

ABSTRACT

Title of Dissertation:

TRIBUTYRIN, A BUTYRATE PRO-DRUG, AS A MUSCLE GROWTH PROMOTER IN A PORCINE MODEL

Robert Len Murray, Doctor of Philosophy, 2018

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Postnatal muscle growth is dependent on myonuclear accretion and subsequent protein synthesis and accumulation. Altering the ability of muscle resident stem cells (satellite cells, SC), which provide nuclei to the growing muscle fiber, to progress through their myogenic lineage can have lifelong effects on muscle growth and repair. The use of butyrate, a histone deacetylase (HDAC) inhibitor, has promoted SC myogenesis *in vitro*. In animal models, dietary butyrate has improved growth performance and has had promising results in treating myopathic conditions, but the impact of dietary butyrate on SC activity and the direct promotion of muscle growth have not been examined. In the first experiment, we investigated whether the use of a physiologically relevant concentration of butyrate (0, 0.1, 0.5, or 1mM) could enhance the myogenic potential of porcine SC. We found increased expression of myogenin, the late stage myogenic regulatory factor, and increased SC fusion in the treated groups, suggesting an increased capacity during terminal differentiation. We further tested this hypothesis by conducting an animal study to test the effects of tributyrin, a butyrate pro-drug, on *in vivo* SC behavior. We found that tributyrin inclusion (0.25% and 0.5%) into the milk replacer of neonatal

piglets led to significant increases in muscle DNA content, suggesting increased myonuclear accumulation. The neonatal portion (21-days) of the study was repeated, and the animals were then weaned and crossed into dry nursery treatment diets. At the end of the 37-day nursery feeding, animals that received tributyrin in their milk replacer formula weighed significantly more and had increased muscle hypertrophy than the untreated animals. Also, SC from treated animals responded similarly *ex vivo* to those in the first experiment, suggesting an increased capacity to progress through the differentiation process. We determined in our final experiment that tributyrin supplementation altered the epigenetic landscape of SC by globally reducing the transcriptionally repressive chromatin mark H3K27me3 around key genes. In addition, we found that this is not the sole mechanism through which butyrate alters SC behavior. These findings help support the notion that tributyrin may be used to enhance muscle growth and could prove useful at treating myopathies.

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PROMOTER IN A PORCINE MODEL

by

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Chapter 1: Literature Review

Introduction

The ability for animals to grow and accumulate muscle mass postnatally relies on a distinct stem cell population residing within the muscle and along the myofibers (17, 74, 75, 87). These stem cells, referred to as satellite cells due to their juxtaposition beneath the basement membrane and outside the sarcolemma of the myofibers (64), are activated and will proliferate, differentiate and fuse into the growing myofiber. This contribution of nuclei to the myonuclear domain can then aid in protein synthesis, accretion, and subsequent muscle hypertrophy (3). It is during the earliest stages of post-natal development that satellite cells are most active and provide the majority of nuclei found in the adult muscle fiber (17, 46, 102). Altering the ability of satellite cells to progress through their myogenic lineage can alter lifetime muscle growth and regeneration (1, 32, 47, 50, 68, 72, 91, 92, 98) which makes them a practical target when trying to enhance muscle hypertrophy. Recently the use of histone deacetylase (HDAC) inhibitors have been shown to be effective at altering muscular homeorhetic characteristics in muscle disease models (27, 37, 71) and exhibited positive effects when supplied to satellite cells *in vitro* (49, 50). Once such HDAC inhibitor, butyrate, has led to improved growth performance measurements when included in the diet of agricultural species (8, 57, 58, 60, 85). Despite what seems to be promising outcomes, research into satellite cell programming effects in response to butyrate treatment in these models have not been evaluated. Tributyrin, a butyrate pro-drug, provides an efficient means to deliver increased amounts of butyrate systemically for a longer duration than butyrate alone (38, 101). The rationale would then

be that butyrate may act on the epigenetic landscape of satellite cells, whereby supplementation through dietary tributyrin may enhance muscle growth through altered satellite cell programming. This chapter summarizes the relevant literature on the effects of butyrate and tributyrin on satellite cell behavior and their applicability as a feed additive, in addition to pertinent satellite cell physiology with regards to muscle growth, myogenic commitment, and epigenetics.

Satellite cell physiology and muscle growth

Satellite cells are a heterogeneous population of stem cells required for post-natal muscle growth and repair and arise embryonically from somites that make up the dermomyotome (11). These embryonic myogenic precursor cells eventually will go into a state of quiescence wedged between the basement membrane and the plasmalemma of the muscle fibers (Figure 1-1) (7). It is this pool of undifferentiated satellite cells that are required for post-natal muscle growth and repair (47, 98) which is orchestrated through a series of tightly controlled regulatory mechanisms. These processes govern the rate of muscle hypertrophy in the growing animal and ultimately have an impact on their lifetime lean growth potential (109) and muscle regenerative capacity (93). Changes in the myogenic potential and the cascade of events that detail satellite cell myogenesis have revealed avenues for possible manipulation to enhance growth, improve health, and prevent myopathies.

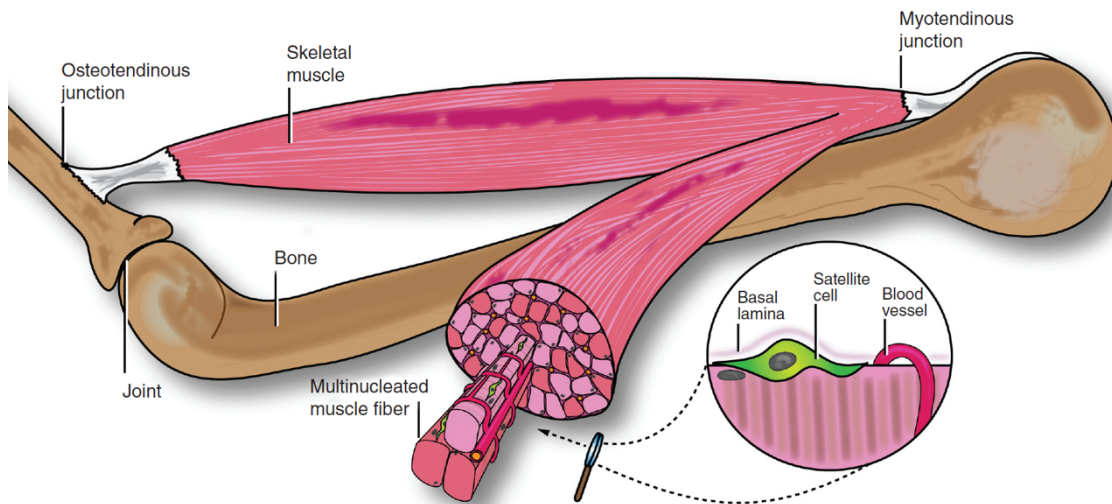


Figure 1-1. Illustration of the anatomical location of skeletal muscle resident stem cells (satellite cells). Skeletal muscles are separated into bundles of multinucleated fibers. Satellite cells reside along these muscle fibers, outside the plasmalemma and underneath the basal lamina (adapted from Dumont et al., 2015).

Fetal myogenesis involves the establishment of primary and secondary myofibers, marked by expansive hyperplasia from the fusion of myogenic precursor cells (MPCs) (22, 83). Pax3-positive (paired box domain-3), and to some extent Pax7-positive, cells represent a pool of MPCs that form primary myofibers and make up a scaffold with which secondary myofibers will align (44). These MPCs will generally have a second wave of migration, differentiation, and fusion to form secondary myofibers surrounding the primary myofibers (92). A subpopulation of MPCs will exit the cell cycle and occupy the satellite cell niche between the basement membrane and sarcolemma (80). The process through which MPCs and ultimately satellite cells progress through their myogenic lineage is under the control of a series of hierarchical transcription factors (9). MPCs are denoted by the expression of Pax3 (4, 12), but not necessarily the expression of Pax7; this contrasts with satellite cells in which Pax7 is an absolute requirement for post-natal myogenesis (81, 98). Both MPCs and satellite cells then differentiate and fuse to form multinucleated myotubes through the

timely expression of muscle regulatory factors (MRFs). Common to both cell types are the primary MRF MyoD and the secondary MRF myogenin which are required for terminal differentiation (47, 68, 105). There is some built in redundancy in the MyoD family of MRFs; however, for this review I will focus the discussion mainly on MyoD and myogenin and their role in post-natal muscle growth (Figure 1-2) (for a detailed review, see Bentzinger et al. 2012 (9)).

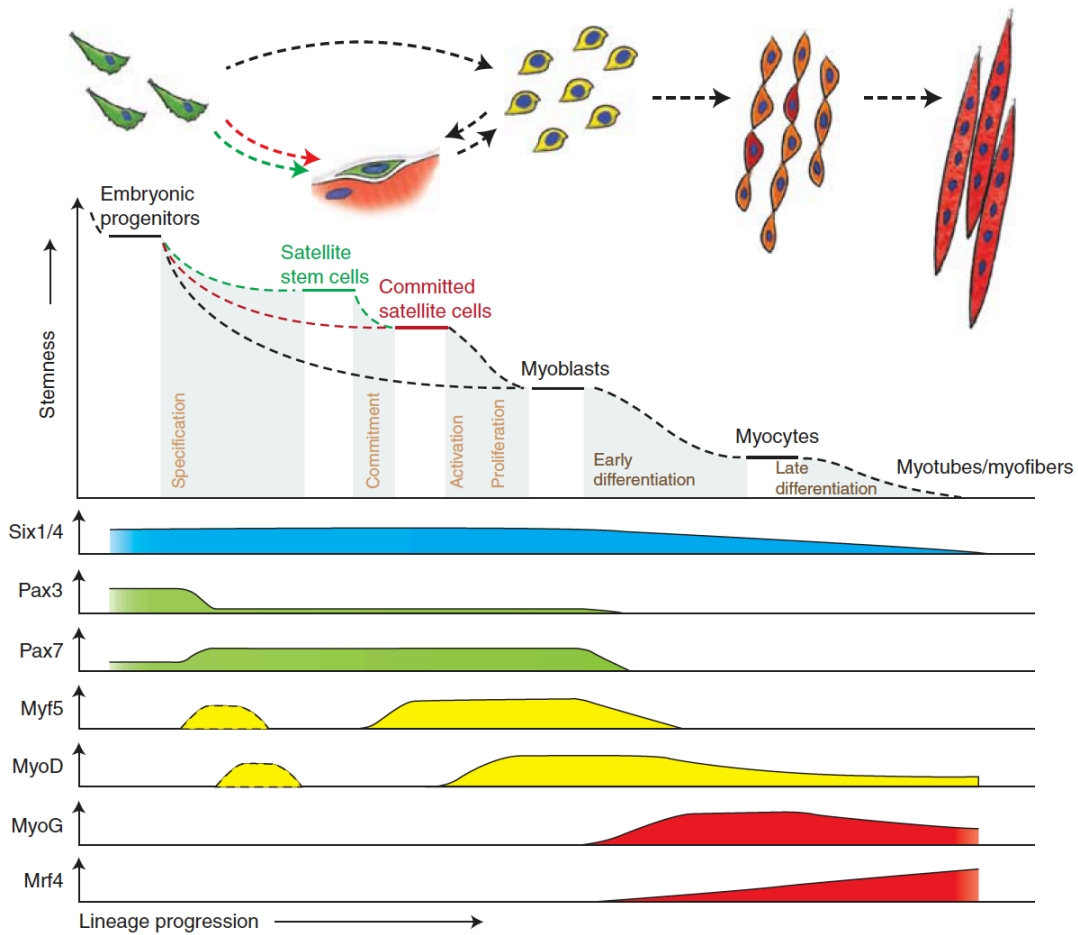


Figure 2-2. Illustration of transcription factors regulating satellite cell lineage progression. Muscle precursor cells (MPC) involved in fetal myogenesis skip straight to myoblasts. Some MPCs will go into a state of quiescence, marked by upregulating Pax7. Once activated, satellite cells will commit to a myogenic lineage and express MyoD. At the onset of differentiation, myoblast downregulate Pax7 and express myogenin through differentiation (adapted from Bentzinger et al., 2012).

Apart from their physical location, satellite cells can be identified by their expression of the Pax7, which has been described as a master regulator of satellite cell specification (15, 56). Satellite cells possess the ability to activate, proliferate, and give rise to either committed myogenic daughter cells or self-renew and eventually return to a mitotically quiescent state. Pax7 plays an important role in the process of lineage fate determination and is upregulated upon activation, specifying myogenic commitment by inducing the expression of Myf5 (66). Those cells expressing Myf5 represent a satellite cell committed to myogenesis that will ultimately start expressing MyoD. Myf5-negative satellite stem cells have the unique ability to asymmetrically divide by producing Myf5-positive daughter cells (54). Once satellite cells start to express MyoD they are generally referred to as myoblasts and it is at this stage where differential expression patterns of Pax7 and MyoD will control the fate between proliferation and determination (79). Where a high Pax7:MyoD expression pattern favors continued proliferation and inhibition of differentiation (78), and a low Pax7:MyoD promotes the differentiation of myoblasts to myocytes. Myocytes are marked by the upregulated expression of myogenin, facilitated by MyoD, and rapidly differentiate (84). Other molecular cues also aid in the myogenic differentiation program by inhibiting Pax7 expression (23, 33). Myogenin and Pax7 expression are mutually exclusive and this cross-inhibitory interaction plays a key role in cell fate determination (79, 94). Once myogenin expression begins, myocytes will terminally differentiate and fuse together to form myotubes, characterized by the expression of the contractile protein myosin heavy chain (70). Myogenin upregulation directly activates myotube associated genes and is required for the expression of most of the sarcomeric proteins (29, 47). The Pax7→MyoD→Myogenin hierarchy of transcription

factors regulate satellite cell fate determination and are hallmarks of their progression through their myogenic lineage. Changes in satellite cell dynamics marked by these transcription factors have shown to have a dramatic effect on post-natal muscle growth and development.

Altering the myogenic potential of satellite cells has shown to have profound effects on the lifetime muscle growth potential and regeneration (1, 72, 93). Post-natal muscle growth is considered a hypertrophic event (increase in muscle fiber size), as the increase in muscle fiber number (hyperplasia) has usually ceased right around the time of birth for most animals (103). In neonatal animals, the ratio of mononuclear satellite cells to multinucleated myotubes is the largest and this ratio steadily declines as an animal ages (2). It is also during this period that muscle growth is this most rapid, marked by enhanced myonuclear proliferation and the muscle protein accumulation (97). As the animal ages, the fractional rate of protein synthesis steadily declines (31). Similar pathways that stimulate satellite cell myogenesis also activate protein translation machinery in parallel during neonatal growth. Nutrition has shown to be a dominant controlling factor with regards to altering both satellite myogenesis and protein synthesis pathways. After the ingestion of a meal, protein synthesis is promoted by altering mammalian target of rapamycin (mTOR) signaling which acts to phosphorylate eIF (eukaryotic initiation factor), 4E binding protein 1 (4EBP1), and the ribosomal protein S6K1 (31). Restriction in total food and protein intake (99), dietary mineral and vitamin restriction (1, 35), and certain amino acid deficiencies (32, 110) during the neonatal period severely impact the myogenic potential of satellite cells as demonstrated by either reduced proliferation or

subsequent fusion into myofibers. Activators of protein translation such as exercise and muscle damage have also shown to be a potent stimulator of satellite cell activation (10, 39), and conversely, disuse and aging leads to muscle atrophy and impaired satellite cell function (13, 93). These known stimuli have aided in the discovery of regulatory networks that provide targets to be manipulated for enhanced muscle hypertrophy.

There has been a great deal of research into the regulatory pathways involved around the epigenetics of satellite cell fate. Epigenetic regulators have been identified as being involved in the chromatin remodeling around Pax7 and the MRFs, ultimately dictating their expression patterns (73, 100). Satellite cells, as most stem cells do, remained “poised” to start the myogenic program once activated (28). The epigenetic marks associated with satellite cells take one of two broad profiles, one which favors proliferation or one which favors differentiation. Once activated, satellite cells begin to proliferate while repressing those genes associated with the differentiation part of the myogenic program (34). In general, this is accomplished by silencing muscle genes through the deposition of methyl groups on lysine 27 of the histone 3 (H3K27me3) (5) by the methyltransferase enzyme, enhancer of zeste-2 (EZH2), a subunit of the polycomb repressive complex-2 (PRC2) (21). The EZH2 subunit plays a critical role in preventing differentiation by associating with the myogenin promoter (5), along with other genes associated with non-muscle lineage fates (108). Concomitant with the silencing of differentiation genes, is the permissive chromatin mark, H3K4me3 which aids in Pax7, MyoD, and cell cycle gene expression (66). Upon induction to differentiate, PRC2-EZH2 leaves the regulatory region of myogenin and deposits repressive H3K27me3 marks around the Pax7 promoter, reducing its transcription

and “switching-off” proliferation (82). The H3K27me3 mark around muscle specific genes is then removed by a demethylase that has been recruited to their promoters (34). Corresponding with demethylase activity is the down regulation of histone deacetylase-1 (HDAC1) (62) and an increase in acetylation of histone tails by histone acetyltransferases (HATs) (67) to allow for chromatin relaxation and access to muscle differentiation target genes. A complex formed by the association of HDAC1 and MyoD is also interrupted which allows for acetylated MyoD to target and upregulate myogenin (20, 90). Taken together, these “switches” allow for the timely regulation of satellite cells to differentiate into proliferating myoblasts, form myocytes, and quickly fuse into myotubes.

HDACs play a key role in the switching on of the differentiation program and switching off cycle/proliferation mechanisms. HDACs target the DNA and compact chromatin structure, repressing transcription directly. They target specific regions of DNA by associating with other regulatory proteins and are integral in the transition of proliferative to the differentiative nuclear state in satellite cells (67). The significance of the role that HDACs play in satellite cell physiology has become more defined recently and offers a potential mechanism that may be exploited for enhanced muscle growth.

Butyrate and histone deacetylase inhibitors

Research has been accumulating over the past several years about the use of HDAC inhibitors in treating muscle myopathies (73, 100). Butyrate, a 4-carbon short chain fatty acid, is a known inhibitor of HDACs (19, 63) that has also recently proved efficacious at treating certain models of myopathy (37, 106, 107) and may serve as an inducer of

differentiation in satellite cell cultures (49, 50). Dietary butyrate has proved beneficial for intestinal health statuses and has been used in the animal agriculture industry since the late 1980's (41). Despite the mounting evidence indicating its beneficial effect on muscle homeorhesis and the development of new butyrate glycerides, research has yet to determine the influence of dietary butyrate on muscle growth.

In 2002, Iezzi and colleagues (49) were the first to report the possible benefits of HDAC inhibitors on satellite cell myogenesis. The researchers noted increases in myoblast fusion and enhanced terminal differentiation when the HDAC inhibitors were added to satellite cell cultures. HDAC inhibitors were originally researched for their ability to arrest growth and force differentiation or apoptosis in cancer cells (30, 63). It has now been found that inhibition of class I HDACs primarily, confers beneficial effects on satellite cell myogenesis through the disruption of the MyoD-HDAC1 complex, allowing the expression of late stage muscle specific genes (62, 67, 90). There is also some evidence that inhibiting class II HDACs aids in the activation of MEF2 whose activity is otherwise blocked by hypoacetylation (61, 90). Treatment of embryonic stem cells with HDAC inhibitors also revealed that not only are HDACs directly inhibited leading to an increase in histone acetylation, but there is also an indirect effect on the epigenetic programming marks associated with histone methylation (52). These results indicate that HDAC inhibitors selectively enhance those genes associated with normal activation during the differentiation process. With the understanding that many of these factors controlling satellite cell fate may be targeted and manipulated, the use of HDAC inhibitors for the treatment of common myopathies such as muscle dystrophy (27, 71), amyotrophic lateral

sclerosis (ALS) (95, 112), and sarcopenia or atrophy (106) has had positive results. Minetti et al. (30) found that MDX mice treated with TSA showed a recovery in exercise performance and an almost 2-fold increase in fiber cross sectional area. When TSA was used to treat a mouse model of ALS, Yoo et al. (112) showed decreased muscle atrophy by exerting a neuroprotective effect, as well as inhibiting HDACs known to promote muscle atrophy. Walsh et al. (107) found similar results in butyrate-fed mice, indicating that butyrate may help in the preservation of muscle mass during aging. The outcomes of these studies have warranted a more comprehensive look at the potential role and benefit that HDAC inhibitors play in enhancing muscle growth.

