HYPOTHYROIDISM AND SKELETAL MUSCLE: AN IN VITRO MODEL OF INVESTIGATING IMPAIRED PATHWAYS OF MUSCLE HEALTH

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BY

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DEDICATION

For my husband, Mark, and children, Caitlin, Rebecca, and Alexandra.

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ABSTRACT

GENA GUERIN

HYPOTHYROIDISM AND SKELETAL MUSCLE: AN IN VITRO MODEL OF INVESTIGATING IMPAIRED PATHWAYS OF MUSCLE HEALTH

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Hypothyroidism is a prevalent metabolic condition in the United States primarily affecting women. Individuals treated for hypothyroidism commonly report symptoms including skeletal muscle (SKM) pain, fatigue, and intolerance to exercise even while under treatment. Exercise is an alternate intervention with potential benefits offsetting hypothyroidism due to overlapping thyroid hormone and exercise signaling pathways.

The purpose of the study was to investigate the effects of hypothyroidism on SKM metabolism, myogenesis, mitochondria, and cellular homeostasis and assess whether an exercise intervention could reduce the detriments caused by intracellular low thyroid hormone availability.

An *in vitro* human SKM cell culture low availability of thyroid hormone model was utilized to represent hypothyroidism. The exercise mimetic, formoterol, was used to provide “exercise” stimulation. This experiment was conducted during the mid- and late stages of myogenesis. The model included three conditions (n = 6), control (CON), thyroid hormone depleted (ThD), and thyroid hormone depleted with three-hour acute

formoterol treatment (ThD+F). Skeletal muscle myocytes were differentiated for four or six days in low thyroid hormone media with the ThD+F group stimulated with formoterol for 3 hours. Extraction of total RNA was performed on days four and six followed by qPCR for gene analysis.

Gene expression was assessed for the following categories: (a) thyroid hormone metabolism, (b) myogenesis, (c) mitochondrial homeostasis, and (d) cellular homeostasis. The $\Delta\Delta$ CT method was used to normalize the data followed by two-way repeated measures ANOVA. Significance was set at $p < .05$.

The low availability of thyroid hormone and formoterol treatment significantly affected the expression of genes related to thyroid hormone metabolism, myogenesis, mitochondrial homeostasis, and cellular homeostatic function in skeletal muscle cells. The intracellular low thyroid hormone availability was compounded by the formoterol treatment leading to decreases in gene expression associated with myogenesis, reactive oxygen species mediation, and metabolism in the skeletal muscle myocytes. Exercise may cause deleterious effects on skeletal muscle in individuals with hypothyroidism. Further research is warranted to determine the safety and prescription of exercise for those with hypothyroidism.

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CHAPTER I

INTRODUCTION

At least 30 million Americans have some form of thyroid disease; it is more prevalent than cardiovascular disease (CVD) and diabetes mellitus (DM) yet is less visible as a serious health issue (American Association of Clinical Endocrinologists, 2019). Thyroid disease is characterized by functional and structural changes to the thyroid gland affecting its ability to produce thyroid hormone (TH). Of the millions with thyroid disease, most have hypothyroidism and according to the American Thyroid Association (ATA, 2019); up to 60% of individuals may remain undiagnosed, mainly due to lack of public awareness about hypothyroidism (Canaris, Tape, & Wigton, 2013). Women have eight times greater risk of developing hypothyroidism than men, making it predominantly a women's health concern (ATA, 2019; Hollowell et al., 2002). Although women may develop hypothyroidism at any age, the incidence increases with age, especially after menopause, primarily due to the connection with autoimmune disease development (Fairweather & Rose, 2004; Morganti et al., 2005). The most common cause of hypothyroidism is Hashimoto's disease, an autoimmune condition whereby the immune system attacks and damages the thyroid gland (Weetman & McGregor, 1994).

Several other causes of hypothyroidism include inadequate intake of dietary iodine, thyroidectomy or partial removal of thyroid gland, genetic predisposition, congenital hypothyroidism, or medications that undermine the thyroid (ATA, 2019). In the case of full or partial thyroidectomy and congenital hypothyroidism, full TH replacement therapy is necessary; however, TH dysregulation may still occur and requires frequent assessment of serum TH concentration and adjustments to medication (Hannouseh & Weiss, 2016).

The clinical definition of hypothyroidism is based on insufficient production of TH by the thyroid gland and resultant low levels of circulating TH, leading to impairments in metabolism, growth and development, and homeostasis of all tissues within the body (Chaker, Bianco, Jonklaas, & Peeters, 2017). Since TH has cellular effects on all tissues, low availability of TH can greatly disrupt whole body homeostasis, creating long-term and cascading symptoms. Perhaps the most recognized symptom is extreme fatigue, which can have a significant impact on activities of daily living, career and family responsibilities, and exercise participation (Watt et al., 2006). In surveys by the ATA (Peterson et al., 2018) and Guerin et al. (2019, in submission), individuals under treatment for hypothyroidism reported the presence of significant ongoing symptoms without resolution, despite TH replacement therapy.

For many individuals, an accurate and timely diagnosis of hypothyroidism is delayed as other co-morbidities present with similar symptoms leading to a missed diagnosis (Jonklaas et al., 2014). The ATA diagnostic guidelines for hypothyroidism include blood tests and a thorough screening of health history including symptomology and a physical examination. The recommended blood tests for hypothyroidism evaluate the levels of thyroid stimulating hormone (TSH) and free tetraiodothyronine or thyroxine (T_4; ATA, 2019). Based on the "normal" reference ranges, elevated TSH and low T_4 indicates hypothyroidism (Jonklaas et al., 2014). The reference ranges have caused contention between clinicians in recent years as the statistically defined ranges do not account for symptoms and increased disease risk even when patients are within the reference range (Surks, 2013; Wartofsky & Dickey, 2005). To further bolster the diagnosis, there are optional blood tests measuring free triiodothyronine (T_3), the unbound, active form of TH, reverse T_3, (rT_3), an inactive form of TH, and antibodies for thyroid peroxidase and thyroxine binding globulin. Taken together, these may present a more accurate picture of the condition and better inform on treatment therapies (Koulouri, Moran, Halsall, Chatterjee, & Gurnell, 2013). At least seven different TH function test patterns as shown in Figure 1 have been reported by Koulouri et al. (2013), demonstrating the complexity in diagnosing thyroid disorders and causes of adverse outcomes in patients.

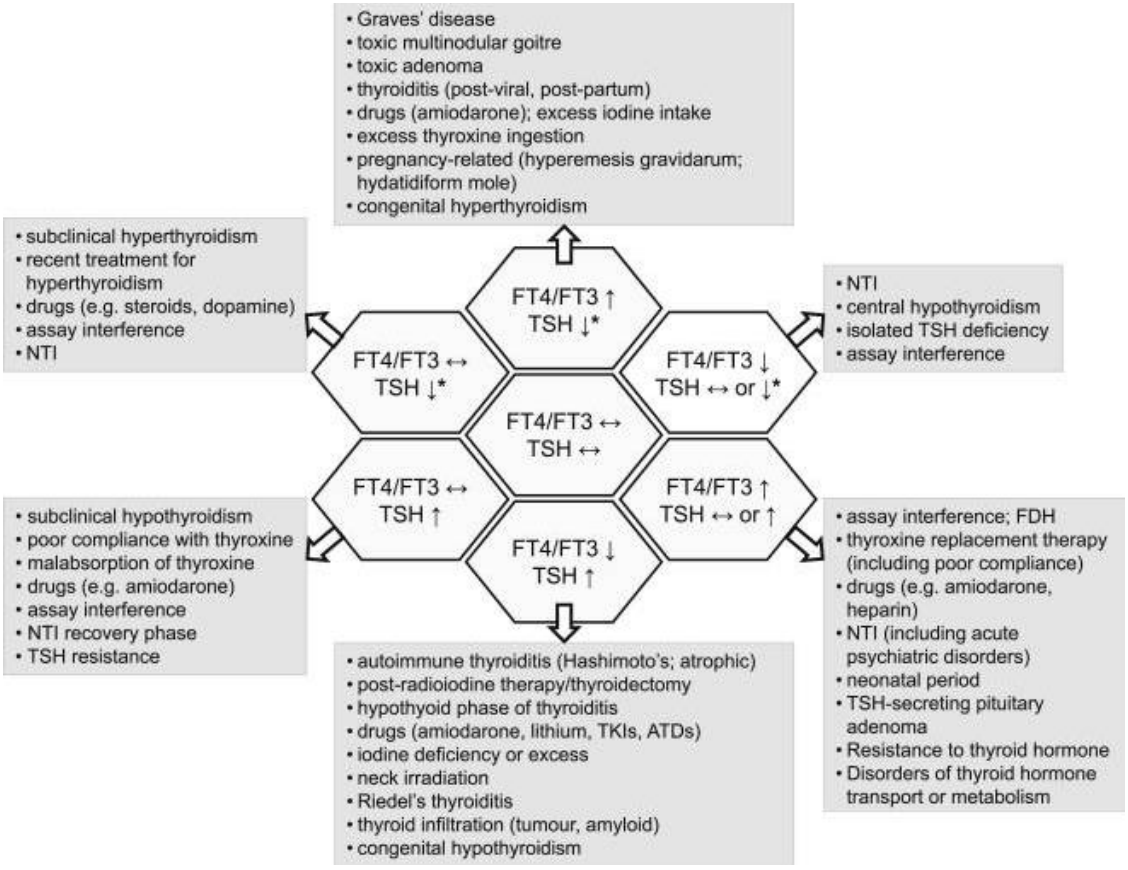

Figure 1. Thyroid function test patterns (Koulouri et al., 2013).

