesis within the body and specific cells (Marton and Pegg, 1995).

1.16 Exogenous availability of polyamines:

When polyamine rich feeds (soybean and corn meal) are fed to cattle, they are digested and absorbed and distributed as a soluble component within the blood plasma until they are selectively taken into the cells through cell surface transporters (Soda et al., 2011). In mammals, selective polyamine transporters have not been identified but it is believed that the amino acid transporter Solute carrier family 3 activator of dibasic, which is a diamine transporter, is important for uptake (Pegg et al., 2011).

1.17 Endogenous synthesis of polyamines:

Polyamine levels are highly regulated in the cell by cellular uptake, de novo synthesis, degradation, interconversion and excretion (Wallace et al., 2003). Their levels are increased within the cell upon various stimuli such as injury, hormonal and anabolic steroidal signaling (Marton and Pegg, 1995; Figure 1.2). Amino acids such as methionine, arginine and ornithine

are the substrates for synthesis of polyamines (e.g., putrescine, spermidine and spermine) (Lee et al., 2010; Miller et al., 2016). L-Arginine is converted to ornithine upon arginase activity. Ornithine is converted into putrescine upon ODC enzyme activity (ornithine decarboxylase enzyme) (Lee et al., 2010). Methionine is converted into decarboxylated s-adenosyl methionine upon s-adenosylmethionine decarboxylase (Mamont et al., 1982). The pathway involving these two enzymes ODC and AdoMetDC are the rate limiting steps in the synthesis of polyamines (Pegg et al., 2009). In mice lethal effects have been observed due to any one of the gene knock-outs (ODC and AdoMetDC), which emphasizes the importance of polyamines (Pendeville et al., 2001; Figure 1.2). AdoMetDC enzyme is expressed in low levels compared to ODC (Theiss et al., 2002). Putrescine is converted to spermidine upon the activity of spermidine synthase (Theiss et al., 2002). Spermidine is further converted into higher polyamine spermine upon spermine synthase activity (Minois et al., 2011). Spermine can be interconverted into spermidine upon spermine oxidase activity (Minois et al., 2011). Spermine/spermidine N-acetyltransferase and polyamine oxidase can convert spermine into spermidine and also spermidine into putrescine (Casero et al., 1993).

ODC and AdoMetDC enzyme levels are regulated at transcription, translation and protein synthesis levels (Dircks et al., 1986; Shantz et al.,1999; Osborne et al., 1991; Nowotarski et al., 2011). For example, translation of ODC is highly regulated by intracellular polyamine concentration (Shantz et al., 1999). In case of increased intracellular polyamine levels, there is a decrease in ODC enzyme translation and decreased intracellular polyamine levels increases ODC enzyme translation rates (Miller et al., 2016; Figure 1.2). Decreased polyamine levels increases ODC homodimerization and produces putrescine polyamine (Sakata et al., 2000). Upon increased levels of polyamines ODC heterodimerizes with antizyme (AZ) and inactivates its action and further degrades ODC through 26S proteasome activity (Pegg, 2009). Antizyme also plays a role in inhibiting polyamine uptake and promoting polyamine excretion (Sakata et al., 2000). Antizyme is regulated by antizyme inhibitor (AZi). Antizyme strongly binds to AZi (high-affinity) than to ODC which allows ODC to dimerize and get away from AZ (Bae et al., 2018). Both AZ and AZi get degraded through ubiquitin-dependent proteasome activity (Igarashi et al., 2010). AdoMetDC is initially synthesized as an inactive proenzyme and gets activated upon putrescine action (Pegg, 2009). AdoMetDC enzyme levels are also dependent upon availability of other intracellular polyamines (Bae et al., 2018). This enzyme

is positively regulated by putrescine and negatively regulated by higher levels of polyamines; spermine and spermidine (Lee et al., 2010). The regulation of AdoMetDC, spermidine synthase and spermine synthase translation is not well studied (Miller et al., 2016). However, AdoMetDC and ODC enzyme levels are increased upon various growth enhancing stimuli such as hormones, steroids and during tissue repair (Cepero et al., 1988). Thus, changes in polyamine levels along with associated rate limiting enzyme, with anabolic stimulus, suggests that it is possible that supplementing polyamines through the diet might have the capacity to increase myogenesis and muscle growth in livestock

1.18 Growth factors increase muscle growth by increasing polyamine levels:

Turchanowa and colleagues in the year 2000 observed increased polyamine levels in rat skeletal muscles following both resistant and endurance exercise, mediated through increased endogenous androgen production along with increased availability of their receptors. The increased testosterone levels activated ODC and AdoMetDC enzyme (2 hours post exercise) levels required for polyamines synthesis (Turchanowa et al., 2000). The same effect is observed even after artificial testosterone administration. In both scenarios, scientists reported an increase in muscle production compared with control (Turchanowa et al., 2000). Cepero and his co-workers in the year 1998 demonstrated a significant increase in rat gastrocnemius and cardiac muscle after stimulated by beta adrenergic hormone (salbutamol). Followed by beta adrenergic hormone stimulation there is an increase in ODC enzyme levels and subsequent increases in putrescine, spermidine and spermine levels (Cepero et al., 1998). To ascertain that observed muscle hypertrophy with increased ODC enzyme is solely because of salbutamol, in next experiment researchers administered beta adrenergic antagonists (propranolol) and ODC antagonist DFMO (α-difluoromethylornithine). This caused a decreased in muscle size and drop in polyamine levels (Cepero et al., 1998). Insulin treatment of H9C2 myoblast cells increased the ODC enzyme activity coinciding with the cells entering differentiation stage, formation of myotubes and expression of myogenin protein (Govoni et al., 2009). With DFMO treatment the ODC enzyme activity is inhibited and myogenin expression is delayed (Govoni et al., 2009). When somatomedins are treated to L-6 myoblast cells they found a similar increase in ODC enzyme and associated muscle differentiation

which is then inhibited by DFMO (Ewton et al., 1984). Additional evidence shows that male orchiectomized mice had decreased muscle mass, kidney size, renal ODC and over all polyamine (putrescine, spermidine and spermine) levels in the body (Goldstone et al., 1982). Supplementation of testosterone to the orchiectomized mice reversed these effects and resumed showing these effects when the ODC enzyme is inhibited using DFMO inhibitor (Goldstone et al., 1982). Androgens perform their functions by binding to the androgen receptors and regulating both polyamine biosynthesis and multiple gene functions (Maclean et al., 2008). A study showed that inhibition of androgens in mice through orchidectomy caused muscle atrophy and decreased ODC levels. whereas androgen knock out mice showed similar results with decrease muscle size and ODC levels (Maclean et al., 2008).

1.19 Depleted polyamine levels and their negative influence:

Polyamines are requiring for cell growth, proliferation, differentiation and maturation, while limited polyamine availability can lead to deleterious effects in cells (Pegg, 2009; Guerra et al., 2016). Anthony and his co-workers in the year 2016 observed an inhibition in cell growth and proliferation after treating with DFMO (ODC inhibitor). Decreased spermidine and spermine levels caused a reduction in cell proliferation rates and increases programmed cell death (Pegg et al., 2016). The cytostatic effect of the cells can be reversed by supplementation of putrescine and spermine exogenously (Pegg et al., 2016). A study showed that when c2c12 myoblast cell lines are deprived of ODC enzyme for 48- and 72-hours using DFMO, decreased myoblast proliferation of 40% and 66% was observed (Lee et al., 2011). Another study showed that cells treated with DFMO (ODC inhibitor), APCHA (N-(3-aminopropyl)-cyclohexylamine) (spermine synthase inhibitor) and deoxy-hypusine synthase inhibitor (hypusine inhibitor), showed a significant decrease in cell growth and proliferation (Pegg et al., 2016). Hypusine is a spermidine derivative that plays a key role in the production of eIf5a (eukaryotic translation initiation factor 5a) involved in DNA translation and protein synthesi (Lee et al., 2011). This indicates that muscle cell proliferation which is essential in muscle protein accretion involves polyamines and their derivatives, such as hypusine (Pegg et al., 2016).

1.20 Polyamines role in muscle regeneration and muscle atrophy:

Polyamines also play a key role in the Muscle regeneration process. This is demonstrated by Kaminska in the year 1982. Mice subjected to tenotomy (surgical transection of a tendon) have increased amounts of putrescine, spermidine and spermine for 3 weeks followed by wound healing and polyamine levels returning to normal levels (Kaminska et al., 1982). When rats are surgically denervated there is an increase in putrescine, spermidine and spermine levels in soleus and gastrocnemius muscles followed by cell proliferation and recovery (Kaminska et al., 1982). Several neuromuscular diseases lead to muscle atrophy and change in polyamine levels, one among them is Duchenne muscular dystrophy characterized by having repeated muscular atrophy (muscle degeneration) and hypertrophy (muscle regeneration) coinciding with increased polyamine levels in regeneration phase (Kaminska et al., 1981). Supporting this, another study found increased polyamine levels in patients suffering with limb girdle dystrophies and the disease is well characterized by degeneration and regeneration process (Rudman et al., 1980).

1.21 Polyamines and myogenesis:

Myogenesis is a complex and highly regulated process involving myogenic regulatory factors (MRFs) such as MyoD, Myf5, myogenin, MRF4, and other transcription factors such as paired box 3 (Pax3) and paired box 7 (Pax7; Soleimani et al., 2012). MyoD and Myf5 are primarily responsible for proliferation and differentiation of myogenic progenitor cells into myoblasts and are expressed prior myogenic differentiation (Gianakopoulos et al., 2011). Myogenin is involved in the differentiation of myoblasts into myotubes while MRF4 leads to cell fate determination (Kassar et al., 2004). Myogenic progenitors such as satellite cells act as stem cells and are involved in the remodeling of muscle (Zanou et al., 2013).

The process of myogenic differentiation is extensively studied in C2C12 cell line. (Buckingham et al., 2003; Bajaj et al., 2011; Sin et al., 2015). Several compounds are demonstrated to be regulating the differentiation of C2C12 myoblasts into fully differentiated post-mitotic myotubes (Sakane et al., 2016). Among these, a polyamine catabolizing enzyme, spermine oxidase (SMO), has been shown to modulate the differentiation of C2C12 myoblasts (Cervelli et al., 2008). Spermine oxidase catalyzes the conversion of spermine to spermidine

and 3-aminopropanal (3-AP)) (Uemura et al., 2017). In muscle tissue, a high transcription and activity of SMO gene has been demonstrated (Cervelli et al., 2004) which rapidly induces response to stress and leads to the production of hydrogen peroxide in different cell lines and malignant tissues (Pledgie et al., 2005). Dysregulation of SMO leads to defective differentiation and can contribute to malignant transformation (Cervelli et al., 2008). Several cancers demonstrate increased SMO expression (Park et al. 2013; Murray et al., 2016). In one study, decreased SMO expression was linked to atrophy of muscle induced by limb immobilization, fasting, muscle denervation, and aging while SMO overexpression resulted in a decrease of the atrophy promoting genes and increase in the genes responsible for maintaining muscle mass (Bongers et al., 2015). In another study, N1, N11-diethylnorspermine, a potent inducer of polyamine catabolism caused an epithelial-mesenchymal transition (EMT) -like dedifferentiation in hepatocyte cultures (HepaRG) (Hepatocyte cultures). There is substantiated evidenced by down-regulation of mature hepatocytes markers and upregulation of classical EMT markers (Ivanova et al., 2018). The authors also found that spermidine was a key regulator of hepatocyte differentiation and DENSpm-triggered dedifferentiation of HepaRG (Hepatocyte cultures) cells. This led to dramatic metabolic adaptations in the several signal transduction pathways leading them to conclude that polyamine metabolism was tightly linked to EMT and differentiation of liver epithelial cells. (Ivanova et al., 2018).

Satellite cells, when co-cultured with adipocytes, express limited myogenic differentiation. (Thornton et al., 2013). However, when these co-cultures were supplemented with polyamines, satellite cells significantly increased the expression of markers of differentiation such as myoD, myogenin, myogenic factor 5 (myf5) and decrease expression of paired box transcription factor 7 (Pax7) and sprouty1 was observed. This suggests a pro-myogenic effect of polyamines in vivo (Thornton et al., 2013).

Polyamines such as putrescine, spermidine and spermine also mediate cell homeostasis and cellular differentiation. Govoni and colleagues (2010) demonstrated that polyamines led to the differentiation of the myogenic cell line H9c2 into a muscle phenotype as evidenced by plasmid mediated overexpression of ornithine decarboxylase, the rate limiting enzyme in the polyamine biosynthesis, that led to spontaneous differentiation into myotubes. In addition,

polyamine depletion with alpha-difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase, annulled the differentiation process (Govoni et al., 2010)

1.22 Polyamines in livestock production:

The polyamines including putrescine, spermidine and spermine are demonstrated to play an important role in basic cellular process in the animal body (Miller et al., 2016). Rapidly growing cells demonstrate enhanced synthesis and content of polyamines and treating with ODC inhibitors such as di-fluoromethylornithine (DFMO) led to the complete loss of putrescine and spermidine and little change in spermine but lead to the inhibition of cell proliferation (Lee et al., 2011). In feeding trails, polyamine supplementation was shown to improve feed efficacy and body weight gain in animals (Smith 1990). Smith (1990) observed that feeding 0.2% putrescine in chicks increased growth rate while further supplementation (0.8 to 1%) was toxic and reduced growth (Smith et al.,1990). Sousadias and colleagues (1995) observed that unlike putrescine, spermine, a biogenic amine and the most cationic of the polyamine, supplementation was toxic even at 0.2% supplementation and growth promoting at lower doses was very small (Sousadias et al., 1995). Hashemia and co-workers (2014) demonstrated that a methionine deficient diet in chickens decreased body weight gain, protein concentration and feed efficacy ratio while putrescine supplementation improved the FCR and blood antibody level (Hashemia et al., 2014).

The performance of Gastrointestinal tract is critical for increasing production in animals. Polyamines are involved in the growth and maturation of intestines (Hashemi et al., 2014). Molina and colleagues (2009) observed that supplementing polyamines (spermine and spermidine) in milk formula enhanced gut growth and maturation in neonatal piglets as evidenced by increased villus length and crypt depth in small intestines and increased the activities of alkaline phosphatase and gamma-glutamyl transferase activities in the jejunum (Molina et al., 2009). Spermine has been recently discovered to be involved in the activation and thermal stabilization of bovine trypsin (Momeni et al., 2016).

Polyamines are necessary for maintaining the integrity of normal GIT epithelium after injury (Wang et al., 2015). The also play vital role in proliferation and differentiation of intestinal mucosa (Wang et al., 2015). Wang and co-workers (2015) reported that oral administration of

putrescine or proline increased the body weight and daily gains of piglets. These findings indicate that polyamine or its precursor could improve mucosal proliferation, intestinal morphology, as well as tight junction and potassium channel protein expressions in early-weaned piglets, with implications for epithelial restitution and barrier function after stress injury (Wang et al., 2015). Similarly, Wang and colleagues (2016) showed that polyamine metabolism and ODC expression were altered by weaning and proline administration, a precursor amino acid for polyamines, in piglets (Wang et al., 2016). Oral administration of proline increased the concentrations of spermidine and spermine in the ileal mucosa and ODC protein expression in the three segments of the intestine (Wang et al., 2016). In an *in vitro* study, Wang along with co-workers (2017) showed that L-proline promotes polyamine synthesis by mediating ODC expression and plays an important role in cell-cycle progression and cell proliferation in the porcine enterocyte IPEC-J2, and polyamine-deficient IPEC-J2 cells, and may protect from apoptosis, thereby improving the health and development of small intestinal mucosa in piglets (Wang et al., 2017).

Reproduction

Polyamines are essential for embryonic and foetal survival, growth, and development. Polyamines are also reported to be involved in the regulation of angiogenesis and early embryogenesis (Reynolds et al., 2001). Zhu along with co-workers (2015) observed that polyamine concentration varied with different foetal compartments, stage of gestation, and the health of the foetus (normal foetus vs intrauterine growth restricted conceptus) (Zhu et al., 2015). Abnormal foetal plasma spermidine levels correlated with increased autophagy which acted as a survival mechanism in Intra uterine growth restriction (Zhu et al., 2015).

Placental insufficiency leads to suboptimal reproductive performance and reduced birth weights in animals especially pigs (Wu et al., 2010). Enhancing placental growth and function through nutritional management is an effective way to improving reproductive performance (Wu et al., 2010). In one study, Wu along with co-workers (2010) showed that dietary supplementation with 0.83% L-arginine to gilts increased the number and litter birth weight of live-born piglets (Wu et al., 2010). In addition, supplementing the gestation diet with 0.4% L-arginine plus 0.6% L-glutamine enhanced the efficiency of nutrient utilization, reduced

variation in piglet birth weight, and increased litter birth weight (Wu et al., 2010). These effects were mediated by regulating syntheses of nitric oxide, polyamines, and proteins, functional amino acids that stimulate placental growth and the transfer of nutrients from mother to embryo or foetus (Wu et al. 2010). In a previous study, Jobgen and co-workers (2008) demonstrated that ewes adapted to nutrient restriction by improving placental efficacy to maintain foetal amino acid supply. In another study, Li and colleagues (2015) showed that supplementation of pro-polyamine amino acid L-arginine (1.3%) during early gestation in gilts and sows improved the reproductive performance through in increased plasma spermidine concentration (Li et al., 2015).