Butyrate is an HDAC inhibitors that has been well researched, but has not been fully evaluated as a muscle growth promoter. Butyrate is the natural byproduct of microbial fermentation of fiber in hind gut (14) and has been utilized for the treatment of human neoplasms (88). It's use on satellite/myogenic cell cultures has been mostly documented as having detrimental effects to the normal myogenic progression (40, 51, 59); however, the study by Iezzi et al. (49) characterized the potential benefit of the butyrate on satellite cell myogenesis. Iezzi and colleagues note that exposure proliferating myoblast to butyrate enhanced the formation of multinucleated myotubes during differentiation after it was removed. These results are in agreeance with the notion that HDAC inhibitors are selective in the genes that are targeted and correlate with proliferating stem cells (26) (Figure 1-3). It is for this reason that the mechanism behind butyrate's action make it a potential modifier of satellite cell fate. Butyrate's use as a pharmacologic for *in vivo* myopathic conditions has exhibited positive indicators at countering disease progression (95, 106, 107); however,

the effect that butyrate has on *in vivo* satellite cell behavior has not been described. As is the case with other HDAC inhibitors, they are currently being researched for the treatment of DMD and other myopathies (27). Although these studies have highlighted the potential benefit to muscle tissue, they have been sparse in describing the potential benefit to promoting muscle growth and regeneration. Likewise, butyrate has been well researched as a feed additive to promote intestinal health (18, 45), but its effect on muscle growth specifically has not been evaluated. Studies dating back to the 1950's have attributed the positive impacts of butyrate on animal growth to some component of intestinal importance. This led to investigations into the benefits of butyrate's modulation of transformed cell lines (6, 48), as well as *in vivo* models of colonic malignancies as fiber in the diet can expose the lower gastrointestinal tract to butyrate and its HDAC inhibitory properties (14, 65). Collectively, these studies indicate that butyrate may have a positive impact on muscle growth through HDAC inhibition.

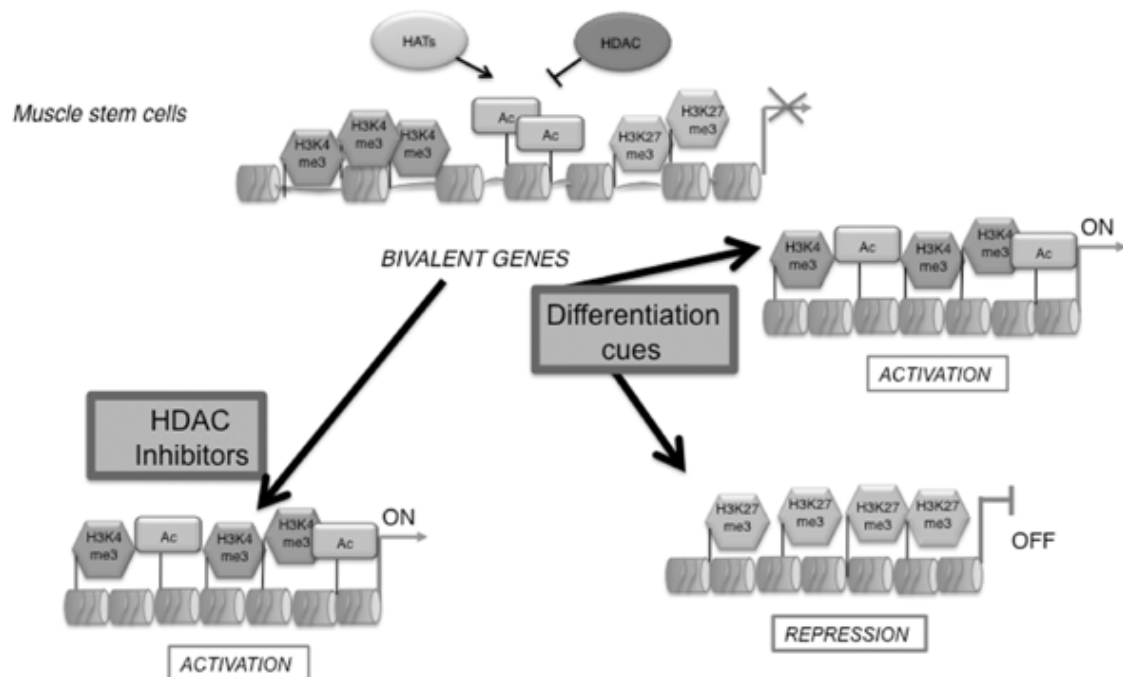


Figure 3-3. Illustration of the epigenetic status of satellite cells in response to HDAC inhibitors. Genes that are susceptible to HDAC inhibition mirror those that are activated during differentiation (adapted from Consalvi et al., 2011).

The results of satellite cell culture studies are unclear and contradictory (40, 49-51, 59), but they allude to an enhanced or forced differentiation of satellite cells. Part of the confusing results may lie with the high concentration (3-7mM) and/or timing of butyrate applied to the cultures. One study involving human patients revealed physiologically relevant concentrations peaked at 59 μ M when administered sodium butyrate at 500mg/kg/day by continuous intravenous infusion (69). Given an intravenous dose (1.25g/kg), rodents also showed a similar clearance rate, and after 30m, plasma butyrate concentrations fell below 1mM (38). Dietary, or enteral, administered sodium butyrate with mice had similar results, with concentrations falling below 1mM after 90m. While the half-life of circulating butyrate is around 5m (69, 86), exposing satellite cells to supraphysiological concentrations butyrate for extended periods of time may be affecting

more than just the myogenic program. It has been shown that butyrate is extremely effective at arresting cell growth and proliferation in culture (88, 89). Therefore, applying high concentrations of butyrate for long periods of time to sub-confluent satellite cells during the proliferative stage would blunt myogenesis as the forced differentiation of sub-confluent satellite cells has been shown to reduce proper myotube formation (104). There has been research into butyrate pro-drugs with superior pharmacokinetics that allow for enhanced delivery of butyrate to systemic tissues for sustained periods (24, 25, 38, 43). Utilizing these butyrate pro-drugs could provide an effective means at exposing satellite cells to physiologically relevant concentrations ($\leq 1\text{mM}$) of butyrate *in vivo*.

One key property of exposing satellite cells to butyrate *in vivo* relies on continuous supplementation throughout periods of muscle growth. While intravenous delivery of butyrate and synthetic butyrate carriers may be effective at treating malignant cell populations (86), oral delivery through food remains the only practical way to enhance post-natal muscle growth for extended periods of time. To this end, the butyrate pro-drug tributyrin may be the most appropriate alternative to promote muscle growth through dietary inclusion.

Tributyrin, a butyrate pro-drug

The beneficial effects of butyrate in treating myopathic models has been demonstrated, but its use as a muscle growth promoter has not been investigated. Utilizing tributyrin to achieve sustained systemic concentrations of butyrate may impact muscle growth and could recapitulate results seen *in vitro* (49). With only one study detailing tributyrin's

effect on muscle tissue (16), most of what is known about the effectiveness of tributyrin and butyrate on peripheral tissues has been from studies involving cancer or gastrointestinal diseases (24, 36, 55). These studies have however, demonstrated that oral tributyrin provides far superior pharmacokinetics over butyrate at reaching systemic tissues as well as the ease of handling without the obnoxious odor that butyrate presents (18). Just as butyrate has been investigated for the potential intestinal health benefits in the animal agriculture industry, so has tributyrin, with some studies indicating increased growth rate. Together, these results support the notion that oral tributyrin would expose muscle tissue to sufficient concentrations of butyrate needed to enhance myogenesis. With regards to outcomes involving the promotion of muscle growth, questions about delivery, inclusion rates, and effectiveness still remain.

Butyrate has been widely used in the animal industry to protect the gut health of animals and improve the trophic status of the intestine (41, 96). To this end, studies involving oral tributyrin have proved to be a more effective means of delivering butyrate to the entire digestive tract aimed at improving growth and/or performance (Table 1-1); the results of which have demonstrated that butyrate and/or tributyrin may influence overall growth as evident by increased final body weights and average daily body weight gain. Apart from an apparent increase in these growth measures seen in the animals receiving treatment diets, is the decrease in adiposity linked to an increased fatty acid oxidation and thermogenesis; suggesting muscle hypertrophy is the cause for increased weight gain (8, 42, 107, 111). Most, if not all, of the animal growth and performance models have been analyzed for some component of intestinal status. While there are no muscle regenerative

models, growing animals can give us an insight into possible actions exerted on satellite cell behavior. The potential for lifetime muscle growth has been linked to when satellite cells are most active and when rapid myonuclear accretion occurs (31, 76), and that hypertrophy cannot occur without satellite cell mediated myonuclear accretion (53, 77). Consequently, it would appear that oral supplementation of butyrate may in fact be promoting muscle growth by altering satellite cell programming through HDAC inhibition and not just an enhanced intestinal trophic status.

Table 1-1: Summary of relevant effects of butyrate and tributyrin on animal growth and performance

Feed Additive/Inclusion Rate	Animal Model	Results		Mechanism of Action	Reference
		Effect on Growth	Effect on Performance*		
Sodium Butyrate/0.17%	Piglet (Weaned)	↑	↑	Improved intestinal health and development	Galfi and Bokori (1990)
Sodium Butyrate/0.08%	Piglet (Weaned)	↑	↑	Improved intestinal development	Piva et al. (2002)
Tributyrin ^a /1.0%	Piglet (Weaned)	↑	↑	Improved intestinal health and development	Piva et al. (2002)
Sodium Butyrate/0.3% ^b	Piglet (Neonatal)	↑	---	Improved intestinal efficiency and development	Kotunia et al. (2004)
Butyrate Glycerides ^c /0.1-4%	Chicken	↔	↔	Improved intestinal development	Leeson et al. (2005)
Sodium Butyrate/0.3%	Piglet (Weaned)	---	↑	Improved intestinal health dynamics	Manzanilla et al. (2006)
Sodium Butyrate/0.1-4%	Piglet (Weaned)	↔	↔	No appreciable improvements	Biagi et al. (2007)
Tributyrin ^a /0.3%	Piglet (Weaned)	↑	↑	Improved intestinal development	Piva et al. (2008)
Sodium Butyrate/0.3%	Calf	↑	↑	Improved intestinal efficiency	Guilloteau et al. (2009)
Sodium Butyrate/0.3% ^b	Piglet (Neonatal & Weaned)	↑	↔	Improved intestinal efficiency	Le Gall et al. (2009)
Sodium Butyrate/0.35%	Calf (Neonatal & Weaned)	↑	↔	Improved intestinal development	Katoh et al. (2011)
Butyrate/0.3%	Piglet (Perinatal) ^e	↑	---	Improved lipid metabolism	Lu et al. (2012)
Sodium Butyrate	Fish	↑	↑	Improved intestinal efficiency	Robles et al. (2013)
Sodium Butyrate ^d /0.15%	Piglet (Weaned)	↑	↑	Improved intestinal health dynamics	Chiofalo et al. (2014)
Sodium Butyrate ^d /0.1%	Piglet (Weaned)	↑	↑	Improved Intestinal permeability and microbiome	Huang et al. (2015)
Tributyrin/0.05%	Piglet (Neonatal)	↑	↔	Improved intestinal development	Dong et al. (2016)
Coated Calcium Butyrate/0.02-4%	Chicken	↑	↑	Improved intestinal efficiency	Kaczmarek et al. (2016)
Sodium Butyrate/1g/day	Piglet (Neonatal)	↔	↔	Altered Microbiome	Xu et al. (2016)
Butyrate Glycerides ^c /0.6%	Chicken	↑	↑	Improved lipid metabolism	Yin et al. (2016)
Butyrate Glycerides ^f /0.05-0.30%	Chicken	↔	↔	Possible improved lipid metabolism	Bedford et al. (2017)

*- Feed efficiency or feed conversion ratio, Feed:Gain

a- Mixture of tributyrin and lactitol

b- On a dry matter basis

c- Mixture of 30% mono-, 50% di, and 20% tributyrin

d- Encapsulated or coated sodium butyrate

e- Mixed 0.3% 1-monobutyrate and 0.3% butyrate glycerides^b

f- Butyrate glycerides: two studies using monobutyrate and mono- and tributyrin

g- Sows fed treatment diet at 77d of gestation through farrowing to end of lactation, or sodium butyrate was supplied just during lactation to neonatal piglets

The butyrate triacylglyceride, tributyrin, is used in many foods as a flavoring agent and adjuvant and is currently approved as a dietary additive (4). Tributyrin was originally synthesized as an oral prodrug aimed at combating neoplastic growths by sustaining systemic levels of butyrate (5, 7, 9, 13). Lipases in the small intestine may cleave off two of the butyric acid moieties, but leave behind the 2-monobutyryl for absorption into the enterocyte (17). The attached butyric acid molecule reaches circulation rather than be immediately utilized for energy by the enterocyte (25). There is also evidence that intact tributyrin can be absorbed and make it into systemic circulation (26), which would allow for gradual hydrolysis of the triglyceride and sustained concentrations of butyrate in circulation. Tributyrin is also well tolerated when given orally (4, 21) and does not have the caustic odor or corrosiveness associated with butyric acid or its salts (primarily sodium or calcium butyrate) that limits their use when included in the diet (19). So, with the aim of targeting muscle tissue, dietary inclusion of tributyrin may not only provide intestinal health benefits but is also more suitable for providing butyrate systemically to alter satellite cell myogenesis.

From a practical stand point, there are several questions that need to be addressed when including tributyrin into the diet for promoting muscle growth. Since tributyrin inclusion increases systemic circulation of butyrate, other proliferating cell populations may be affected. There is some evidence that the mesenchymal stem cell (MSC) population, another tissue resident stem cell population linked to lifetime bone and adipose (as well as other) tissue growth, may adopt an osteogenic lineage when exposed to butyrate (3). Tributyrin may direct growth potential towards musculoskeletal development with reduced

adiposity. Another question that remains is the rate of inclusion of tributyrin into the diet. It has been noted that at certain amounts of butyrate or tributyrin, feed intake declines (15) which has been linked to certain states of mild ketosis caused by intake of exogenous ketone bodies (22). Overall, this could limit the supply of available nutrients needed for muscle hypertrophy, masking the effects of tributyrin supplementation. From this standpoint, inclusion rates of tributyrin necessary to have a positive effect on muscle growth without experiencing a decline in feed intake would need to be determined. Some authors have also looked at and suggested that the timing (pre- or post-weaning) of butyrate/tributyrin inclusion in the diet may have certain periods of effectiveness with regards to growth (1, 14, 20). It may be that continual supplementation of tributyrin into the diet may not necessarily be needed to achieve positive effects on muscle growth and lifetime lean tissue accretion.

Conclusion

Altering the ability of satellite cells to progress through their myogenic lineage is key to enhancing the post-natal growth of muscle and lifelong potential for lean tissue accretion. As evidenced by the positive outcomes seen when animals are given a diet supplemented with butyrate or tributyrin, early-life intervention with a potential hypertrophic stimulus could have lifelong impacts on muscle growth and regeneration (24). Butyrate and other compounds have shown to positively influence satellite cell myogenesis when treated in culture (10, 11); although, the concentration and timing of butyrate application may not have been physiologically relevant. These changes; however, have been linked to their ability to inhibit HDACs and subsequently make changes to the epigenetic status of satellite

cells (6, 18). The beneficial effects on muscle tissue seen in some animal disease models treated with butyrate (2, 8, 23, 27, 28) highlight the potential for therapeutics by inhibiting HDACs. The potential for oral butyrate to affect muscle growth has been presented in a variety of animal models (Table 1-1), but these studies were aimed at improving growth through improved intestinal dynamics and did not investigate the impact of butyrate on satellite cells or their contribution to the observed measures of growth. It is thought that through changes in the epigenetic landscape, butyrate treatment may epigenetically modify the myogenic potential of tissue resident stem cells (12, 16). The butyrate prodrug tributyrin is a better alternative to dietary butyrate inclusion due to its pharmacokinetics (9) and ease of use (4). It is my hypothesis that when included into the diet, tributyrin may promote muscle growth by altering satellite cell myogenesis. It is important to determine what effect butyrate has on satellite cells at physiologically relevant concentrations and during all periods of the myogenic lineage. Also, it appears that butyrate treatment may have a more profound effect on growth when supplemented earlier in life (14, 20) which would be indicative of when satellite cells are most active. Ascertaining the effects of tributyrin supplementation during the early stages of life is central to understanding the degree to which tributyrin may be able to promote muscle growth. The outcomes generated by investigating these issues may lead to a more efficient and sustainable means of lean meat in the production industry and also have an impact on the potential treatment of muscle diseases.

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Chapter 2: The HDAC inhibitor sodium butyrate promotes late stage differentiation of porcine satellite cells

Abstract

The ability of satellite cells, (muscle specific stem cells) to proliferate and differentiate through their myogenic lineage is critical for muscle growth, regeneration and repair. Understanding the underlying mechanisms responsible for satellite cell myogenesis will improve our understanding of muscle growth and may provide targets for therapeutics for musculoskeletal diseases. Muscle injury models treated with histone deacetylase (HDAC) inhibitors have had positive results on muscle growth, but the underlying mechanisms have not been elucidated. Previous studies have reported pleotropic effects of the HDAC inhibitor, butyrate, on the progression of satellite cells through their myogenic program. We investigated whether the use of a physiologically relevant concentration of butyrate could enhance the myogenic potential of porcine satellite cells. Satellite cells isolated from neonatal piglets were treated with sodium butyrate (0, 0.1, 0.5, or 1mM) and myogenic potential was analyzed during proliferation and differentiation. Proliferation was reduced in a dose-dependent manner with increasing sodium butyrate concentrations. The relative expression of Pax7 significantly decreased during proliferation, while the late stage myogenic regulatory factor, myogenin, increased in the sodium butyrate treated groups. Satellite cells fused and formed myotubes more rapidly with increasing sodium butyrate concentrations. These findings suggest that butyrate may serve as a muscle growth promoter and increase regenerative capacity of muscle by enhancing the terminal differentiation of satellite cells.

Introduction

Satellite cells are a heterogeneous population of muscle progenitor and stem cells, which are responsible for post-natal muscle development and self-renewal (14). Satellite cells reside along the muscle fiber outside the sarcolemma under the basal lamina and provide myonuclei to the growing muscle fiber (39). Enhancing the ability of satellite cells to progress through their myogenic lineage can have profound effects on an animal's lifetime muscle growth and regeneration (1, 8). As with other tissues, the neonatal period represents a crucial time for muscle development and growth through enhanced satellite cell proliferation (2, 10) and rapid myonuclear accumulation (18, 50). The process through which satellite cells proliferate and fuse to become myofibers is tightly regulated by the timely expression of transcription factors and signaling molecules (6); these key factors provide potential targets for epigenetic modification to enhance myogenesis (38, 54). Butyrate, a short chain fatty acid, is known to alter epigenetic regulators in stem cells (11, 45, 46), but its effectiveness at promoting myogenesis of satellite cells remains unclear.

Satellite cells, first described by Mauro (1961) were identified as a population of mononuclear cells responsible for the skeletal muscle regeneration. Since being first described, it is now known that this population of muscle resident cells are indeed stem cells, capable of self-renewal and providing muscle progenitors (14). Postnatally, muscle growth is considered a hypertrophic event and satellite cells contribute new nuclei to these growing muscle fibers (48). Subsequent protein synthesis is incumbent on myonuclear accretion, which can limit the fractional rate of muscle growth and repair (40, 50). The process through which satellite cells activate, proliferate, and differentiate and fuse into

growing myofibers is under the control of a hierarchal transcriptional network (6). Satellite cells are marked by their universal expression of the paired-homeobox transcription factor Pax7 (43), a key transcription factor that is required for satellite cell specification (31, 49, 52) and the prevention of terminal differentiation (42). Pax7 aids in the regulating the expression of a network of downstream myogenic regulatory factors (MRFs), namely MyoD and myogenin (60). The interaction of the expression patterns of Pax7 and MyoD have been determined to have an impact on satellite cell commitment fate, where an upregulation of Pax7 will prevent differentiation (44). A reduction in MyoD expression results in a state resembling satellite cell quiescence or self-renewal (36) while the upregulation of MyoD marks the beginning of differentiation and myoblast commitment (4). Downstream and a target of MyoD, is the MRF myogenin, whose expression marks terminal differentiation into myocytes (51). Loss of myogenin results in pools of undifferentiated mononuclear cells and the inability to complete late stage myogenesis by forming multinucleated muscle fibers (25, 56). Myogenin works synergistically with MyoD at common set of muscle genes (12) and alone in the promotion of muscle specific gene transcription (7, 16). This network of transcription factors that make up the myogenic program of satellite cells and the epigenetic mechanisms that regulate their expression have been offered as potential targets for improving muscle growth and disease outcomes (38, 53, 54).

Of particular interest are epigenetic modifiers that inhibit HDACs as they have shown to be beneficial in treating some myopathy models (9, 21, 37, 57, 58). Butyrate has also had positive effects at promoting growth in animal models, but the benefits have been attributed

to an improved intestinal immune response and lipid metabolism (26, 59). The HDAC inhibitory properties of butyrate were initially described as a way to treat human malignancies of the colon (33). Butyrate is a 4-carbon fatty acid that is the byproduct of fiber fermentation in the gut (45) and inhibits the activity of class I and II HDACs (17). Butyrate is a potent inducer of cell cycle arrest in cancer cells (41), and this led to the *in vitro* application of butyrate on satellite cells with mixed results as to its benefit at promoting myogenesis (27-30). In satellite cells, HDACs are expressed during proliferation so as to maintain muscle specific gene silencing (35). Not only do HDACs remodel the chromatin around muscle specific genes in a repressive way, but HDAC1 is known to associate with MyoD and inhibit target gene expression (5). Neither the myopathy or growth models treated with butyrate have investigated the impact on satellite cells or HDAC activity.