Currently, the standard prescribed treatment by the ATA is synthetic TH replacement by levothyroxine, a T_4 only compound (Garber et al., 2012), with the goal of attaining TSH and T_4 levels within "normal" range. The recommendations for T_4 only treatment were revisited by the American Task Force on Approaches and Strategies to Investigate Thyroid Hormone Economy and Action (Bianco et al., 2014) to determine if T_4 only treatment was still the best standard of care. The Task Force concluded there was a lack of evidence supporting the use of alternative medications like combined T_4/T_3

or desiccated TH. However, recent studies have investigated the use of combined T_4/T_3 and conclude these may be viable alternatives for patients not responding well to T_4 alone. As discussed further in this chapter, some individuals cannot convert T_4 to T_3, but caution that these patients should be monitored for adverse outcomes (Dayan & Panicker, 2018; Tariq, Wert, Cheriyath, & Joshi, 2018). More research is needed in this area to explore alternatives. Presently, there is no cure for hypothyroidism and lifetime-use of medication is necessary for management of the disease, especially for those who have had a thyroidectomy performed (ATA, 2019). Currently, no other interventions beyond TH replacement therapy are recommended for management of the disease despite the many reported uncontrolled metabolic impairments and wide spread symptoms even while being on hormone therapy (Jonklaas et al., 2014; Peterson et al., 2018).

Thyroid Hormone Regulation, Synthesis, and Transport

In order to gain understanding of the complexity of hypothyroidism, here we will briefly review normal TH regulation. The thyroid gland is a butterfly-shaped, endocrine gland located near the base of the front of the neck. Even though it is relatively small, the thyroid gland is the master regulator of whole-body metabolism producing about a teaspoon of very potent TH every year (Mullur, Liu, & Brent, 2014). This process is finely regulated by the hypothalamic-pituitary-thyroid axis and kept within tight ranges of production; therefore, modest changes in TH production greatly affect the body.

As a function of the hypothalamic-pituitary-thyroid axis, the thyroid gland's production of TH is regulated by a negative feedback loop (Ortiga-Carvalho, Chiamolera, Pazos-Moura, & Wondisford, 2016). Low T_4 in the circulation signals the paraventricular nucleus of the hypothalamus to excrete thyrotropin releasing hormone (Ortiga-Carvalho et al., 2016). Once released, thyrotropin releasing hormone stimulates the thyrotropes within the anterior pituitary to excrete TSH which signals TH production (Ortiga-Carvalho et al., 2016). TSH stimulates its receptor on the baso-lateral membrane of the thyroid follicular cells initiating activation of the adenylyl cyclase (AC)-cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) pathway (Rousset, Dupuy, Miot, & Dumont, 2015). Once activated, the AC-cAMP-PKA pathway stimulates the expression and function of the sodium/iodide symporter (NIS), thyroid peroxidase, and thyroglobulin; all key participants in TH production (Rousset et al., 2015). In Figure 2, the synthesis of TH within the thyroid gland is reviewed.

After exocytosis through the membrane into the blood, 99% of TH are bound to the protein carrier thyroxine-binding globulin (Refetoff, 2015). Bound TH are not transported through plasma membranes into cells; however, free T_4 and free T_3, each of which constitute less than 0.05% of their respective total, enter cells by monocarboxylate transporters or organic anion transporters (Mayerl et al., 2018; Muller et al., 2014). Most important to thyroid hormone cell signaling, the availability of free T4 in the circulation and subcellular conversion within various tissues provides the

functional effects of this hormone. A potential problem in the clinical setting is that standard blood work analyzing T4 only gives a picture of what is happening in the circulation, but does not give an accurate indication of what is happening within target cells. The overall effects of low TH may be most noticeable due to its many roles in skeletal muscle (SKM) tissue.

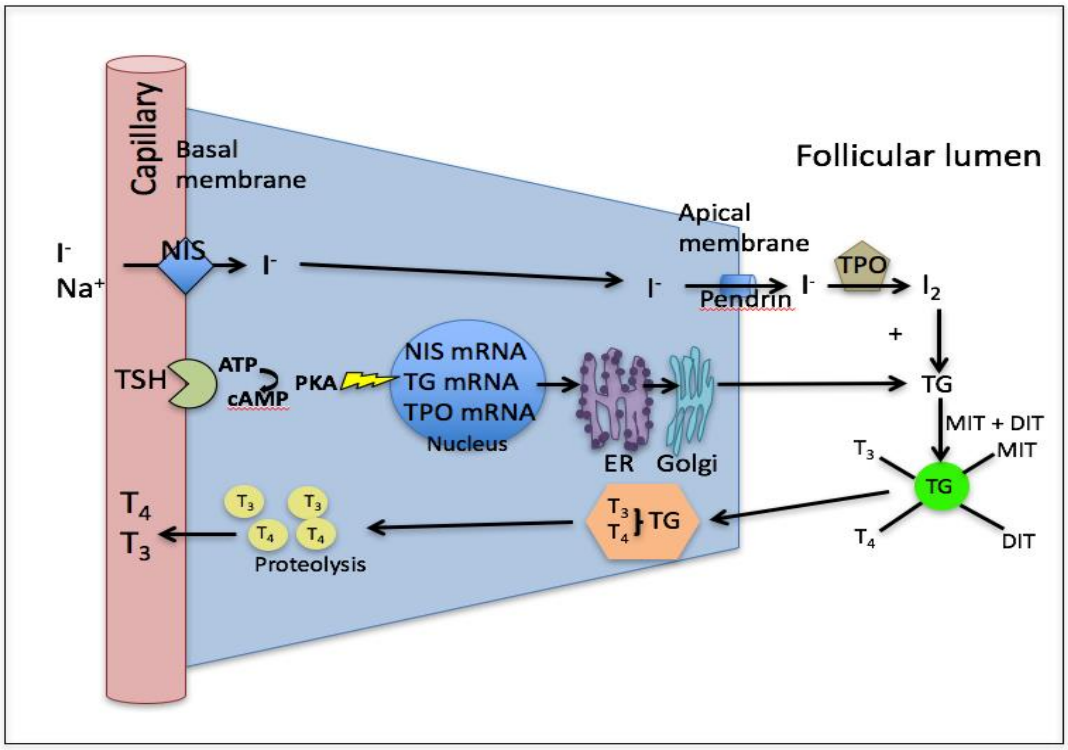

Figure 2. Thyroid hormone synthesis. The NIS transport of iodide across the baso-lateral membrane of thyroid cells is called iodine trapping. Next, pendrin pumps the iodide through the apical membrane into the follicular lumen where it is oxidized by thyroid peroxidase into iodine, and incorporated into TH. Thyroglobulin is synthesized by the ribosomes, glycosylated by the endoplasmic reticulum, and packaged by the golgi apparatus before exocytosis into the follicular lumen. Once in the colloid, thyroglobulin's tyrosine residues are iodinated by thyroid peroxidase producing monoiodotyrosine (MIT) and diiodotyrosine (DIT). Thyroid peroxidase couples MIT and DIT for packaging and endocytosis into the follicular cell. The MIT and DIT-contained vesicle fuses with a lysosome cleaving the segments into tetraiodothyronine (T_4) and triiodothyronine (T_3) ready to be transported throughout the body.

Thyroid Hormone Action in Skeletal Muscle Cells

The cell signaling effects of TH are perhaps the most palpable in SKM due to its prolific role in metabolism, function, and myogenesis (Mullur et al., 2014) and is the major focus of this study. About 40% of body mass is comprised of SKM and it

contributes the highest percentage of energy expenditure to whole-body metabolism (Zurlo, Larsen, Bogardus, & Ravussin, 1990). The primary purpose of SKM is to support and move the skeleton, which requires the conversion of chemical energy into mechanical energy, thus leading to further increases in bioenergetics beyond its effects on basal energy metabolism (Westerblad, Bruton, & Katz, 2010). An overview of TH signaling in SKM is displayed in Figure 3. TH enters the SKM cell via transporters, where T4 is converted to T_3 thus promoting effects on gene expression.