Based on the literature reviewed herein the hypotheses tested within this thesis research project are as follows:

1.23 Hypotheses:

1) Physiological doses of polyamines influence the mRNA levels of myogenic transcription factors in cultured muscle cells.
2) Physiological doses of polyamines influence the de novo polyamine synthesis in cultured muscle cells by altering mRNA abundance of the rate limiting enzyme; ornithine decarboxylase.
3) Polyamine treatment of commercial immortalized muscle cell lines; c2c12 and sol8 will affect myogenic transcription factors in the same manner

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Figure 1.1. postnatal skeletal myogenesis

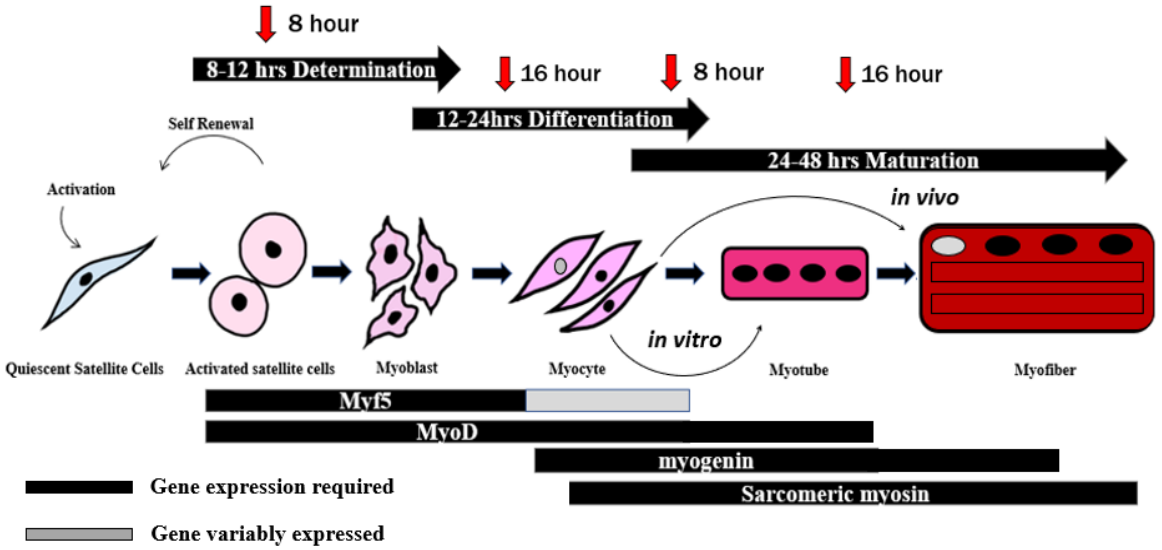


Figure 1.1. Schematic representation of postnatal myogenesis. This illustrate the quiescent satellite cell upon activation enter determination phase to become myoblast (High Myf5 and MyoD expression), further myoblasts enter the differentiation (High MyoD and partial or no Myf5 expression indicated by uncolored rectangle) and maturation phase (peak Myogenin and Sarcomeric myosin expression) and convert into myocytes and myotubes. This progression is highly dependent on action of various myogenic transcription factors that turn on and off during this process of myogenesis.

Figure 1.2. Endogenous polyamine synthesis pathway

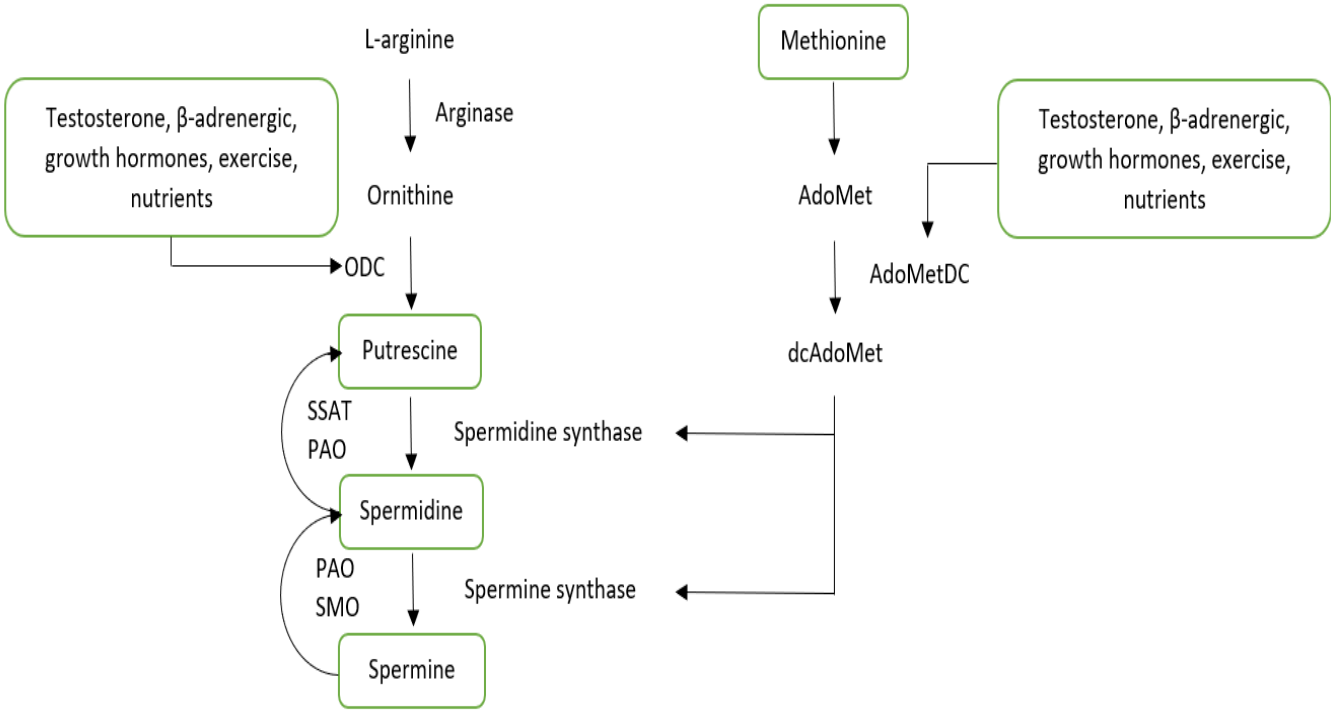


Figure 1.2. Endogenous polyamine metabolic pathway and interconversion. ODC=Ornithine decarboxylase;AdoMetDC=S-adenosylmethionine decarboxylase; dcAdoMet=decarboxylated adenosyl methionine; SMO= spermine oxidase; PAO= Polyamine oxidase; SSAT=spermidine/spermine-N -acetyl transferase. Anabolic signals along with nutrients influence ODC and AdoMetDC enzymes. (Modified from Pegg, 2009)

Chapter 2: Materials and Methods

2.1 Materials:

Mouse c2c12 myoblasts (CRL-1772) and sol8 myoblasts (CRL-2174) cells were acquired from ATCC and stored in liquid nitrogen. Dulbecco's modified eagle's medium (DMEM media) (Lot# 1663988) with high glucose was obtained from Gibco Life technologies. Fetal bovine serum (Lot# 1939752), horse serum (Lot# 1365591) and 0.25% Trypsin-EDTA (Lot# 903931) were obtained from Gibco. PES filter membrane 0.2um (Batch No: W150624) was purchased from VMR. Realtime TaqMan based probes, forward and reverse primers for 18S, MyoD, Myogenin, Myf5, ODC, were designed using (primer express 3.0) and acquired from Invitrogen Life technologies. Turbo DNAase Free (p/n: AM1907) and High-capacity cDNA reverse transcription kit (part no: 4368813, lot no. 00283768) were obtained from Ambion and Applied Biosystems. TaqMan fast master mix with ROX normalization fluorescent dye, was purchased from Applied Biosystems. Six well plates (Lot# 08390151) (Cat. -N0: 657 160) from Greiner Bio one and T75 flasks are purchased from corning company (REF 430641U-5/Bag). VMR micro centrifuge tubes, VMR sterile aerosol pipet tips, VMR micro pipettes and Pipet-aid pipette are used in entire experiment. For PCR 0.2ML thin wall strip tubes and caps are purchased from Genuine Axygen quality brand. TRIzol Reagent (lot# 990066) was supplied from Ambion life technologies. Supplies of 2-Propanol ≥99% pure (Batch# 64296DM) and absolute ethanol (Lot# SHBC9546V), both are molecular biology grade purchased from Sigma-Aldrich and used in RNA extraction method. Chloroform (molecular purpose) (Lot# u220719) is obtained from Fisher Bio Reagents. Spermine (Lot# BCBG3281V, MW: 202.34 g/mol and P code: 101186157) stored at 4ºc and putrescine dihydrochloride stored at room temperature (Lot# BCBF3048V, MW: 161.07 g/mol and P code: 101143518) are purchased from Sigma life science and were resuspended in sterile Millipore RNase free water prior to use.

2.2 Cell culture of c2c12 cells:

a. Undifferentiated cells:

Mouse c2c12 myoblasts were obtained from ATCC and stored in liquid nitrogen until use. Stored vials are thawed for 2 min in a warm water bath (37ºC). Based on commercial information regarding the cell concentration we removed an estimated $1x10^6$ cells and cultured them in DMEM media containing high glucose and 10% FBS. The preparation of growth media for c2c12 cells included adding 12g of powdered DMEM medium and 3.33 grams of sodium bicarbonate to 900ml of purified distilled water. Contents were mixed thoroughly using a magnetic stir bar and stir plate. The growth media pH was adjusted to 7.1 by addition and titration with 1N HCl. Inside the laminar air flow 100 ml of fetal bovine serum was added and the media was filtered using 0.2um PES membrane under hood supply vacuum. Cells were cultured under humidified atmospheric conditions in HEPA cell incubator maintained at 37^0C temperature and 5% CO_2. When cultured cells were assessed to have reached 70% confluence the cells were trypsinized with 0.25% trypsin for division. Live c2c12 cell count was assessed using Life technologies Countless II automated cell counting chamber. Each well in the 6 well culture plate was subsequently seeded with approximately 78,000 live cells. Following 18 h of c2c12 cells incubation and equilibration in DMEM containing 10% fetal bovine serum, the cells were treated with 0.5 mM spermine (spermine treated cells), or 5 mM putrescine (putrescine treated cells) or 10 mM methionine (amino acid control) or no polyamine or amino acid (control) Stock solutions: 0.5 mM spermine were prepared by dissolving 21.24 mg of spermine in 5ml of sterile Millipore water, 84.55 mg of putrescine was dissolved in 5ml of sterile Millipore water and 156.67 mg of methionine was dissolved in 5ml of sterile Millipore water. Each 6 well plate was comprised of 3 controls and 3 treatments per plate to allow for direct within plate treatment versus control comparison. Control and test samples are arranged as illustrated in the figure (Figure 1.3.) below in the plate. Each Test well consisted of 2 ml growth media and 100 µl of polyamines treatment while control well consist of 2 ml of growth media and 100 µl of sterile Millipore water. After 8 and 16 hours of the treatment, culture media was removed, and cells are treated with TRIzol reagent (0.3 ml). After TRIzol addition, lysed cells and nucleic acid was transferred into sterile 1.5mL microcentrifuge tubes using Gilson micropipettes with sterile tips.

b. Differentiated cells:

Mouse c2c12 myoblasts were obtained from ATCC and stored in liquid nitrogen until use. Stored vials are thawed for 2 min in a warm water bath containing 37ºC temperature. Cells were grown and cultured in 6 well plate (78,000 live cells/well) following the undifferentiated c2c12 cells protocol. To differentiate c2c12 cells differentiating media is prepared by taking 12 g of powdered DMEM medium with high glucose and 3.33 g of sodium bicarbonate to dissolved in 880 ml of distilled water using magnetic stirrer and the pH was adjusted to 7.2 by adding 1N HCL. 20 ml of horse serum is added, and contents are filtered using 0.2 µm PES membrane with vacuum inside laminar air flow hood. Following 18 h of c2c12 cells incubation with DMEM containing 10% fetal bovine serum, the cells are treated with differentiated media (DMEM media containing 2% horse serum) for 24 hours to differentiate, after that cells were treated with 0.5mM spermine (spermine treated cells), or 5 mM putrescine (putrescine treated cells) or 10 mM methionine (amino acid control) or no polyamine or amino acid (control). Stock solutions: are prepared as detailed in undifferentiated c2c12. Each 6 well plate was comprised of 3 controls and 3 treatments per plate to allow for direct within plate treatment versus control comparison. Control and test samples are arranged as illustrated in the figure (Figure 1.3.) below in the plate. Each Test well consisted of 2 ml growth media and 100 µl of polyamines treatment while control well consist of 2 ml of growth media and 100 µl of sterile Millipore water. After 8 and 16 hours of the treatment, culture media was removed, and cells are treated with TRIzol reagent (0.3 ml). After TRIzol addition, lysed cells and nucleic acid was transferred into sterile 1.5mL microcentrifuge tubes using Gilson micropipettes with sterile tips.

2.3 Cell culture of sol8 cells:

a. Undifferentiated cells:

Mouse sol8 myoblasts were obtained from ATCC and stored in liquid nitrogen until use. Stored vials are thawed for 2 min in a warm water bath containing 37ºC temperature. Based on commercial information regarding the cell concentration we removed an estimated $1x10^6$ cells and cultured them in DMEM media containing high glucose and 20% FBS. The preparation of growth media for c2c12 cells included adding 12g of powdered DMEM

medium and 3.33 grams of sodium bicarbonate to 900ml of purified distilled water. Contents were mixed thoroughly using a magnetic stir bar and stir plate. The growth media pH was adjusted to 7.1 by addition and titration with 1N HCl. Inside the laminar air flow 200 ml of fetal bovine serum was added and the media was filtered using 0.2um PES membrane under hood supply vacuum. Cells were cultured under humidified atmospheric conditions in HEPA cell incubator maintained at 37^0C temperature and 5% CO_2. When cultured cells were assessed to have reached 65% confluence the cells were trypsinized with (0.25% trypsin) for division. Live c2c12 cell count was assessed using Life technologies Countless II automated cell counting chamber. Each well in the 6 well culture plate was subsequently seeded with approximately 78,000 live cells. Following 16 h of sol8 cells incubation and equilibration in DMEM containing 20% fetal bovine serum, the cells were treated with 0.5mM spermine (spermine treated cells), or 5 mM putrescine (putrescine treated cells) or 10 mM methionine (amino acid control) or no polyamine or amino acid ("control"). Stock solutions: are prepared as detailed in undifferentiated c2c12. Each 6 well plate was comprised of 3 controls and 3 treatments per plate to allow for direct within plate treatment versus control comparison. Control and test samples are arranged as illustrated in the figure (Figure 1.3.) below in the plate. Each Test well consisted of 2 ml growth media and 100 µl of polyamines treatment while control well consist of 2 ml of growth media and 100 µl of sterile Millipore water. After 8 and 16 hours of the treatment, culture media was removed, and cells are treated with TRIzol reagent (0.3 ml). After TRIzol addition, lysed cells and nucleic acid was transferred into sterile 1.5mL microcentrifuge tubes using Gilson micropipettes with sterile tips.

b. Differentiated cells:

Mouse sol8 myoblasts were obtained from ATCC and stored in liquid nitrogen until use. Stored vials are thawed for 2 min in a warm water bath (37ºC). Cells were grown and cultured in 6 well plate (78,000 live cells/well) following the undifferentiated sol8 cells protocol. To differentiate sol8 cells differentiating media is prepared by taking 12 g of powdered DMEM medium with high glucose and 3.33 g of sodium bicarbonate to dissolved in 880 ml of distilled water using magnetic stirrer and the pH was adjusted to 7.2 by adding 1N HCL. 20 ml of horse serum is added, and contents are filtered using 0.2 µm PES membrane with vacuum inside laminar air flow hood. Following 16 h of c2c12 cells incubation with DMEM containing 20%

fetal bovine serum, the cells are treated with differentiated media (DMEM media containing 2% horse serum) for 24 hours to differentiate, after that cells were treated with 0.5mM spermine (spermine treated cells), or 5 mM putrescine (putrescine treated cells) or 10 mM methionine (amino acid control) or no polyamine or amino acid (control). Stock solutions: are prepared as detailed in undifferentiated c2c12. Each 6 well plate was comprised of 3 controls and 3 treatments per plate to allow for direct within plate treatment versus control comparison. Control and test samples are arranged as illustrated in the figure below in the plate. Each Test well consisted of 2 ml growth media and 100 µl of polyamines treatment while control well consist of 2 ml of growth media and 100 µl of sterile Millipore water. After 8 and 16 hours of the treatment, culture media was removed, and cells are treated with TRIzol reagent (0.3 ml). After TRIzol addition, lysed cells and nucleic acid was transferred into sterile 1.5mL microcentrifuge tubes using Gilson micropipettes with sterile tips. All experiments contain 3 test (polyamine or amino acid) and 3 controls (no addition of polyamine or amino acid) for each time point (8 and 16 h) (Figure 1.3.). Each of such experiments were performed thrice (3 test samples and 3 control samples per plate x 3 experimental plates = 9 test samples + 9 control samples for each time point).

2.4 RNA Extraction and quantification:

Total RNA extraction was carried out using TRIzol extraction method. 0.3 ml of TRIzol reagent is used directly to lyse the ($1x10^7$) cells on culture plate. 60 µl of chloroform is incubated for 2-3 minutes. Samples were centrifuged for 15 minutes at 12,000 x g at 4^{o}C to separate the mixture into different layers containing red phenol-chloroform in the bottom middle interphase and upper aqueous layer. The upper aqueous phase containing RNA was carefully transferred into fresh tubes and 150 µl of isopropanol was added to the aqueous phase containing RNA and precipitated for 3 hours by placing it at -20ºC freezer. Later samples were subsequently centrifuged at 15,000 x g for 30 min at 4^{o}C (Avanti model-1688) this causes RNA to form a pellet at the bottom of the tube. Supernatant was carefully discarded using micro pipettor and pellet was resuspended with 300 µl of 75% Ethanol. Samples were vortexed for few seconds and centrifuged at 12000 x g for 10 min. Obtained supernatant was discarded using micro pipettor. Extracted RNA pellet at the bottom is air dried for 5-10 minutes and dissolved in 15 µl of Nuclease free water and kept in -20ºC freezer. Each RNA

sample was quantified using Nanodrop 1000 UV-V spectrophotometer. Each RNA sample was quantified using Nanodrop 1000 UV-V spectrophotometer.