It has been reported that butyrate may enhance muscle gene expression at different stages of myogenesis (27) as well as inhibit the myogenic program (23, 29, 30). These conflicting reports are at odds and further investigation is warranted. These studies differed in timing of butyrate treatment and butyrate concentration. Given the differing techniques and application of butyrate on satellite cells in culture, we investigated the degree to which butyrate can alter the myogenic program. At physiologically relevant concentrations butyrate was applied during proliferative and differentiative conditions and satellite cell myogenic potential was assessed.

Materials and Methods

Isolation of satellite cells

All animal protocols were approved by the University of Maryland-College Park's Institutional Animal Care and Use Committee. Primary satellite cells were isolated and cultured individually from 4 piglets (14±2 days of age) according to a procedure modified from Doumit and Merkel (19) and Allen et al. (3). Briefly, loin muscle was excised, trimmed of connective tissue and minced with scissors. Tissue fragments were digested with 1.25mg/ml protease from *Streptomyces griseous* (Pronase, Sigma-Aldrich, St. Louis, MO) for 1h at 37°C. Satellite cells were disassociated from tissue fragments by trituration and differential centrifugation. Cells were pre-plated on uncoated 15cm tissue culture dishes for 2h (37°C, 5% CO₂) in proliferative growth media (PGM, DMEM + 10% FBS + antibiotics- 100U/mL penicillin, 100µg/mL streptomycin, 10µg/mL gentamycin; Gibco) and then seeded on tissue cultured treated dishes coated with Poly-L-lysine (100µg/mL ddH₂O, Sigma-Aldrich) and fibronectin (10µg/mL PBS, Sigma-Aldrich) in proliferative growth medium until they reached 50% confluence (37°C, 5%CO₂). Cells were then released with 0.05% Trypsin (Gibco) and plated for our studies.

Cell culture

Effect of butyrate during proliferative conditions- Satellite cells were seeded at 2,500 cells/cm² in PGM on to plates coated with Poly-L-lysine and fibronectin. After a 24h attachment period, complete media changes were made daily and cells received PGM supplemented with sodium butyrate (NaBu) at 0mM, 0.1mM, 0.5mM, or 1mM and allowed to proliferate for 48hrs in the presence of NaBu. Gene expression, immunocytochemistry,

and *in vitro* proliferation and apoptosis were measured at 24h or 48h after addition of NaBu.

Effect of butyrate during differentiative conditions- Satellite cells were allowed to proliferate in PGM until confluent and then switched to differentiation media (DM, DMEM + 2% horse serum; Gibco + antibiotics) for 96h in the presence of 0, 0.1, 0.5, or 1mM NaBu. Gene expression was measured at all time points during differentiation. At the 48h and 96h time points *in vitro* apoptosis and immunocytochemistry was measured.

In vitro proliferation was analyzed at the 24h and 48h proliferative time points using the Click-iT EdU Alexa Fluor 488 imaging kit (Molecular Probes). Satellite cells were pulsed for 2h and then fixed and stained according to the manufacturer's protocol. The rate of apoptosis was also measured during proliferation (48h) and differentiation (48h and 96h) using the Click-iT TUNEL Alexa Fluor 594 assay (Molecular Probes) according to the manufacturer's protocol.

Analysis of gene expression

Total RNA was isolated using the RNAqueous Micro kit (Ambion) and quantified using the Quant-iT RiboGreen assay (Molecular Probes) according to the manufacturers' protocols. Harvested RNA was reversed transcribed with the SuperScript IV First-Strand Synthesis System (Invitrogen) and treated with the RNase H to ensure removal of RNA. The resulting cDNA was quantified with the Quant-iT OligoGreen assay (Molecular Probes). Total RNA and cDNA quantification were detected on the Synergy HTX microplate reader using the Gen 5.0 v3.0 software (BioTek Instruments, Winooski, VT). 10ng of cDNA was used for multiplex qRT-PCR using Bio-Rad's CFX96 Touch Real-

Time PCR Detection System and iQ Multiplex Powermix (Hercules, CA). Primers and probes were designed by Integrated DNA Technologies (Coralville, IA) (Table 2-1). Genes of interest were normalized to the reference gene, RPL4, using the $2^{-\Delta\Delta CT}$ method. Reactions were amplified for 45sec at 60°C for 40 cycles. Analysis and amplification plots were executed with the CFX Manager Software (version 3.1, Bio-Rad).

Table 2-1. Primers and probe sequences used for gene expression analysis by multiplex quantitative RT-PCR.

Gene Symbol	Gene ID	Primer Sequence 5'-3'	Probe and Sequence 5'-3'
PAX7	100625823	F: CAGCAAGCCCAGACAGG R: TCGGATCTCCCAGCTGAA	(HEX): TTGAGGAGTACAAGAGGGAGAACCCA
MYOD1	407604	F: CCGACGGCATGATGGATTATAG R: CGACACCGCAGCATTCTT	(FAM): AATAGGTGCCGTCGTAGCAGTTCC
MYOG	497618	F: AGTGAATGCAGTTCCACAG R: AGGTGAGGGAGTGCAGATT	(Texas Red): CAACCCAGGGGATCATCTGCTC
RPL4	100038029	F: TGGTGGTTGAAGATAAAGTTGAAAAG R: TGAGAGGCATAAACCTTCTTGAT	(Cy5): AACCAAGGAGGCTGTTCTGCTTCT

Immunocytochemistry

Satellite cells were fixed in 4% paraformaldehyde in PBS for 15min and permeablized with 0.1% Triton X-100 in PBS for 20min. Fixed cells were blocked in 10% goat serum in PBST (0.1% Tween-20 in PBS) for 1h at room temperature. The cells were incubated overnight at 4°C in the primary antibodies diluted in PBST at the following concentrations: (1) mouse monoclonal anti-Pax7, 10µg/mL, Sigma-Aldrich; (2) rabbit polyclonal anti-MyoD1, 5µg/mL, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; (3) mouse monoclonal anti-myogenin, 5µg/mL, AbCam, Cambridge, MA; and (4) rabbit polyclonal anti-fast myosin skeletal heavy chain (MyHC), 5µg/mL, AbCam. Primary antibodies were removed and incubated with secondary antibodies (AlexaFluor 488 goat anti-mouse IgG and AlexaFluor 594 goat anti-rabbit IgG at 1:500 dilutions, Jackson ImmunoResearch, West

Grove, PA) in 1% BSA in PBS for 1h at room temperature. Nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). Images were visualized on a Leica DM5500 with software (Leica Microsystems, Wetzlar, Germany).

Statistical Analysis

The effect of NaBu treatment on satellite cell behavior was examined using an F-test in ANOVA. In the case of a significant F-test, multiple mean comparisons were analyzed using Dunnett's test. Proliferation data was examined by regression analysis with linear and quadratic contrasts to determine the nature of a dose response. Four biological replicates were included for all comparisons; a probability of $P \leq 0.05$ was considered significant. Statistical procedures were analyzed using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA). Data reported as least square means \pm SEM.

Results

Treatment with NaBu reduced proliferation rates the 24h time point (quadratic; $R^2=.77$, $P<.05$), but not the 48h time point (quadratic; $R^2=.66$, $P>.05$), in a dose-dependent manner (Figure 2-1). After exposure to 0.1, 0.5, or 1mM NaBu for 24h, satellite cell proliferation was reduced by 15%, 36%, and 41%, respectively, as compared to the control. After 48h of continuous exposure, the reduction in satellite cell proliferation rates did not exhibit a dose response when treated with NaBu; however, proliferation rates were significantly reduced by 50% ($P<.05$) and 64% ($P<.01$) in the 0.5mM and 1mM treatment groups, respectively, as compared to the control. During proliferation and differentiation, no

apparent increase in cells undergoing apoptosis could be observed in any treatment group via TUNEL assay (Figure 2-2).

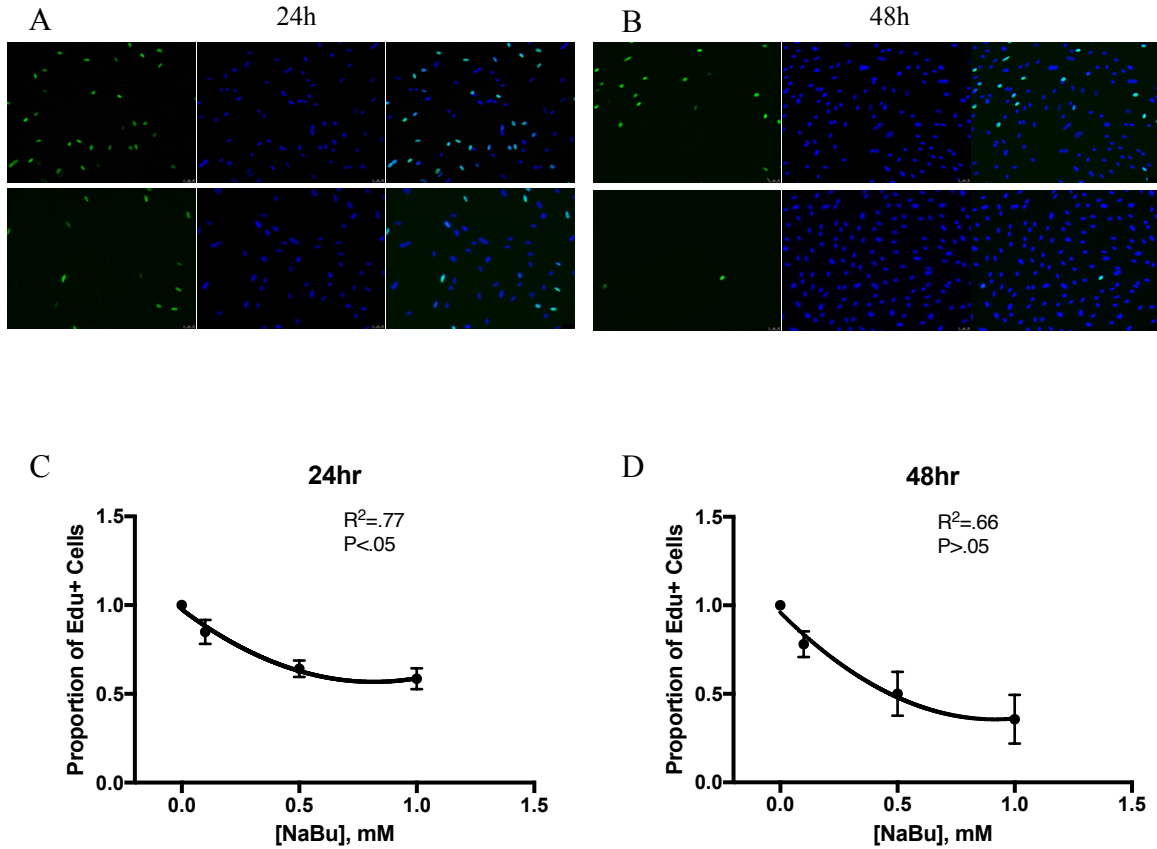


Figure 2-1. Effect of sodium butyrate on proliferation of satellite cells after 24h (A & C) and 48h (B & D). Satellite cells were pulsed with EdU for 2h and allowed to proliferate. Representative images (top row: 0mM; bottom row: 1mM) detecting the newly synthesized DNA in replicating cells (green) and total nuclei were counted (blue) and compared (composite). The percentage of EdU⁺ cells in the 0mM, control treatments, were set at 1 for each animal and number of EdU⁺ cells of the treatment groups were normalized as a proportion thereof within each animal. At least 300 cells were counted and averaged from two technical replicates for each animal. Values depicted are means with standard error plotted on an interpolated quadratic function.

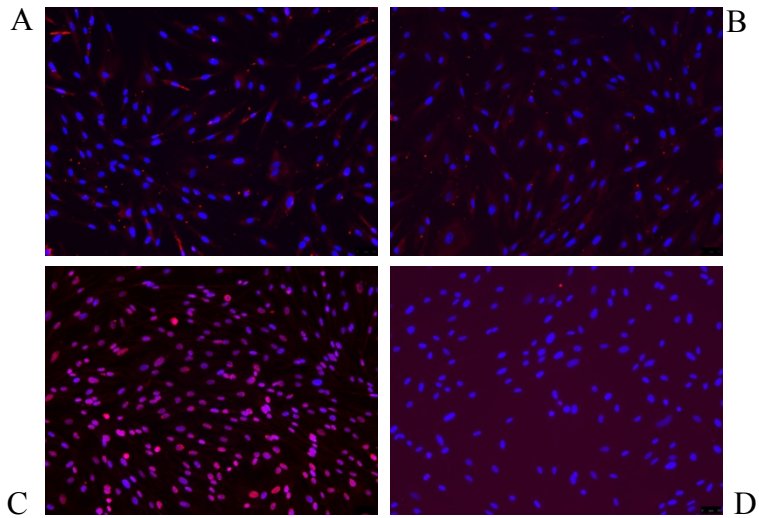
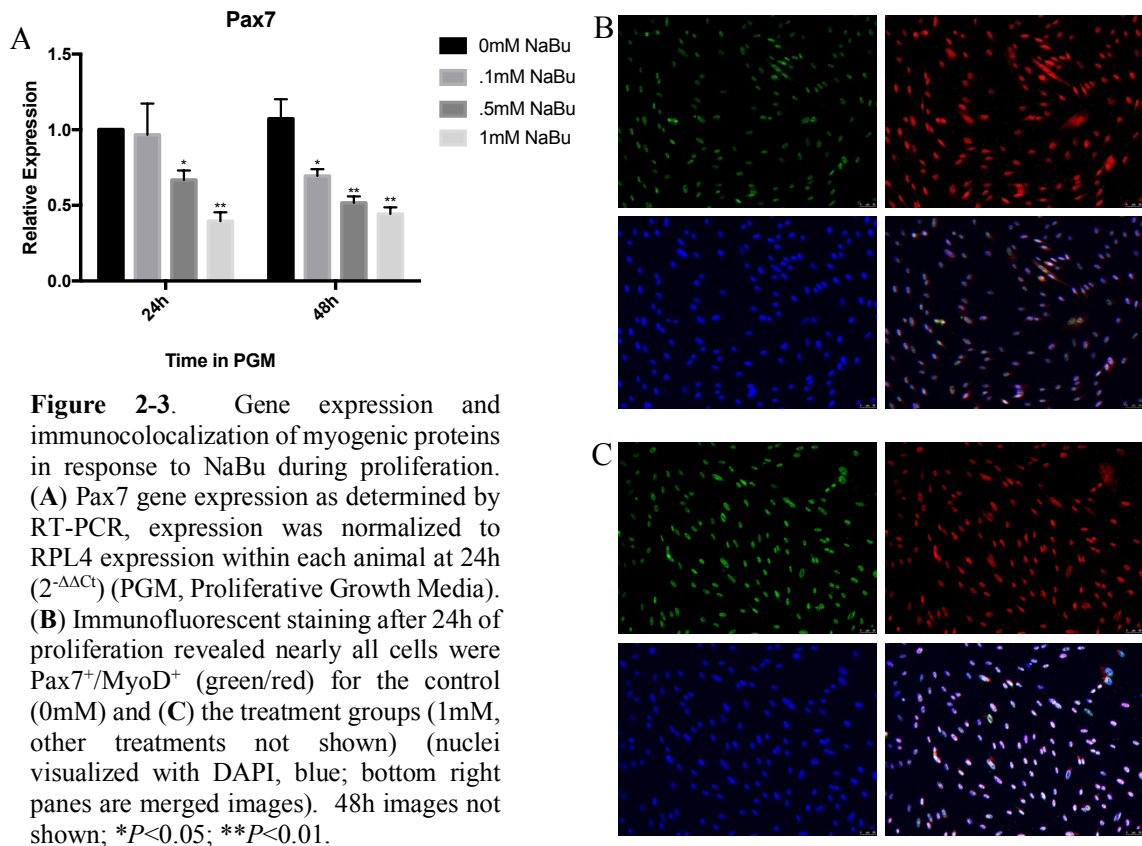


Figure 2-2. Effect of sodium butyrate on apoptosis in satellite cells. A TUNEL assay was performed to determine the rate at which butyrate may be affecting cell apoptosis at 48h of proliferation (image not shown) and at the 48h and 96h time points of differentiation (96h time point not shown). Across all time points and all treatments, apoptosis was undetectable. Images A: 0mM, B: 1mM indicating TUNEL⁻ staining. Images C: Satellite cells treated with DNase as positive control (TUNEL⁺, red), D: Negative control lacking the TdT (terminal deoxynucleotidyl transferase) enzyme. All images are composite, nuclei visualized with DAPI (blue).

Relative gene expression and immunocolocalization of Pax7 and the MRFs MyoD and myogenin were determined to examine the effect of NaBu on the progression of satellite cells through their myogenic lineage. During proliferation, we found a significant reduction ($P < 0.05$) in Pax7 transcript with increasing NaBu concentration at both the 24h and 48h time points (Figure 2-3). Satellite cell expression of MyoD during proliferation did differ significantly from the NaBu treatment as compared to the control (data not shown). Localization of these two transcription factors indicated nearly all cells were Pax7⁺/MyoD⁺ in all NaBu treatments (Figure 2-3). There was no relative difference among treatment groups in myogenin expression or MyoD⁺/Myogenin⁺ satellite cells during proliferation (data not shown).



Untreated satellite cells were then switched to differentiation media after 90% of confluence and subjected to the same NaBu treatment regimen for 96h. Pax7 was reduced at the 24hr time point of differentiation, but not significantly as this is when Pax7 expression is typically reduced. Myogenin expression was strongly upregulated in the 0.5mM and 1mM NaBu treatments after 48h of differentiation as compared to endogenous expression in the control ($P < .05$) (Figure 2-4). The NaBu treatments did not have a significant effect on MyoD expression. Immunostaining for MyoD⁺/Myogenin⁺ cells did not reveal any significant differences between the treatment groups. Differentiated cells were also immunostained for MyHC, a late stage contractile protein of differentiated satellite cells (20), so as to analyze the number of fused cells and myotube formation. There

was an increase in the number of multinucleated cells with increasing concentrations of NaBu at both the 48h and 96h time points (Figure 2-5).

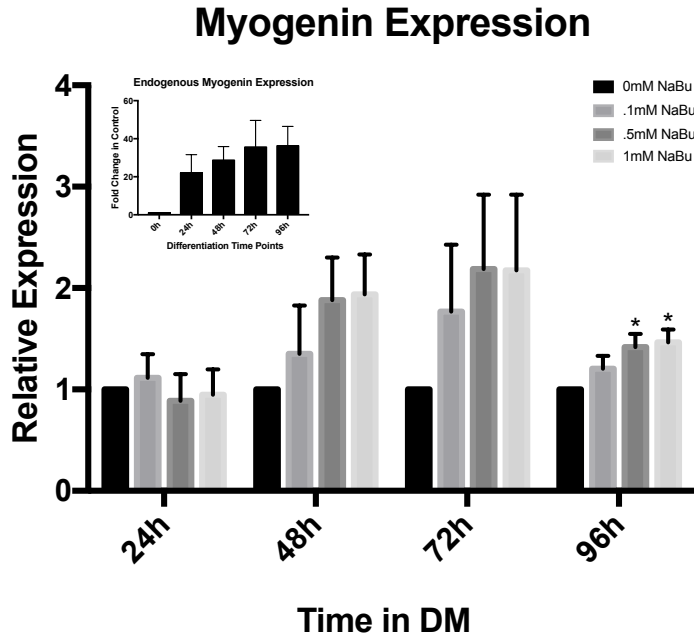


Figure 2-4. Myogenin expression in differentiating satellite cells as determined by RT-PCR. Expression was normalized to RPL4 expression within animal at each time point ($2^{-\Delta\Delta Ct}$) (DM, Differentiation Media). Inset: Endogenous expression of myogenin in the control dose, normalized to time point 0h when induced to differentiate; * $P < 0.05$.

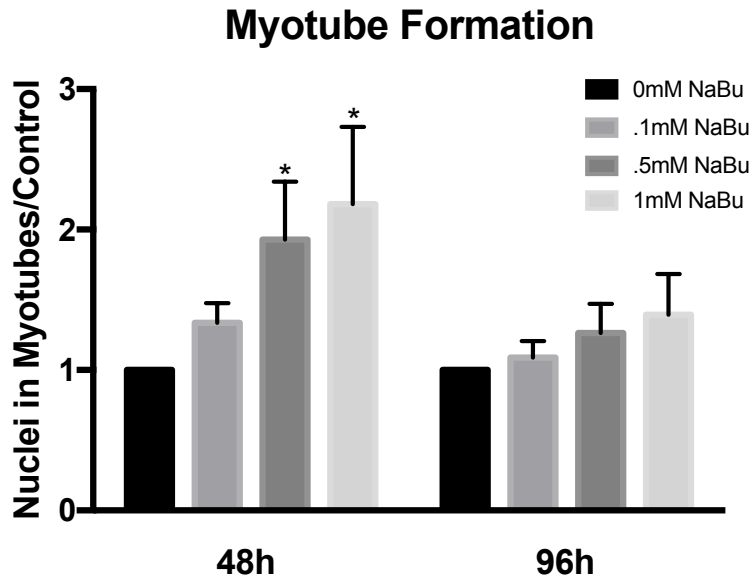


Figure 2-5. The fusion percentages of differentiating satellite cells were assessed by immunostaining for MyHC to determine the number nuclei in MyHC⁺ cells/total nuclei. Multinucleated (2 or more nuclei in the same MyHC⁺ cell) myotubes were counted and compared to total nuclei after 48h and 96h post-differentiation. Fusion percentages were calculated and the control was set to 1. Two fields of view from two technical replicates were averaged for each animal; * $P < 0.05$.