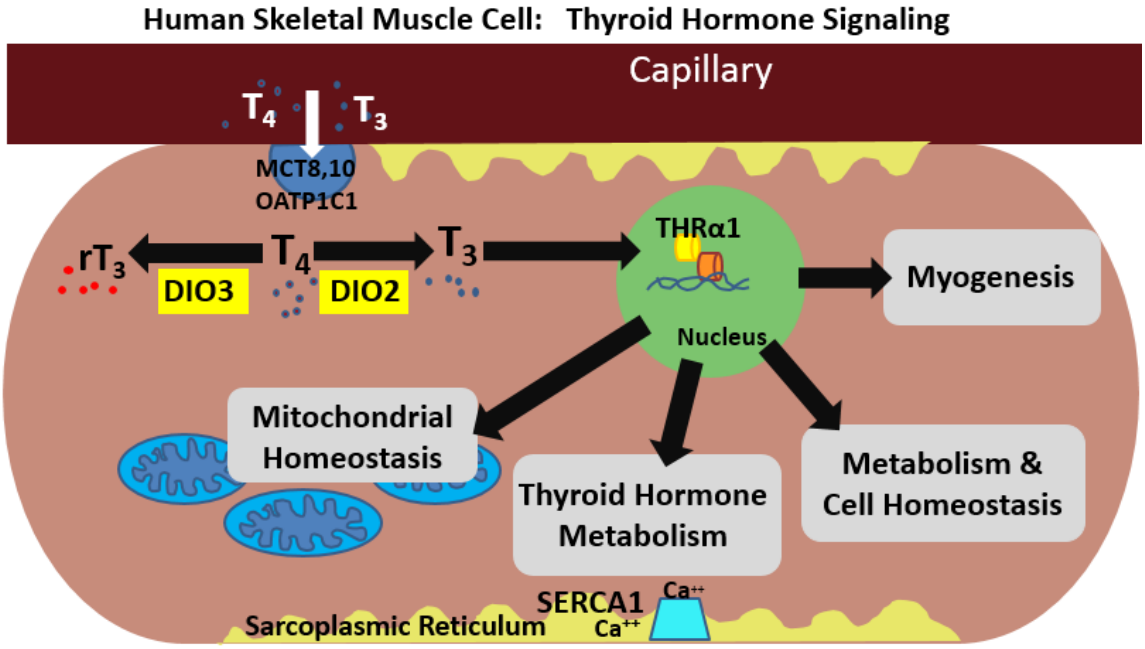

Figure 3. Thyroid hormone is transported into the SKM cell. T_4 is activated by deiodinase 2 (DIO2) into T_3. T_4 becomes inactive if converted into reverse T_3 by deiodinase 3 (DIO3). Active T_3 stimulates nuclear receptors in promotor regions of specific thyroid hormone-related target genes responsible for the regulation of metabolism, myogenesis, and calcium homeostasis.

Both T_4 and T_3 enter SKM cells (from here on we are focusing on subcellular effects of T4 and T3 and are referring to the unbound "free" versions of these hormones, as these are the forms able to enter the cell); however, only T_3 is active and able to bind to nuclear targets (Brent, 2012). Conversion and activation of T_4 into T_3 in SKM occurs by the enzyme, deodinase 2 (DIO2; Lee, Kim, & Milanesi, 2014). Activated T_3 stimulates TH nuclear targets specific to metabolism, contractility, and myogenesis by binding to thyroid hormone receptor-α (THRα) in SKM (Lee et al., 2014). Stimulation of specific gene targets by T_3 requires a TH response binding element (TRE) to be present on a short segment of DNA (Brent, 2012). It is here that the THRα is occupied by a heterodimer consisting of a retinoid x receptor (RXR) and a nuclear co-repressor (NCoR; Yamamoto, Kakuta, Miyachi, & Sugimoto, 2011). Once T_3 enters the nucleus, it will dislodge the NCoR and bind with THRα to stimulate gene transcription of those T_3-related targets, which influence SKM metabolism, contractility, and myogenesis (Kupr, Schnyder, & Handschin, 2017).

Peripheral SKM Hypothyroidism

Due to the complexity of TH interactions at the level of intracellular mechanisms, tissue-specific hypothyroidism diagnosis and treatment is challenging (Kansagra, McCudden, & Willis, 2010). Peripheral hypothyroidism, especially within SKM, occurs in tissues outside of the hypothalamic-pituitary-thyroid axis. Possible causes leading to peripheral hypothyroidism in SKM include faulty cell transporters (Dumitrescu &

Refetoff, 2015), overactive DIO3 activity elevating rT_3 levels (van Mullem, Visser, & Peeters, 2014), a genetic polymorphism in DIO2 inhibiting intracellular TH activation (Bianco & Kim, 2018), or a THRα mutation restricting transcription of nuclear targets (Bianco, Salvatore, Gereben, Berry, & Larsen, 2002). While these factors are not measurable via circulating blood markers, they may present as muscle symptoms related to hypothyroidism. Currently, literature elucidating these mechanisms is lacking and further research specifically investigating thyroid-related SKM signaling is warranted.

Effects of Exercise on SKM T_3 Targets

In SKM, exercise and T_3 both stimulate many of the same pathways related to metabolism, function, and myogenesis (Louzada & Carvalho, 2018; Mullur et al., 2014). In hypothyroidism, however, it is assumed that SKM transcripts typically upregulated by T_3 signaling are instead downregulated by the low availability of T_3 leading to impaired metabolism, function, and myogenesis (Bloise, Oliveira, Cordeiro, & Ortiga-Carvalho, 2018; Guglielmi et al., 2016; Salvatore et al., 2014). However, the research to fully substantiate the global and specific effects of hypothyroidism in human SKM is lacking. As displayed in Figure 4, the stimulatory effects of exercise are also important to consider for TH-related SKM metabolism and functional physiology.

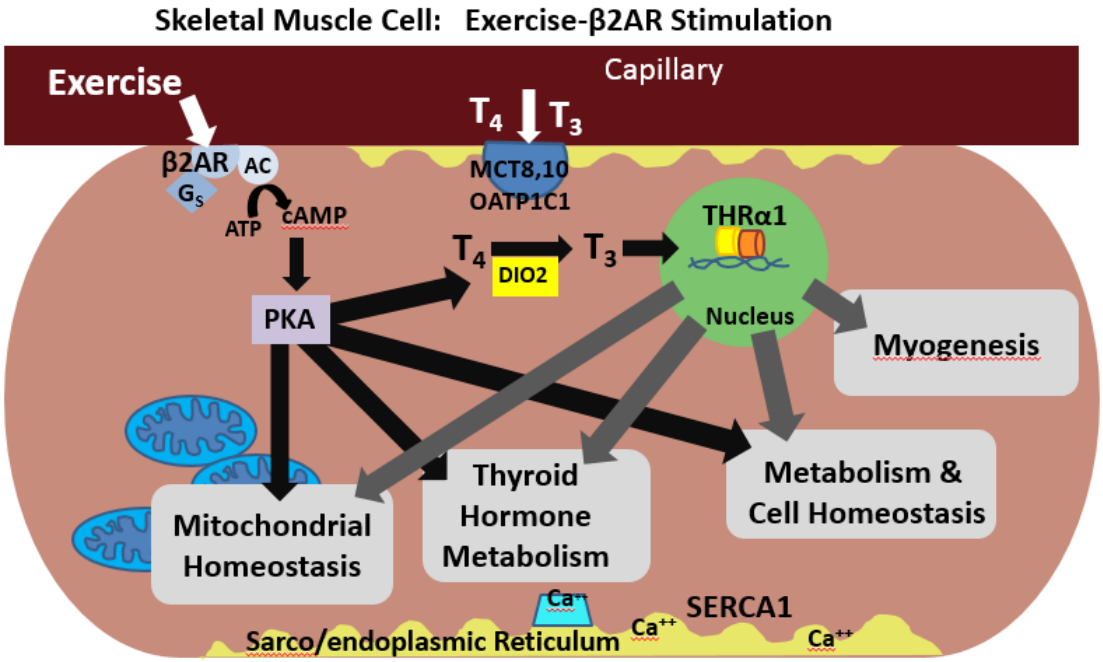

Figure 4. Exercise and thyroid hormone stimulation of SKM myocyte.

During exercise, the catecholamines, epinephrine and norepinephrine, are released into the blood and act as ligands, binding to β-2 adrenergic receptors (B2AR) on the SKM membrane. Stimulation of B2AR activates the cAMP-PKA pathway causing downstream effects on metabolism, function, and myogenesis. For a guide to the many effects of TH signaling and exercise regulation, refer to the overview of TH signaling and exercise regulation displayed in Table 1.

Table 1

Thyroid hormone signaling and exercise stimulation of SKM

Thyroid hormone Signaling	Thyroid hormone Action	Thyroid hormone Regulation	Exercise Regulation
DIO2	Conversion and activation of $T_4 \rightarrow T_3$	Downregulated by T_4 and T_3	Increases Activity
DIO3	Inactivates $T_4 \rightarrow$ rT3; early proliferation during myogenesis	Unknown if TH regulate DIO3?	Overtraining may increase DIO3 expression?
THRα	TH nuclear receptor in promotor regions of TH-responsive genes	Increases expression	Increases activity
PGC-1α	Mitochondrial biogenesis, FA oxidation	Increases expression and activity	Increases expression and activity
AMPK	Energy sensor of myocyte	Increases activity	Increases activity
GLUT4	Glucose transport into myocytes	Increases expression and translocation	Increases expression and translocation
SERCA1	Type II twitch phenotype Ca^{++}ATPase	Increases expression	Increases expression
SERCA2a	Type I twitch phenotype Ca^{++}ATPase	Increases expression	Increases expression
MYOD1	Myoblast proliferation & differentiation	Increases expression	Increases expression
Myogenin	Myoblast differentiation	Increases expression	Increases expression

Exercise and TH activation and nuclear transcription. Perhaps the most-studied TH intermediaries are DIO2 and THRα. Their actions predict the efficacy of TH on metabolism, function, and myogenesis and they have the most potential to improve intracellular mechanisms related to TH signaling. DIO2 conversion and activation of T_4 to T_3 is associated with the cAMP-PKA and AMPK pathways (Egan et al., 2010; Lira, Benton, Yan, & Bonen, 2010). Stimulation of the B2AR by catecholamines leads to cAMP-PKA pathway upregulation causing DIO2 activities and expression to increase (Bocco et al., 2016; Narkar et al., 2008) and in fact, cAMP signaling is the main determinant of DIO2 transcription (Drigo, Fonseca, Werneck-de-Castro, & Bianco, 2013). Also of note, SKM is the main site of extra-thyroidal T_3 production because of its mass and DIO2 activity (Maia, Kim, Huang, Harney, & Larsen, 2005) and yet, 15% of the population may have a DIO2 gene mutation affecting local TH concentrations (Drigo et al., 2013).