2.5 cDNA synthesis:

1 µg of RNA was dissolved in 10 µl of nuclease free water and the Samples were then treated with Turbo DNAse free to remove all traces of DNA contamination. Then 1 µl of TURBO DNase buffer and 1 µl of TURBO DNase were added to the RNA samples and incubated at 37ºc for 30 min. After the incubation period 2 µl of DNase inactivation reagent was added and incubate for another 5 min at room temperature by flicking it 2-3 times during incubation. Later the samples are centrifuged at 10,000 x g for 1.5 min and transfer the RNA into a fresh tube. Turbo DNAse treatment has produced 10 µl of the solution for each sample and this is used to synthesis cDNA. Using high capacity reverse transcription kit cDNA is synthesized with 1 µg of RNA for each sample. RT master is prepared with 2 µl of 10 x RT buffer/sample, 0.8 µl of 25 x dNTP mix/sample, 2.0 µl of 10 x RT random primers/sample, 1.0 µl of Multiscribe Reverse Transcriptase/sample, 1.0 µl of RNase inhibitor/sample, 3.2 µl of nuclease free H_20. Using micro pipette 10 µl of 2 RT master mix is pipetted into 0.2 ml thin wall strip tubes and then 10ul of RNA sample was pipetted into those tubes. Samples are centrifuged briefly to spin down the contents and eliminate the air bubbles. Strip tubes are placed on Thermal cycler and programed it to run for 25ºc for 10min in step 1, 37ºc for 120min in step 2, 85ºc for 5 min in step 3 and can place in 4ºc for infinite time. Store it in -20ºC Finally, 1 µl containing 50 ng of cDNA has been used to analysis gene in real time PCR.

2.6 Real-time PCR:

Relative quantification using real time PCR (qRT-PCR) was performed on using TaqMan MGB probe, reverse and forward primers. Real-time PCR assays were run in duplicate for each sample for all genes along with 18S as the endogenous control (also in duplicate). We included negative controls for all genes, these were wells without the addition of cDNA but with all other master mix components. Each reaction consists of 7.5 µl of master mix, 1.5 µl of reverse primer (conc: 10 µm/lit), 1.5 µl of forward primer (conc: 10 µm/lit), 1.5 µl of probe cDNA (conc: 2.5 µm/lit), 2 µl of Nuclease free water and 1 µl of cDNA. Real time PCR cycle

conditions include holding time of 90ºc for 20 sec, 40 cycles of 90ºc for 3 sec followed by 60ºc for 30 sec of melting and extension temperatures respectively.

2.7 Statistical analysis:

All statistical analyses were run using R software (Version 3.5.1). Data obtained from 3 experiments for each time point and polyamine dose (3 test samples and 3 control samples per plate x 3 experimental plates = 9 test samples + 9 control samples for each time point and dose) are pooled. Two tailed t-test were performed on pooled data set to measure the significant difference between treatments and control. Data was analyzed using relative C_T (ΔCt) method (Pfaffl 2001). In all cases duplicates for each sample were measured and the mean (average) of the two values was utilized for analyses. Delta Ct values of each gene were obtained by deducting average Ct values of target gene (18S) from average Ct values of endogenous control (18S). Set threshold is consistent among all the experiments with specific to gene is 18S - 0.044122, ODC - 0.06274, Myf5 - 0.032756, MyoD - 0.031881, Myogenin – 0.02242. Necessary base line corrections are performed in linear phase and they change with every experiment. In Standard error of means was used to calculate the error bars. Significance was calculated using p-value. A $p \leq 0.05$ was considered significant and labeled with * whereas a $p \leq 0.01$ was also considered significant and indicated with **

Figure 2.0. Template of 6 well plate

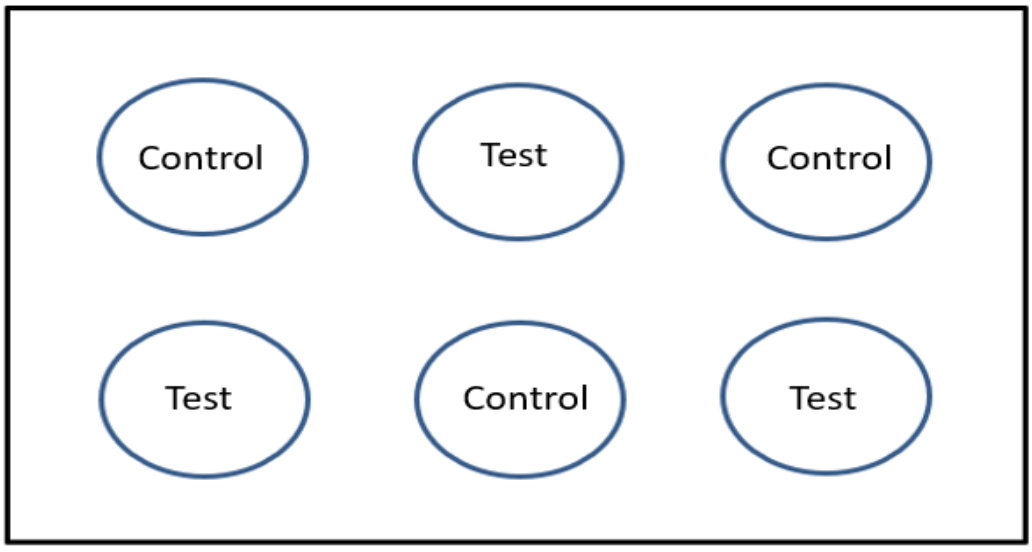


Figure 2.0. The test and control wells alternately arranged in 6 well plates.

Results and Discussion

2.1 Putrescine influence on Myf5 mRNA in undifferentiated and differentiated c2c12 cells:

Putrescine (5 mM) (Figure 2.1) treatment significantly increases the Myf5 gene transcription at determination (P = 0.040) and differentiation stages (16h) (P = 0.030) of myogenesis in undifferentiated cells. There is no significant difference in Myf5 mRNA at early (P = 0.100) and late (P = 0.100) maturation phases in differentiated c2c12 cells.

Increase in the Myf5 mRNA upon treatment with physiological doses of putrescine is considered a promyogenic signal to undifferentiated c2c12 cells. The significant increase in Myf5 mRNA upon putrescine supplementation made us reject the null hypothesis in undifferentiated cells. Myf5 is one of the predominant genes that is expressed in the activated satellite cells and helps them to enter determination and differentiation stages of myogenesis (Chal et al., 2017). This increase in Myf5 mRNA can cause more muscle cells to enter the determination and differentiation stages of myogenesis yielding efficient muscle protein when translated to live animals. According to a previous study when putrescine was supplemented to broiler chicken at 0.05 and 0.1%, there was a significant increase in intestinal villi and growth rate at early post hatch life of chicken (Devipriya et al., 2015). Though the animal model and dose rates differ to the experimental model of this study, the increased Myf5 mRNA in undifferentiated cells of this study (promyogenic sign), agrees with the increased growth rate of chicken.

Lack of significant change of Myf5 mRNA in differentiated cells led to the failure of rejecting the null hypothesis. Given that only two-time points were measured, this study might have missed a critical time point or alternatively putrescine treatment at the differentiation stage might not cause considerable difference as the cells are not rapidly dividing compared to determination stage. Polyamine effects are significantly observed in rapidly diving cells than in normal cells. Their levels quickly increase during the growth phase and inhibiting them will cease the growth (Igarashi et al., 2009; Landau et al., 2010; Lee et al., 2011; Mandal et al., 2013). This elucidates that polyamines are require for the growth and cells require to synthesis

them during this phase (Igarashi et al., 2009). It has been observed that cells in rapidly dividing stage and in cancerous stages have increases their growth rate when supplemented with polyamines compared to normal cells (Minois et al., 2011; Soda et al., 2011). This demonstration that polyamines might be showing a significant effect on rapidly diving cells and this effect might not be seen in normal cells.

2.2 Putrescine influence on MyoD mRNA in undifferentiated and differentiated c2c12 cells:

Putrescine (5mM) (Figure 2.2) treatment does not significantly change the MyoD gene transcription at determination stage ($P = 0.100$) whereas significant difference is observed in differentiation stage (16 h) ($P = 0.040$) of myogenesis in undifferentiated cells. There was no significant difference in MyoD mRNA at early ($P = 0.100$) while an increasing trend was observed in the late ($P = 0.060$) maturation phases of differentiated c2c12 cells.

The significant increase in MyoD mRNA upon putrescine supplementation in differentiation stage made to reject the null hypothesis. This increase in MyoD mRNA upon supplementing physiological doses of putrescine acted as a promyogenic signal for the cell to proceed towards becoming a mature muscle cell. It is well established that MyoD, which is a myogenic determination factor, is expressed in satellite cells as they successfully enter into determination phase and transition to myoblasts (Ustanina et al., 2007; Natasha et al., 2016). MyoD continues to be expressed in differentiation stages of myogenesis and the expression of this gene supports the transcriptomic profile essential for the progression towards a phenotypically mature myocyte (Marshall et al., 2008, Garcia et al., 2011). This increase in the transcription of MyoD mRNA is a direct stimulus that recruits more satellite cells to become myoblasts, and subsequently myocytes; hence, it is a promyogenic signal. Further, MyoD promotes successful myogenesis, and its absence typically causes myoblasts to undergo apoptosis (Angelo et al., 2002; Hirai et al., 2010). Therefore, expressing significant increased levels of MyoD mRNA can potentially increase the myoblast and myocyte number, thereby subsequently increasing the efficiency of muscle production or growth.

Lack of significant difference in differentiated cells led to the failure of rejecting the null hypothesis. The reasons for this observation are the same as discussed for “putrescine effects on Myf5 mRNA in c2c12 differentiated cells”.

A study published in 2001 by Mireia and colleagues stated that putrescine treatment of colon carcinoma cell line at 100, 550 and 1000 µm concentration for 24 hours significantly increased the proliferation of colon cells compared to the control. Though the cell line and dosage used in this study are different from the present study, increased proliferation due to putrescine supplementation is corroborated by our current finding that increased MyoD mRNA was an essential step for cell proliferation.

2.3 Putrescine influence on myogenin mRNA in undifferentiated and differentiated c2c12 cells:

Putrescine (5 mM) (Figure 2.3) treatment does not significantly change the myogenin gene transcription at determination stage ($P = 0.100$) whereas significant difference was observed in the differentiation stage (16h) ($P = 0.003$) of myogenesis in undifferentiated cells. There was no significant difference in the myogenin mRNA at early ($P = 0.800$) and late ($P = 0.400$) maturation phases in differentiated c2c12 cells.

The significant increase in the myogenin mRNA in differentiation stage of undifferentiated cells upon putrescine supplementation led to the rejection of the null hypothesis. Myogenin gene is usually expressed during the differentiation stage and its expression increases in early and late maturation stages (Le Grand et al., 2007). The increasing trend of myogenin mRNA in the differentiation stage is consequent to the cells entering the maturation phase which require myogenin to transcribe other muscle-specific proteins.

Lack of significant difference in the differentiated cells led to the failure of rejecting the null hypothesis. The reasons for this observation are similar to “putrescine effects on Myf5 mRNA in c2c12 differentiated cells”.

According to a study in 2014 by Kong (Kong et al., 2014) and other fellow scientists putrescine supplementation to porcine trophectoderm cell line 2 cells cultured for 2 to 4 days at 10, 25 or 50 µM concentration increased the cell proliferation and protein synthesis by increasing activation of mTOR signaling pathway. Though this experimental design is different in dose, time and cell line chosen to the above study, increased cell proliferation and protein synthesis corroborates the experimental finding that increased myogenin mRNA is an essential step for cell proliferation and protein synthesis.

2.4 Putrescine influence on ODC mRNA in undifferentiated and differentiated c2c12 cells:

Putrescine (5 mM) (Figure 2.4) treatment significantly increased the ODC gene transcription at determination (P = 0.010) and differentiation stages (16h) (P = 0.020) of myogenesis in undifferentiated cells. There was also a significant increase in ODC mRNA at early (P = 0.004) maturation phase. There was no significant change in late (P = 0.200) maturation phases of differentiated c2c12 cells.

Physiological doses of putrescine (5 mM) supplementation to undifferentiated c2c12 cells significantly increased the ODC mRNA (the enzyme required to synthesize polyamines). This suggests that supplementation of physiological doses of polyamines may increase their *de novo* synthesis but not inhibit it by negative feedback. Endogenous polyamine synthesis along with exogenous polyamine availability increases the chances of the cells to be in promyogenic state. It is proven that polyamines play multiple crucial roles in growth and regeneration phase and increased expression of ODC has been correlated with increased proliferative ability (Pietila et al., 1997; Palanimurugan et al., 2004). So, increased ODC mRNA transcription upon polyamine treatment, can be a positive or favorable sign for the cells to grow and be in the promyogenic state.

In accordance with literature, it is widely accepted that ODC is downregulated during differentiation stage (Roni et al., 1989; Rosander et al., 1995) and this down regulation of ODC serves as a good marker for non- proliferating cells (Cervelli et al., 2008). But, the continued synthesis of ODC mRNA upon putrescine supplementation in determination, differentiation and early maturation stages gives an evidence that polyamines cause additional cells to enter proliferating stage. This could lead to increased muscle production when translated to animals.

A study (Farriol et al., 2001) showed that when putrescine was supplemented at 100,500,1000µm concentration to colon carcinoma cell line for 24 h, rapid tumor growth was observed with a decreased intracellular spermidine and spermine levels without affecting the ODC enzyme activity or ODC mRNA content. This study found a significant increase in ODC mRNA at determination, differentiation and early maturation stages of myogenesis which does not agree with the above study. The reasons for the apparent contradictory findings could

be because of differences in sampling time points, dose rate and cell line chosen. At 24 h post putrescine treatment, the cells used in our study might have already translated enough mRNA necessary for transcribing the protein. The cancerous cell lines have different morphology, physiological properties and enzyme activation compared to normal cell lines and this could explain the unchanged ODC mRNA or that the dose of putrescine might have been sufficient for the cells and inhibited *de novo* polyamine synthesis and ODC mRNA transcription.

2.5 Fate of cells in the absence of myogenic transcription factors:

According to experimental observations, putrescine supplementation to differentiated cells decreased the Myf5 (early maturation) and MyoD (late maturation) mRNA which was not a promyogenic sign. Decreased Myf5 and MyoD mRNA transcription can cause cells to undergo apoptosis rather than contribute to muscle development. In a normal muscle cell of live (in vivo) animal, during early maturation phases, MyoD should continue to express until it transcribes myogenin gene and other muscle related proteins like myosin heavy chain, troponin, etc (Charge et al., 2004) but this does not occur in cell lines. It is documented that Myf5 and MyoD genes are downregulated when c2c12 cells are treated with differentiation medium (Yoshida et al., 1998). However, in this study upon polyamine treatment, there was a further significant decrease in Myf5 and MyoD mRNA at different maturation stages of myogenesis compared to control. myogenin mRNA which should significantly increase during the maturation phase did not occur at the specific time points measured in the study.

2.6 Spermine influence on Myf5 mRNA in undifferentiated and differentiated c2c12 cells:

Spermine (0.5 mM) (Figure 2.5) treatment caused an increasing trend in Myf5 gene transcription at determination ($P = 0.090$) phase. No significance is noticed at differentiation stages (16 h) ($P = 0.100$) of myogenesis in undifferentiated cells. Similarly, increasing trend is observed in Myf5 mRNA at early maturation ($P = 0.080$) and doesn't show any significance at late ($P = 0.400$) maturation phases in differentiated c2c12 cells.

A recent study in 2018 by Linlin and colleagues showed that polyamine and spermine analogues (spermine @ 0, 10, 20, 30, 50 and 80 μM) treatment to drug resistant cancerous cell line showed a dose dependent cytotoxic effect excepting putrescine treatment. It is interesting

to find that cytotoxic (cell apoptosis) effect was not because of the spermine, as no cell death was evident in spermine treated cells without FBS. Therefore, this study suggested that cytotoxicity activity of spermine was related to its metabolites in FBS. Lack of significance in the spermine treated cells might be because of the cytotoxic affect related to FBS.

2.7 Spermine influence on MyoD mRNA in undifferentiated and differentiated c2c12 cells:

Spermine (0.5 mM) (Figure 2.6) treatment showed an increasing trend in MyoD gene transcription at determination (P = 0.090) phase. A significant increase in MyoD mRNA at differentiation stage (16 h) (P = 0.020) of myogenesis was observed in undifferentiated cells. There was no significant difference in MyoD mRNA at early (P = 0.100) maturation stage. Increasing trend was observed in late (P = 0.080) maturation stages of myogenesis in differentiated c2c12 cells. A study on (Guirard et al., 1964) lactobacillus in 1964 showed that on supplementing spermine at 0.1,0.2,0.3,0.5 and 1.0 µg concentration to lactobacillus bacteria, an increased growth rate and slight decrease in their lag phase growth duration was observed. Though our study design was very different from the above study. The growth of the lactobacillus agrees with the current finding of increased MyoD mRNA in differentiation stage, which is essential for the growth in cattle.