Discussion

The progression of satellite cells through their myogenic lineage is a tightly controlled process under the regulation of a hierarchal network of transcription factors. In the present study, we examined the ability of the HDAC inhibitor butyrate to alter proliferation and differentiation of primary porcine satellite cells in vitro. While HDAC inhibitors have been shown to both enhance and obstruct myogenesis (27-30), our results demonstrate that butyrate can accelerate myogenesis through increased upregulation of the MRF myogenin during differentiation and an increase in satellite cell fusion. Our findings also help further establish that at low doses, butyrate can serve as a potential effector molecule for muscle hypertrophy and regeneration.

In concurrence with previous findings, in a dose dependent manner NaBu forced satellite cells to exit the cell cycle and slowed the rate of proliferation. Relative Pax7 expression was markedly decreased when treated with NaBu. When exposed to butyrate, or any other HDAC inhibitor, satellite cell expression of Pax7 has yet to be investigated. While Pax7 is crucial for the survival, proliferation, and self-renewal of satellite cells (52, 60), over expression in activated satellite cells down-regulates MyoD and prevents differentiation through an inhibition of myogenin expression (42). Our results support the assertion that Pax7 and myogenin expression are mutually exclusive, whereby butyrate's negative influence on Pax7 expression during proliferation may aid in the upregulation of myogenin during differentiation. The cell culture methods used by Iezzi et al. (27) demonstrate this ability of NaBu to enhance satellite cell myogenesis when applied for a 24h period during proliferation and then removed when switched to differentiation media; in part, priming the satellite cells for differentiation by pulsing with NaBu.

The apparent increase in myogenin expression and satellite cell fusion reinforce the major theme, in that butyrate can enhance terminal differentiation. While not all muscle differentiation genes are under the control of myogenin (56), a substantial number of sarcomeric, contractile and enzymatic components of muscle require myogenin and cannot rely on MyoD or the myocyte enhancer factor 2 (MEF2) family of genes for transcriptional activation (16). Furthermore, almost all satellite cells had begun to express MyHC even if they had not yet fused to neighboring cells. When NaBu was applied to subconfluent cells, myogenin expression was upregulated; however, myotube formation was hindered. The ability of subconfluent (<50%) satellite cells to form myotubes is reduced (55) and

treatment with NaBu at reduced confluency may have confounding effects, as seen by Iezzi et al. (27). Inherent to satellite cell fusion and progression through the myogenic program, the timing of the differentiation process and the necessity of an *in vitro* satellite cell niche may be the reason that such incongruity on the definitive role that butyrate plays in myogenesis.

Butyrate acts by inhibiting the chromatin remodeling activity of class I and II HDACs, ultimately leading to hyperacetylated histones and a relaxed chromatin structure (17). HDACs have also been implicated in associating with both the MRF and MEF2 family of genes and repressing their activity (24, 35). Also of note, in undifferentiated satellite cells, MyoD is known to associate with class I HDACs which represses myogenesis (47). This underscores our assertions that butyrate enhances terminal differentiation by inhibiting the repressive HDACs. However, forcing satellite cells to differentiate too early in the myogenic program results in poor myotube formation as was also indicated by our results. Under normal conditions, the acetylation of MyoD only takes place when satellite cells are triggered to differentiate (32), explaining the observed increase in myogenin expression without a parallel increase in MyoD expression. NaBu may have accelerated the rate at which MyoD was acetylated while also preventing the repressive action of MyoD-recruited HDACs. To what degree of interaction MEF2 and myogenin may have warrants further investigation, but in sum, the synergistic effects of unrepressed MyoD-mediated transcriptional activation of both myogenin and MEF2 appears to hasten and amplify satellite cell differentiation and fusion.

Our results demonstrate that at low (0.1-1mM) concentrations of NaBu, primary satellite cell behavior is altered. Previous *in vitro* studies (27, 29, 30) have exposed satellite cells to concentrations of butyrate at 5mM or greater. From a translational aspect, exposing satellite cells *in vivo* to this level of butyrate becomes a significant technical challenge in non-ruminant species. It should also be taken into consideration the clearance rate of butyrate once it reaches peripheral circulation. Egorin et al. (22) found that intravenous dosing of sodium butyrate was unable to maintain plasma butyrate concentrations above 1mM for more than 30min in mice. Similarly, orally administered butyrate is rapidly metabolized, has a short plasma half-life and even the salt form of the volatile fatty acid produces a strong and an unpleasant odor, making it unsuitable for maintaining effective butyrate plasma concentrations. Tributyrin, a butyrate prodrug, which has 3 butyrate molecules esterified to a glycerol backbone, has shown to be a suitable alternative to orally ingested butyrate, maintaining concentrations above 0.1mM for 3h (13, 15, 22). Collectively, this warrants a more detailed investigation into butyrate's influence on myogenesis and the satellite cell myogenic program *in vivo*.

In conclusion, we examined to what effect varying concentrations of the HDAC inhibitor NaBu have on satellite cells during their myogenic lineage. *In vivo* and *in vitro* experimentation models treated with butyrate have had positive results on myogenesis, but it is unclear from these studies as to how the myogenic program is affected. Our work presents a detailed view of the potential mechanism through which butyrate alters satellite cell behavior and programming at physiologically relevant doses. Conversely, we note that by inhibiting satellite cell proliferation with butyrate will also cause a reduction in myotube

formation, and that inherent satellite cell characteristics must be taken into account when developing an appropriate cell culture system. We offer that tributyrin may serve as a viable vehicle to expose satellite cells *in vivo* to requisite amounts of butyrate in order to promote muscle growth.

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Chapter 3: Dietary tributyrin, a HDAC inhibitor, promotes muscle growth through enhanced terminal differentiation of satellite cells

Abstract

Muscle growth and repair rely on two main mechanisms, myonuclear accretion and subsequent protein accumulation. Altering the ability of muscle resident stem cells (satellite cells) to progress through their myogenic lineage can have a profound effect on lifetime muscle growth and repair. The use of the histone deacetylase (HDAC) inhibitor, butyrate, has had positive outcomes on the *in vitro* promotion of satellite cell myogenesis. In animal models, the use of butyrate has had promising results in treating myopathic conditions as well as improving growth efficiency, but the impact of dietary butyrate on satellite cells and muscle growth have not been elucidated. We investigated the impact of tributyrin, a butyrate pro-drug, on satellite cell activity and muscle growth in a piglet model. Satellite cells from tributyrin treated piglets had altered myogenic potential, and piglets receiving tributyrin had a ~40% increase in myonuclear accretion after 21d, indicating the potential for enhanced muscle growth. To assess muscle growth potential, piglets were supplemented tributyrin (0.5%) during either the neonatal phase (d1-d21) and/or the nursery phase (d21-d58) in a 2x2 factorial design. Piglets who received tributyrin during the neonatal phase had improved growth performance at the end of the study and had a ~10% larger loin eye area and muscle fiber cross-sectional area. Tributyrin treatment in the nursery phase alone did not have a significant effect on muscle growth or feed efficiency. These findings suggest that tributyrin is a potent promoter of muscle growth via altered satellite cell myogenesis.

Introduction

Post-natal muscle growth and repair rely on the ability of satellite cells, muscle resident stem cells, to activate, proliferate, and fuse into growing myofibers (16, 53). During the neonatal stage of life, the fractional rate of protein synthesis contributing to muscle growth is highest (13), marked by increased satellite cell activation, proliferation (2, 10), and myonuclear accretion (52). It has also been found that interventions that target satellite cells at this age can have lifetime effects on muscle growth and regenerative capacity (1, 8). The myogenic program is under the regulation of a hierarchy of transcription factors that govern satellite cell lineage fate (51) and these regulators have proven to be prospective targets in promoting muscle growth (5). It has recently been suggested that histone deacetylase (HDAC) inhibitors may serve as a viable tool to epigenetically alter satellite cell behavior (41, 54). Butyrate is a potent and broad-spectrum inhibitor of HDACs that has shown to be beneficial at treating models of muscle pathology (40, 57), but its effects on satellite cell activity (26, 28, 32) is controversial. Dietary butyrate, along with tributyrin (a butyrate pro-drug), have had positive effects on growth performance (as measured by growth rate and efficiency of growth per unit of feed) (45, 47, 61), but these findings have been attributed to improved intestinal and digestive functions (14, 23, 25, 29, 30). While the favorable use of dietary butyrate seems clear, investigating whether it could be used as a muscle growth promoter would have profound impacts for human health and animal production.

Post-natal muscle growth and protein accretion is considered a hypertrophic event, as the number of muscle fibers is set for most species at the time of birth (50). The activity of

satellite cells and their fusion with growing muscle fibers govern the rate of muscle growth and regeneration (5). As expected, due to their central role in lifetime muscle growth and regeneration, the activity of satellite cells is tightly regulated via the expression of multiple transcription factors. The paired-homeobox transcription factor Pax7 is universally expressed in satellite cells and allows for satellite cell proliferation by preventing precocious differentiation (44, 53). Additionally, Pax7 plays a role in the regulation of the downstream network of myogenic regulatory factors (MRFs), specifically myogenic differentiation 1 (MyoD) and myogenin (42). Changes in the Pax7 and MRF expression patterns have been shown to regulate satellite cell commitment fate, where a decrease in the Pax7:MyoD ratio leads to terminal differentiation and myogenin expression (43). Upregulating Pax7 expression prevents myogenin expression and allows for satellite cells to either self-renew or enter into a quiescent state (42). Activation of MyoD is necessary for proliferating satellite cells to continue through their myogenic lineage and to trigger terminal differentiation of myoblasts (38). The downstream target of MyoD, myogenin, is necessary for fiber development embryonically and for post-natal muscle growth (22, 56). Defects in the myogenin gene lead to pools of undifferentiated satellite cells without an apparent effect on the expression of MyoD. The alteration of the expression and timing of these myogenic regulatory genes has been presented as a practical means to increase muscle hypertrophy and regeneration (6, 41).

Epigenetic modifiers that inhibit HDACs in myoblasts are gaining increasing interest in the fields of muscle growth and regeneration (54). The beneficial effects of HDAC inhibitors were initially described as a method to treat neoplasms in humans and animal

models (see (36) for review). Only recently has it been suggested that inhibiting HDACs could alter myogenic programming (37). When HDACs are expressed in undifferentiated satellite cells, they bind to MyoD and the late stage MRF, myogenin, is not expressed (37). In culture, the HDAC inhibitors have shown to increase myotube hypertrophy without an increase in satellite cell proliferation (27). Sodium butyrate has also had positive effects at influencing satellite cell fusion and increasing myotube hypertrophy in culture (26).

The 4-carbon fatty acid, butyrate, is an inhibitor of many HDACs (11, 12). Butyrate is produced naturally in the body from the fermentation of dietary fiber and has been examined as an antitumor agent since the 1970's (39, 48). There have been conflicting reports on the effect of *in vitro* application of butyrate on satellite cell behavior, from enhancing muscle gene expression at different stages of myogenesis (26) as well as inhibiting some parts of the myogenic program (20, 28, 32). Within the animal production industry, butyrate has been used as an aid in improving intestinal health (23, 61) and growth performance through inclusion as the more palatable version, tributyrin, in the diet (25, 30). While muscle hypertrophy through satellite cell programming has not been elucidated, butyrate has had promising effects on muscle healing in some injury models (18, 57, 58). Using neonatal and nursery piglets as model of rapid muscle growth, we characterized the effects of butyrate on satellite cell activity and their myogenic progression. We hypothesize that supplementation of dietary tributyrin may serve as an effective promoter of muscle growth through enhanced satellite cell myogenesis.

Materials and Methods

Animals, diets and experimental protocol

All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Maryland-College Park. In two animal feeding trials, we investigated the effects of tributyrin supplementation on muscle growth during both the neonatal (birth to 21-days of age) and nursery (22 to 58-days of age) phases of growth. During first animal feeding trial, tributyrin was supplemented at two different levels for 21 days to establish an inclusion rate for the second animal feeding trial that would extend into the nursery phase.

Study 1- To assess the impact of dietary tributyrin inclusion on *in vivo* satellite cell programming, 30 cross-bred female piglets (24±6h old; 1.79±.25kg body weight) were assigned to 1 of 3 treatments ($n=10$ /group) and balanced by body weight and litter. Piglets received either a standard commercial milk replacer formula (Advance Liqui-Wean, Milk Specialties Co., Dundee, IL) where 175g of dry milk replacer was reconstituted in water to 1kg total formula (C), or the milk replacer formula supplemented with 0.25% (T_{.25}) or 0.5% (T_{.5}) total butyric acid in the form of spray dried tributyrin (AviPremiumD, Vetagro SpA, Reggio Emilia, Italy). Tributyrin inclusion was on a dry matter basis and diets across treatments were made isoenergetic by the addition coconut oil. Piglets were housed individually and received formula every 2hrs (0900-2300) at a limit-fed rate to match sow reared growth. One piglet from T_{.5} was removed from the study due to non-treatment related health issues. Body weight and feed intake were recorded daily for the duration of the 21-day feeding trial. Piglets were orally administered 20mg/kg body weight

bromodeoxyuridine (BrdU, Sigma Aldrich, St. Louis, MO) 18h prior to euthanasia to determine *in vivo* proliferation of satellite cells. Longissimus dorsi (LD) muscle was used for all tissue analysis and satellite cell isolation.

After the 21-day neonatal feeding trial, LD was snap frozen in liquid N₂ for total DNA, protein abundance, and gene expression analysis. Total muscle protein extractions were performed on ice in NP-40 buffer (20mM Tris-HCl pH 8, 125mM NaCl, 1% NP-40, 2 mM ethylenediaminetetra-acetic acid (EDTA), 10% glycerol) for 30 min containing cOmplete protease inhibitor and PhosSTOP phosphatase inhibitor cocktails (Roche, Basel, Switzerland). Insoluble material was pelleted by centrifugation at 15,000g for 15 min at 4°C. Protein concentrations were determined using BCA assays (ThermoFisher Scientific, Waltham, MA).

Total DNA was extracted (DNeasy, Qiagen), fluorescently quantified (Quant-iT dsDNA assay kit) and compared to the total protein content of the LD muscle. Total RNA was isolated by homogenization using tri-reagent (ThermoFisher Scientific) with phase separation achieved by chloroform wash. RNA was precipitated with 70% ethanol and transferred to RNeasy spin column and purified according to the manufacturers protocol (Qiagen).

For immunohistological analysis to determine fiber cross-sectional area (FCA), LD samples were embedded in a 1:1 10% tragacanth gum OCT mixture and snap frozen in liquid N₂-cooled isopentane. Muscles sections (8µm thick) were cut and mounted on

Superfrost Plus slides for immunostaining. FCA and fiber number were determined by anti-dystrophin staining of the sarcolemma described below.

Study 2- We investigated the impact of continual dietary tributyrin inclusion through the nursery phase of growth in a 2 x 2 factorial treatment structure. Piglets, 30 cross-bred females (24±6h old; 1.54±.32kg body weight), were either fed either a standard commercial milk replacer formula (C, *n*=15) or the milk replacer formula supplemented with 0.5% total butyric acid in the form of spray dried tributyrin (T, *n*=15) (as performed above) for 21-days. Piglets were then weaned at 22-days of age and crossed into their respective nursery phase dry diet: either commercial dry nursery control diet (CC or TC; *n*=6/group) (TechMix, Stewart, MN) or a control diet supplemented with 0.5% total butyric acid in the form of spray dried tributyrin (CT or TT; *n*=9/group) (AviPremiumD, Vetagro SpA). The control dry diet was supplemented with microencapsulated palm oil to ensure equivalent energetics to the treatment diet. Piglets were fed ad libitum and had access to ad libitum water. Three piglets (1 from the TC group and 2 from the TT group) were removed from the study for failing to wean. After weaning, body weight and feed intake were recorded weekly for the duration of the 37-day nursery feeding trial. At the end of the 58-day feeding trial, LD muscle was removed at the 12th rib to measure total loin area, evaluate gene expression, and for immunohistochemical analysis to determine FCA (as described above).

Satellite cell isolation and culture

Satellite cells from the neonatal piglets (Study 1) were isolated according to a procedure modified from Doumit and Merkel (15) and Allen et al. (3). Briefly, LD muscle was

excised from neonatal piglets after their prescribed 21-day feeding regimen, trimmed of connective tissue, and minced with scissors. Tissue fragments were digested with 1.25mg/ml protease from *Streptomyces griseous* (Pronase, Sigma-Aldrich) for 1h at 37°C. Satellite cells were disassociated from tissue fragments by trituration and differential centrifugation. Cells were pre-plated on uncoated 15cm tissue culture dishes for 2h (37°C, 5% CO₂) in proliferative growth media (PGM, DMEM + 10% FBS + antibiotics- 100U/mL penicillin, 100µg/mL streptomycin, 10µg/mL gentamycin; Gibco) and then seeded on tissue cultured treated dishes coated with Poly-L-lysine (100µg/mL ddH₂O, Sigma-Aldrich) and fibronectin (10µg/mL PBS, Sigma-Aldrich) in PGM until they reached ~50% confluence (37°C, 5%CO₂) or analyzed for *in vivo* proliferation (described below). Cells were then released with 0.05% Trypsin (Gibco) and plated for our studies.

The effect of dietary tributyrin inclusion (0.25% and 0.5%, n=10) effect on *ex vivo* satellite cell dynamics was analyzed under proliferative and differentiative conditions. Satellite cells were seeded at 2,500 cells/cm² in PGM on to plates coated with Poly-L-lysine and fibronectin. After a 24h attachment period, satellite cells were given 48hrs in PGM and then induced to differentiate (DM, DMEM + 2% horse serum; Gibco + antibiotics) for an additional 72h with complete media changes daily. Total RNA was isolated (RNeasy, Qiagen) at each 24h time point for gene expression analysis. Satellite cell fusion was measured at 48h post-differentiation by immunostaining.

In vitro proliferation was analyzed 24h after plating using the Click-iT EdU Alexa Fluor 488 imaging kit (Molecular Probes). Satellite cells were pulsed for 2h with EdU and then

fixed and stained according to the manufacturer's protocol. *In vivo* proliferation was assayed by identifying proliferating cells with BrdU. Satellite cells that had been direct plated were fixed 4% paraformaldehyde and permeablized in 0.1% Triton X-100 (Sigma-Aldrich) in PBS. Antigen retrieval was performed with 2N HCl for 45m at 37°C, acid was buffered for 10m with sodium tetraborate, and cells were blocked with 10% goat serum in PBST. Satellite cells were incubated with anti-BrdU (6µg.mL, Bio-Techne, Minneapolis, MN) overnight at 4°C. Nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). Images were visualized on a Zeiss AxioObserver Z.1 and analyzed with ZenPro automated image analysis suite (Carl Zeiss AG, Oberkochen, Germany).

Western blot analysis

Protein from neonatal LD muscle homogenate was quantified for total protein content by BCA assay (Pierce) and subjected to western blotting. Equal amounts of protein were electrophoresed and separated on 7.5% Mini-PROTEAN TGX Precast Gels, transferred to an Immun-Blot PVDF Membrane (Bio-Rad, Hercules, CA) and stained with SimplyBlue SafeStain (ThermoFisher Scientific) to ensure protein transfer. The membrane was then incubated at 4°C overnight with the following the primary antibodies at a 1:1000 dilution, rabbit anti-phospho mTOR (Ser-2448) and rabbit anti-phospho-AMPK α (Thr-172) (Cell Signaling Technology, Danvers, MA). Membranes were incubated for 1h with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA), and developed with SuperSignal West Pico Chemiluminescent Substrate Kit (ThermoFisher Scientific). Densitometry analysis was performed using a ChemiDoc XRS system and Image Lab Software (BioRad). Equal loading of proteins was confirmed

by reprobing with anti-AMPK α and anti-mTOR antibodies (1:1000, Cell Signaling Technology). Optical density was normalized to a pooled treatment sample as a loading control.