The conversion and activation of TH by DIO2 is also implicated in myogenesis (Dentice et al., 2010). Forkhead box O3 (FOXO3) induces the expression of DIO2, thus stimulating TH conversion during early satellite cell activation (Dentice et al., 2010). In opposition, DIO3 maintains the quiescent satellite cell state restricting TH activation (Ambrosio et al., 2013). Low TH may delay and impair the myogenic process due to the changes in expression of T_3 –dependent myogenic regulatory factors (MRFs; Bloise et al., 2018). Until more recently, it was thought that DIO3 activity was not present in adults; however, it was found that inflammatory illness and postinjury induce elevated DIO3

expression in adults causing conversion of rT_3 (Huang & Bianco, 2008). Two other TH-related illnesses involving central and local dysregulated deiodinase activity, non-thyroidal illness syndrome and low T_3 syndrome, mimic hypothyroidism and exhibit reduced DIO2 activation of T_4 and increased rT_3 generation by DIO3 (Dentice et al., 2010). The T_3: rT_3 is an available blood lab test, which can assess DIO3 activity and may indicate impaired intracellular TH activation.

In SKM, THRα is recognized as the dominant nuclear receptor and responsible for much of the genomic T_3 signaling, especially in type 1, oxidative fibers (Bahi et al., 2005). Stimulation of the targets glucose transporter (GLUT4), sarco/endoplasmic reticulum Ca^{2+}-ATPase 1/2a (SERCA 1/2a) and type II fast fiber phenotype isoforms, myosin heavy chain 1, 2, and 4, and MRF are all dependent upon T_3 stimulation of THRα (Milanesi et al., 2016) The stimulation of THRα by T_3 has direct effects on metabolism, function, and growth of SKM as does exercise. This is mainly due to the stimulatory effects on PGC-1α, a master regulator of mitochondrial biogenesis, which also has important effects on the transcription of genes related to the aforementioned metabolic functions. The expression of THRα is increased with exercise (Kinugawa et al., 2001) leading to downstream effects on the same transcripts. Interestingly, the only other known upregulator of THRα expression is myogenic differentiation 1 (MYOD1) during myogenesis (Busson et al., 2006). There is a negative feedback loop between THRα and MYOD1 whereby, MYOD1 expression is suppressed by THRα in the absence of T_3 (Daury

et al., 2001), yet MYOD1 promotes THRα expression (Busson et al., 2006). As will be discussed further, exercise also upregulates myogenesis and MYOD1, thus providing another pathway to increase nuclear transcription activity (Caldow et al., 2015).

Exercise and SKM metabolism (PGC-1α & AMPK). In SKM, the two principle regulators of metabolism associated with both exercise and TH stimulation are peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and 5' adenosine monophosphate-activated protein kinase (AMPK). As a member of the peroxisome proliferator-activated receptor family, PGC-1α has multifaceted transcription capabilities. Catecholamine stimulation of the B2AR stimulates expression of PGC-1α, thus upregulating many downstream gene targets (Egan et al., 2010; Lira et al., 2010; Miura et al., 2007). Outside its conical role as a major regulator of mitochondrial biogenesis, PGC-1α carries other duties within myocytes including modulating proteins related to oxidative phosphorylation and fatty acid oxidation (Bonen, 2009), angiogenesis (Chinsomboon et al., 2009), and fiber phenotype modifications (Zhang et al., 2017) with many new discoveries emerging in recent years. The regulation of the expression of PGC-1α differs between humans and rodents. In rodents, PGC-1α is stimulated directly by T_3 in the nucleus; however, this is not the case in humans (Barbe et al., 2001). Upregulation of PGC-1α occurs indirectly via T_3 stimulation of AMPK, an upstream mediator of PGC-1α (Irrcher, Walkinshaw, Sheehan, & Hood, 2008) and transcriptionally, T_3 stimulates the mitochondrial truncated form of THRα, p43 leading

to mitochondrial nuclear transcription of PGC-1α (Casas et al., 1999; Lombardi et al., 2015; Psarra, Solakidi, & Sekeris, 2006).

During exercise, the rapid changes in energy requirements are mediated by AMPK. Known as energy sensor of the SKM myocyte, AMPK is the master of SKM energy homeostasis (Viollet, 2018). The adenosine diphosphate (ADP): adenosine triphosphate (ATP) and adenosine monophosphate (AMP): ATP provides the signals indicating fuel levels within the cell. During exercise, the increasing ratio of ADP: ATP leads to an elevated AMP: ATP as the result of the adenylate kinase reaction which activates AMPK regulation (Hardie, Ross, & Hawley, 2012; Richter & Ruderman, 2009). There are widespread downstream effects of AMPK on catabolic and anabolic pathways important for maintaining energy status (Kjobstead et al., 2018). As discussed previously, in the cell, T_3 exerts direct stimulation on AMPK, thus positively impacting its downstream effects (de Lange et al., 2008; Irrcher et al., 2008). Signaling from AMPK activates GLUT4 translocation to the plasma membrane (Ren, Semenkovich, Gulve, Gao, & Holloszy, 1994; Richter & Hargreaves, 2013). The energy and contractile requirements of SKM during exercise are strong stimulators of GLUT4 expression (Richter & Hargreaves, 2013) as is T_3. There is a THRα in the promotor region of GLUT4 stimulated by T_3 (Brunetto, Teixeira, Giannocco, Machado, & Nunes, 2012) and Teixeira et al. (2012) found that T_3 stimulation alone was able to increase translocation of GLUT4 to the SKM plasma

membrane unrelated to transcription. Low stimulation by intracellular T_3 negatively affects GLUT4 expression and translocation (Brenta, 2011).

Exercise and calcium homeostasis. Tightly regulated calcium homeostasis is imperative for the function of SKM. The prime roles of SKM are posture and movement requiring optimal regulation of contractile speed and duration. Intracellular calcium handling is controlled in the sarcoplasmic reticulum (SR) by the SERCA. There are two main isoforms of SERCA. In SKM, SERCA1 is most abundant in the fast twitch fiber phenotype while SERCA2a dominates the slow twitch phenotype (Morissette et al., 2014). Both exercise and T_3 upregulate SERCA expression in SKM (Kubo et al., 2003; Salvatore et al., 2014). In HT, many individuals experience SKM myopathy causing symptoms like cramping and tightness that worsen with exercise (Bloise et al., 2018). Poor calcium sequestration into the SR may delay relaxation rates causing "pseudohypertrophy" which has been reported in those with hypothyroidism (Klein, Parker, Shebert, Ayyar, & Levey, 1981).

Exercise and myogenesis. In myogenesis, quiescent satellite cells progress to mature myofibers. Myogenesis is recognized predominantly during growth as in the fetal stage (Yan, Zhu, Dodson, & Du, 2013), regeneration after injury (Musaro, 2014), and acute bouts of exercise (Caldow et al., 2015). The growth of SKM myocytes is dependent upon transcription of MRF. Exercise has been found to increase the expression of the MRF, MYOD1 and myogenin (Drummond et al., 2010; Wilborn, Taylor,

Greenwood, Kreider, & Willoughby, 2009). We have found similar effects on MYOD1 and myogenin expression utilizing acute and chronic exercise mimetic treatment on human SKM cells in culture in our lab. The growth of SKM cells is dependent upon TH during myogenesis (Hughes et al., 1993; Muscat, Mynett-Johnson, Dowhan, Downes, & Griggs, 1994). In a study by Dentice et al. (2010), FOXO3 was found to induce DIO2 activation leading to the downregulation of DIO3 and the activation of T_3, thus stimulating the expression of MYOD1 and myogenin. Therefore, both exercise and TH have pivotal roles in SKM growth and development.

Exercise Mimetics and Dose Responses in SKM

Many *in vitro* and *in vivo* investigations have utilized the stimulatory effects of exercise mimetics to elucidate SKM cellular pathways. Stimulation of the B2AR upregulates the cAMP-PKA pathway leading to downstream effects similar to exercise (Miura et al., 2007). The exercise mimetic, Formoterol (FORM), is a B2AR agonist and has been shown to upregulate PGC-1α with resultant increases in mitochondrial biogenesis, cellular respiration, and reduced inflammation (Duplanty, Simon, & Molina, 2018; Wills et al., 2012). Based on preliminary results from our lab using a human SKM cell culture model and *in vitro* FORM treatments, we found significant increases in the expression of PGC-1α and genes related to TH signaling, mitochondrial regulation, and myogenesis.

This dissertation proposal is the culmination of several projects beginning with a comprehensive survey of women with hypothyroidism (Guerin et al., 2019 in submission – *Women and Health*). The survey garnered 1,093 respondents of which 580 qualified for the study investigating the effect of exercise on SKM symptoms. The results showed that women currently under treatment for hypothyroidism experience significant SKM symptoms both at rest and during exercise. Also of note, those women performing a combination of aerobic exercise and resistance training weekly had significantly ($p < .05$) lower symptoms than those performing only aerobic exercise, only resistance training, or no exercise at all. Specific to the generation of this study, we have characterized the effects of FORM on gene expression related to thyroid signaling, mitochondria, myogenesis, and metabolic signaling in human SKM cells (data in preparation for publication). Our model of investigation is focused on collecting data during mid-myogenesis (established by our lab as Day 4 of myotube differentiation) and terminal differentiation (mature myotubes at Day 6), which allows for a translational approach when comparing this model to *in vivo* stages of growth and repair. From these previous studies, we have evidence to support the feasibility of this study that aims to establish and characterize a SKM cell culture model of hypothyroidism. This dissertation will inform future investigations including *in vivo* human studies involving exercise modalities to reduce the detriments and symptoms of hypothyroidism.