2.8 Spermine influence on myogenin mRNA in undifferentiated and differentiated c2c12 cells:

Spermine (0.5 mM) (Figure 2.7) treatment did not significantly change the myogenin gene transcription at determination (P = 0.100) and differentiation stages (16 h) (P = 0.200) of myogenesis in undifferentiated cells. There is a significant decrease in myogenin mRNA at early maturation phase (P = 0.020). No significance was found in the late (P = 0.300) maturation phase of differentiated c2c12 cells. The conversion of c2c12 myoblasts to myotubes involves several gene expressions and spermine oxidase (enzyme that converts spermine into spermidine) is one of the novel genes to express during this phase (Cervelli et al., 2008). This implies that spermine is an essential compound for cells to enter from myoblast to myotube stage. Decreased supplementation of spermine might decreased the production of spermine oxidase which could lead to decrease muscle cells and decreased muscle production in animals.

Given that only two-time points were measured, this study might have missed a critical time point or alternatively spermine treatment at the differentiation stage might have caused additional stress to cells as the cells are not rapidly dividing in this stage compared to determination stage, or perhaps putrescine treated cells might have already synthesized sufficient myogenic transcription factor protein at these measured time points. Furthermore, spermine treated cells might have accelerated or decelerated the maturation process, or they might have negatively influenced the maturation process, which is not possible to determine within our experimental design as this study did not measure the actual protein levels to exclude the above possibilities. However, it was subjectively noted that the spermine treated cells fused more quickly forming multinucleated cells and myotubes more rapidly compared to control cells which may support the hypothesis of accelerated myogenesis.

2.9 Spermine influence on ODC mRNA in undifferentiated and differentiated c2c12 cells:

Spermine (0.5 mM) (Figure 2.8) treatment caused an increasing trend in ODC gene transcription at determination (P = 0.070) and differentiation stages (16 h) (P = 0.060) of myogenesis in undifferentiated cells. There is a significant decrease in ODC mRNA at early (P = 0.030) maturation phase. No significance was noticed in late (P = 0.090) maturation phases in differentiated c2c12 cells.

In c2c12 cells experiments, exogenous polyamine supplementation increased the endogenous ODC mRNA which was required for *de novo* polyamine synthesis in undifferentiated c2c12 cells. Supporting the trend results of this study, an experiment conducted on 11-day old postnatal mice showed that oral supplementation of 0.3- 0.4 mM spermine per kg body weight could stimulate the ODC enzyme linked to polyamine synthesis in pancreatic acinar cells (Romain et al., 1998). Another study showed similar increase in the activity of ODC enzyme in pancreatic cells of 3-day old unweaned rat pups upon spermine treatment (Romain et al., 1998). This suggests that spermine when supplemented during the rapid proliferating phase, can stimulate the ODC enzyme to synthesize more polyamines leading to tissue growth. The significant increase trend in ODC enzyme mRNA after supplementing spermine in physiological doses at both the determination and differentiation stages might increase endogenous polyamine synthesis and may contribute to efficient and increased muscle

production because our study also observed a significant increase in myogenic transcription factors in addition to ODC enzyme. This might not be true in differentiated cells as polyamines downregulated the ODC mRNA transcription at this phase with few exceptions. Therefore, more research is required to clarify the physiological role and doses of polyamines that impact cell division and growth.

2.10 Comparison between spermine and putrescine effects on differentiated and undifferentiated cells:

Spermine and putrescine treatments showed significant difference in their mRNA transcription of different myogenic transcription factors at the time points chosen for experimental study. Putrescine is found to significantly increase gene transcriptions of Myf5 (Figure 2.1 (a)) at both time points, MyoD (Figure 2.2 (a)) and myogenin (Figure 2.3 (a)) at 16 h a time point in undifferentiated c2c12 cells. While spermine significantly increases only MyoD (Figure 2.6 (a)) at 16 h time point in undifferentiated c2c12 cells. Putrescine does not show any significance in differentiated c2c12 cells, while spermine decreased myogenin at 8 h (Figure 2.7 (b)) in differentiated c2c12 cells.

Similar kind of difference in mRNA of myogenic transcription factors is also noticed in sol8 cells. Putrescine significantly increases the myogenin mRNA (Figure 2.15 (a)) at 8 h in undifferentiated sol8 cells while Myf5 mRNA (Figure 2.17 (a)) is significantly decrease at differentiation stage in undifferentiated sol8 cells after spermine treatment. Differentiated cells show decrease in myogenin mRNA (Figure 2.15 (b)) at 8 h leaving in putrescine treated cells while no change in spermine treated differentiated sol8 cells (Figure 2.19 (b)).

Polyamines are highly positively charged ions with spermine being strongly charged than putrescine. They bind and interact with negatively charged macromolecules such as RNA, DNA, ribosomes, proteins, nucleic acids and phospholipids with different affinities. They also influence the opening of ion channels and gap junctions in normal physiological functioning cell. These interactions result in different physiological outcomes in different cells. Polyamines and their availability influence many genes transcription and translation rates. As putrescine and spermine are different polyamines their response and functions various to stimuli. It has been shown that when ODC is inhibited by DFMO this caused to reduce only putrescine and spermidine, leaving no change to the spermine levels. (Desiderio et al.,1997).

Another experiment found similar results that inhibiting ODC with ODC inhibitor decreased putrescine, spermidine and spermine levels by 74,78,10% respectively. Whereas, cells treated with spermidine and spermine are most effective in restoring the normal growth pattern than putrescine treated cells. (Thomas, 1994). Thus, it explains that polyamines are different in nature and shows different effects in cell lines.

2.11 Comparison between c2c12 and sol8 cells:

C2c12 and sol8 cells are not identical in terms of their response to polyamine as evidenced by myogenic transcription factors mRNA at the time points chosen in the study. In undifferentiated c2c12 cells, putrescine caused significant increase in gene transcriptions of Myf5 mRNA (Figure 2.1 (a)) at both time points, MyoD (Figure 2.2 (a)) and myogenin (Figure 2.3 (a)) at differentiation stage. While, spermine significantly increases only MyoD (Figure 2.6 (a)) at differentiation stage in undifferentiated c2c12 cells. In undifferentiated sol8 cells, putrescine significantly increases myogenin (Figure 2.15 (a)) at determination and spermine shows significant decrease effect on Myf5 at differentiation stage (Figure 2.17 (a)).

Putrescine treatment of differentiated c2c12 cells did not show any significance in myogenic transcription factor mRNA. Spermine treatment resulted in significant decrease of myogenin mRNA (Figure 2.7 (b)) at early maturation stage. Differentiated sol8 cells only decreases myogenin at early maturation stage upon putrescine treatment. (Figure 2.15 (b)).

This indicates that c2c12 and sol8 cells are in fact phenotypically different in their response to polyamines or it may simply reflect that the growth rate of these cells is different, and the timing of sampling resulted in the differences in the observed myogenic transcription factor mRNA. It is observed that c2c12 cells grows faster than sol8 cells (Forterre et al., 2013) and origin of c2c12 mouse myoblast cells is from thigh muscle of C3H mice after crush injury (Yaffe et al., 1977) while sol8 cells has been isolated from soleus muscle of normal C3H mouse (Daubas et al., 1988). These differences might have caused a significant difference in their response to polyamines.

2.12 Methionine does not significantly affect myogenic transcription factors mRNA (Myf5, MyoD, myogenin) in undifferentiated and differentiated c2c12 cells:

Methionine (10 mM) (Figure 2.9) treatment doesn't significantly change the Myf5 gene transcription at determination ($P = 0.700$) and differentiation stages ($P = 0.100$) of myogenesis in undifferentiated cells. There is an increasing trend in Myf5 mRNA at early ($P = 0.070$) maturation phase. No significance is observed in late ($P = 0.500$) maturation phases of myogenesis in differentiated c2c12 cells.

MyoD gene transcription was not significantly affected by methionine (10 mM) (Figure 2.10) treatment at determination (P=0.800) and differentiation stages (P=0.100) of myogenesis in undifferentiated cells. Similarly, there is no significant differences in MyoD mRNA at early (P=0.200) and late (P=0.100) maturation phases in differentiated c2c12 cells.

Myogenin gene transcription was not significantly affected by methionine (10 mM) (Figure 2.11) treatment at determination (P=0.800) and differentiation stages (P=0.600) of myogenesis in undifferentiated cells. Similarly, there is no significant differences in myogenin mRNA at early (P=0.100) and late (P=0.600) maturation phases in differentiated c2c12 cells.

Methionine supplementation does not significantly affect the myogenic transcription factors mRNA in sol8 cell line. When the broiler chicken is treated with 0.38 and 0.28 g/100 g ,0.51 and 0.42g/100g, 0.65 and 0.52g/100g for 1-21 and 22-42 days respectively (Wen et al., 2017). High concentration methionine diets showed increased weight gain with increasing in mRNA levels of Myf5 and Mrf4 along with increased phosphorylation of mTOR pathway. Present study's experimental findings do not support this observation, this might be because of the difference in cell line, dosage of methionine and time of sampling.

2.13 Methionine doesn't significantly affect ODC mRNA in undifferentiated and differentiated c2c12 cells:

Methionine (10 mM) (Figure 2.12) treatment doesn't significantly change the ODC gene transcription at determination ($P = 0.200$) phase. In the differentiation stage significant increase in ODC mRNA ($P = 0.010$) is observed. There are no significant differences in

myogenin mRNA at early (P = 0.700) and late (P = 0.100) maturation phases in differentiated c2c12 cells.

Methionine treatment significantly increases the ODC mRNA in only differentiation stage of myogenesis in c2c12 cell lines. Supplementing dietary methionine at 0.55% to week old chicks for 2 weeks showed an increasing trend for ODC activity in liver (Smith, 1981). Current study results on differentiation stage does not accepts with this finding this might be because of the difference in species, dose, timing and the choice of sample collection.

3.14 Putrescine influence on Myf5 mRNA in undifferentiated and differentiated sol8 cells:

Putrescine (5 mM) (Figure 2.13) does not significantly change the Myf5 gene transcription at determination (P = 0.300) and differentiation stages (P = 0.200) of myogenesis in undifferentiated cells. There is an increasing trend found in Myf5 mRNA at early (P = 0.090) maturation stage. No significance is found in late (P = 0.600) maturation phases in differentiated sol8 cells.

Putrescine treatment does not significantly affect the Myf5 mRNA in sol8 cell line. Oral supplementation of putrescine @ 5 mg/kg BW to neonatal suckling piglets for 14 days significantly increased final body weight, average daily intake compared to the control. (Wang et al., 2015) Increased growth rate of the piglets does not agree with the study's experimental findings as there is no significant difference in Myf5 mRNA which is essential for the growth to occur.

2.15 Putrescine influence on MyoD mRNA in undifferentiated and differentiated sol8 cells:

Putrescine (5 mM) (Figure 2.14) treatment significantly increases the MyoD gene transcription at determination (P = 0.300) and differentiation stages (P = 0.200) of myogenesis in undifferentiated cells. There are no significant differences in MyoD mRNA at early (P = 0.100) and late (P = 0.600) maturation phases in differentiated sol8 cells.

Putrescine treatment does not significantly affect the MyoD mRNA in sol8 cell line. According to the study by Murdoch and colleagues (Murdoch et al., 2013) showed that

polyamines when treated to satellite cells in monoculture and coculture with adipocytes there is significant increase in MyoD gene expression at 24 and 48 h. This study is not supported by our findings, and the reason might be difference in the time points.

2.16 Putrescine influence on myogenin mRNA in undifferentiated and differentiated sol8 cells:

Putrescine (5mM) (Figure 2.15) treatment significantly increases the myogenin gene transcription at determination ($P = 0.001$) and differentiation stages ($P = 0.500$) of myogenesis in undifferentiated cells. There is significant decrease in myogenin mRNA at early ($P = 0.050$) maturation phase. No difference is observed in late ($P = 0.300$) maturation phases in differentiated sol8 cells.

Putrescine supplementation causes significant increase in only myogenin mRNA at determination stage. A study by Murdoch in the year 2013 showed that (Murdoch et al., 2013) when polyamines treated to satellite cells in monoculture and coculture there is significant increase in myogenin gene expression at 24 and 48 h. This study is supported by our findings in determination stage, as we observed significant increase in the myogenin at this stage.

2.17 Putrescine influence on ODC mRNA in undifferentiated and differentiated sol8 cells:

Putrescine (5 mM) (Figure 2.16) treatment significantly increases the ODC gene transcription at determination stage ($P = 0.050$). No significant difference is observed in differentiation stages ($P = 0.200$) of myogenesis in undifferentiated cells. There is significant decrease in ODC mRNA at early ($P = 0.020$) maturation stage. In the late ($P = 0.400$) maturation phases, significance is not observed.

The experimental findings agree with previous literature which indicates that when a polyamine; putrescine or spermidine are supplemented at 1mM concentration to myoblasts cells (L6 Rat myoblasts cell line) treated with 10% horse serum, there is a decrease in the ODC enzyme activity without concurrent decreases in cell number as compared to control (Stoscheck et al., 1980). In the current study, ODC activity was not measured beyond the cell differentiation phase to compare with the ODC mRNA results in late maturation. Though there

are differences in the concentration of horse serum used, the timing of the experiment performed, the dose of polyamine treatment and the cell line chosen between their experiments and this study, caused decreases in ODC mRNA in response to putrescine treated sol8 cells at early maturation phase. However, ODC enzyme activity was not measured in the current study. It is plausible that the exogenous supply of polyamines might have maintained required polyamine levels within the differentiated cells and thus cells do not require transcription of the ODC enzyme to synthesize them. It is well established that the presence of polyamines themselves can downregulate the ODC enzyme through a negative feedback mechanism thereby preventing the unnecessary polyamine accumulation. Therefore, differentiated cells supplemented with polyamines may have downregulated the ODC enzyme levels because there is a reduced need for *de novo* synthesis. There are also other possible explanations for these observations. As with any real-time PCR analyses of mRNA data only represent the short sampling timeframe. Hence the exogenous polyamines might have increased or decreased the ODC mRNA. The study does not have data relating to the primary regulation of ODC activity mediated by ODC antizyme. The transcription of antizyme (AZ) involved in the downregulation of ODC enzyme and its cognate protein levels were not assessed in this study.

2.18 Spermine influence on myogenic transcription factor mRNA in undifferentiated and differentiated sol8 cells:

Spermine (0.5 mM) (Figure 2.17) treatment doesn't significantly change the Myf5 gene transcription at determination (P = 0.300) stage. There is significant decrease in Myf5 gene transcription at differentiation stages (P = 0.020) of myogenesis in undifferentiated cells. There are no significant differences in Myf5 mRNA at early (P = 0.100) and late (P = 0.100) maturation phases in differentiated c2c12 cells.

Spermine (0.5 mM) (Figure 2.18) treatment showed increasing trend in MyoD gene transcription at determination stage (P = 0.090). No significant difference is observed in differentiation stages (P = 0.300) of myogenesis in undifferentiated cells. Similarly, there is no significant differences in MyoD mRNA at early (P = 0.600) and late (P = 0.200) maturation phases in differentiated c2c12 cells.

Whereas spermine (0.5 mM) (Figure 2.19) treatment doesn't significantly change the myogenin gene transcription at determination (P = 0.100) and differentiation stages (P = 0.100) of myogenesis in undifferentiated cells. Similarly, there is no significant differences in myogenin mRNA at early (P = 0.600) and late (P = 0.900) maturation phases in differentiated c2c12 cells.

Upon supplementing physiological doses of spermine, there is only significant decrease in Myf5 mRNA at differentiation stage of myogenesis. This indicates that though a significant decrease in Myf5 mRNA is not a promyogenic sign, no significance is obtained in other gene mRNA transcriptions. Given that only two-time points are measured, the study might have missed a critical time point or spermine treated cells might have not caused significant difference in the mRNA transcription of myogenic transcription factors especially in the maturation stages of myogenesis.

Contrasting to our study, previous literature shows that spermine supplementation at 0.2-0.4 mM per kg body weight in 11-day old rats, increased the differentiation of the pancreas acinar cells by decreasing the maturation time (Romain et al., 1998). Supporting this study, another observation showed that spermine at 0.5-1.0 mM concentration promoted differentiation of mouse embryonic stem cells to multi-layer muscle fiber sheet and formation of contractile muscle fibers. The lack of spermine supplementation ceased the differentiation process and halted formation of muscle fibers (Sasaki et al., 2008). Other supporting data shows that the precocious maturity of the intestines occurred by oral administration of either spermidine (10 µmol) or spermine (6 µmol) to 12-day old rats. The authors observed a change in the structural and biochemical composition of the villi in small intestines compared to control (Dufour et al., 1988). All the above findings show that spermine significantly increases the growth and proliferation in early stages of growth rather than at maturation phases and at physiological doses can decrease the maturation time and increases the number and rate of cells undergoing differentiation. Thus, it seems plausible that spermine might have accelerated the maturation process of myogenesis in differentiated cells and this could explain missing a critical time point. To confirm this future study would benefit from quantifying the myogenic transcription factor protein levels.

2.19 Spermine influence on ODC mRNA in undifferentiated and differentiated sol8 cells:

Spermine (0.5 mM) (Figure 2.20) treatment showed increasing trend in ODC gene transcription at determination stage (P = 0.090). There is no considerable difference in differentiation stages (P = 0.300) of myogenesis in undifferentiated cells. No significant differences are observed in ODC mRNA at early (P = 0.500) and late (P = 0.700) maturation phases in differentiated c2c12 cells.

Spermine supplementation is not affected by the ODC gene transcription at the time points measured in sol8 cell lines. A study showed that when spermine and spermidine (Theiss et al., 2002) is supplemented at 0.05 mmol concentration to the *Chlamydomonas reinhardtti* (green algae) it has decreased ODC activity to 30 to 35%. Current experimental results don't accept these results, and the difference might be due to diverse experimental approach.