Analysis of gene expression

Total RNA isolated from neonatal piglet LD muscle and satellite cells were quantified using the Quant-iT RiboGreen assay (Molecular Probes) according to the manufacturer's protocol. Harvested RNA was reversed transcribed with the SuperScript IV First-Strand Synthesis System, using equal concentrations of OligodT₍₂₀₎ and random hexamers (Invitrogen) and treated with the RNase H to ensure removal of RNA. The resulting cDNA was quantified with the Quant-iT OligoGreen assay (Molecular Probes). Total RNA and cDNA quantification were detected on the Synergy HTX microplate reader using the Gen 5.0 v3.0 software (BioTek Instruments, Winooski, VT). cDNA was used for multiplex qRT-PCR using Bio-Rad's CFX96 Touch Real-Time PCR Detection System and iQ Multiplex Powermix. Analysis of gene expression (Pax7, MyoD, myogenin) and amplification plots were executed with the CFX Manager Software (version 3.1, Bio-Rad). Primers and probes for the gene expression analysis were designed by Integrated DNA Technologies (Coralville, IA) (Table 3-1). After optimization, a 2:1 primer-to-probe ratio was utilized for genes of interest while a 1:1 ratio was used for the reference gene, RPL4. For each assay, samples were amplified for 45s at 60°C for 40 cycles.

Table 3-1. Primers and probe sequences used for gene expression analysis by multiplex quantitative RT-PCR.

Gene Symbol	Gene ID	Primer Sequence 5'-3'	Probe and Sequence 5'-3'
PAX7	100625823	F: CAGCAAGCCCAGACAGG R: TCGGATCTCCCAGCTGAA	(HEX): TTGAGGAGTACAAGAGGGAGAACCCA
MYOD1	407604	F: CCGACGGCATGATGGATTATAG R: CGACACCGCAGCATTCTT	(FAM): AATAGGTGCCGTCGTAGCAGTTCC
MYOG	497618	F: AGTGAATGCAGTCCCACAG R: AGGTGAGGGAGTGACAGATT	(Texas Red): CAACCCAGGGGATCATCTGCTC
RPL4	100038029	F: TGGTGGTTGAAGATAAAGTTGAAAG R: TGAGAGGCATAAACCTTCTTGAT	(Cy5): AACCAAGGAGGCTGTTCTGCTTCT

Immunostaining

Satellite cell cultures and LD muscle sections were immunostained to determine myotube formation and FCA respectively. Satellite cells were analyzed for the expression of the contractile protein myosin heavy chain (MyHC) after 48h of differentiation. Satellite cells were pre-fixed and nursery LD muscle sections were post-fixed in 4% paraformaldehyde and permeabilized with Triton X-100. Samples were blocked with 10% goat serum in PBST (0.1% Tween-20 in PBS) for 1h at room temperature. Cells and slides were incubated overnight at 4°C with primary mouse monoclonal anti-MyHC at 10µg/mL (Roche) or anti-dystrophin at 5µg/mL (R&D Systems, Minneapolis, MN) respectively. Primary antibodies were removed and incubated with the secondary antibody (AlexaFluor 488 goat anti-mouse IgG at 1:500 dilution, Jackson ImmunoResearch) in 5% goat serum for 1h at room temperature. Myotube formation and FCA images were collected with Zeiss AxioObserver Z.1 and analyzed with ZenPro automated image analysis suite (Carl Zeiss AG).

Statistical Analysis

Study 1- The effects of tributyrin concentration on the response variables were analyzed using an F-test in ANOVA (GraphPad Prism 7, GraphPad Software, Inc., La Jolla, CA).

Study 2-Data were analyzed as a two-way ANOVA using the PROC MIXED procedure in SAS (version 9.3; SAS Institute Inc., Cary, NC). Milk replacer treatment, dry nursery dietary treatment, and their interaction were analyzed as fixed effects while covariate(s) (initial body weight and/or weaning weight) were analyzed as random effects in the MIXED procedure of SAS (9.3).

In the case of a significant F-test, multiple mean comparisons were analyzed using a Tukey's adjustment. A probability of $P \leq 0.05$ was considered significant and a P -value between 0.05 and 0.10 ($0.05 < P \leq 0.10$) was considered a trend. Data reported as least square means \pm SEM.

Results

Tributylin inclusion on growth performance

Study 1-After the 21-day, neonatal feeding trial, there was no effect of treatment on final body weight, average daily gain (ADG), or feed efficiency. There was a nonsignificant decrease in feed intake in the T₅ group. LD muscle was harvested to analyze treatment effects on cellular mechanisms of muscle growth rate. Tissue homogenate was examined for total protein and DNA content to assess the DNA:protein as a measure of myonuclear accretion (Figure 3-1). There was a significant increase in the DNA:protein in the T₅ group (5.5 ± 0.5 mg/g, DNA/protein) compared to the other two treatment diets (T₂₅= 4.1 ± 0.4 mg/g; C= 3.8 ± 0.5 mg/g) ($P < .05$). There was no treatment effect on the ratio of phosphorylated to total mTOR or AMPK α protein expression revealed by western

blotting (data not shown). Based on these findings, we supplemented the milk replacer with 0.5% tributyrin for the nursery feeding trial in order to investigate the potential for enhanced muscle growth.

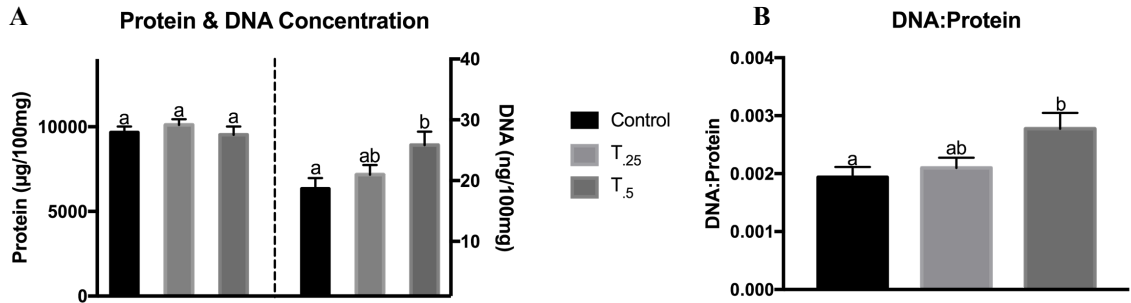


Figure 3-1. Total protein and DNA were extracted from *Longissimus dorsi* muscle of piglets fed either a basal milk replacer ($n=10$), or the milk replacer supplemented with 0.25% ($T_{.25}$, $n=10$) or 0.5% ($T_{.5}$, $n=9$) tributyrin for 21 days. (A) Protein and DNA concentrations in LD muscle tissue. (B) Bar graph depicting the ratio of DNA:Protein. Bars not sharing a common superscript differ significantly, $P<.05$.

Study 2-At the end of the 58-day feeding trial (neonatal + nursery), there was a significant increase in final body weight and ADG in animals that received tributyrin in the milk replacer before weaning (TT and TC) compared to animals that received control milk replacer diet (CC and CT) ($P<.05$; Table 3-2). After completion of the trial, piglets treated with tributyrin during the neonatal phase (D1-D21) weighed 8% more than the control piglets, $30.8\pm 0.6\text{kg}$ and $28.4\pm 0.6\text{kg}$, respectively, and had a 9% increase in their ADG ($659\pm 17\text{g}$ compared to the control $603\pm 16\text{g}$). There were no treatment effects seen in final body weight or feed efficiency between the four nursery diet treatment comparisons. Loin area and FCA from the piglets treated with tributyrin during the neonatal phase was significantly larger compared to those piglets that did not receive tributyrin in their milk replacer ($P<.05$; Figure 3-2A). At the end of the 58-day feeding trial, piglets supplemented with tributyrin during the neonatal period had a loin area of $25.3\pm 0.7\text{cm}^2$ compared to the control piglets, $22.7\pm 0.6\text{cm}^2$. Similarly, muscle histology sections stained with anti-

dystrophin (Figure. 3-2) revealed a 25% increase in the FCA of TT and TC piglets ($1790 \pm 120 \mu\text{m}^2$) compared to CC and CT piglets ($1420 \pm 60 \mu\text{m}^2$) ($P < .05$; Figure 3-2).

Table 3-2. Effects of tributyrin supplementation during neonatal and nursery phases on growth performance.

	Treatments*†				SEM	P‡		
	CC	CT	TC	TT		Milk	Dry	Milk X Dry*
n	6	9	5	7				
Initial Body Weight (kg)	1.6	1.5	1.4	1.6	0.06	----	----	----
Weaning Weight (kg)	6.4	6.3	5.9	6	0.1	0.11	-----	-----
Final Body Weight (kg)	29.6	29.3	30.4	29.6	1.1	0.03	0.66	1.00
Post-weaning Average Daily Gain (g)	627	618	658	641	28	0.03	0.66	0.98
Feed Efficiency After Weaning‡	1.41	1.41	1.37	1.41	0.04	0.37	0.52	0.99

*- Value represented as least square means of main effects

†- CC (milk replacer control; nursery control), CT (milk replacer control; nursery diet with tributyrin), TC (milk replacer with tributyrin; nursery control diet), and TT (milk replacer with tributyrin; nursery diet with tributyrin).

‡- Feed efficiency = Average daily feed intake/average daily gain (day 22-58)

§- Effect of tributyrin inclusion in the milk replacer, in the nursery diet, and their interaction

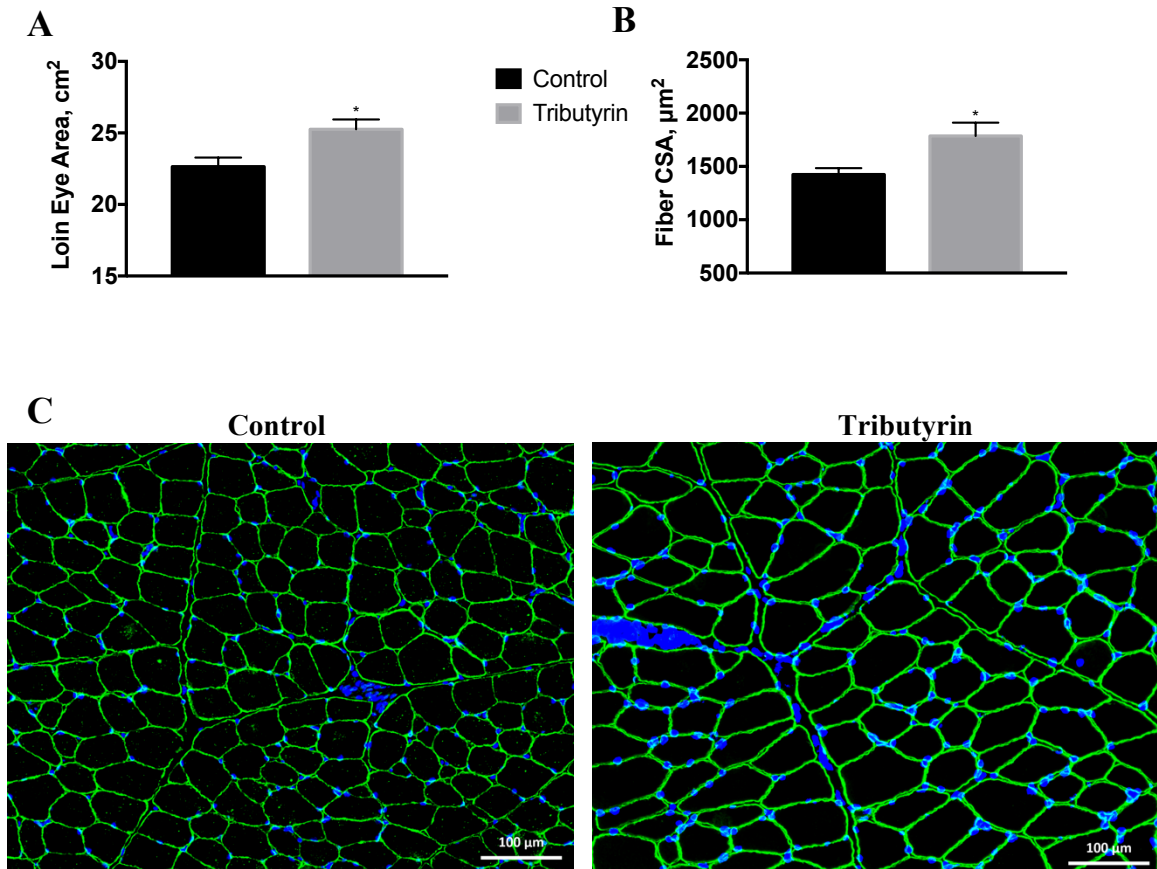


Figure 3-2. At 58d of age, cross section of *Longissimus dorsi* (LD) muscle was taken at the 12th rib and utilized for immunohistochemical analysis to determine fiber cross-sectional area (FCA). Values depicted are based off pooled neonatal control (C, $n=12$) or tributyrin (T, $n=12$) treatment groups. (A) The cross-sectional area of the LD at the 12th rib (loin eye). (B) LD muscle FCA as determined by immunohistochemistry. (C) Immunohistochemical analysis of FCA of the LD muscle. Muscle fibers were cryosectioned and stained with anti-dystrophin to visualize sarcolemma (green), >400 fibers/animal were counted using Zeiss ZEN Pro automated image analysis. Nuclei were visualized with DAPI. Significance was declared at $P<.05$ (*).

Satellite cell myogenesis

Satellite cells were harvested from neonatal piglets after 21d of milk feeding with (T_{.25} or T_{.5}) or without (C) tributyrin supplementation. There was no treatment effect on *in vivo* or *in vitro* satellite cell proliferation, as assessed by BrdU and EdU staining, respectively. Satellite cells were cultured under proliferative conditions until confluent and induced to

differentiate with gene expression analyzed every 24h to determine myogenic progression. Throughout differentiation, there was an average 7-fold ($T_{.25}$; $P < .01$) and 4-fold ($T_{.5}$; $P < .05$) increase in the expression of the late stage MRF myogenin in satellite cells from those animals treated with tributyrin compared to control animals (Figure 3-3). There was a not a significant change in Pax7 or MyoD expression in satellite cells from treated animals compared to the control animals (data not shown). When LD muscle sections of neonatal piglets from study 1 were analyzed for myogenic gene expression, a similar trend was revealed. Myogenin was upregulated 1.4-fold in the $T_{.25}$ group compared to the control ($P < .10$) without changes in Pax7 or MyoD expression (Figure 3-4). To further examine the effect of tributyrin on *ex vivo* satellite cell myogenesis, myotube formation was determined 48h after satellite cells were induced to differentiate (Figure 3-5). There was a 1.7-fold increase in the number of MyHC⁺ fused nuclei from $T_{.25}$ animals compared with the control animals ($P < .05$; Figure 3-5).

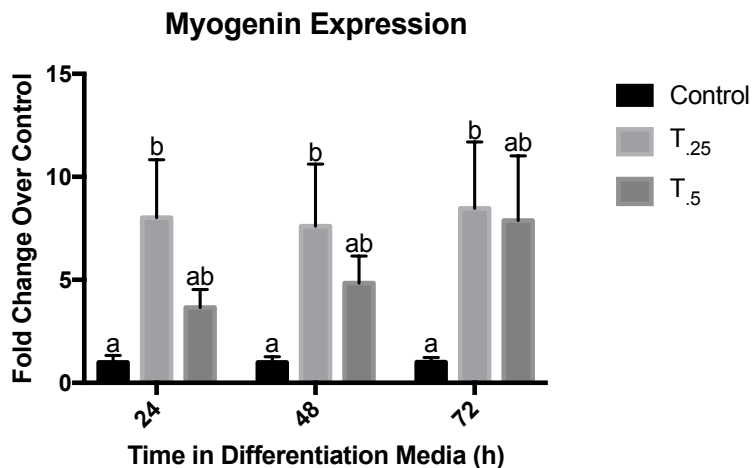


Figure 3-3. Myogenin gene expression in cultured satellite cells from neonatal piglets treated with either control diet ($n=10$), or a control diet supplemented with 0.25% ($T_{.25}$, $n=10$) or 0.5% ($T_{.5}$, $n=9$) tributyrin for 21 days. After induced to differentiate, total RNA was harvested and myogenin expression was quantified by RT-PCR. Expression was normalized within animal to RPL4 at each time point. Bars not sharing superscripts differ significantly, $P < .05$.

Gene Expression in LD Muscle

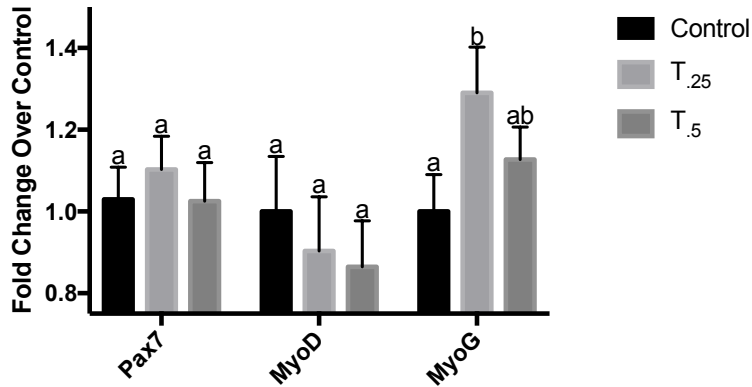
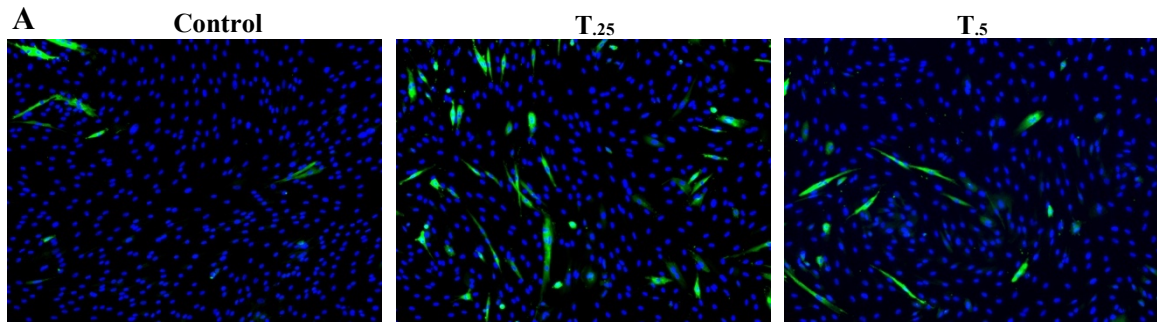


Figure 3-4. Total RNA was harvested from *Longissimus dorsi* muscle from neonatal piglets treated with either control diet ($n=10$), or a control diet supplemented with 0.25% (T_{.25}, $n=10$) or 0.5% (T_{.5}, $n=9$) tributyrin for 21 days. Gene expression of Pax7 and the myogenic regulatory factors MyoD and myogenin (MyoG) was measured by quantitative RT-PCR. Expression was normalized within animal to RPL4. Bars not sharing superscripts showed a trend, $P<0.10$.



B Satellite Cell Fusion

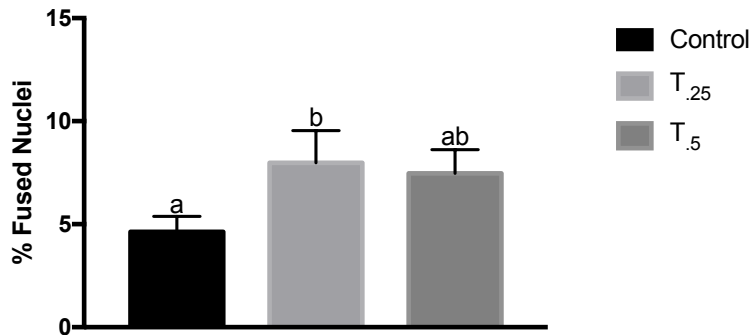


Figure 3-5. Satellite cells harvested from neonatal piglets treated with a control milk replacer diet ($n=10$), or a control diet supplemented with 0.25% (T_{.25}, $n=10$) or 0.5% (T_{.5}, $n=9$) tributyrin for 21-days were induced to differentiate for 48h. (A) Representative images of immunofluorescently stained myotubes with anti-MyHC (green). Nuclei were visualized with DAPI and counted using Zeiss ZEN Pro automated image analysis. (B) Bar graph depicting the fusion percentages (≥ 2 nuclei expressing MyHC/total nuclei) in satellite cells from those animals treated with either control diet, or a control diet supplemented with 0.25% (T_{.25}) or 0.5% (T_{.5}) tributyrin. Bars not sharing superscripts differ significantly, $P<0.05$.

Discussion

The benefits of dietary inclusion of butyrate or tributyrin on animal health (21, 24, 45, 47) and growth performance (4, 31, 33, 46) have been demonstrated; however, the role that butyrate has on the mechanisms behind muscle growth has yet to be elucidated. In the present study, we examined the ability of dietary tributyrin to enhance muscle hypertrophy and its effect on satellite cell programming. Our results demonstrate that early life supplementation with tributyrin can promote muscle growth through increased satellite cell myogenic potential.

The objective of study 1 was to assess what dietary tributyrin inclusion level is necessary to impact muscle growth parameters. Also, satellite cells were harvested from the neonatal animals and we investigated the effect of tributyrin supplementation on *in vivo* and *ex vivo* satellite cell programming. At the completion of study 1, the results of tributyrin inclusion during neonatal feeding were suggestive that an inclusion rate of tributyrin at 0.5% in study 2 may improve muscle growth through enhanced myonuclear accretion.