Statement of the Problem

Even though diagnosis and treatment guidelines for hypothyroidism are unambiguous, many individuals do not find relief of SKM symptoms after treatment. The standard blood tests detect circulating TH levels while the intracellular TH dysregulation continues (Kansagra et al., 2010). Impairment in SKM metabolism, bioenergetics, and contractility, lead to pain, fatigue, weakness, tightness, and stiffness (Bloise et al., 2018; Duyff et al., 2000; Sindoni, Rodolico, Pappalardo, Portaro & Benvenga, 2016). Based on preliminary data from our lab and the 2018 national survey conducted by the ATA (Peterson et al., 2018), individuals reported significant symptoms while under treatment for hypothyroidism due to the insufficient stimulation of TH nuclear targets adversely affecting metabolism, function, and growth in SKM (Salvatore et al., 2014). This is particularly pronounced during exercise when SKM symptoms may be exacerbated (Bloise et al., 2018; Guerin et al., 2019, in submission), thus potentially limiting movement and leading to sedentary behavior. Inactivity may worsen the illness by further reducing metabolism leading to insulin resistance and glucose intolerance and increasing the risk of developing co-morbidities like CVD and DM (Vyakaranam, Vanaparthy, Nori, Palarapu, & Bhongir, 2014). In hypothyroidism, exercise as a therapeutic treatment has been overlooked possibly due to metabolic and functional detriments reported (Bloise et al., 2018; Finsterer, Stollberger, Grossegger, & Kroiss, 1999; Kahaly, Kampmann, & Mohr-Kahaly, 2002; Monzani et al., 1997; Reuters et al.,

2009). However, first and foremost, exercise is a powerful stimulator of SKM metabolism, function, and growth; thus, potentially abating hypothyroidism impairment and, by virtue of these effects, exercise should be considered as treatment.

Hypotheses

Based on preliminary data from our lab and reports in the literature, we hypothesized that hypothyroidism results in many connected and unexplored metabolic effects on SKM health. Further, we hypothesized that exercise can be an effective treatment to attenuate potential detrimental effects. Therefore, this study used a human SKM cell culture model to investigate the effects of low TH availability and exercise stimulation on genes related to SKM metabolism, function, and myogenesis. To test these hypotheses, the specific aims of this study were:

AIM 1: Using an *in vitro* model, myocytes were cultured in TH depleted media and the effects on genes related to metabolism, myogenesis, and mitochondrial function within the myocyte were assessed.

AIM 2: Using an *in vitro* model of hypothyroidism in SKM, the differential effects of exercise mimetic stimulation in a TH depleted environment was tested on gene targets related to TH and exercise SKM signaling.

Significance

The majority of the *in vitro* and *in vivo* research on hypothyroidism has been conducted on specific pieces of the cellular puzzle, predominantly in rodent models.

These models have significant differences in TH metabolism versus human models. This study used human SKM myocytes to more closely replicate the human model of hypothyroidism. This study focused on putting the smaller pieces of cell signaling pathways together to see the bigger picture of the connections between thyroid metabolism, exercise stimulus, and factors that influence SKM health. The results from this study will inform future investigations on the overall effects of hypothyroidism on SKM health and provide insights on the potential therapeutic benefits of exercise.

CHAPTER II

LITERATURE REVIEW

Individuals with hypothyroidism report significant SKM symptoms like pain, fatigue, weakness, tightness, and cramps collectively known as hypothyroid myopathy resulting from intracellular metabolic and functional impairment (Bloise et al., 2018). In a case study of severe hypothyroidism, Finsterer, Stollberger, Grossegger, and Kroiss (1999) found extremely elevated creatine kinase values (9,000 U/L), fatigue, gait disturbance, myxedema, and reduced deep tendon reflexes. Fortunately, after treatment with T_4 for 3 months, the symptoms subsided except for sensory disturbances lingered. Even in those with subclinical hypothyroidism, clinical and biochemical changes to SKM were found by Monzani et al. (1997). In this *in vivo* study, the participants reported neuromuscular complaints such as fatigue, cramps, paresthesia, and weakness. An exercise protocol was performed using a handgrip dynamometer with 1-min bouts of maximal voluntary contraction followed by 2 min rest with blood glucose, lactate, and pyruvate concentrations collected at rest and during each interval. At rest, blood glucose, blood lactate, and pyruvate levels were the same between the subclinical participants and controls; however, during exercise, the blood lactate and pyruvate levels were significantly higher in the subclinical participants. Based on the findings from

the study and thyroid disease history and treatment, Monzani et al. (1997) concluded that early treatment to resolve metabolic defects might stave off the onset of overt hypothyroidism.

Similarly, in a study by Khushu, Rana, Sekhri, Sripathy, and Tripathi (2010), SKM bioenergetics were investigated in the calf muscles of hypothyroid, hyperthyroid, and control participants. Participants performed plantar flexion against a pedal timed with a metronome while inside nuclear P magnetic resonance spectroscopy (MRS). Every 15 s, MRS analysis was performed measuring metabolic ratios for phosphocreatine/inorganic phosphate, phosphocreatine/ATP, Pi/ATP, and phosphodiesters/ATP during rest, exercise, and recovery. During rest, the hypothyroid phosphocreatine/Pi was decreased, while the Pi/ATP, and phosphodiesters/ATP were increased and the hyperthyroid participants had increased phosphocreatine/Pi and decreased Pi/ATP, and phosphodiesters/ATP demonstrating that TH status affected energy homeostasis during exercise and that hypothyroidism decreased oxidative capacity by 50% indicating dysfunction in mitochondrial high energy phosphate metabolism.

In a study by Duyff et al. (2000), the neuromuscular symptoms in hypothyroid patients were characterized. Neuromuscular complaints were reported in 79% of patients with hypothyroidism with 38% having SKM weakness as confirmed by manual muscle testing. After 1 year of physical therapy, 13% of hypothyroid patients still experienced weakness and myopathy. Results of these studies confirm hypothyroidism

is problematic in SKM and may not be resolved easily with treatment by TH replacement. There have been few studies that have utilized biopsies from individuals with hypothyroidism to examine the metabolic and functional detriments of hypothyroidism. However, to investigate the real-time effects of exercise and TH dysregulation in SKM, use of biopsies would be the most illuminating method. However, the potentially impaired regeneration in SKM postbiopsy may pose a significant risk and therefore inhibit this model of research. Alternative models to examine the effects of impaired TH-related signaling in SKM utilize cell culture and rodent experiments (*in vivo* and *in vitro* models).

Investigations utilizing exercise mimetics to stimulate β_2-adrenergic (epinephrine and norepinephrine) receptors *in vitro* have informed on the exercise upregulation of SKM intracellular pathways *in vivo*. The exercise mimetic, formoterol (FORM) has been used successfully in SKM research to stimulate the β-adrenergic receptors-cAMP-PKA pathway (Duplanty et al., 2018; Wills et al., 2012). In a study by Lynch and Ryall (2008), the exercise mimetic FORM was used as treatment to determine its effects on aging rats, rats with cancer cachexia, and mice with muscular dystrophy. The study found that those animals treated with FORM had increases in body mass, SKM mass and strength, and fast twitch fiber type. These findings were also found in a study by Conte et al. (2012) of aging rats and SKM regeneration postinjury. Using FORM increased the phosphorylation of mammalian target of rapamycin (mTOR) leading to increases in

protein synthesis, cross-sectional size, and force production. Mirroring these findings, Ametller et al. (2011) found that muscle wasting in rats caused by cancer cachexia could be treated with FORM and showed significant improvement in SKM mass and protein. Also of note, the expression of myogenic regulatory factors, MYOD1 and myogenin were also increased indicating muscle regeneration and satellite cell activation. As seen in these studies, the use of exercise mimetics to stimulate SKM cells provides a method to investigate the effects of exercise on the regulation of subcellular metabolism, function, and growth.

Investigating the cause of hypothyroid SKM symptoms involves examining the effects of low TH availability on TH-related target genes and proteins. Both knockout (KO) and mutations to key TH mediators have been developed to complete these investigations. In an early deiodinase 2 (DIO2) study conducted by Hosoi et al. (1999), human SKM cells in culture were used to investigate DIO2 activity and mechanisms responsible for its regulation. The cells were treated with forskolin or $(Bu)_2$cAMP for 6 hr and then mRNA and total RNA was extracted and polymerase chain reaction (PCR) and Northern blot techniques were used for analysis. The activity of DIO2 was significantly stimulated by Forskolin (10^{-5} mol/L). It was concluded that DIO2 activity is indeed stimulated via the cAMP pathway in SKM. Next, the β-adrenergic agonists, isoproterenol or norepinephrine were also used to examine the effects on DIO2. Both the activity and mRNA of DIO2 were rapidly increased indicating β-adrenergic mechanisms have a role in

the regulation of DIO2 expression. Lastly, TH was added to the culture to examine the effects of TH on DIO2. The activity of DIO2 was inhibited by T_4 and reverse T_3, however, T_3 inhibited the expression of DIO2; therefore, a negative feedback loop downregulates DIO2 activity in the presence of TH. In hypothyroid rodents, DIO2 activity increases due to the negative feedback to compensate for low intracellular TH levels; however, this action has not been found in humans and the underlying mechanisms are in need of further investigation (Heemstra et al., 2009).