2.20 Methionine influence on myogenic transcription factors mRNA in undifferentiated and differentiated sol8 cells:

Methionine (10 mM) (Figure 2.21) treatment causes an increasing trend in Myf5 gene transcription at determination (P = 0.070) phase. There is no significance found in differentiation stages (P = 0.500) of myogenesis in undifferentiated cells. There are no significant differences in Myf5 mRNA at early (P = 0.100) and late (P = 0.100) maturation phases in differentiated sol8 cells.

Methionine (10 mM) (Figure 2.22) treatment doesn't significantly affect the MyoD gene transcription at determination (P=0.900) and differentiation stages (P=0.900) of myogenesis in undifferentiated cells. There is significant increase in MyoD mRNA at early (P=0.050) maturation phase. There is no difference in late (P=0.100) maturation stage in differentiated sol8 cells.

There is no significant change in the myogenin gene transcription at determination (P=0.600) (Figure 2.23) and differentiation stages (P=0.100) of myogenesis in undifferentiated cells. Similarly, there is no significant differences in myogenin mRNA at early (P=0.500) and late (P=0.600) maturation phases in differentiated sol8 cells.

Except the MyoD mRNA in early maturation phase no other myogenic transcription factor is significantly affected by methionine supplementation in sol8 cell lines. An experimental study (Wen et al., 2014) showed that methionine supplemented to day old Arbor Acres broiler chicks (192) at 0.60% and 0.53% starter and finisher diet caused increased overall growth with increased breast muscle size along with increasing myogenic factor 5 (Myf5) and myocyte enhancer factor2B mRNA expression. Current experimental study did not observe the mRNA expression of myogenic transcription factors to compare with this study because though the mRNA is not transcribing at the specific time points, it might be translating the protein. Considering the mRNA transcription alone, present experimental results doesn't accept with this study and the difference might be due the changes in experimental study.

2.21 Methionine doesn't significantly affect ODC mRNA in undifferentiated and differentiated sol8 cells:

Methionine (10 mM) (Figure 2.24) treatment doesn't significantly change the ODC gene transcription at determination ($P = 0.900$) phase. There is an increasing trend in ODC mRNA at differentiation stages (16 h) ($P = 0.090$) of myogenesis in undifferentiated cells. There are no significant differences in ODC mRNA at early ($P = 0.200$) and late ($P = 0.200$) maturation phases in differentiated sol8 cells.

This states that methionine does not significantly influence the ODC mRNA transcription at determination and differentiation stages of myogenesis at the specified time points chosen. A study in 2019 (Zhongyue et al., 2019) showed that methionine treated to teleost fish @ 0.85,1.82 and 2.80% did not significantly change the ODC mRNA expression, showing ODC gene expression is not affected by methionine supplementation. Though the species, dose and timing of the samples performed varies between the experiments, the current experimental results accepts the unchanged ODC mRNA observation.

Conclusion

Based on experimental results, the first hypothesis that states physiological doses of polyamines influence the mRNA levels of myogenic transcription factors in cultured muscle cells is accepted since in putrescine treated undifferentiated c2c12 cells and spermine treated undifferentiated c2c12 cells MyoD mRNA is found significant at 16hr, myogenin mRNA at 8hr in differentiated c2c12 cells. Whereas in putrescine treated undifferentiated and differentiated sol8 cells myogenin mRNA is found significant at 8hr and spermine supplementation caused significance in Myf5 mRNA at 16hr in undifferentiated sol8 cells. The second hypothesis states that physiological doses of polyamines influence the *de novo* polyamine synthesis in cultured muscle cells by altering mRNA abundance of the rate limiting enzyme; ornithine decarboxylase is accepted as we note that spermine treated undifferentiated and differentiated sol8 cells and in spermine treated undifferentiated c2c12 cells ODC mRNA expression was altered. According to these results, the experiment found differential influence of polyamines on different stage of myogenesis (decreased effect observed in differentiated c2c12 and sol8 cells whereas increased effect observed in undifferentiated c2c12 and sol8 cells) which could be difficult to conclude polyamine effects on livestock. Finally, the third hypothesis that states treating physiological doses of polyamine to commercial immortalized muscle cell lines (c2c12 and sol8) will affect myogenic transcription factors in the same manner is rejected. This hypothesis is not supported by the experimental data, as c2c12 and sol8 cell lines influence the mRNA abundance of different myogenic transcription factors at the specific timepoints measured. This is an important finding which suggests that using a single immortalized cell line could potentially bias the effects of on myogenic transcription factors and may not exactly represent the in vivo behavior of muscle cells. In conclusion, physiological doses of polyamine supplementation do influence the myogenic transcription factor mRNA in c2c12 and sol8 cells. Polyamines also affect ODC mRNA (*de novo* polyamine synthesis) in differentiated cells. The current experiment also supports the need to and the advantage of using multiple cell types and multiple time points to ascertain the effects of polyamines in future *in vitro* experiments. The distinguishing effect between putrescine and spermine are, putrescine can significantly increase Myf5, MyoD and myogenin mRNA in undifferentiated c2c12 cells, while spermine shows significance in only MyoD mRNA. The

key difference among undifferentiated and differentiated cells is, c2c12 cells showed significant increased effect in Myf5, MyoD and myogenin mRNA after putrescine supplementation, whereas differentiated cells lack significance on myogenic transcription factors and there is also a significant decreased affect observed in differentiated cells (spermine treated c2c12- myogenin 8 h, Putrescine treated sol8 - myogenin 8 h) compared to increased effect in undifferentiated cells. The key difference noticed between c2c12 and sol8 cells is, putrescine increases MyoD, Myf5 and myogenin mRNA in c2c12 cells, while it increases only myogenin in sol8 cells and this provides evidence that c2c12 and sol8 have differential effect on cell lines.

Figures

Figure 2.1. The effect of putrescine on Myf5 mRNA in c2c12 cells

a) Undifferentiated cells b) Differentiated cell

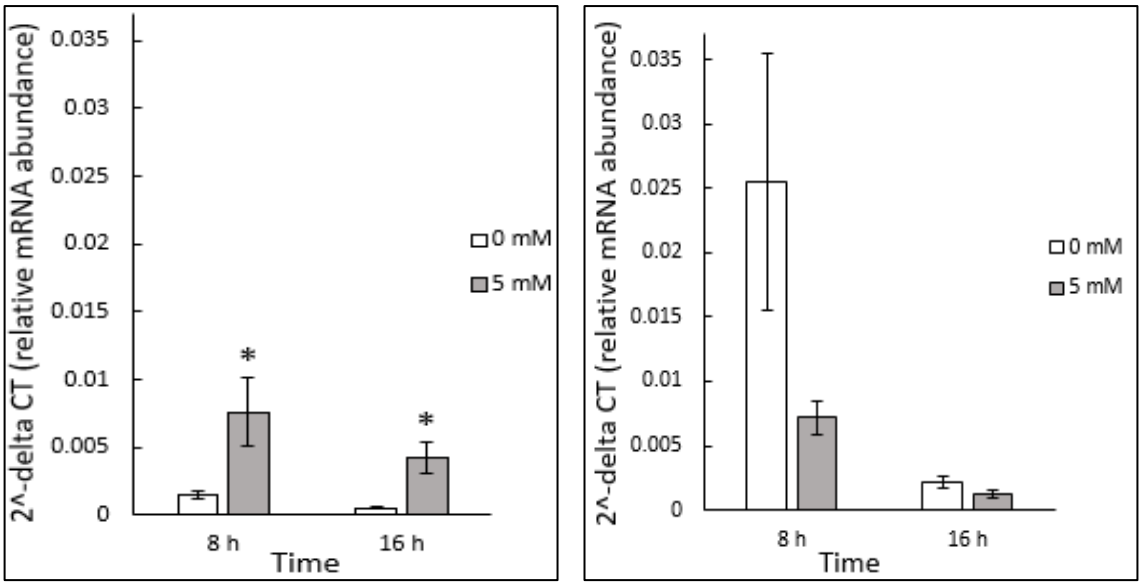


Figure 2.1. Real-time PCR quantification of *Myf5* mRNA expression performed using a) undifferentiated and b) differentiated c2c12 cells after treating them with 5mM putrescine after 8 and 16 h. *Myf5* samples are normalized using respective 18S controls and data was analyzed using relative C_T (ΔC_T) method. Bar-graphs represent the mean ± S.E.M of *Myf5* mRNA. Significance is determined through comparison of treated (5mM putrescine) with control, ** indicates significance $p \leq 0.01$ using a two-tailed t-test.

Figure 2.2. The effect of putrescine on MyoD mRNA in c2c12 cells

a) Undifferentiated cells b) Differentiated cells

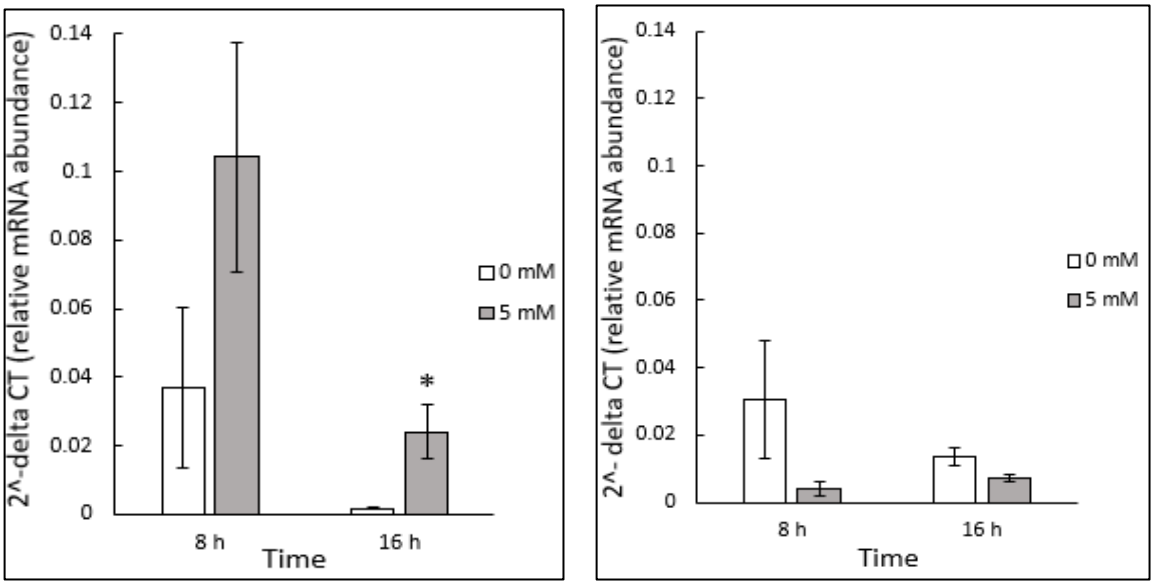


Figure 2.2. Real-time PCR quantification of *MyoD* mRNA expression performed using undifferentiated c2c12 cells after treating them with 5mM putrescine after 8 and 16 h. *MyoD* samples are normalized using respective 18S controls and data was analyzed using relative C_T (ΔC_T) method. Bar-graphs represent the mean ± S.E.M of *MyoD* mRNA. Significance is determined through comparison of treated (5mM putrescine) with control, * indicates significance $p \leq 0.05$ using a two-tailed t-test.

Figure 2.3. The effect of putrescine on myogenin mRNA in c2c12 cells

a) Undifferentiated cells b) Differentiated cells

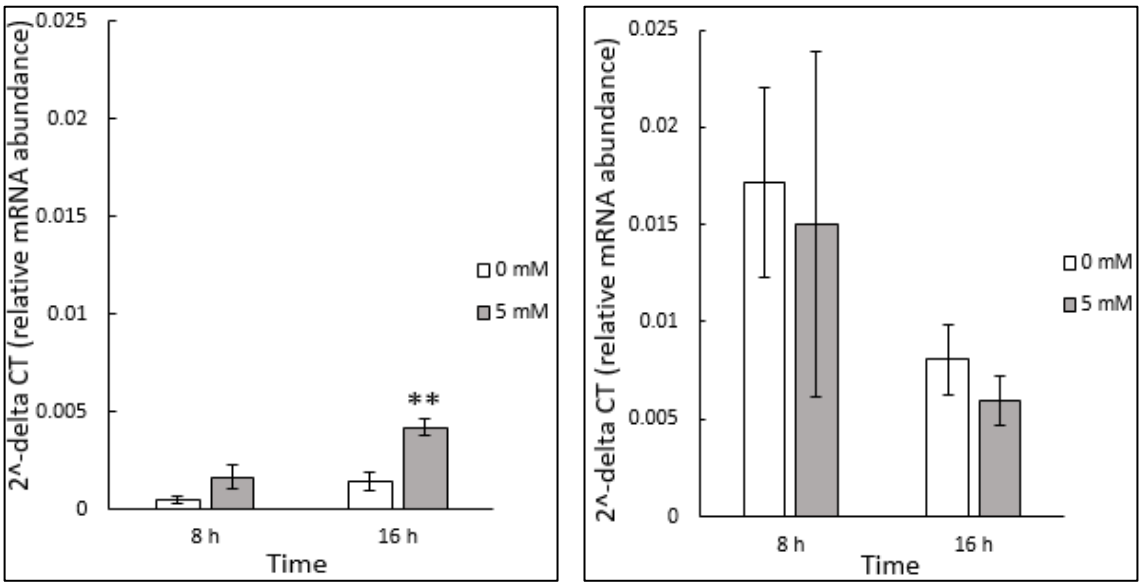


Figure 2.3. Real-time PCR quantification of *myogenin* mRNA expression performed using a) undifferentiated and b) differentiated c2c12 cells after treating them with 5mM putrescine after 8 and 16 h. *Myogenin* samples are normalized using respective 18S controls and data was analyzed using relative C_T (ΔC_T) method. Bar-graphs represent the mean ± S.E.M of *myogenin* mRNA. Significance is determined through comparison of treated (5mM putrescine) with control, ** indicates significance $p \leq 0.01$ using two-tailed t-test

Figure 2.4. The effect of putrescine on ODC mRNA in c2c12 cells

a) Undifferentiated cells b) Differentiated cells

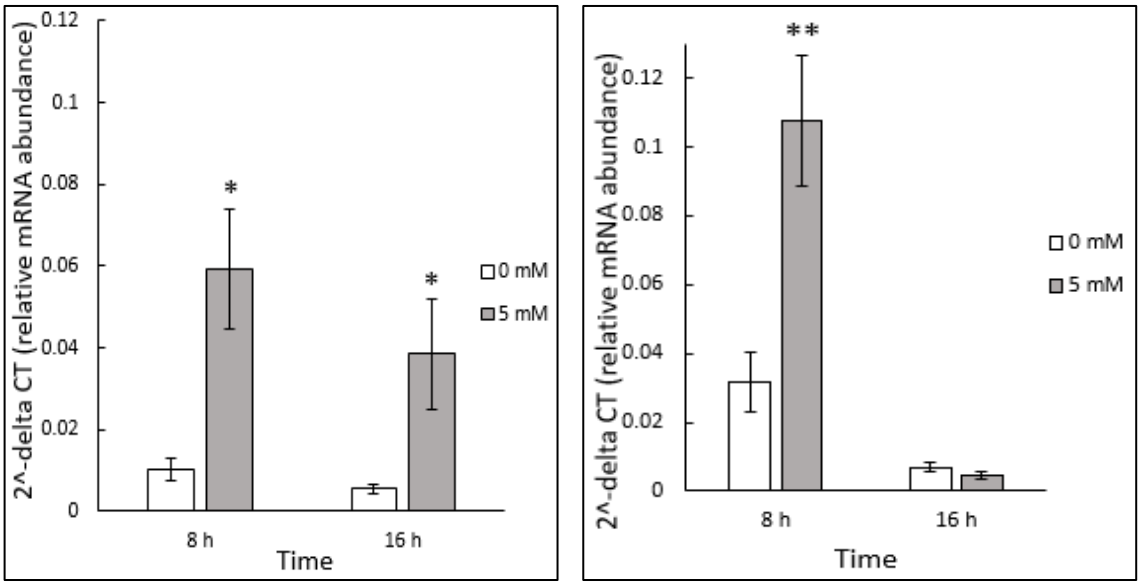


Figure 2.4. Real-time PCR quantification of *ODC* mRNA expression performed using a) undifferentiated and b) differentiated c2c12 cells after treating them with 5mM putrescine at 8 and 16 h. *ODC* samples are normalized using respective 18S controls and data was analyzed using relative C_T (ΔC_T) method. Bar-graphs represent the mean ± S.E.M of *ODC* mRNA on log2 scale. Significance is determined through comparison of treated (5mM putrescine) with control, ** indicates significance $p \leq 0.01$ using two-tailed t-test.