Studies have revealed the beneficial performance and growth effects of butyrate and tributyrin supplementation, all the while linking these positive results to an enhanced trophic status of the GI tract. Tributyrin provides an efficient means to deliver butyrate to systemic circulation for a sustained duration (19). Our results indicate that butyrate may act as a molecular signal, with a direct effect on satellite cell myogenesis. Our primary objective was to determine the effect of tributyrin supplementation on muscle growth at different stages of early life (pre-weaning/post-weaning). Post-natal muscle growth and

regeneration are dependent upon satellite cell activation and proliferation followed by consequent differentiation and fusion into the growing myofiber (13). After being fed a milk replacer supplemented with tributyrin for 21d, piglets had an increase in total DNA content and the DNA:protein. While there were no differences in weight gain at the end of the neonatal feeding trial, an increase of almost 40% in total DNA content and the DNA:protein ratio suggest an amplification of myonuclear accretion and a propensity for enhanced muscle growth (9). Secondary to myonuclear accretion is the subsequent protein synthesis and muscle fiber hypertrophy. During the neonatal feeding trial, piglets were limit-fed to match normal, sow reared growth. Given this feeding regimen, it is possible that the piglets were limited in amino acid availability for maximal protein synthesis. While it seems that tributyrin supplementation altered satellite cell behavior, there did not appear to be any changes in the mTOR signaling pathway, which governs protein synthesis (7) and has shown regulate satellite cell fusion machinery (55). In intestinal epithelial cells, butyrate has been shown to reduce the amount of phospho-AMPK which is known to inhibit mTOR (60); however, we did not see any differences by tributyrin treatment. The time required for the accumulated myonuclei to synthesize protein and contribute to the growing fiber may not have been sufficient to realize increased muscle hypertrophy. These results imply that prolonged, post-weaning ad-libitum feeding in the nursery would be necessary to assess whether dietary tributyrin inclusion would effectively promote muscle growth.

Neonatal piglets were once again fed a milk replacer supplemented with 0.5% tributyrin and then weaned into the nursery on a dry diet supplemented with 0.5% tributyrin. After

the nursery feeding trial, animals that had received tributyrin supplementation in their milk had a significant increase in ADG and final body weight. Interestingly, there was not a treatment effect with regards to nursery diet. These results are similar to those obtained by Le Gall et al. (30), where those animals that had received butyrate during milk feeding had enhanced growth, but the authors again attribute the increase in growth to increased feed digestibility and increased feed intake. Our data showed no differences in feed efficiency, and feed intake was reduced in both of the groups that received tributyrin during their respective stages. Butyrate supplementation has been associated with decreased feed intake while on treatment diets in other studies (24, 31); there is also a body of evidence linking mild ketosis with decreased appetite and increased satiety through unknown mechanisms (49). It may be that exogenous supplementation of butyrate induces a mild ketosis of which decreases overall feed intake and may confound the beneficial effects of tributyrin supplementation pre-weaning. However, the positive effects of tributyrin supplementation in the piglet milk replacer resulting in an increase in growth performance (Final BW = 8%, ADG = 9%) and muscle growth (Loin area = 11%, FCA = 25%) were unmistakable after piglets had reached the end of the nursery feeding.

We found that the hypertrophic benefit of tributyrin supplementation altered satellite cell behavior and enhanced terminal differentiation. The increase in myogenin expression found in the LD muscle of the tributyrin treated neonatal piglets may be indicative of satellite cells beginning to differentiate quicker and fuse into present myofibers (59). This was marked by the enhanced terminal differentiation seen in the *ex vivo* satellite cell cultures. Satellite cells from those piglets treated with tributyrin (both T_{.25} and T_{.5}) had an

increase in the proportion of those cells expression the contractile protein MyHC. This was associated with the increased upregulation of myogenin after satellite cell cultures were induced to differentiate. Although there were no noticeable treatment effects on either *in vivo* or *ex vivo* satellite cell proliferation, this is noteworthy due to butyrate's ability to halt cell proliferation seen in satellite cell culture experiments (26, 32). These results indicate that the increased myonuclear content may not come from a significant increase in the proliferative potential of satellite cells, but rather a temporal acceleration of the differentiation process. The lack of differences seen in the *in vivo* proliferation of satellite cells also suggests that tributyrin's effect on satellite cell behavior may be most salient at the earliest stages of life.

The differences in gene expression and myogenic potential displayed in the satellite cell cultures from the tributyrin treated groups also provides acceptance that tributyrin, and its ensuing metabolite butyrate, may be serving as an epigenetic modifier of satellite cell behavior (54). The HDAC inhibitory properties of butyrate may be leading to improved muscle differentiation through histone modifications that result in increased myogenin expression. This compliments findings that decreased HDAC activity has resulted in increased acetylation of non-histone proteins, such as MyoD, which is necessary for myogenesis and promotes myogenic differentiation (17, 34, 35). In this regard, it appears that dietary tributyrin may serve as a viable inhibitor of HDACs for pharmacological manipulation of myogenic genes.

Using a neonatal piglet model of muscle growth, we have shown that early dietary inclusion of the butyrate prodrug, tributyrin, resulted in an increase in muscle mass by muscle fiber hypertrophy. Our findings also indicate that the accelerated muscle growth triggered by tributyrin is due to increased myonuclear accretion and subsequent myofiber hypertrophy. Supplementing tributyrin in the milk replacer formula of neonatal piglets resulted in enhanced muscle growth driven by enhanced satellite cell myogenesis. Contradictory with some previous findings (30, 46), dietary supplementation of tributyrin to the older weaned pigs did not increase muscle growth or improve growth performance. This suggests that there is a window of opportunity to utilize tributyrin to impact muscle growth via alterations in satellite cell activity and that early-life interventions with tributyrin may be able to ameliorate deficits in muscle growth caused by limitations in the myogenic activity of satellite cells.

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Chapter 4: The HDAC inhibitor butyrate primes satellite cells for differentiation by altering the epigenetic landscape

Abstract

Satellite cells (SC) are a heterogeneous population of muscle resident stem cells that are responsible for postnatal muscle growth and repair. With the investigation into the genomic regulation of satellite cell fate, we now have an idea as to how the epigenome governs SC activation, proliferation, and differentiation. Interestingly, histone deacetylase (HDAC) inhibitors have proven to be effective at enhancing the SC myogenesis, but their precise mode of action in altering the epigenetic landscape of SC remains undetermined. Our objective was to determine how a HDAC inhibitor, butyrate, promotes myogenic differentiation. Neonatal piglets were fed a standard milk replacer or one supplemented with 0.5% spray dried Tributyrin (TB), a butyrate pro-drug. Satellite cells from TB supplemented piglets showed a decrease in the expression of EZH2 (enhancer of zeste homolog 2), the methyltransferase subunit of the PRC2 (polycomb repressive complex 2) *ex vivo*. SC ChIP-Seq analysis revealed that TB treatment resulted in a global reduction of the H3K27me3 repressive chromatin mark, specifically around those sites associated with genes involved in myogenic differentiation, negative regulation of cell proliferation, and micro-RNAs normally upregulated during differentiation. SC were transfected with siRNA targeting EZH2, treated with 0.5mM butyrate, or both to determine if this was the primary mechanism through which butyrate alters SC myogenesis. Treatment with butyrate significantly reduced the expression of genes associated with satellite cell proliferation 24h after treatment, while EZH2 ablation had no effect on the expression of

these transcription factors. Immunostaining revealed that EZH2 depletion resulted in an increase in differentiating SC, but not myotube hypertrophy. These results indicate that while EZH2 ablation may force myogenic differentiation, butyrate may operate through a parallel mechanism to enhance the myogenic program. These findings support the notion that butyrate may be used to enhance muscle growth and could prove useful at treating myopathic conditions.

Introduction

Tissue-specific stem cells have the potential to self-renew or differentiate into mature cells, contributing to tissue growth and homeostasis. The muscle specific stem cells, satellite cells, maintain the ability to self-renew or differentiate and are required for post-natal muscle growth and repair (3, 15, 22). The use of novel gene sequencing technology in the investigation of the genomic regulation of satellite cell fate, has provided insight into how the satellite cell epigenome governs myogenesis (31, 42). Specifically, that changes in the chromatin structure and the regulators that direct these changes modulate satellite cell activation, proliferation, and differentiation (2, 8, 12, 35). Interestingly, histone deacetylase (HDAC) inhibitors have proven to be effective at enhancing the myogenic program of satellite cells (19, 20, 30), but their precise mode of action in remodeling the epigenetic landscape of satellite cells remains undetermined. One such HDAC inhibitor, butyrate, is a short-chain fatty acid that has been validated as an effective promoter of myogenic differentiation (19), and while the mechanism remains uncertain, indications allude to a modification of the epigenetic status (21). Determining whether these presumptive alterations to the satellite cell epigenome triggered by butyrate can be affected *in vivo* would be of great significance in combating muscle disease and improving muscle growth.

Satellite cells lie along the muscle fiber beneath basal lamina and plasma membrane (28) and remain in a “poised” state, ready to activate, proliferate, and differentiate upon molecular and mechanical cues (2, 6, 9). The epigenetic status of satellite cells has been

well defined through their myogenic lineage (5, 42), marked by their timely expression of a hierarchical network of transcription factors (15, 47). The paired-box 7 (Pax7) transcription factor is a hallmark of satellite cell identification and is required for postnatal muscle growth and repair (36, 41). Upon satellite cell activation, Pax7 is upregulated and cells will proliferate and begin expressing the basic helix-loop-helix myogenic regulatory factor (MRF), MyoD (33). The transcription factor MyoD is a master regulator of satellite cell lineage fate and is responsible for the expression of muscle specific genes through its initiation of the differentiation program (45). Satellite cells that do not express MyoD, or have high levels of Pax7 expression compared to MyoD, will self-renew and go back to a quiescent state, reserved for the next round of stimulation and activation (34, 49). MyoD has also been found to play a role in repressing the muscle differentiation genes that it is associated with until differentiation cues are present (26), and that not all genes that MyoD associates with are expressed at the same time (4). These findings describe the integral arrangement and temporal spacing of gene expression during the myogenic program. Another MRF, myogenin is also required for proper satellite cell terminal differentiation (16) and is regulated by MyoD. In both a direct and indirect manner, MyoD recruits chromatin remodeling enzymes to the myogenin promoter allowing for the binding of MyoD to the promoter and allowing for transcription (11, 46). Myogenin target genes include those responsible for appropriate satellite cell fusion and contractile protein formation (10, 29). Myogenin expression marks the transition from myoblast proliferation to differentiation (7) and the down-regulation of Pax7, as the two are mutually exclusive (32). Upon pro-myogenic differentiation cues, regulatory remodeling of chromatin around

these transcription factors has been shown to govern the rate in which satellite cells progress through their myogenic program (31, 42).

The subset of myogenic progenitor cells that make up the satellite cell pool after embryogenesis lie in quiescence and rely on a highly coordinated transcriptional network that governs maintenance, activation, and differentiation. Satellite cell commitment to its myogenic lineage is regulated and defined by the epigenome (43), and with new chromatin immunoprecipitation sequencing (ChIP-Seq) technology, these epigenetic signatures have been defined (24). Satellite cell quiescence is maintained by the absence of the repressive trimethylated lysine 27 residue on the histone H3 protein (H3K27me3) and the presence of H3K4me3 (24). Upon satellite cell activation, most of the H3K4me3 marks remain, but repressive H3K27me3 marks are deposited on muscle specific genes (MyoD, MyoG) by the Polycomb group (PcG) proteins in association with the transcriptional repressor HDAC1 (12). Satellite cells will then continue to either self-renew, or asymmetrically divide and give rise to myogenic daughter cells that begin to express MyoD (22). These committed daughter cells (termed myoblasts) retain the permissive H3K4me3, but lose the bivalent H3K27me3 mark, allowing for increased MyoD expression (12). Prior to myoblast differentiation, MyoD associates with HDAC1 resulting in silencing of MyoD-dependent transcription of muscle specific genes (27, 37). Myoblasts continue to proliferate until pro-differentiation cues lead to a slowing of Pax7 transcription through the removal of H3K4me3 and deposition of the repressive H3K27me3 marks by the Polycomb repressive complex 2 (PRC2) (35). Concomitant removal of the H3K27me3 marks around the myogenin promoter allow for transcriptional activation and terminal differentiation to

begin (44). This removal is consistent with a loss of the catalytic subunit, EZH2, of the PRC2 protein which regulates the methyltransferase capability of PRC2 and ultimately mediates satellite cell differentiation (48). The HDAC inhibitor butyrate has shown to disrupt and downregulate EZH2 expression, resulting in a reduction of H3K27me3 marks around lineage specific genes (21).

The use of HDAC inhibitors in satellite cell cultures and animal growth studies have yielded positive results with regards to muscle differentiation (19) and growth (13, 17, 25), respectively. Also, EZH2 depleted satellite cells have shown an increased expression of myogenin compared in satellite cell cultures (44). While the benefits of using butyrate on *in vitro* myogenesis are clear, *in vivo* studies are lacking. Determining the molecular regulators affected by butyrate with regards to muscle growth, may provide targets for manipulation in treating myopathies and promoting muscle growth. In this study, we attempt characterize the epigenetic alterations in satellite cells seen by feeding tributyrin, a butyrate prodrug. Also, we attempted to characterize the interaction butyrate has on the epigenetic enzyme EZH2 and to determine whether butyrate modulates satellite cell myogenesis through this epigenetic regulator.

Materials and Methods

Animals, diets and experimental protocol

All animal protocols were approved by the University of Maryland-College Park's Institutional Animal Care and Use Committee. To assess the impact of dietary tributyrin inclusion on *in vivo* satellite cell epigenetics, 20 cross-bred female piglets (n=10, 24±6h old; 1.79±.25kg body weight) were assigned to a treatment diet of either a standard commercial milk replacer formula (Advance Liqui-Wean, Milk Specialties Co., Dundee, IL) where 175g of powder was reconstituted in water to 1kg total formula (C), or the milk replacer formula supplemented with 0.5% (T) total butyric acid in the form of spray dried tributyrin (AviPremiumD, Vetagro SpA, Reggio Emilia, Italy). Tributyrin inclusion was on a dry matter basis and coconut oil was used to ensure the diets were isoenergetic. Piglets were housed individually and received formula every 2hrs (0900-2300) at a limit-fed rate to match sow reared growth. One piglet from the T group was removed from the study due to non-treatment related health issues. Body weight and feed intake were recorded daily for the duration of the 21d feeding trial. At sacrifice, *Longissimus dorsi* (LD) muscle was collected and used for all tissue analysis and satellite cell isolation.

Satellite cell isolation and culture

Satellite cells were isolated according to a procedure modified from Doumit and Merkel (14) and Allen et al. (1). Briefly, LD muscle was excised from neonatal piglets, trimmed of connective tissue and minced with scissors. Tissue fragments were digested with 1.25mg/ml protease from *Streptomyces griseous* (Pronase; Sigma-Aldrich, St. Louis, MO)

for 1h at 37°C. Satellite cells were disassociated from tissue fragments by trituration and differential centrifugation. Cells were pre-plated on uncoated 15cm tissue culture dishes for 2h (37°C, 5% CO₂) in proliferative growth media (PGM, DMEM + 10% FBS + antibiotics- 100U/mL penicillin, 100µg/mL streptomycin, 10µg/mL gentamycin; Thermo Fisher Scientific, Waltham, MA) and then seeded on tissue cultured treated dishes coated with Poly-L-lysine (100µg/mL ddH₂O, Sigma-Aldrich) and fibronectin (10µg/mL PBS, Sigma-Aldrich) in PGM until they reached 50% confluence (37°C, 5%CO₂). Cells were then released with 0.05% Trypsin (Thermo Fisher Scientific) and either plated for our studies or used for ChIP-Seq analysis.

The effect of tributyrin inclusion on *ex vivo* satellite cell expression of EZH2 was analyzed through their myogenic progression under proliferative and differentiative conditions. Satellite cells were seeded at 2,500 cells/cm² in PGM on to plates coated with Poly-L-lysine and fibronectin. After a 24h attachment period, satellite cell proliferated for 48hrs and were then induced to differentiate (DM, DMEM + 2% horse serum; Sigma-Aldrich, + antibiotics) for an additional 48h with complete media changes daily. Total RNA was isolated (RNeasy; Qiagen, Hilden, Germany) at each 24h time point for gene expression analysis.

Satellite cell transfection and butyrate treatment

Satellite cells were plated and allowed to proliferate in PGM until ~80% of confluence, were transfected, treated with 0.5mM sodium butyrate (NaBu), or both (Mixed) for 24h and then induced to differentiate. Cells were transfected with 100nM duplexed siRNA

targeting the EZH2 transcript (siEZH2) (Dharamcon, Lafayette, CO) or a scrambled control (siScrambled) (MISSION Universal Negative Control; Sigma-Aldrich) using Lipofectamine 2000 (Thermo Fisher Scientific) for 24h according to the manufacturer's protocol. Transfection efficiency was ~80% with ~70% knockdown efficiency in EZH2 transcript. Total RNA was collected at the time of treatment (D-24) and every 24h for 96h (72h after differentiation, D+72). At the D+72h time point, satellite cells were fixed and immunostained for myosin heavy chain expression as an indicator of terminal differentiation and myotube formation.

Neonatal piglet muscle tissue analysis

After the 21d neonatal feeding trial, piglet muscle (LD) was snap frozen in liquid N₂ for EZH2 gene expression analysis. Total RNA was isolated by homogenization using tri-reagent (Thermo Fisher Scientific) with phase separation achieved by chloroform wash. RNA was precipitated with 70% ethanol and transferred to RNeasy spin column (Qiagen) and purified according to the manufacturer's protocol.

Analysis of gene expression

Total RNA isolated from both neonatal LD muscle and satellite cells were quantified using the Quant-iT RiboGreen assay (Thermo Fisher Scientific) according to the manufacturer's protocol. Harvested RNA was reverse transcribed with the SuperScript IV First-Strand Synthesis System (Thermo Fisher Scientific) and treated with the RNase H to ensure removal of RNA. The resulting cDNA was quantified with the Quant-iT OligoGreen assay (Thermo Fisher Scientific). Total RNA and cDNA quantification were detected on the

Synergy HTX microplate reader using the Gen 5.0 v3.0 software (BioTek Instruments, Winooski, VT). cDNA was used for qRT-PCR using Bio-Rad's CFX96 Touch Real-Time PCR Detection System and iQ Multiplex Powermix or iQ SYBR Green Supermix. Analysis of gene expression and amplification plots were executed with the CFX Manager Software (version 3.1, Bio-Rad). Primers and probes for the multiplexed myogenic genes of interest and EZH2 expression (singleplex) were normalized to RPL4 using $\Delta\Delta CT$ method (Table 4-1). For each assay, samples were amplified for 45s at 60°C for 40 cycles. Primers and probes were designed by Integrated DNA Technologies (Coralville, IA).

Table 4-1. Primers and probe sequences used for gene expression analysis by quantitative RT-PCR.

Gene Symbol	Gene ID	Primer Sequence 5'-3'	Probe and Sequence 5'-3'
PAX7 ^α	100625823	F: CAGCAAGCCCAGACAGG R: TCGGATCTCCCAGCTGAA	(HEX): TTGAGGAGTACAAGAGGGAGAACCCA
MYOD1 ^α	407604	F: CCGACGGCATGATGGATTATAG R: CGACACCGCAGCATTCTT	(FAM): AATAGGTGCCGTCGTAGCAGTTCC
MYOG ^α	497618	F: AGTGAATGCAGTTCACACAG R: AGGTGAGGGAGTGCAGATT	(Texas Red): CAACCCAGGGGATCATCTGCTC
RPL4 ^α	100038029	F: TGGTGGTTGAAGATAAAGTTGAAAG R: TGAGAGGCATAAACCTTCTTGAT	(Cy5): AACCAAGGAGGCTGTTCTGCTTCT
EZH2 ^β	100625497	F: GCGGAAGCGTGAAAAATCAGA R: CCTTCGCTGTTCCACTCTT	-----
RPL4 ^β	100038029	F: CAAGAGTAACTACAACCTTC R: GAACTCTACGATGAATCTTC	-----

α- Multiplex, probe based qRT-PCR

β- Singleplex, SYBR Green qRT-PCR

Immunostaining

Satellite cell cultures were immunostained to determine terminal differentiation and reveal myotube formation and satellite cell fusion. Satellite cells were analyzed for the expression of the contractile protein myosin heavy chain (MyHC) after 72h of differentiation. Satellite cells were fixed in 4% paraformaldehyde and permeablized in 0.1% Triton X-100 (Sigma-Aldrich) in PBS. Samples were blocked with 10% goat serum in PBST (0.1% Tween-20 in PBS) for 1h at room temperature. Cells were incubated overnight at 4°C with primary

mouse monoclonal anti-MyHC at 10 μ g/mL (Roche, Indianapolis, IN). Primary antibodies were removed and then incubated with the secondary antibody (AlexaFluor 488 goat anti-mouse IgG at 1:500 dilution; Jackson ImmunoResearch, West Grove, PA) in 5% goat serum for 1h at room temperature. Nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). Images were visualized on a Zeiss AxioObserver Z.1 and analyzed with ZenPro automated image analysis suite (Carl Zeiss AG, Oberkochen, Germany).