In a study by Grozovsky et al. (2009), both human and rodent primary myoblasts were cultured to examine the effects of pioglitazone, a peroxisomal proliferator-activated receptor-γ (PPAR-γ) agonists on DIO2 activity. After 8 hr of treatment with pioglitazone, DIO2 activity in the human SKM cells increased 1.7 to 1.9 fold with similar effects using other PPAR agonists, ciglitazone and troglitazone, resulting in 1.6-fold increases after each treatment. Next, rodent myoblasts from DIOKO and wild-type mice treated with forskolin. The DIO2 activity significantly increased in the myotubes as did iodide release (10-fold) and levels of both T_2 and T_3. After 24 hr of pioglitazone treatment, the DIO2 activity in myotubes increased 2.7-fold at 5 ηM and 1.7-fold at 10 ηM. Grozovsky et al. (2009) concluded that PPAR-γ ligands upregulate DIO2 in SKM and that their effects may alter TH signaling; a PPAR response element in the DIO2 promotor region reinforces this conclusion (Fiorito et al., 2007). Due to the normally very low activity of DIO2 after peak expression (48 hr differentiation [Dentice et al., 2010]), it was

suggested that the DIO activation of T_3 may not significantly add to plasma levels and this may be the case with long-term, high intensity exercise stimulation in humans. The intracellular TH demands decrease plasma T_4 during and for a short time after exercise with T_3 plasma levels decreasing over the course of several weeks of intense training (Arkader, Rosa, & Moretti, 2016; Ciloglu et al., 2005). The effect of exercise upregulating DIO2 activity leading to increases in both circulating and intracellular levels of TH research is lacking and more investigation is needed in this area.

In a seminal study by Bocco et al. (2016), the effects of DIO2 activation of TH and exercise performance were investigated. DIO2 conversion of TH provides the only extra-thyroidal T_3 production and as discussed this occurs primarily in myogenesis and during exercise in SKM (Salvatore, Bartha, Harney, & Larsen, 1996). In this study, adult male Wistar rats, SKM-DIO2KO mice, MYF5-DIO2KO mice were used. The rats performed treadmill exercise for 20 min at 70-75% of maximal speed as predicted from testing. Some rats were injected with the β-adrenergic receptor antagonist, propranolol, 1 hr prior to exercise to reduce the effects of exercise on PGC-1α post exercise. The control group received Iopanic acid to inhibit DIO2 activity 2 hr prior to exercise. The VO_{2Max} of both groups was the same. The mice performed either acute 20 min bouts of exercise similar to the rats or chronic treadmill exercise 5 days per week for 30 min at 55-60% of maximum speed for 6 weeks.

The DIO2 activity of the exercise group of rats increased 2 hr after exercise 2.4-fold in the slow twitch soleus muscle (ST) while the DIO2 expression increased 2.7-fold in the fast twitch white gastrocnemius muscle (FT). PGC-1α expression increased 5.5-fold in the ST and 1.8-fold in the FT. By blocking the effects of β-adrenergic stimulation with propranolol, there was no upregulation of DIO2 and PGC-1α, and serum T_3 was reduced by 25% at 30 min of exercise but returned to baseline 2 hr after exercise. It was concluded that β-adrenergic stimulation by exercise induces DIO2. Regarding PGC-1α mediation by DIO2 activity, the use of Iopanic acid to block 100% of DIO2 activity during exercise reduced PGC-1α by 50% at 2 hr post-exercise in the ST and 30% in FT. In the exercise group of mice, acute treadmill exercise induced a 1.5-fold increase of DIO2 expression in the ST and increased PGC-1α expression 1.6-fold after 1 hr of exercise and 3.8-fold after 2h of exercise without any changes in TH serum concentrations. In the SKM-DIO2KO mice, there was a reduction of 60% DIO2 activity at baseline in ST yet there was no reduction in maximal exercise capacity. After acute exercise, DIO2 activity increased in both the SKM-DIO2KO (1.8-fold) and control (2.8-fold) in ST.

Next, In the SKM-DIO2KO mice, PGC-1α expression was 35% lower than controls in the ST, while the PGC-1α increased 1.3-fold in FT of controls. There was no change in PGC-1α expression in the SKM-DIO2KO mice. Taken together, it was found that DIO2-mediated T_3 activation induced PGC-1α expression in SKM. It is important to note, however; that T_3 does not directly upregulate PGC-1α in humans as found in rodents. In

humans, PGC-1α is upregulated via AMPK and p43 in humans (Barbe et al., 2001). Further, in chronic exercise conditions, both SKM-DIO2KO and controls completed the 6 weeks of exercise. PGC-1α expression increased 25% in the ST of the controls but not the SKM-DIO2KO mice. In the FT muscles of both controls and SKM-DIO2KO, PGC-1α expression did not increase.

The key points of this comprehensive study are: a) exercise stimulation of β-adrenergic receptors induced DIO2 in SKM, b) exercise and TH stimulated PGC-1α, c) blocking DIO2 in SKM reduced PGC-1α expression, and d) DIO2 disruption lead to decreases in PGC-1α in chronic exercise conditions. Clearly, there is an association between DIO2 and PGC-1α, however, as will be discussed in the Werneck-de-Castro et al. (2015) and Carmondy et al. (2019) studies, the effects on downstream targets like PGC-1α may not entirely be attributed to DIO2.

Additional investigations on DIO2 and its effects were conducted by Werneck-de-Castro et al. (2015) and Carmody et al. (2019). Both studies used DIO2KO mice and found that the changes to downstream DIO2 targets may not be from the lack of DIO2 expression and activity. Werneck-de-Castro et al. (2015) found that despite the lack of SKM DIO2, the mice performed treadmill testing as well as wild type and in excised SKM tissue, the genes related to T_3 signaling (SERCA 1, SERCA2, myosin heavy chain 1, myosin light chain, α-actin, and tropomyosin) were mostly unaffected by the lack of DIO2 as was the intracellular T_3 level. Werneck-de-Castro et al. (2015) concluded that there must

be other sources of DIO2 inside SKM i.e. fibroblasts, brown adipose tissue, smooth muscle cells, etc. that may affect SKM DIO2. Finally, Werneck-de-Castro et al. (2015) concluded DIO2 is more important during myogenesis than in adult myofibers as its expression decreases to almost undetectable levels.

One possible reason for the low DIO2 activity and expression in adult myofibers may be the overstimulation and excess conversion of TH potentiated by physical work or exercise. In early stages of growth, DIO2 and T_3 are necessary to drive the myogenic program. Once terminal differentiation has been attained, the metabolic and functional mechanisms operate in homeostasis and do not require strong signals for daily processes. However, if demands by metabolic and functional processes far exceed normal homeostasis, as in heavy exercise or physical labor and if DIO2 expression and activity were to remain elevated (as in myogenesis), then the increased conversion process would undoubtedly lead to TH excess or depletion of intracellular T_4.

In a similar conclusion, Carmody et al. (2019) found that the effects of low intracellular TH conversion in the SKM DIOKO mouse model were of less consequence than the 50% reduction of PGC-1α expression downstream. The SKM fiber phenotype and contractile changes were caused by the decrease in PGC-1α and not from the DIO2 lack of conversion of TH within the cell.

In a study by Wouters et al. (2017), it was found that about 10% of the population and 11% of individuals with hypothyroidism have the DIO2 gene mutation,

Thr92Ala, as has been confirmed by other studies as well (Mentuccia et al., 2002). The Thr92Ala polymorphism causes reductions to DIO2 activity and intracellular T3 pools (Castagna et al., 2017), yet Wouters et al. (2017) concluded that the treated patients and the general population in the study did not have significant differences in circulating TH levels or comorbidities. These studies taken all together demonstrate that exercise stimulation via β_2-adrenergic receptors increases the role of DIO2 as a TH mediator and as an upstream and downstream target of PPAR/PGC-1α, thus assisting in both TH-related gene expression and metabolism in general.

The connections being made between SKM TH activation and signaling directly impact its function and focusing on calcium homeostasis may elucidate the impact of hypothyroidism on SKM contractility. In individuals with hypothyroidism, low T_3 availability effects SKM contractility by its transcription of the sarco/endoplastic reticulum Ca^{2+} ATPase isoforms SERCA1 and SERCA2a. In a study by van der Linden et al., (1996), the soleus and extensor digitorum longus muscles from hypothyroid, euthyroid, and hyperthyroid treated rats were processed to investigate the SERCA isoforms. Both fast twitch (myosin heavy chain II) and slow twitch fibers (myosin heavy chain I) were examined based on thyroid status. In both muscles, the absence of T_3 stimulation lead to the development of slow twitch fiber phenotype which was then affected by T_3 stimulation towards a fast twitch phenotype. There was a full conversion of one-half the slow twitch fibers to fast twitch phenotype and the other half of the slow twitch fibers

were partially converted to fast twitch causing a mixed phenotype. T_3 increased the total SERCA expression in the SKM fibers was indicated by the upregulation of SERCA activity. The stimulation of SERCA1 by T_3 leads to a rise in Ca^{2+}ATPase activity and a downregulation of SERCA2a expression. Similarly, in a study by Sayen, Rohrer, and Dillman (1992), thyroid status determined the SERCA isoform expression. These studies show the importance of T_3 in fiber type and contractile function. Unfortunately, the SKM symptoms do not always respond as well to TH treatment in humans and this research needs more emphasis.