Figure 2.5. The effect of spermine on Myf5 mRNA in c2c12 cells

a) Undifferentiated cells b) Differentiated cells

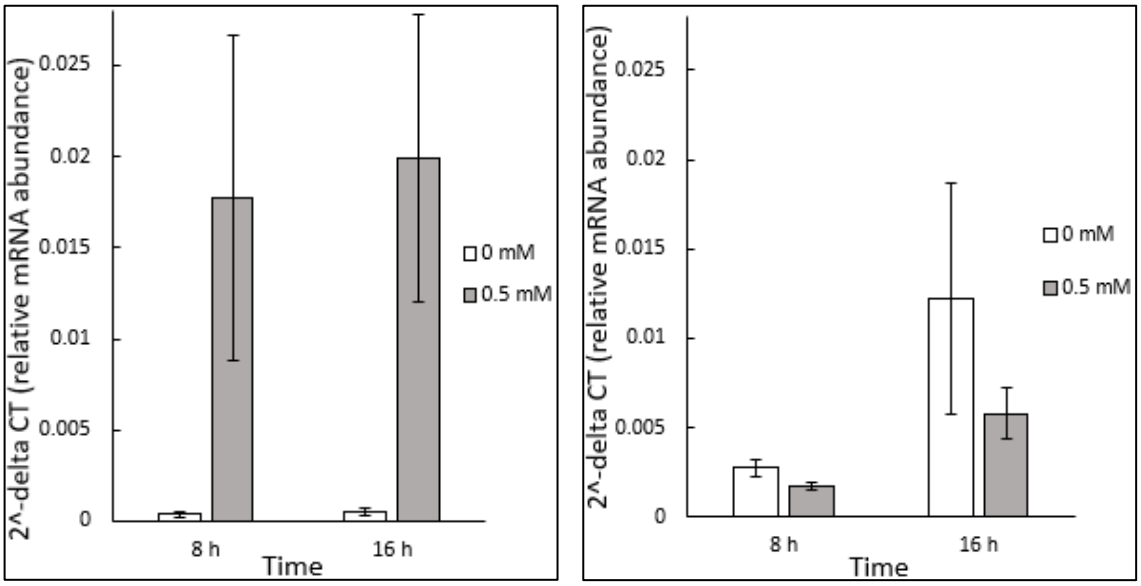


Figure 2.5. Real-time PCR quantification of *Myf5* mRNA expression performed using a) undifferentiated and b) differentiated c2c12 cells after treating them with 0.5mM spermine after 8 and 16 h. *Myf5* samples are normalized using respective 18S controls and data was analyzed using relative C_T (ΔC_T) method. Bar-graphs represent the mean ± S.E.M of *Myf5* mRNA. Significance is determined through comparison of treated (0.5mM spermine) with control, * indicates significance $p \leq 0.05$ using two-tailed t-test.

Figure 2.6. The effect of spermine on MyoD mRNA in c2c12 cells

a) Undifferentiated cells b) Differentiated cells

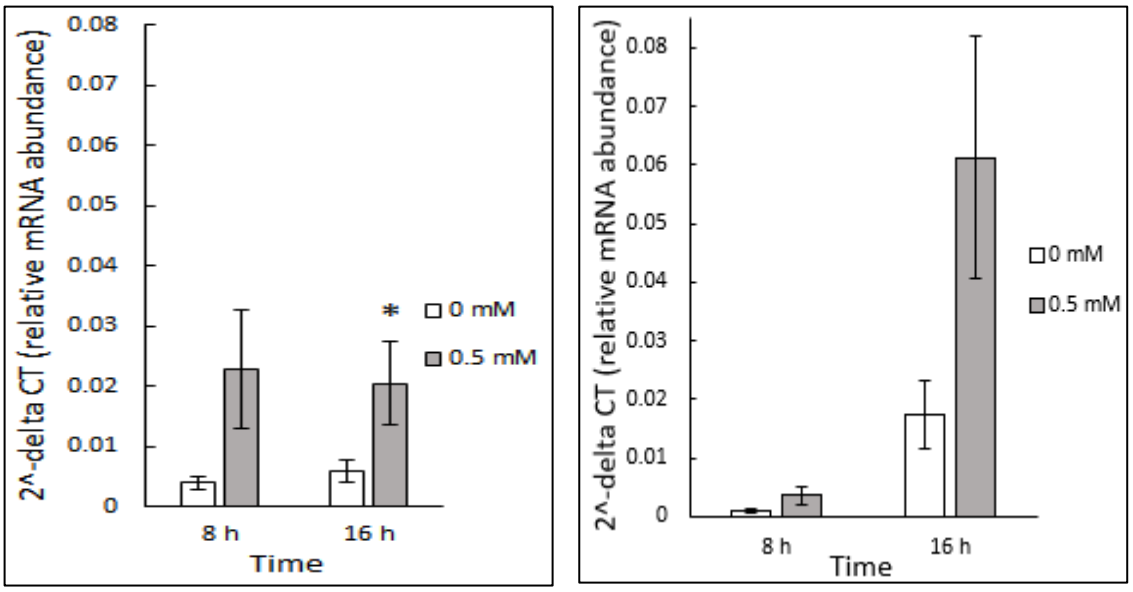


Figure 2.6. Real-time PCR quantification of *MyoD* mRNA expression performed using a) undifferentiated and b) differentiated c2c12 cells after treating them with 0.5 mM spermine after 8 and 16 h. *MyoD* samples are normalized using respective 18S controls and data was analyzed using relative C_T (ΔC_T) method. Bar-graphs represent the mean ± S.E.M of *MyoD* mRNA. Significance is determined through comparison of treated (0.5 mM spermine) with control, * indicates significance $p \leq 0.05$ using two-tailed t-test.

Figure 2.7. The effect of spermine on myogenin mRNA in c2c12 cells

a) Undifferentiated cells b) Differentiated cells

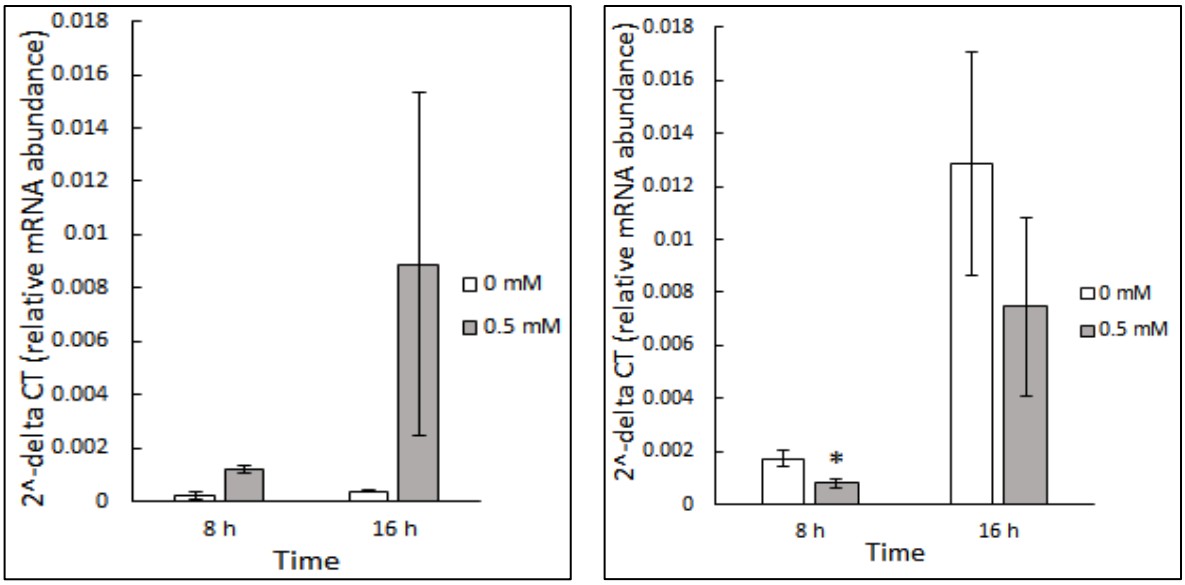


Figure 2.7. Real-time PCR quantification of *myogenin* mRNA expression performed using a) undifferentiated and b) differentiated c2c12 cells after treating them with 0.5 mM spermine after 8 and 16 h. *Myogenin* samples are normalized using respective 18S controls and data was analyzed using relative C_T (ΔC_T) method. Bar-graphs represent the mean ± S.E.M of *myogenin* mRNA. Significance is determined through comparison of treated (0.5 mM spermine) with control, * indicates significance $p \leq 0.05$ using two-tailed t-test.

Figure 2.8. The effect of spermine on ODC mRNA in c2c12 cells

a) Undifferentiated cells b) Differentiated cells

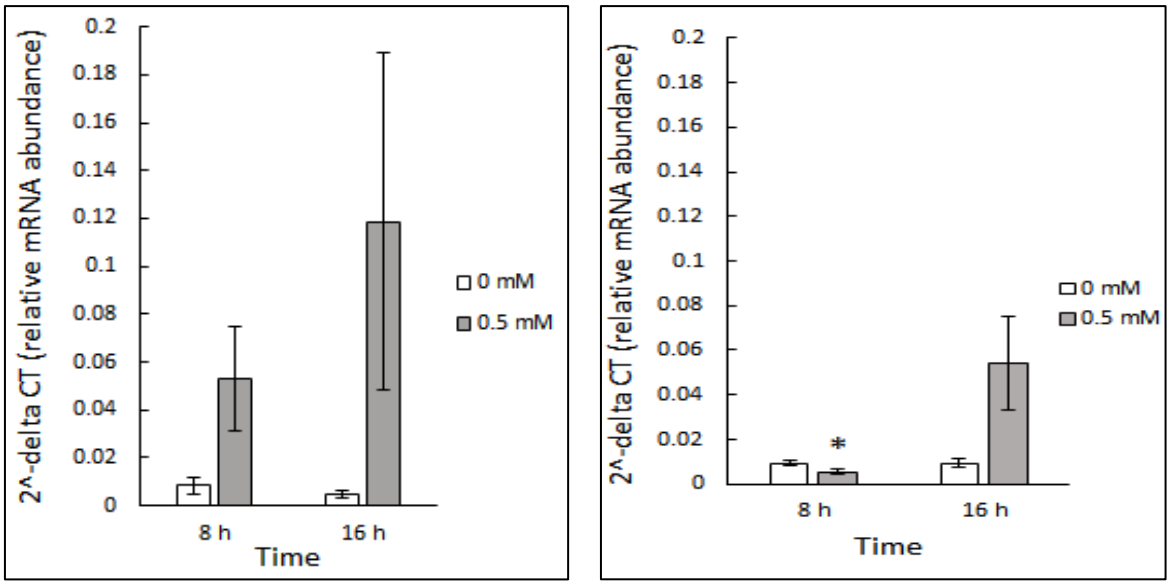


Figure 2.8. Real-time PCR quantification of *ODC* mRNA expression performed using a) undifferentiated and b) differentiated c2c12 cells after treating them with 0.5 mM spermine after 8 and 16 h. *ODC* samples are normalized using respective 18S controls and data was analyzed using relative C_T (ΔC_T) method. Bar-graphs represent the mean ± S.E.M of *ODC* mRNA. Significance is determined through comparison of treated (0.5 mM spermine) with control, * indicates significance $p \leq 0.05$ using two-tailed t-test.

Figure 2.9. The effect of methionine on Myf5 mRNA in c2c12 cells

a) Undifferentiated cells b) Differentiated cells

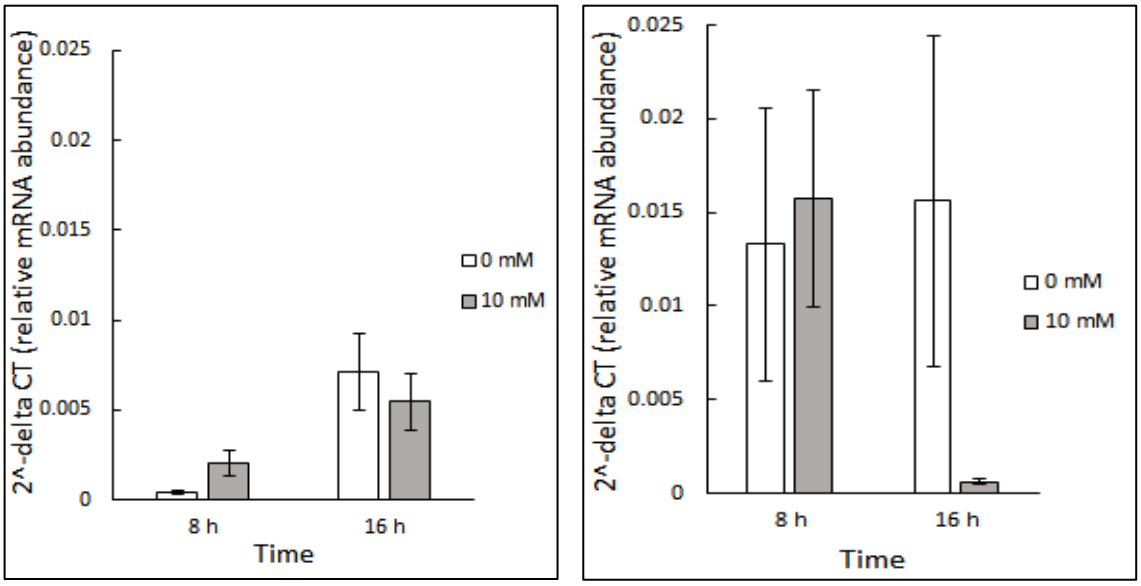


Figure 2.9. Real-time PCR quantification of *Myf5* mRNA expression performed on c2c12 a) undifferentiated and b) differentiated cells after treating them with 10mM methionine after 8 and 16 h. *Myf5* samples are normalized using respective 18S controls and data was analyzed using relative C_T (ΔC_T) method. Bar-graphs represent the mean ± S.E.M of *Myf5* mRNA.

Figure 2.10. The effect of methionine on MyoD mRNA in c2c12 cells

a) Undifferentiated cells b) Differentiated cells

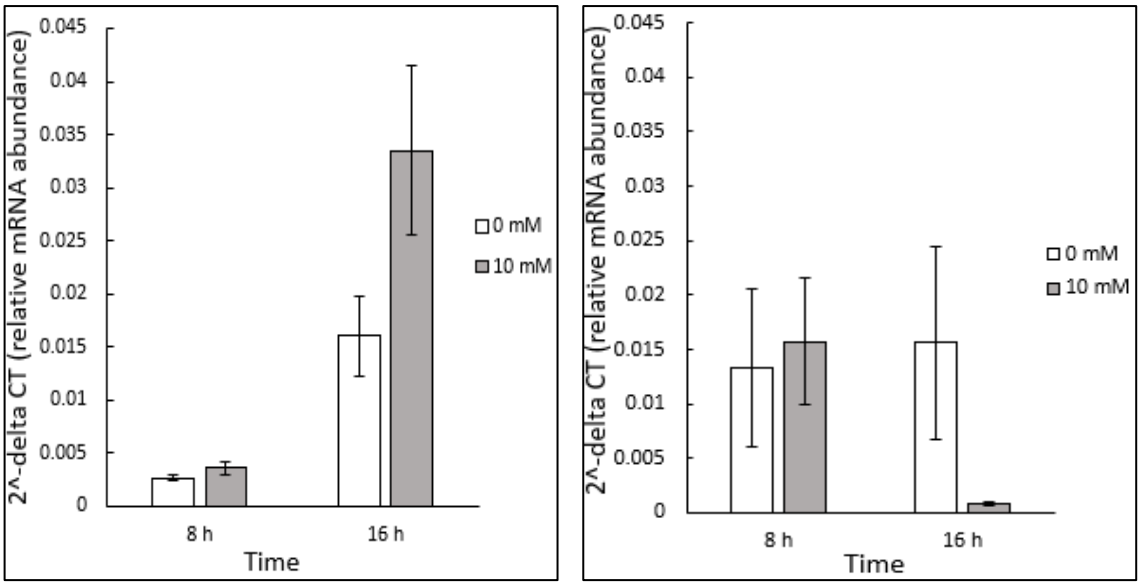
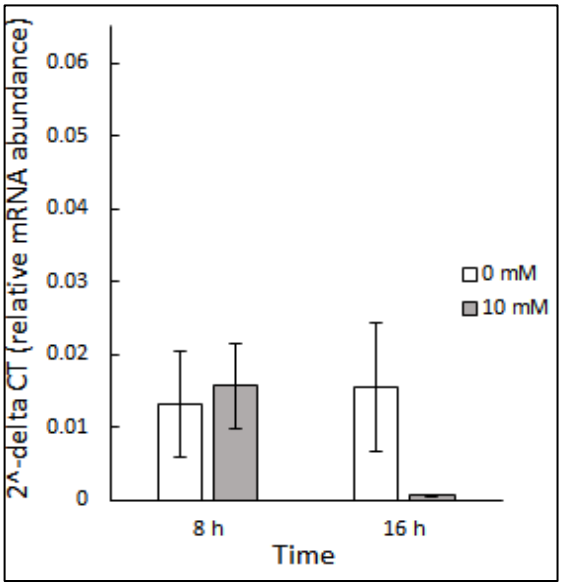


Figure 2.10. Real-time PCR quantification of *MyoD* mRNA expression performed on c2c12 a) undifferentiated and b) differentiated cells after treating them with 10mM methionine after 8 and 16 h. *MyoD* samples are normalized using respective 18S controls and data was analyzed using relative C_T (ΔC_T) method. Bar-graphs represent the mean ± S.E.M of *Myf5* mRNA.

Figure 2.11. The effect of methionine on myogenin mRNA in c2c12 cells

a) Undifferentiated cells



b) Differentiated cells

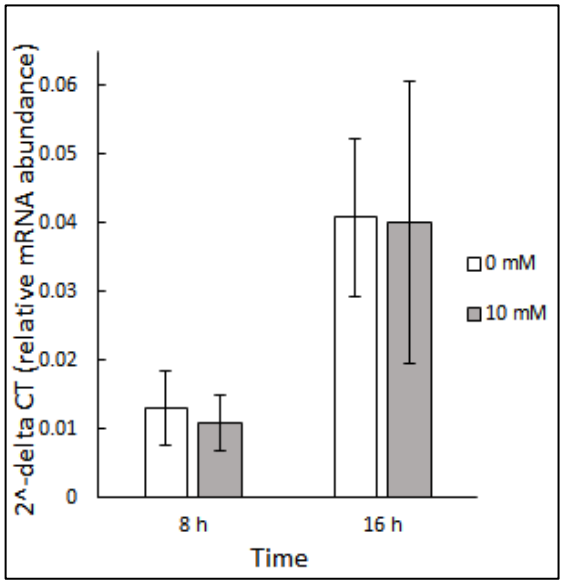


Figure 2.11. Real-time PCR quantification of *myogenin* mRNA expression performed on c2c12 a) undifferentiated and b) differentiated cells after treating them with 10mM methionine after 8 and 16 h. *Myogenin* samples are normalized using respective 18S controls and data was analyzed using relative C_T (ΔC_T) method. Bar-graphs represent the mean ± S.E.M of *myogenin* mRNA.