ChIP Library Construction and Sequencing

ChIP libraries were constructed from primary satellite cells (n=3/treatment group) and were utilized to perform ChIP-Seq analysis as previously described (39). Briefly, approximately one million cells/sample were used to obtain formaldehyde-crosslinked chromatin that was fragmented into smaller sizes (~150–300 base pairs) by sonication (Cell Disrupter 350; Heat Systems Ultrasonics, Plainview, NY) and micrococcal nuclease (New England Biolabs, Ipswich, MA) digestion. Chromatin was then immunoprecipitated with ChIP-grade H3K27me3 (ab6002) and H3K27ac (ab4729) antibodies (Abcam, Cambridge, United Kingdom) and purified to obtain immunoprecipitated DNA. ChIP DNA was repaired by using NEBNext End repair module (New England Biolabs) and 3'A addition was performed using NEB Klenow Fragment (New England Biolabs). Then, adaptors (Illumina Inc, San Diego, CA) were ligated to the repaired end with T4 DNA ligase (New England Biolabs). After PCR amplification, DNA fragments of 200 – 500bp were selected from the agarose gel. The resulting library was sequenced on the Illumina HiSeq 2000 single-end system (Illumina Inc, San Diego, CA).

Statistical Analysis

ChIP-Seq Data

Raw sequence data quality was checked by FastQC (40). Bowtie, an ultrafast memory-efficient short read aligner was used to align reads to the pig reference genome Sscrofa10.2 (Swine Genome Sequencing Consortium) (23). No trimming process was used because of high data quality. After quality control and alignment, peak-calling was achieved by using Model Based Analysis of ChIP-Seq (MACS) (50). Identification of differentially enriched regions between treatment and control groups were accomplished by DiffBind package in R (version 3.2.3) (38). Peaks identified with MACS (BAM) were used as input data for DiffBind. EdgeR analysis was run in the DiffBind package and used for Trimmed Mean of M-values normalization. The significance cut-off value for identification of differential regions was set to < 0.1 false discovery rate (FDR) for H3K27ac and < 0.05 FDR for H3K27me3. Next, the ChIPpeakAnno package in R was used to annotate genomic features of those identified differential enrichment regions (51). ChIPpeakAnno allows the extract of information overlaps, distances, and relative positions of requested genetic features. Gene ontology (GO) enrichment analysis was performed on the online DAVID Bioinformatics Resources 6.8 to highlight the most relevant GO terms from the list of related gene annotated by ChIPpeakAnno (18).

Gene expression and satellite cell fusion data

General statistical procedures were analyzed using an F-test in ANOVA (GraphPad Prism 7, GraphPad Software, Inc., La Jolla, CA). In the case of a significant F-test, multiple mean comparisons were analyzed using a Tukey's adjustment. A student's t-test was used

to analyze treatment mean differences where indicated. A probability of $P \leq 0.05$ was considered significant and a P -value between 0.05 and 0.10 ($0.05 < P \leq 0.10$) was considered a trend.

Results

Tributylin's effect on EZH2 gene expression

Muscle tissue and isolated satellite cells were assessed for tributyrin's impact on EZH2 gene expression. Primary satellite cells were allowed to progress through their myogenic lineage *ex vivo* for 96h. EZH2 gene expression was analyzed every 24h after plating for 96h; after 48h of proliferation, and just prior to induction to differentiate, expression was reduced in satellite cells from the tributyrin treated piglets as compared to the control ($P < .10$) (Figure 4-1). EZH2 transcript expression was not significantly reduced 24h after plating or 24h after differentiation, and by 48h of differentiation, there were no treatment effects on EZH2 gene expression. Total RNA extracted from whole muscle tissue showed no difference in EZH2 gene expression with tributyrin feeding.

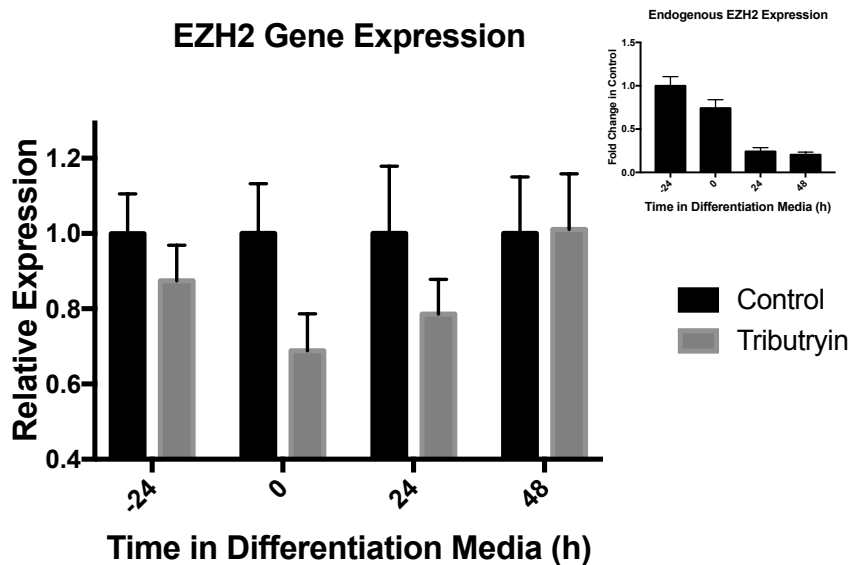


Figure 4-1. EZH2 expression in primary satellite cells isolated from neonatal piglets fed a control milk replacer or a milk replacer supplemented with 0.5% Tributyrin. Satellite cells were cultured under proliferative conditions until ~90% confluence and then induced to differentiate. Total RNA was harvested and relative EZH2 expression was analyzed by qRT-PCR and normalized to RPL4 expression ($2^{-\Delta\Delta CT}$). (Inset) Endogenous fold-change in the expression of EZH2 in satellite cells (control) during the differentiation program.

ChIP-Seq Results

Satellite cells from tributyrin treated piglets (n=3) and control piglets (n=3) were analyzed for global levels of genome binding for H3K27me3 and H3K27ac histone marks by ChIP-Seq. Tributyrin treatment resulted in 358 of the 532 differentially bound sites being significantly reduced for H3K37me3 enrichment based on edgeR ($FDR \leq 0.05$). Analysis of H3K27ac revealed 162 differentially enriched regions with an even distribution of annotated regions between the treatment groups ($FDR \leq 0.10$) (Figure 4-2). GO enrichment analysis from differentially bound sites in satellite cells from tributyrin treated animals revealed significant decreases in the enrichment of repressive H3K27me3 marks associated with increased myoblast differentiation, negative regulators of cell proliferation, and microRNAs involved in satellite cell differentiation as compared to cells from the

control animals. Conversely, sites involved with positive regulators of cell proliferation and increased stem cell maintenance were enriched for H3K27me3 in the tributyrin group (Table 4-2). We performed the same analysis with H3K27ac differentially bound peaks found in control and tributyrin clusters; Tributyrin cluster genes were enriched for GO terms related to increase cell adhesion, TORC1 complex and microRNAs upregulated during myoblast differentiation. H3K27ac control cluster genes were enriched for GO terms related to increased ATP and calcium binding (Table 4-3).

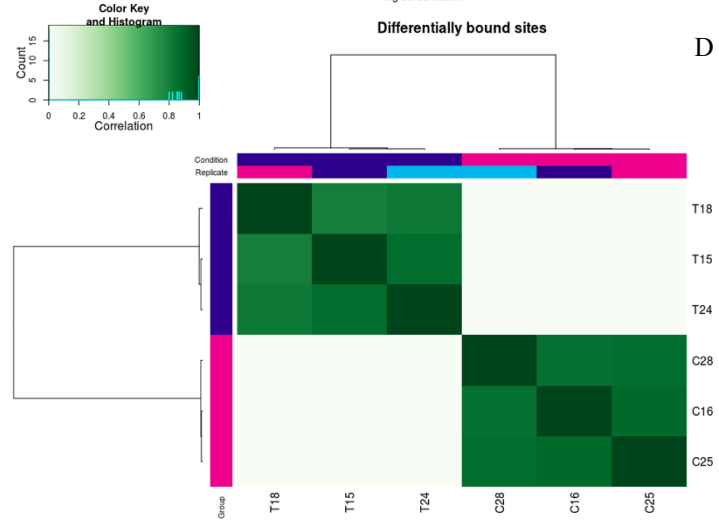
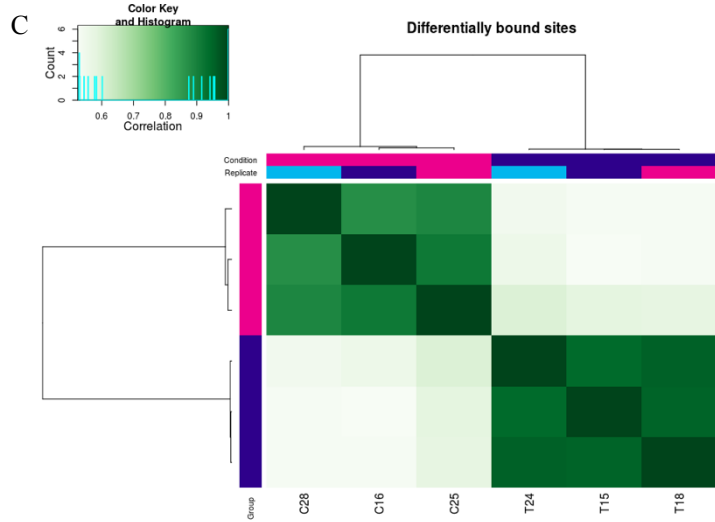
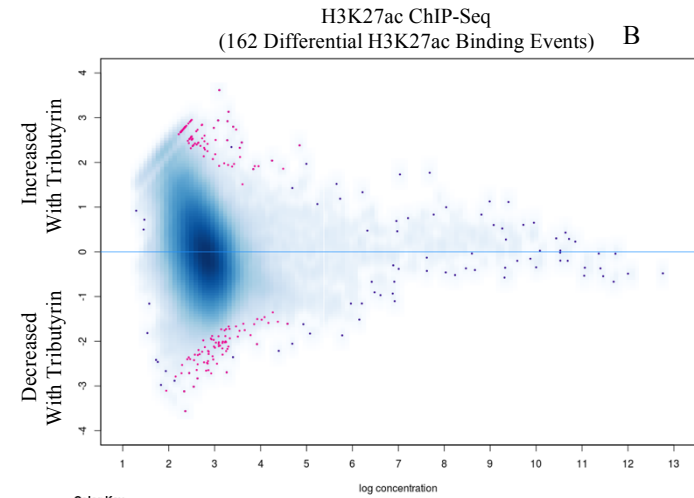
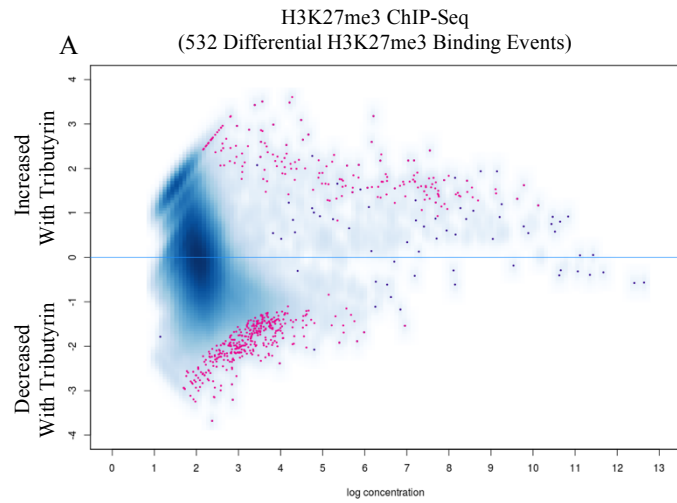


Figure 4-2. ChIP-Seq of satellite cells selected randomly from control (C, n=3) and tributyrin treated (T, n=3) animals for H3K27me3 and H3K27ac histone marks. (A&B) MA plot of tributyrin-control contrast. Significantly differentially bound sites shown in red. (C&D) Unsupervised hierarchical clustering of differentially bound sites using H3K27me3 (C) and H3K27ac (D) profiles are shown with Pearson correlation. Grades are color coded with replicate numbers in the labels.

Table 4-2: Gene Ontology (GO) Analysis of H3K27me3 Differently Bound Peaks

GO Categories Enriched in H3K27me3 from Satellite Cells Treated with Tributyrin				
GO Term	Associated Gene ID	Gene Name/Function	Fold Change	P-value
Skeletal Muscle Cell/Myoblast Differentiation/Cell Differentiation	MYOD1	myogenic differentiation 1	-2.059	4.2E-04
	SOX11	SRY-box 11	-2.653	3.0E-04
	QKI	KH domain containing RNA binding	-1.398	4.7E-04
	NTRK3	neurotrophic tyrosine kinase, receptor, type 3	-2.023	1.9E-04
Negative Regulation of Cell Proliferation	DRD2	dopamine receptor D2	-2.508	3.1E-04
	BTG4	BTG anti-proliferation factor 4	-2.002	1.3E-05
	INSM1	INSM transcriptional repressor 1	-1.486	3.0E-04
Negative Regulation of Cell Response to Growth Factor Stimulus	SLIT2	slit guidance ligand 2	-2.227	4.7E-04
	CASK	calcium/calmodulin dependent serine protein kinase	-1.308	2.5E-04
Cell Cycle Arrest	THBS1	thrombospondin 1	-1.753	4.9E-04
	IL-12B	interleukin 12B	-2.251	1.8E-07
Homophilic cell adhesion via plasma membrane adhesion molecules	CDH6	cadherin 6	-2.002	1.5E-05
	CDH10	cadherin 10	-1.853	2.5E-04
	FAT3	FAT atypical cadherin 3	-2.201	3.3E-04
Micro-RNAs	MIR206	-----	-2.574	4.1E-04
	MIR214	-----	-1.757	4.5E-04
	MIR208B	-----	-1.525	5.9E-04
	MIR130A	-----	2.862	2.0E-04
Positive Regulation of Cell Proliferation	FGF17	fibroblast growth factor 17	2.496	1.2E-08
	RASGRP4	RAS guanylyl releasing protein 4	1.743	2.9E-05
Germ-line stem cell population maintenance	PIWIL2	piwi-like RNA-mediated gene silencing 2	2.502	1.1E-06

Table 4-3: Gene Ontology (GO) Analysis of H3K27ac Differently Bound Peaks

GO Categories Enriched in H3K27ac from Satellite Cells Treated with Tributyrin				
GO Term	Associated Gene ID	Gene Name/Function	Fold Change	P-value
Regulation of Cell Proliferation	DMNT1	DNA (cytosine-5-)-methyltransferase	-1.732	1.2E-04
	MYO5A	myosin VA	-3.563	3.8E-05
ATP Binding	PLK2	polo like kinase 2	-2.695	9.1E-07
	EGFR	epidermal growth factor receptor	-2.643	1.3E-04
Calcium Ion Binding	SCUBE3	signal peptide, CUB domain, EGF-like 3	-2.407	1.3E-04
	ANXA10	annexin A10	-2.233	4.2E-05
	ITPR3	inositol 1,4,5-triphosphate receptor 3	-2.022	2.7E-05
Micro-RNAs	MIR128-1	-----	-2.360	1.4E-04
	MIR181A-1	-----	1.964	3.8E-06
	MIR210	-----	2.922	5.7E-07
Cell Adhesion Via Plasma Membrane	CDH7	cadherin 7	2.819	5.6E-05
	PCDH15	protocadherin 15	3.133	4.6E-07
TORC1 Complex	RPTOR	regulatory associated protein of MTOR, complex 1	1.916	9.8E-07

Effect of EZH2 knockdown on differentiation

In order to determine if butyrate works through, or in conjunction with, EZH2 reduction to enhance myogenic differentiation, satellite cells isolated from control animals were exposed to either butyrate (0.5mM, NaBu), RNAi targeting the EZH2 transcript (siEZH2), or both butyrate and siEZH2 (Mixed) during proliferation. After 24h of exposure to butyrate, satellite cell expression of the EZH2 transcript was not significantly reduced as compared to the control. The siEZH2 treatment resulted in a 60% reduction ($P < .01$) in EZH2 transcript expression as compared to siScrambled control. An additive effect of the combination of butyrate and siEZH2 (Mixed) was seen with a 75% reduction ($P < .01$) in EZH2 gene expression compared to the siScrambled treatment (Figure 4-3). After 24h of differentiation (D+24), EZH2 expression was reduced, as compared to levels during proliferation, with no significant differences between treatments. Total RNA was also analyzed for the expression of the myogenic genes Pax7, MyoD, and myogenin. EZH2

knockdown did not have a significant effect on the expression of the myogenic genes after transfection or during differentiation. Treatment with NaBu alone and NaBu+siEZH2 did however, result in reducing Pax7 ($P<.01$) and MyoD ($P<.05$) expression ~40% 24h after treatment (Figure 4-3), but did not affect myogenin expression (data not shown). After being induced to differentiate, no statistically significant differences in myogenic gene expression was found between treatment groups. After 72h of differentiation, satellite cells were fixed and stained for the contractile protein MyHC as an indicator of terminal differentiation. Satellite cells treated with siEZH2 tended ($P<.10$) to have ~75% increase in MyHC⁺ cells relative to the siScrambled control, but EZH2 knockdown did not result in more multinucleated myotubes (Figure 4-4).

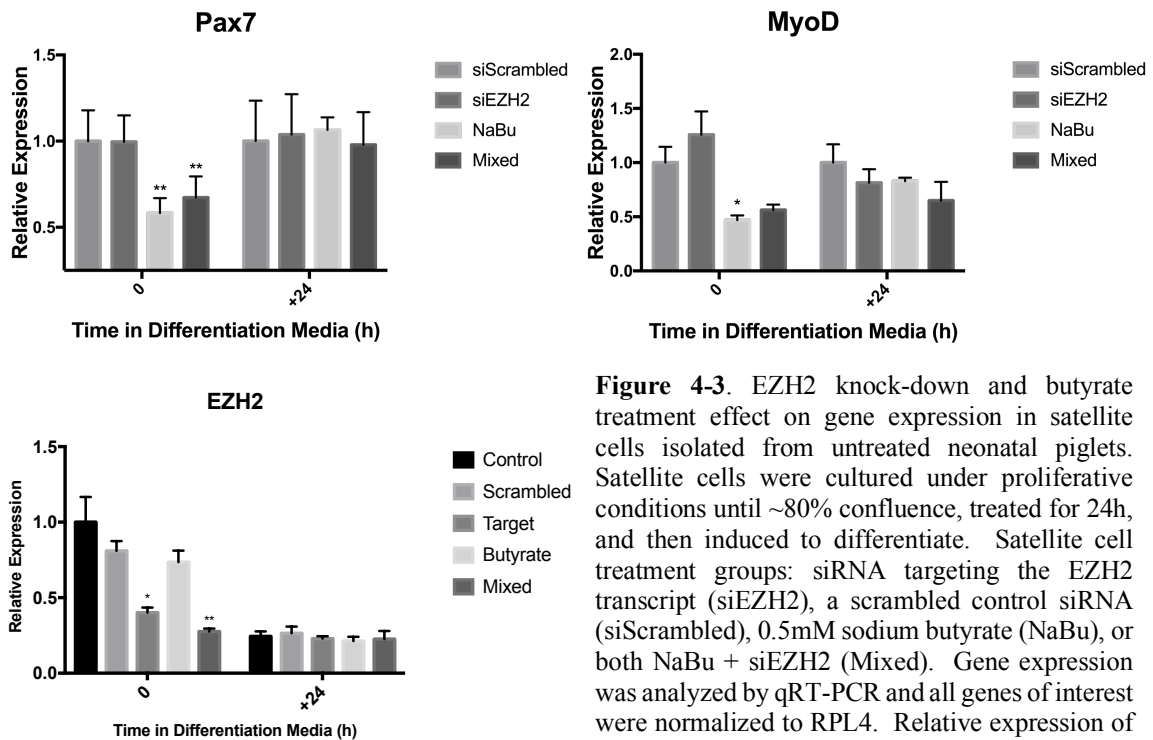


Figure 4-3. EZH2 knock-down and butyrate treatment effect on gene expression in satellite cells isolated from untreated neonatal piglets. Satellite cells were cultured under proliferative conditions until ~80% confluence, treated for 24h, and then induced to differentiate. Satellite cell treatment groups: siRNA targeting the EZH2 transcript (siEZH2), a scrambled control siRNA (siScrambled), 0.5mM sodium butyrate (NaBu), or both NaBu + siEZH2 (Mixed). Gene expression was analyzed by qRT-PCR and all genes of interest were normalized to RPL4. Relative expression of the genes of interest (EZH2, Pax7, and MyoD) from the transfected treatments (siEZH2 and Mixed) were normalized to siScrambled control while the NaBu treatment was normalized to the untreated control ($2^{-\Delta\Delta CT}$). ($P<.05^*$; $P<.01^{**}$).