In hypothyroidism, the metabolic impairments may increase the risk of developing glucose intolerance and insulin resistance in SKM. Exercise has been an effective treatment to increase glucose disposal in individuals with glucose dysregulation primary by the stimulation of glucose transport into SKM by AMPK and contractile activities (Sylow, Kleinert, Richter, & Jensen, 2017). Glucose transporter 4 (GLUT4) is a non-insulin dependent glucose transporter specific to SKM and assists in the disposal of more than 85% of plasma glucose (DeFronzo, Ferranninni, Sato, Felig, & Wahren, 1981). Torrence, Devente, Jones, and Dohm (1997) examined the effect of T_3 on GLUT4 expression. Male, Sprague-Dawley rats (250-275 g) and Zucker rats (lean, 304 $\pm$ 17 g; obese, 393 $\pm$ 9 g) were used. Hypothyroidism was induced in the Sprague-Dawley rats with propylthiouracil administered in their drinking water. Animals were injected daily with T_3 (100 μg/100 g body weight) for 4 hr, 3 days, or 10 days with final

injections 4 hr prior to death. Controls received a sham injection. Gastrocnemius and quadriceps muscles were removed and processed for RNA or protein. Nuclei were isolated from SKM. The Sprague Dawley rats with hypothyroidism treated with T_3 were also utilized to examine the time line of T_3 stimulation of GLUT4 transcription and mRNA. The expression of GLUT4 was found to be higher at 10 days of treatment T_3 treatment solely in the red fibers of the gastrocnemius and quadriceps muscles and nuclear run-on analysis of GLUT4 transcription showed a 2.5-fold increase also only in red muscle. Next, total membrane protein from the same muscle preparations was analyzed and resulted in 5-fold GLUT4 protein expression increases in both red and white SKM fibers with 10 days of T_3 treatment. Regarding the lean and obese Zucker rats, T_3 stimulation did not significantly increase GLUT4 in the lean rats with 3 days of treatment; however, in the obese rats, there was a 2-fold increase in GLUT4 mRNA. Interestingly, the GLUT4 protein expression in obese rats was 75% lower than the lean rats, yet with 3 days of T_3 treatment, there was a 3-fold increase in the GLUT4 protein. The lean rats treated with T_3 also experienced a 2-fold increase in GLUT4 protein. Torrance et al. (1997) concluded that GLUT4 gene expression is induced by transcription, especially in red fibers with other mechanisms contributing to white SKM GLUT4 increases. The stimulation of GLUT4 expression and protein by T_3 may have therapeutic effects in cases of obesity and disease, which have implications for hypothyroidism.

The effects of T_3 on GLUT4 go beyond stimulating expression. In a cell culture model using TH depletion, Teixeira et al. (2012) demonstrated, using L6 rat myotubes, that T_3 treatment rapidly activates GLUT4 transporters and increases glucose uptake at the cell membrane. The TH depleted cells that did not receive T_3 treatment, decreased glucose uptake into the cells was found and there was lower GLUT4 protein content at the surface versus controls. To stimulate the uptake of glucose into the myotubes, the cells were treated with T_3 for 30 min. The GLUT4 cells treated with T_3 had significant increases in glucose uptake versus the untreated, control type cells. The total amount of GLUT4 protein was not changed by the absence of T_3 or the treatment with T_3 until 40 min, whereby an increase in GLUT4 protein content was seen. GLUT1 protein was not affected by T_3 stimulation. The translocation of GLUT4 and subsequent uptake of glucose were increased with T_3 stimulation. In hypothyroidism, reduced metabolism contributes to glucose intolerance (Peppa, Koliaki, Nikolopoulos, & Raptis, 2010) and this is compounded by reduced GLUT4 expression (Torrance, Devente, Jones, & Dohm, 1997) and reduced GLUT4 translocation. It can be postulated that low T_3 will negatively affect the uptake of glucose due to the lack of stimulatory effects on GLUT4. Exercise is a viable way to improve glucose disposal while upregulating the expression of key anti-inflammatory regulators like PGC-1α.

The classic pathway of TH signaling in SKM cells involves nuclear stimulation of THRα by T_3. Milanesi et al. (2016) used a mouse, C2C12, cell culture model with THRα

and THRβ knockouts for this experiment. Cells were stimulated by T_3 or a THRβ agonist to investigate the effects on THRα myogenic targets. The T_3-stimulated myoblasts displayed increased differentiation, including myofiber number, and spontaneous contraction versus those simulated by the THRβ agonist which were delayed in development. The cells with THRα knockout presented reduced proliferation and differentiation compared to controls. The expression of PAX7, myoblast determination protein (MyoD), myogenic factor 5 (Myf5) was reduced more than 50% in the THRα knockout myoblasts. Another experimental model included mice with THRα and THRβ mutation. In this model, mice with the THRα mutation had significantly lower type II fibers and PAX7 expression versus both THRβ mutation and wildtype mice. Lastly, muscle injury was induced in THRα and THRβ mutated and wild type mice. The THRα mutation led to reduced proliferation, decreased expression of MyoD and Myf5, smaller myofibers and cross-sectional area, and fewer nuclei versus both the THRβ mutated and wild type mice. The results from this study show the importance of THRα to myogenesis and the health of SKM cells. Impairment during myogenesis may continue into poor mature SKM quality and health.

During healthy myogenesis, DIO2 and T_3 activity is imperative for inducing the expression of myogenic factors. In a study by Dentice et al. (2010), the importance of DIO2 activation of T_3 and its role in myogenesis was investigated. *In vivo* and *in vitro* mouse models were used for this study. DIO2 expression in adult mice was found to be

low, while in newborn mice, DIO2 was highly expressed, but decreased rapidly over the next 12 days until it was lower than the DIO2 expression in the adults. There were similar findings for the expression of PAX7. In the same study, cell culture experiments were used to further examine SKM myoblast proliferation and differentiation. DIO2 expression was discovered to be increased during differentiation. Further, inducing DIO2 expression during differentiation caused potent increases in intracellular T_3 and MYOD1 expression. Also, mice with SKM DIO2KO exhibited hypothyroidism impairments with decreases in both expression and protein of MYOD1, myogenin, myosin, and myosin heavy chain 2.

Even in the presence of T_3, DIO2KO mice had effects of mild hypothyroidism on MYOD1, troponin1/2, and SERCA2. Therefore, DIO2 is necessary for normal differentiation during myogenesis. The regulation of DIO2 expression during differentiation in SKM is controlled by the gene forkhead box O3 (FOXO3); however, in DIO2KO, FOXO3 did not induce differentiation. The study also found that post-injury, the myonuclei of DIO2KO remained centrally located much longer than in wild type mice that had already moved to the periphery by 15 days showing delayed recovery. The FOXO3/DIO2 pathway regulates local T_3 production and the myogenic program. In hypothyroid myocytes, myogenesis may be impaired due to low availability of T_3 and depressed DIO2 activity thus reducing the expression of MYOD1 and myogenin delaying the myogenic process, further, low intracellular T_3 post-injury may lead to impaired or

incomplete healing. More research is necessary to elucidate the effects of hypothyroidism on regeneration and possible therapeutic targets like FOXO3.

Summary

In summary, there are adverse effects on metabolism, function, and growth caused by hypothyroidism within SKM cells that lead to symptoms like pain, fatigue, weakness, tightness, and cramps. These symptoms manifest due to effects of low availability of TH and the resultant decreases in expression of genes crucial for healthy SKM function. The activation of TH by DIO2 activity is important for effects throughout the SKM myocyte as is the concomitant effect on THRα signaling, however, over-emphasizing the role of DIO2 activation of TH has limited the research on the far-reaching effects of the global low T_3 and this needs to be addressed. Additionally, the use of rodent models may be misleading due to the differences in DIO2 regulation in hypothyroidism (Heemstra et al., 2009) and stimulation of PGC-1α expression in humans (Barbe et al., 2001). The significance and innovation of the current proposal is highlighted by the experimental use of human SKM cells in culture, which may ameliorate the possible irregularities in TH mechanisms characteristic of rodent models. Finally, stimulation of β_2-adrenergic receptors with exercise and exercise mimetics increased TH signaling, thus increasing expression of T_3 targets and related signal transduction. Research on the potential benefits of exercise-related improvements to

hypothyroid detriments to SKM is still largely unexplored and much more work needs to be conducted to elucidate effects on individual and overlapping cellular mechanisms.

CHAPTER III

METHODS

Experimental Approach to the Problem

The proposed study aimed to elucidate the underlying mechanisms of dysregulated SKM cell signaling by using an *in vitro* model of low TH availability (via depleted TH culture media). Additionally, a group of cells was also treated with the exercise mimetic formoterol, a β2-adrenergic receptor agonist to investigate the effects of stimulating exercise-related pathways on SKM physiology in the context of low TH availability. Formoterol has been previously established in the literature to upregulate exercise pathways similar to epinephrine's effects during both endurance and resistance exercise, providing modest external validity when comparing its stimulation effects to exercise in general. Previously data produced in our lab indicates that using 30 nM formoterol treatment in SKM cell culture results in stimulation of target genes typically associated with *in vivo* exercise stimulation. For each experimental group, the expression of genes related to TH metabolism, myogenesis, and mitochondrial homeostasis (Table 2) were collected and normalized to a control group (standard culture media).