Figure 2.12. The effect of methionine on ODC mRNA in c2c12 cells

a) Undifferentiated cells b) Differentiated cells

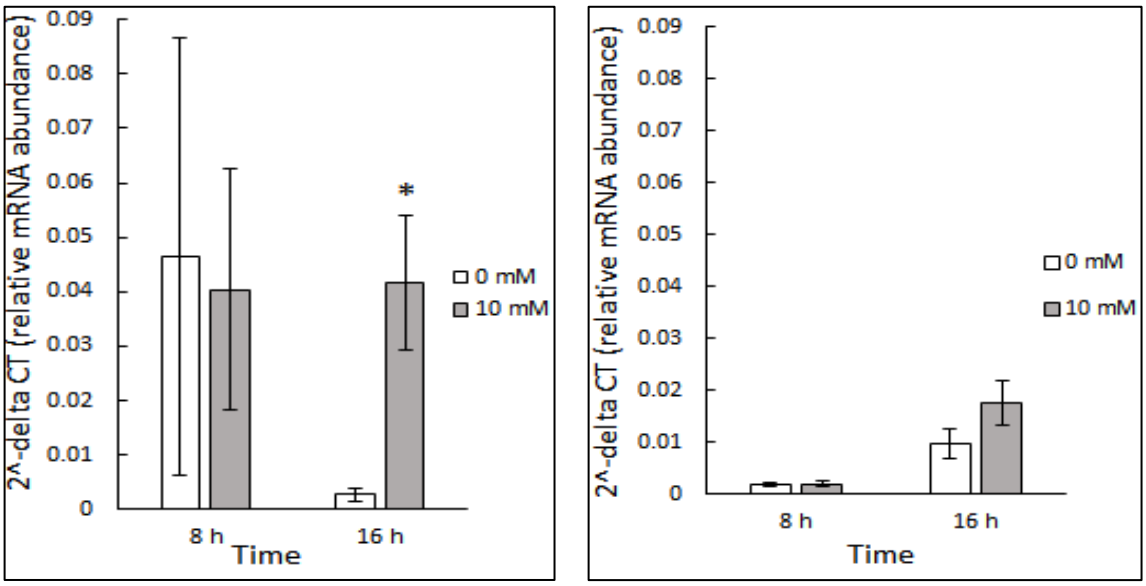


Figure 2.12. Real-time PCR quantification of *ODC* mRNA expression performed using c2c12 a) undifferentiated and b) differentiated cells after treating them with 10mM methionine after 8 and 16 h. *ODC* samples are normalized using respective 18S control and data was analyzed using relative C_T (ΔC_T) method. Bar- graphs represent the mean ± S.E.M of *ODC* mRNA. Significance is determined through comparison of treated (10mM methionine) with control, * indicates significance $p \leq 0.05$ using two-tailed t-test

Figure 2.13. The effect of putrescine on Myf5 mRNA in slo8 cells

a) Undifferentiated cells b) Differentiated cells

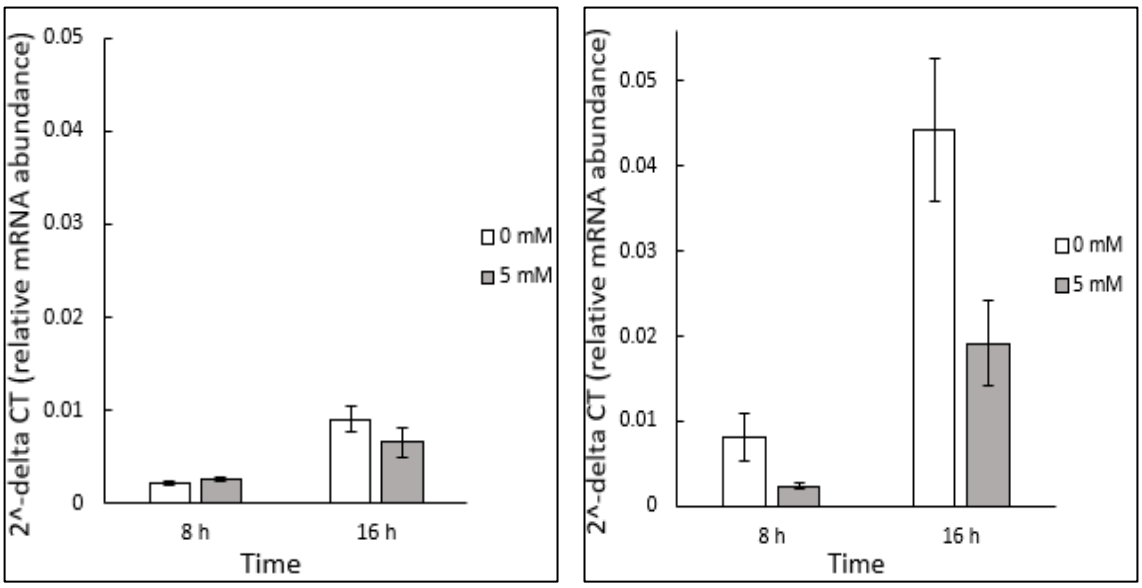


Figure 2.13. Real-time PCR quantification of *Myf5* mRNA expression performed using a) undifferentiated and b) differentiated sol8 cells after treating them with 5mM putrescine after 8 and 16 h. *Myf5* samples are normalized using respective 18S control and data was analyzed using relative C_T (ΔC_T) method. Bar-graphs represent the mean ± S.E.M of *Myf5* mRNA.

Figure 2.14. The effect of putrescine on MyoD mRNA in slo8 cells

a) Undifferentiated cells b) Differentiated cells

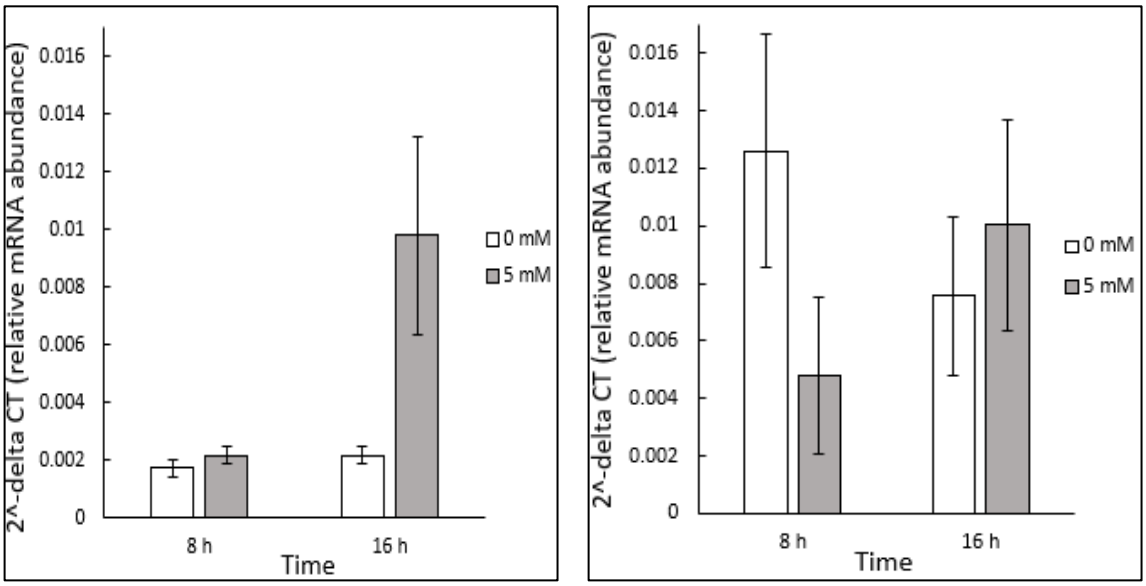


Figure 2.14. Real-time PCR quantification of *MyoD* mRNA expression performed using a) undifferentiated and b) differentiated sol8 cell line after treating them with 5mM putrescine after 8 and 16 h. *MyoD* samples are normalized using respective 18S control and data was analyzed using relative C_T (ΔC_T) method. Bar-graphs represent the mean ± S.E.M of *MyoD* mRNA.

Figure 2.15. The effect of putrescine on myogenin mRNA in slo8 cells

a) Undifferentiated cells b) Differentiated cells

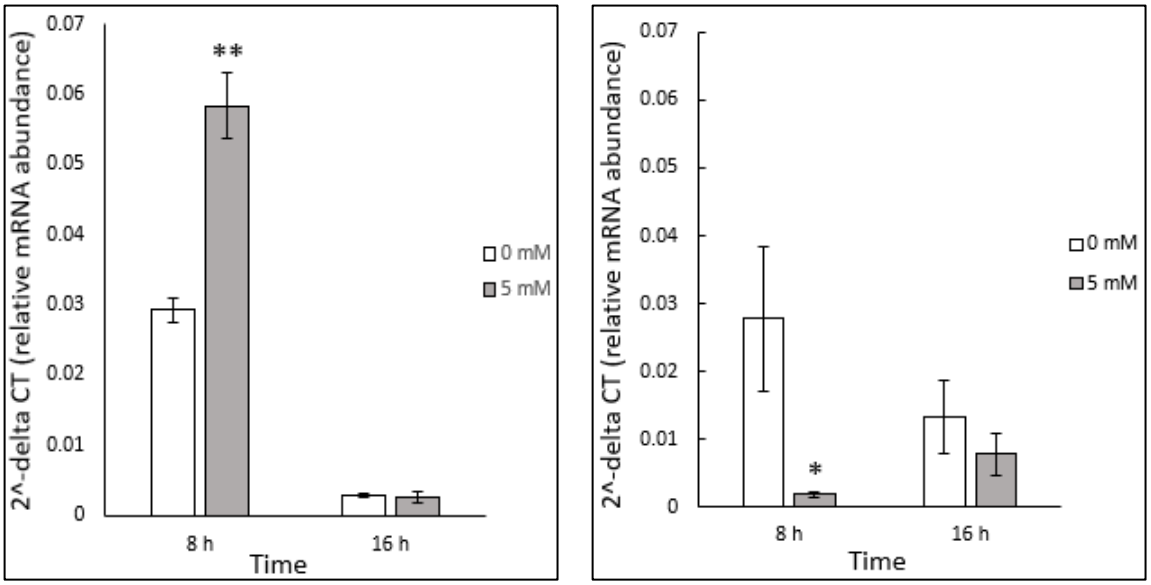


Figure 2.15. Real-time PCR quantification of *myogenin* mRNA expression performed using a) undifferentiated and b) differentiated sol8 cells after treating them with 5mM putrescine after 8 and 16 h. *Myogenin* samples are normalized using respective 18S controls and data was analyzed using relative C_T (ΔC_T) method. Bars graph shows the mean ± S.E.M of *myogenin* mRNA. Significance is determined through comparison of treated (5mM putrescine) with control, * indicates significance $p \leq 0.05$ and ** indicates significance $p \leq 0.01$ using two-tailed t-test

Figure 2.16. The effect of putrescine on ODC mRNA in slo8 cells

a) Undifferentiated cells b) Differentiated cells

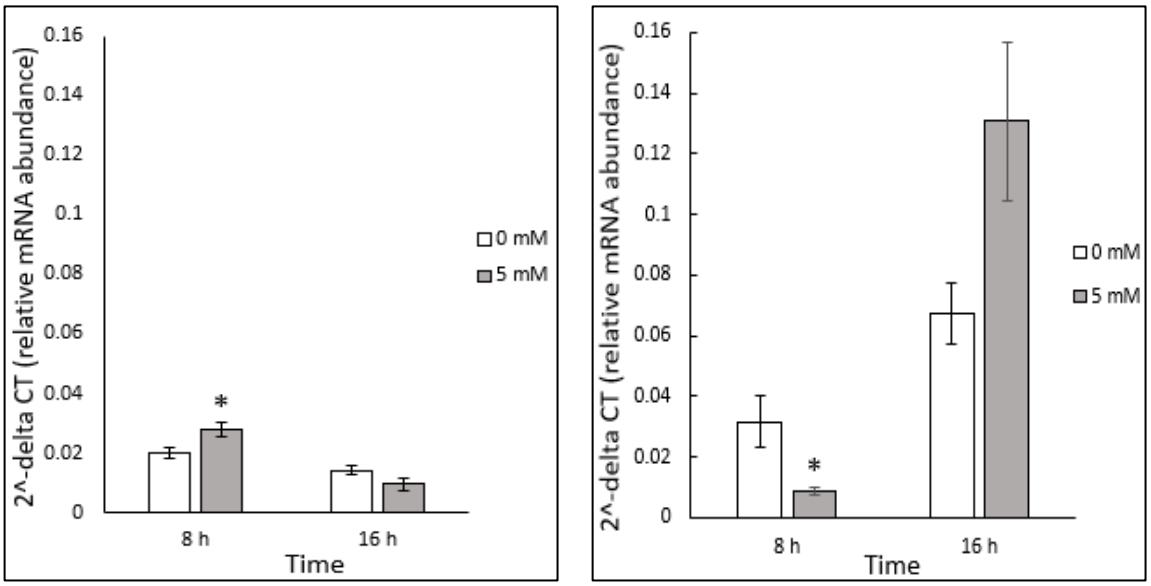


Figure 2.16. Real-time PCR quantification of *ODC* mRNA expression performed using a) undifferentiated and b) differentiated sol8 cells after treating them with 5mM putrescine after 8 and 16 h. *ODC* samples are normalizing using respective 18S controls and data was analyzed using relative C_T (ΔC_T) method. Bar-graphs represent the mean ± S.E.M of *ODC* mRNA. Significance is determined through comparison of treated (5mM putrescine) with control, * indicates significance $p \leq 0.05$ using two-tailed t-test.

Figure 2.17. The effect of spermine on Myf5 mRNA in slo8 cells

a) Undifferentiated cells b) Differentiated cells

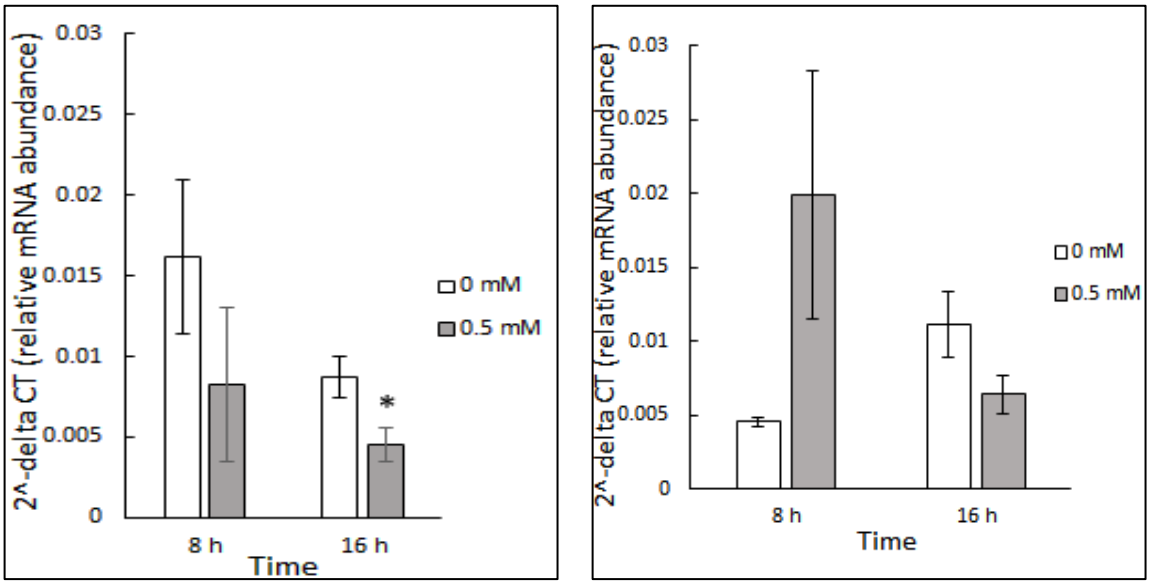


Figure 2.17. Real-time PCR quantification of *Myf5* mRNA expression performed using a) undifferentiated and b) differentiated sol8 cells after treating them with 0.5 mM spermine after 8 and 16 h. *Myf5* samples are normalized using respective 18S controls and data was analyzed using relative C_T (ΔC_T) method. Bar-graphs represent the mean ± S.E.M of *Myf5* mRNA. Significance is determined through comparison of treated (0.5 mM spermine) with control, * indicates significance $p \leq 0.05$ using two-tailed t-test.