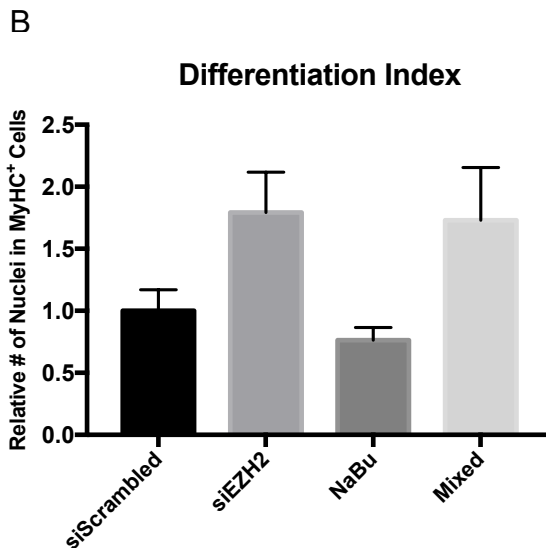
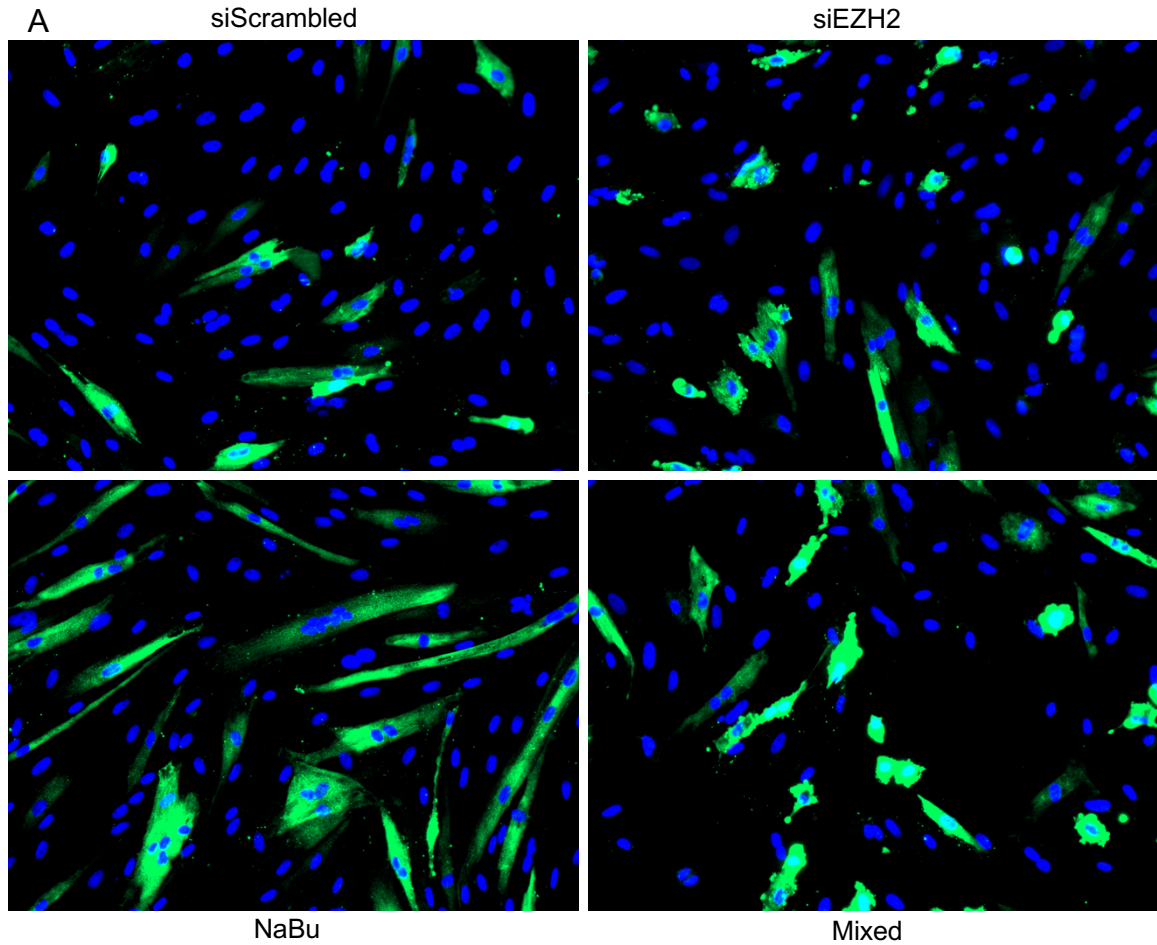


Figure 4-4. EZH2 knock-down and butyrate treatment effect on satellite cell differentiation as assessed by myosin heavy chain (MyHC) expression 72h after differentiation. Satellite cells were cultured under proliferative conditions until ~80% confluence, treated for 24h, and then induced to differentiate. Satellite cell treatment groups: siRNA targeting the EZH2 transcript (siEZH2), a scrambled control siRNA (siScrambled), 0.5mM sodium butyrate (NaBu), or both NaBu + siEZH2 (Mixed). (A) Representative images of myotube formation revealed by immunostaining with anti-MyHC (green); nuclear counterstain with DAPI (blue). (B) The number of MyHC⁺ cells were counted and compared to total nuclei. The number of MyHC⁺ cells from the transfected treatments (siEZH2 and Mixed) were normalized to the siScrambled control while the NaBu treatment was normalized to the untreated control.

Discussion

The transcriptionally repressive H3K27me3 histone mark deposited by the Polycomb EZH2 methyltransferase protein has been implicated in regulating satellite cell specification and the differentiation process (2). It has been demonstrated in other tissue specific stem cells that EZH2 expression is regulated by HDACs and can be altered with HDAC inhibitors *in vitro* (7). Animals treated with butyrate have shown increased functionality in models of muscle pathology (14) and increased growth (8); however, the molecular mechanisms leading to these outcomes has remained generally unaddressed. Our results suggest that dietary inclusion of butyrate is sufficient to alter the expression of EZH2 and the epigenetic landscape of satellite cells. Our data does not indicate that the reduction in EZH2 expression caused by butyrate is the sole mechanism through which butyrate may alter the myogenic program of satellite cells.

Dietary inclusion of tributyrin resulted in a hastened reduction in EZH2 expression in satellite cells *ex vivo*. Primary satellite cells from tributyrin treated piglets showed a reduction in EZH2 expression just prior to differentiation as compared to the control piglets. After 24h in differentiation media, EZH2 expression was similar between the two groups. These findings indicate that those cells may start the differentiation program earlier or may be primed to initiate the differentiation machinery more quickly. There are published data that are consistent with this scenario, showing that an auto-regulatory loop exists in which the microRNA miR-214 (discussed below) targets the 3'UTR of EZH2 just prior to differentiation (6). Given the reduction in EZH2 seen in satellite cells from treated

animals, we performed ChIP-Seq to determine global genome enrichment of the H3K27me3 and H3K27ac histone marks.

ChIP-Seq was performed on proliferating primary satellite cells and revealed that treatment with tributyrin resulted in a global reduction of H3K27me3 marks, but intriguingly did not result in hyperlactation of H3K27. It would appear that other differentiation cues must be necessary in order for histone acetyltransferases (HATs) to be recruited to those locations, despite HDAC inhibition. Our results showed that those regions with differential enrichment of H3K27me3 were associated with the GO terms related specifically to myoblast differentiation. Most importantly among these genes was the MRF MyoD which had a differentially enriched region 10kb upstream of the transcription start site that was reduced for H3K27me3 with tributyrin treatment. There is evidence that the homeoprotein Msx1, Msh homeobox-1, recruits the PRC2 complex to MyoD regulatory regions and results in increased repressive H3K27me3 being deposited in several regions (16). Other genes that were associated with regions showing an overall reduction in H3K27me3 were related to the GO terms negative regulation of cell proliferation, cell cycle arrest, and plasma membrane adhesion molecules. Conversely, tributyrin treatment resulted in fewer H3k27me3 enriched regions as compared to the control; however, those regions that were enriched over the control were associated with genes related to the positive regulation of cell proliferation. These results underscore the anti-proliferative and pro-differentiative properties attributed to butyrate (12) while also highlighting the epigenetic nature of these changes. Interestingly, our ChIP-Seq results indicate that tributyrin treatment resulted in differentially enriched regions around key miRNAs. Of note, miR-214 which regulates

EZH2 expression (6), as detailed above, was enriched for the repressive H3K27me3 in the satellite cells of the control animals. This may explain one way in which EZH2 expression was reduced in the satellite cells from the treatment group. Also, H3K27me3 marks around miR-206 were reduced in satellite cells from the tributyrin treatment group. miR-206 has been implicated in repressing Pax7 and allowing for differentiation to take place (3). Complementary to this, tributyrin treatment resulted in H3K27ac enrichment near both miR-181a and miR-210 which are strongly upregulated during satellite cell differentiation (4, 9). It appears that the beneficial effects of butyrate on satellite cells reported previously (5) may be due, at least in part, to a modulation of EZH2 activity.

To investigate whether butyrate's primary mechanism of action occurs through the down regulation of EZH2 expression and subsequent hypomethylation of H3K27, we assessed the interaction of EZH2 depletion alone or in concert with butyrate exposure in proliferating satellite cells just prior to differentiation. We found that EZH2 knockdown alone had no effect on myogenic gene expression at any time point; however, butyrate significantly reduced Pax7 and MyoD gene expression, both required for proper satellite cell proliferation and activation (15, 17). Pax7 is required for satellite cell specification (13), but is also crucial for preventing precocious differentiation (10). It is clear that butyrate must act through some other mechanism to silence Pax7 and force myogenic cell commitment. It has been determined that EZH2 is required for Pax7 gene silencing through progressive H3K27me3 deposition at the Pax7 promoter (11). Immunostaining for MyHC⁺ satellite cells revealed that treatment with siEZH2 forced differentiation; however, it did not result in proper activation of the differentiation program as indicated by the failure to

form multinucleated myotubes. These findings are consistent with conditional ablation of SUZ12 (another component of PRC2) in myoblasts, with the exception that SUZ12 ablation resulted in enhanced myotube formation (1). This may be in part due to the inability to assemble H3K27me3 marks about the Pax7 gene. In the absence of other differentiation cues, it was apparent that forcing the differentiation program too early had severe repercussions on myotube formation. The combined action of butyrate and siEZH2 was not enough to recover appropriate myotube formation. These results indicate that premature EZH2 ablation will inadvertently initiate the differentiation program without the appropriate mechanisms in place for satellite cell fusion or myotube hypertrophy.

In our study, we have shown that tributyrin supplementation had a genome-wide impact on H3K27me3 mark deposition, primarily due to the interaction of butyrate with the PRC2 methyltransferase enzyme EZH2. Our *in vitro* work demonstrates that a reduction in EZH2 gene expression is not the only pro-myogenic differentiation cue that may be taking place. It has been suggested that an interaction exists where in which HDAC1 complexes with EZH2 and they function collectively to trimethylate H3K27 (2). It is possible that the HDAC inhibitory effects may reduce the effect and recruitment of EZH2 to areas responsible for muscle gene expression, and that these marks are passed on to subsequent daughter cells, as shown in our results (Figure 4-5). Further investigation is warranted into the hyperacetylation of MyoD, as well as the co-immunoprecipitation of the genome that is bound by both EZH2 and HDAC1. Taken together, these observations detail one way in which butyrate through dietary supplementation may be accelerating the myogenic program.

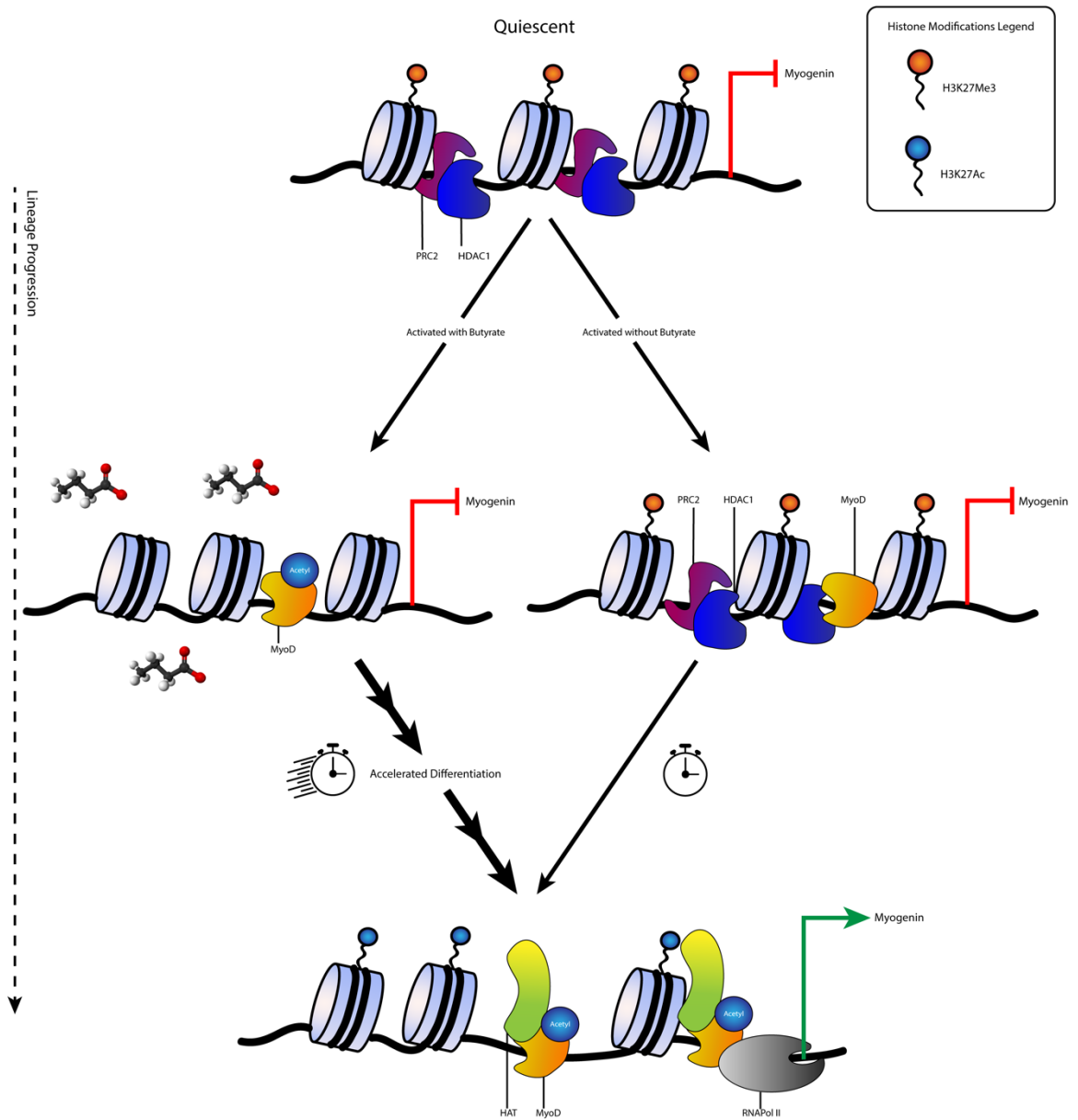


Figure 4-5. Regulation of satellite cell activation and differentiation in the presence of butyrate. In undifferentiated, quiescent satellite cells lineage determination genes (i.e. myogenin) are not expressed due to the repressive H3K27me3 heterochromatin marks and lack of MyoD locating to the promoter region. Upon activation, MyoD is transcribed and locates to the promoter region of muscle genes (i.e., myogenin). Under normal conditions, MyoD is associated with HDAC1 and H3K27me3 still marks relevant muscle genes which prevent precocious differentiation. Satellite cells that are activated in the presence of butyrate allow the early acetylation of MyoD and removal of H3K27me3 marks. Butyrate inhibits HDAC1 and may interfere with PRC2 mediated trimethylation of H3K27. Inhibition of HDAC1 may also cause early PRC2 dislocation from muscle gene promoter regions. During differentiation cues, histone acetyl transferases (HATs) are recruited to muscle genes for deposition of the active H3K27ac euchromatin mark. Histone tail acetylation takes place during differentiation, but at a faster pace in the presence of butyrate while repressive PRC2 that has already disassociated from muscle gene promoters can now be recruited to the promoters of stemness genes.

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Chapter 5: General Conclusions

The objective of these studies was to determine what impact butyrate has on satellite cell myogenesis and its efficacy as a muscle growth promoter in the form of tributyrin. Positive results from previous cell culture experiments with butyrate and other histone deacetylase (HDAC) inhibitors, our own satellite cell culture study, and animal growth studies warranted further exploration as to the effect of butyrate on muscle growth and satellite cell behavior. Our results from the first of two successive animal trials indicated that butyrate may alter the ability of satellite cells to differentiate into myofibers, whereby increasing myonuclear content. The second animal trial supported our hypothesis, that tributyrin promoted muscle growth. Further analysis revealed satellite cells from tributyrin treated animals had a greater myogenic capacity and were “poised” for enhanced differentiation. A reduction in repressive H3K27me3 histone mark around key differentiation genes confirmed previous reports that butyrate interferes with the methyltransferase ability of the polycomb repressive complex-2 (PRC2). However, through our knock-down experiment, we determined that this may not be the sole mechanism through which butyrate alters the ability of satellite cells to progress through their myogenic lineage. Taken together, these results highlight the potential impact that butyrate and tributyrin has on the promotion of muscle growth through altered satellite cell programming.

Previous *in vitro* application of sodium butyrate on satellite cell myogenesis has had conflicting results. We found that satellite cell behavior can be altered when exposed

to physiologically relevant concentrations (0-1.0mM) of sodium butyrate. When examining satellite cell proliferation, cells demonstrated the typical response of cell cycle arrest to butyrate treatment. Most importantly, we found an increase in terminal differentiation ability of satellite cells treated with butyrate, marked by the upregulation of the terminal differentiation gene, myogenin. These results warranted further analysis to determine if this effect could be obtained from *in vivo* supplementation of butyrate.

The results of the first animal trial revealed that neonatal piglets treated with tributyrin, a less caustic way to sustain circulating butyrate concentrations, for 21-days had significant increase in DNA content in muscle tissue. An increased DNA:protein ratio suggested that an increase in myonuclear content had resulted from tributyrin treatment. This warranted a second animal trial of sustained duration to last through weaning. Animals that had received tributyrin in their milk replacer ended the study weighing significantly more than animals that received just the standard milk replacer. We attribute this increase in weight to enhanced muscle growth, supported significant increases in the loin area and fiber cross-sectional area from the tributyrin treated pigs. We did not find any discernable differences in feed efficiency, nor tributyrin's effect on muscle growth when supplemented post-weaning. Satellite cells from the first animal study we then analyzed for any programming effect that tributyrin supplementation may have caused. Similar to our *in vitro* study, satellite cells from treated animals showed enhanced terminal differentiation and increased myogenin expression, supporting the assertion that tributyrin supplementation has a lasting change to the myogenic program through a possible epigenetic mechanism.

Given that the most dramatic differences on muscle growth was seen between the neonatal tributyrin treatment groups, we analyzed satellite cells from tributyrin treated animals to ascertain changes in the epigenetic landscape. We found that tributyrin treatment reduced the expression of the enzymatic subunit of PRC2 (enhancer of zeste homolog-2, EZH2) just prior to differentiation. EZH2 is responsible for the trimethylation of H3K27 around key muscle specific genes. To further investigate whether this had an effect on deposition of the repressive H3K27me3 mark, and if the HDAC inhibitory properties of tributyrin affected acetylation at H2K27 (H3K27ac), we performed ChIP-Seq analysis. We found a global reduction of H3K27me3 marks in those cell from tributyrin treated animals with specific reductions around those genes with associated the gene ontology (GO) terms myoblast differentiation, negative regulation of cell proliferation, and miRNAs upregulated during differentiation. There did not seem to be any differences in the number of H3K27ac histone mark enrichment sites due to tributyrin treatment; however, regions associated with the GO terms calcium ion binding and ATP binding were reduced by tributyrin treatment as compared to the control. This prompted us to determine what interaction existed between butyrate and PRC2. To this end we treated satellite cells with butyrate, a short-interfering RNA (siRNA) targeting the EZH2, or both butyrate and the siRNA. While butyrate may be interfering with EZH2 expression, we assert that another mechanism must act in concert with this interference. Butyrate treatment resulted in significant reduction of Pax7 expression while EZH2 depleted satellite cells showed no changes in Pax7 expression despite forced differentiation. Together, it appears that dietary

butyrate can alter the epigenetic landscape through EZH2 modulation, but that HDAC inhibition is also key for enhanced terminal differentiation.

In closing, we have shown that dietary inclusion of butyrate in the form of tributyrin has the potential to serve as a possible promoter of muscle growth and that the myogenic programming of those satellite cells from treated animals may be altered. The benefit of dietary tributyrin inclusion is most apparent during the earliest stages of life, when satellite cells are in a constant state of activation and proliferation. Given the similarities between fetal myogenesis and post-natal myogenesis governed by satellite cell activation, proliferation, and differentiation, it would seem that tributyrin supplementation during gestation may be warranted. Satellite cells also go through periods of intense activation during bouts of injury, tributyrin or butyrate treatment may be offered as a therapeutic to enhance healing and functional outcomes during such afflictions.

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