Figure 2.18. The effect of spermine on MyoD mRNA in slo8 cells

a) Undifferentiated cells b) Differentiated cells

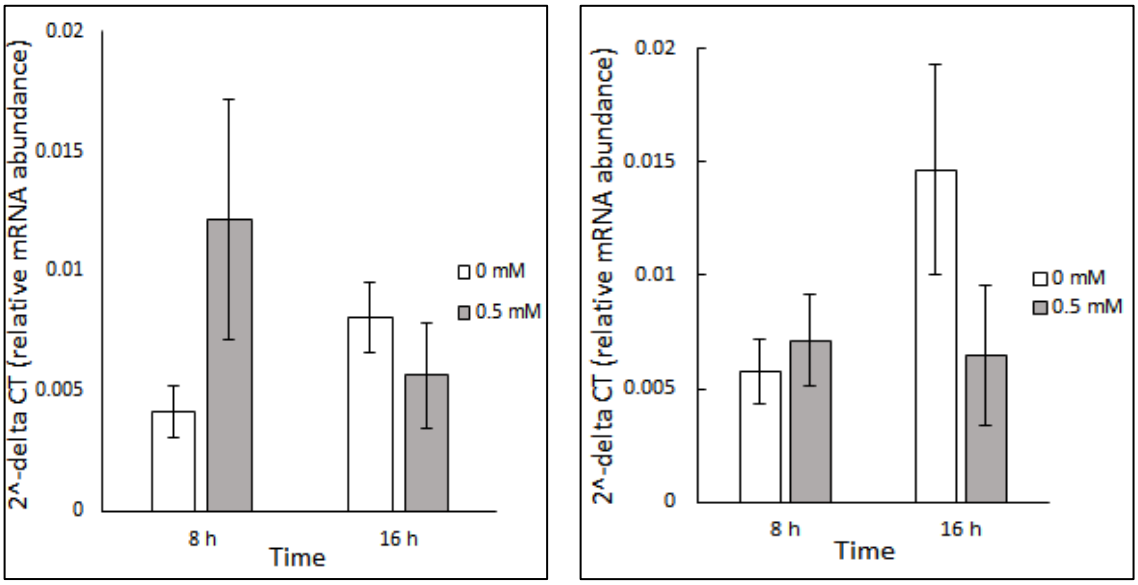


Figure 2.18. Real-time PCR quantification of *MyoD* mRNA expression performed using a) undifferentiated and b) differentiated sol8 cells after treating them with 0.5mM spermine after 8 and 16 h. *MyoD* samples are normalized using respective 18S control and data was analyzed using relative C_T (ΔC_T) method. Bar-graphs represent the mean ± S.E.M of *MyoD* mRNA.

Figure 2.19. The effect of spermine on myogenin mRNA in slo8 cells

a) Undifferentiated cells b) Differentiated cells

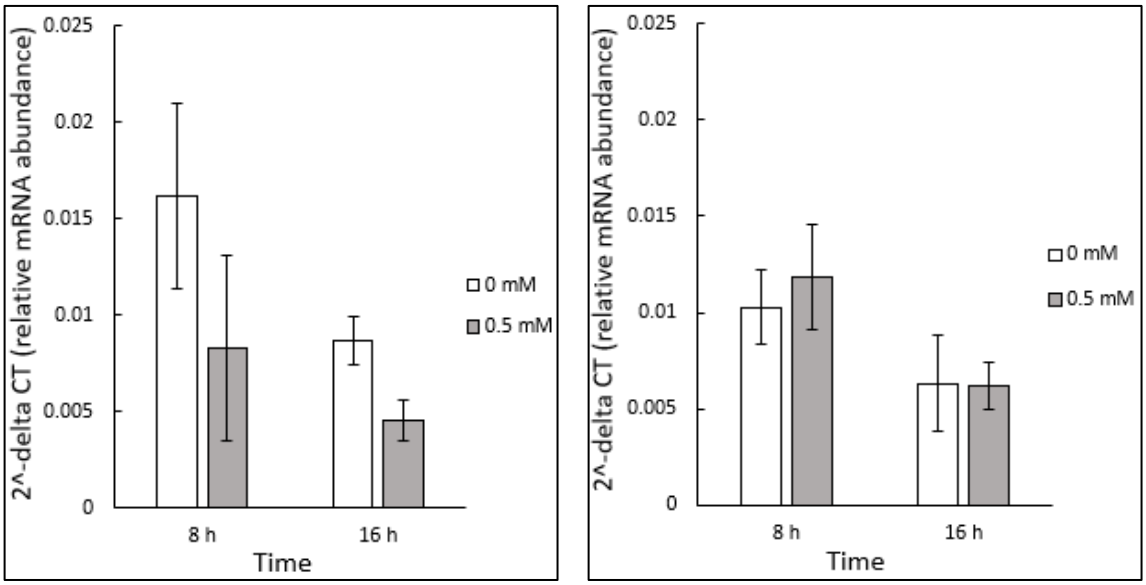


Figure 2.19. Real-time PCR quantification of *myogenin* mRNA expression performed using sol8 a) undifferentiated and b) differentiated cells after treating them with 0.5mM spermine at 8 and 16 h. *Myogenin* samples are normalized using respective 18S controls and data was analyzed using relative C_T (ΔC_T) method. Bar-graphs represent the mean ± S.E.M of *myogenin* mRNA.

Figure 2.20. The effect of spermine on ODC mRNA in slo8 cells

a) Undifferentiated cells b) Differentiated cells

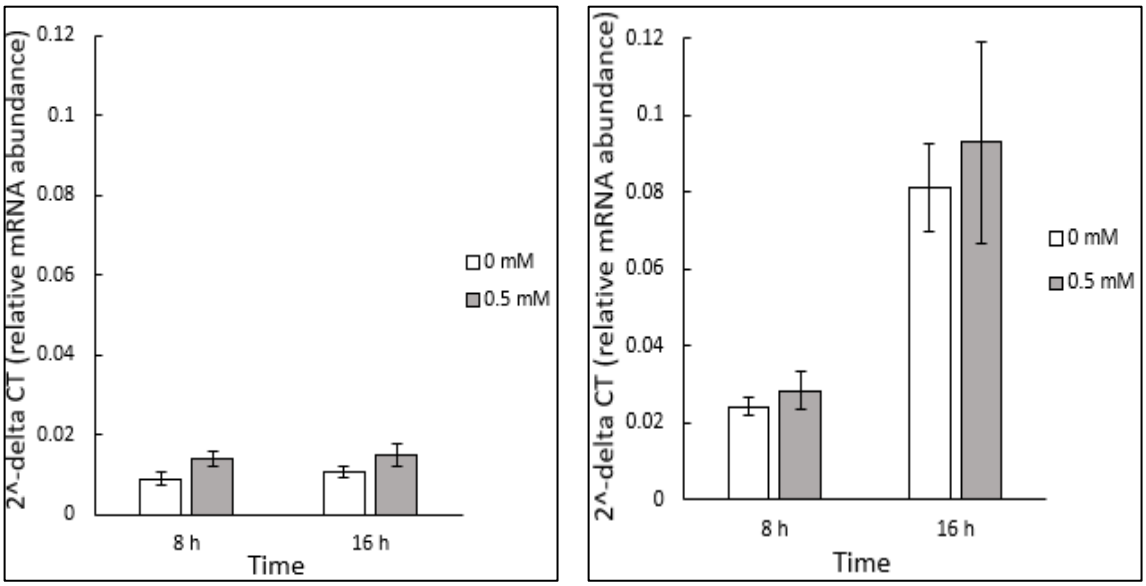


Figure 2.20. Real-time PCR quantification of *ODC* mRNA expression performed using a) undifferentiated and b) differentiated sol8 cells after treating them with 0.5 mM spermine after 8 and 16 h. *ODC* samples are normalized using respective 18S controls and data was analyzed using relative C_T (ΔC_T) method. Bar-graphs represent the mean ± S.E.M of *ODC* mRNA.

Figure 2.21. The effect of methionine on Myf5 mRNA in sol8 cells

a) Undifferentiated cells b) Differentiated cells

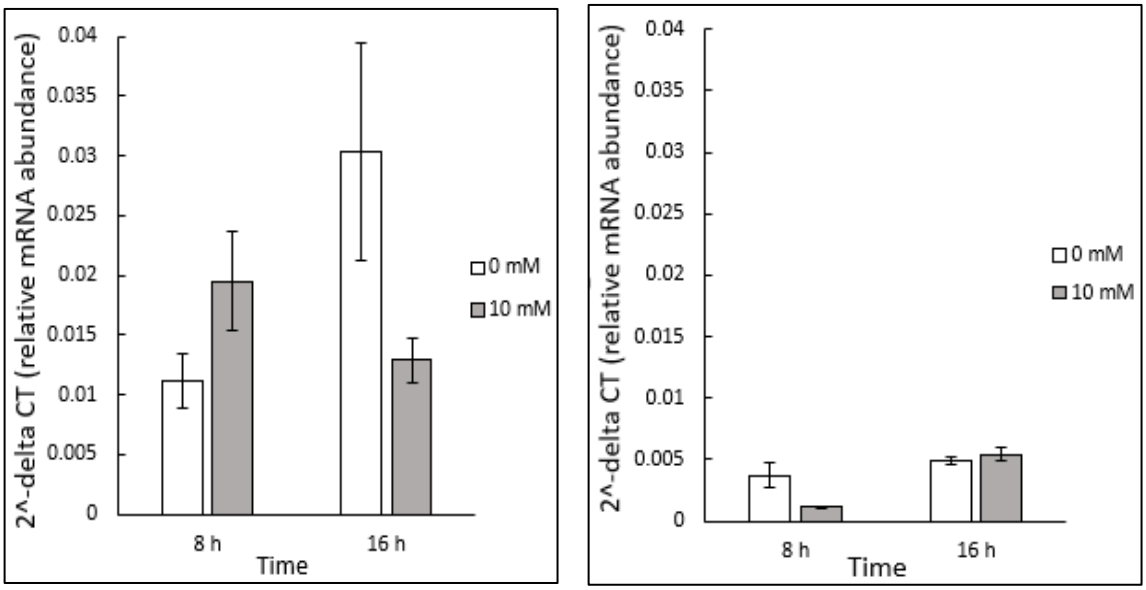


Figure 2.21. Real-time PCR quantification of *Myf5* mRNA expression performed using a) undifferentiated and b) differentiated sol8 cells after treating them with 10mM methionine at 8 and 16 h. *Myf5* samples are normalized using respective 18S controls and data was analyzed using relative C_T (ΔC_T) method. Bar-graphs represent the mean ± S.E.M of *Myf5* mRNA.

Figure 2.22. The effect of methionine on MyoD mRNA in slo8 cells

a) Undifferentiated cells b) Differentiated cells

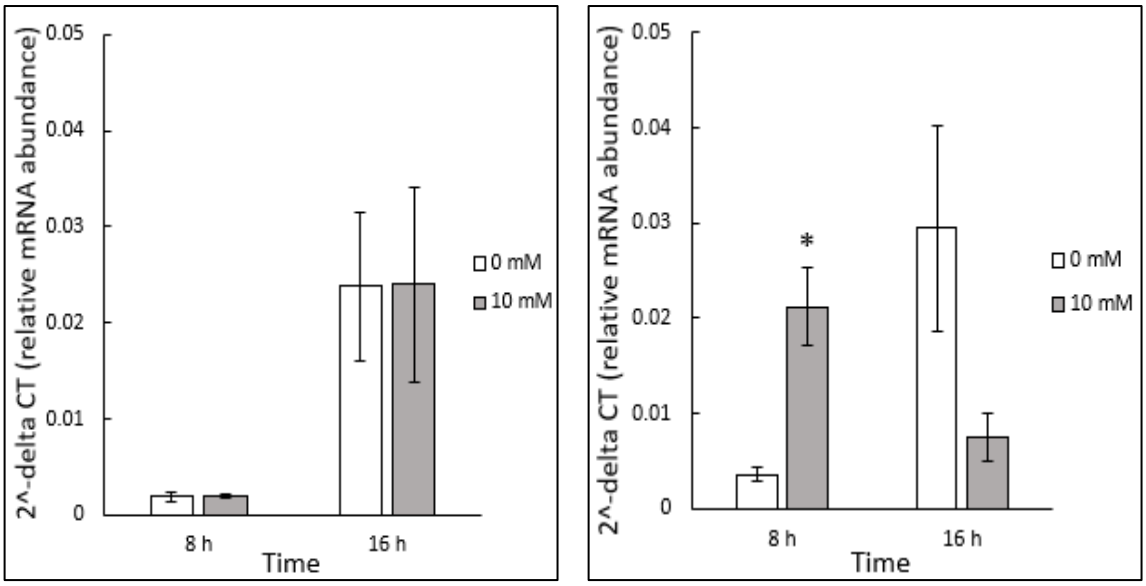


Figure 2.22. Real-time PCR quantification of *MyoD* mRNA expression performed using a) undifferentiated and b) differentiated sol8 cells after treating them with 10mM methionine after 8 and 16 h. *MyoD* samples are normalized using respective 18S controls and data was analyzed using relative C_T (ΔC_T) method. Bar-graphs represent the mean ± S.E.M of *MyoD* mRNA. Significance is determined through comparison of treated (10mM methionine) with control, * indicates significance $p \leq 0.05$ using two-tailed t-test.

Figure 2.23. The effect of methionine on myogenin mRNA in sol8 cells

a) Undifferentiated cells b) Differentiated cells

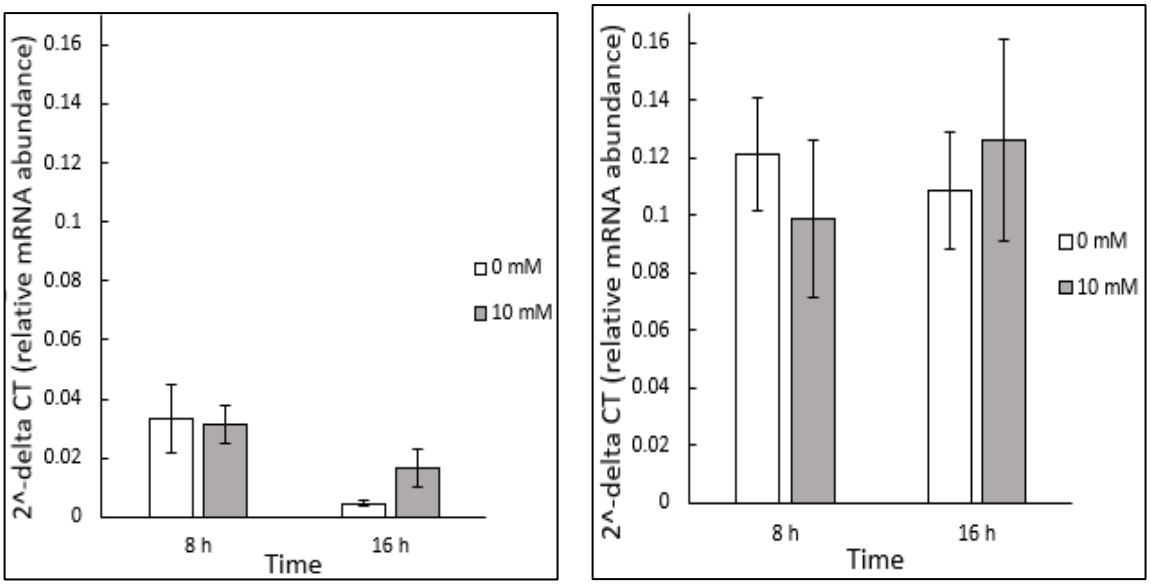


Figure 2.23. Real-time PCR quantification of *myogenin* mRNA expression performed using a) undifferentiated and b) differentiated sol8 cells after treating them with 10mM methionine after 8 and 16 h. *Myogenin* samples are normalized using respective 18S controls and data was analyzed using relative C_T (ΔC_T) method. Bar-graphs represent the mean ± S.E.M of *myogenin* mRNA.

Figure 2.24. The effect of methionine on ODC mRNA slo8 cells

a) Undifferentiated cells b) Differentiated cells

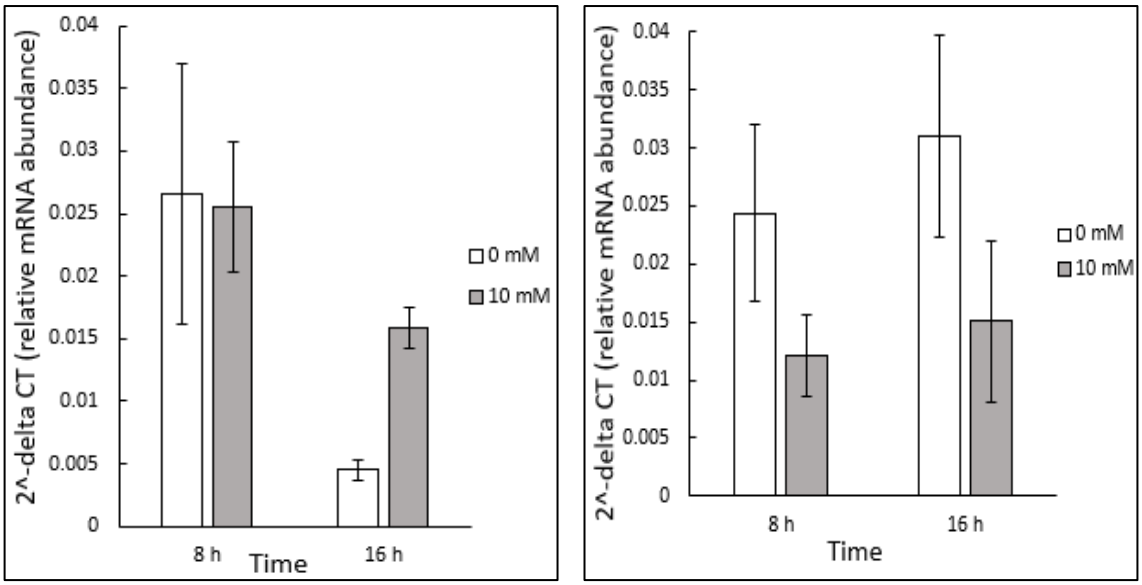


Figure 2.24. Real-time PCR quantification of *ODC* mRNA expression performed using a) undifferentiated and b) differentiated sol8 cells after treating them with 10mM methionine after 8 and 16 h. *ODC* samples are normalized using respective 18S controls and data was analyzed using relative C_T (ΔC_T) method. Bar-graphs represent the mean ± S.E.M of *ODC* mRNA.

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