Study Design

The study was approved by the Texas Woman's University Institutional Review Board. This study was performed using commercially available (Sigma-Aldrich, St. Louis, MO), primary Human SKM myoblasts obtained from healthy adult donors that have been de-identified of personal information. A sample size of six (n = 6) per condition was utilized based on previous results in our lab indicating that six biological replicates is sufficient to detect differences in SKM subcellular targets related to metabolism, myogenesis, and mitochondrial function.

The cells were cultured in a Thermo Fisher Midi40 incubator (Thermo Fisher Scientific, Asheville, NC) with controlled temperature (37 °C), humidity, and 5% CO_2 (representing physiological conditions). Firstly, myoblasts from passage 5 were cultured in 35 mm, six-well collagen-coated plates with Human Skeletal Muscle Growth Media (Cell Applications, San Diego, CA), which included specific nutrients and hormones that mimic *in vivo* conditions for healthy SKM cell growth. Myoblasts were seeded at a density of 80,000 cells per well, as previously established to ensure ample proliferation and to attain 80% confluency within 72 hr of growth. Representative images of SKM cells in culture wells were captured for each condition on Day 4 and Day 6 using a Leica DMi1 inverted microscope cell imaging system at 10x objective magnification (Leica Microsystems Inc., Boulder Grove, IL).

Once the SKM myoblasts attained desired confluency, cells were exposed to differentiation media – thus initiating the process of myoblast differentiation into mature myotubes. Experimental data were collected during days 4 (mid-myogenesis) and 6 (mature myotubes) of differentiation to assess our outcome measures during phases of myogenesis. To accomplish the goals of our specific aims, three culture conditions were utilized: (a) control conditions (CON), (b) TH depleted culture media (ThD), and (c) ThD culture media + acute formoterol (FORM: Sigma-Aldrich, St. Louis, MO) treatment (3 hr before extraction: ThD+F). The CON condition utilized standard Human Skeletal Muscle Differentiation Media (Cell Applications, San Diego, CA) while both the ThD and ThD+F conditions used customized TH depleted differentiation media purchased from BBI Solutions, Inc. (BBI Solutions, Crumlin, UK) to represent low availability of TH and low availability of TH and exercise.

At Day 0 of differentiation, standard proliferation media was removed from culture plates, cells were washed with potassium buffered saline (Gibco, New York, NY), and the appropriate differentiation media was added for each condition. From this point, cells were maintained and checked daily under uniform incubation conditions. After 4 days of differentiation had elapsed (only Day 4 experimental groups), media was emptied, cells were washed, collected, and total RNA was extracted and stored for future analysis. This process was repeated again at Day 6 for cells that were plated separately and not interrupted at Day 4 (Day 6 experimental groups). For both of the

ThD+F conditions (Day 4 and Day 6), at 3 hr before initiation of RNA extraction 6, the cells were exposed to 30 nM formoterol in culture media. The overview of the study design is depicted in Figure 5.

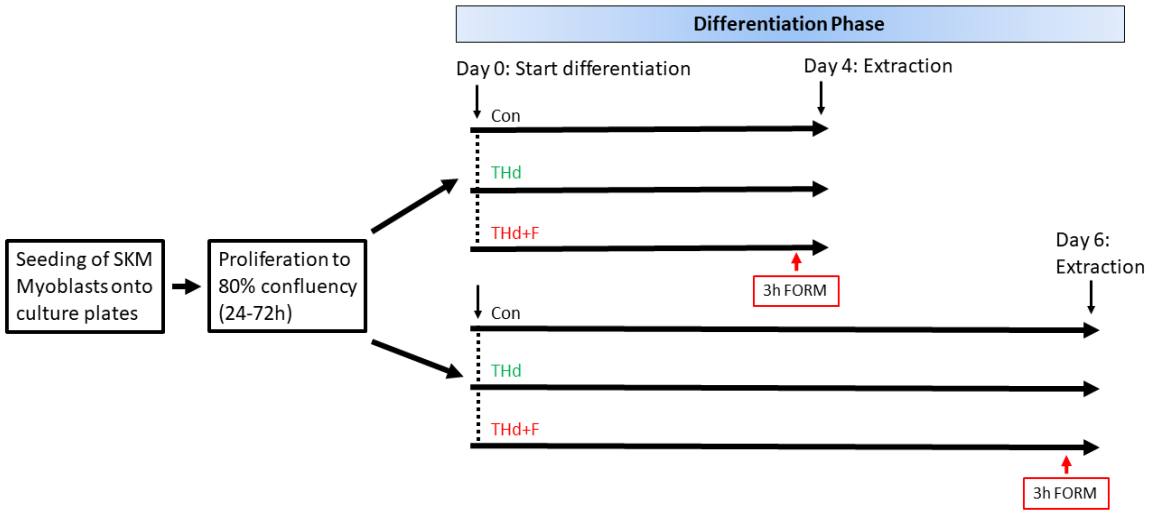

Figure 5. Overview of Study Timeline

Gene Expression Analysis

The total RNA concentration was analyzed using a BioTek Take3/Synergy HTX spectrophotometer (BioTek Instruments, Inc., Winooski, VT). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA for the 20 μl reverse transcriptase-polymerase chain reaction (qPCR) in the Bio-Rad T100 Thermocycler (Bio-Rad Laboratories, Inc., Hercules, CA) following instructions per the Applied Biosystems' High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). Primers for gene expression targets were designed to span exon-exon junction and purchased from IDT (Integrated DNA Technologies, Skokie, IL) as shown in Table 2. The 20 μl total

volume reaction utilized PowerUp SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA). All reactions were completed in duplicate using a QuantStudio RealTime PCR System (Applied Biosystems, San Francisco, CA). Data were analyzed using the comparative CT method ($\Delta\Delta$CT). Target genes were compared with ribosomal protein S13 (RPS13) as an endogenous control and all group values were normalized to D4 CON.

Table 2

Target genes for analysis related to thyroid hormone metabolism (TH), myogenesis (Myo), mitochondrial homeostasis (Mito), and homeostatic-general cell function (Cell)

Target gene	**Abbreviation**	**Related to:**
AMP-activated protein kinase	AMPK	Cell, TH
Autophagy related 5	ATG5	Cell, Mito
Beta 2 adrenergic receptor	B2AR	Cell
Deiodinase 2	DIO2	TH, Myo
Deiodinase 3	DIO3	TH
Estrogen related receptor alpha	ERRα	Cell, Mito
Forkhead box O3	FOXO3	Myo, TH
Glucose transporter 4	GLUT4	Cell
Glutathione synthetase	GSS	Cell, Mito
Myogenic differentiation 1	MyoD	Myo
Myogenic factor 5	Myf5	Myo
Myogenin	MyoG	Myo
Nuclear factor, erythroid 2 like 2	Nrf2	Mito
Nuclear respiratory factor 1	NRF1	Mito
Peroxisome proliferator-activated receptor gamma coativator1-alpha	PGC-1α	Mito, TH, Cell
Peroxisome proliferator-activated receptor gamma coativator1-beta	PGC-1β	Mito, TH, Cell
Ribosomal protein S13	RPS13	Normalization
Sarco/endoplasmic reticulum Ca2+ ATPase 1	SERCA1	TH, Cell
Sarco/endoplasmic reticulum Ca2+ ATPase 2a	SERCA2a	TH, Cell
Superoxide dismutase 2	SOD2	Mito
Thyroid receptor alpha	THRα	TH
Transcription factor A, mitochondrial	TFAM	Mito

Statistical Analysis

Data was analyzed using SPSS v24.0 (IBM, Armonk, NY). Two-way repeated measures analysis of variance was used to determine significant differences between experimental conditions. Statistical significance was established at $p < .05$.

CHAPTER IV

RESULTS

This study utilized an *in vitro* model of low TH availability (via depleted TH culture media) to investigate the effects of low TH and an exercise mimetic, formoterol, on human SKM cell gene expression. To increase the efficacy of interpretation, the results analyzed from this study have been organized into the following categories of genes related to: (a) TH metabolism (see Figure 6), (b) myogenesis (see Figure 7), (c) mitochondrial homeostasis (see Figure 8), and (d) homeostatic cell function (see Figure 9).

Gene Expression Related to TH Metabolism

In SKM, the primary TH nuclear receptor, THRα, regulates much of TH targeted gene transcription. There was no change in THRα (see Figure 6A) for CON from D4 to D6. However, the ThD and ThD+F groups were decreased compared to CON for both days, with ThD+F having the lowest THRα expression for each day. Within the ThD+F group, D4 expression of THRα was lower than D6. For DIO2 (see Figure 6B), CON expression increased from D4 to D6 and ThD and ThD+F were both decreased from D4 to D6. Within D4, ThD+F was increased compared to CON and ThD. For D6, ThD was lower than both CON and ThD+F. There was no change in DIO3 (see Figure 6C) for CON

from D4 to D6. Within D4, ThD+F was increased compared to CON and ThD. ThD+F was decreased at D6 compared to ThD+F at D4. SERCA1 (see Figure 6D) expression decreased from D4 to D6 for all groups. Within D4, ThD+F was lower than CON and at D6 both ThD and ThD+F were lower than CON. There was an increase in SERCA2 (see Figure 6E) and ThD+F for CON from D4 to D6. Within D4, ThD+F was lower than CON and ThD. For D6, both ThD and ThD+F were lower than CON.

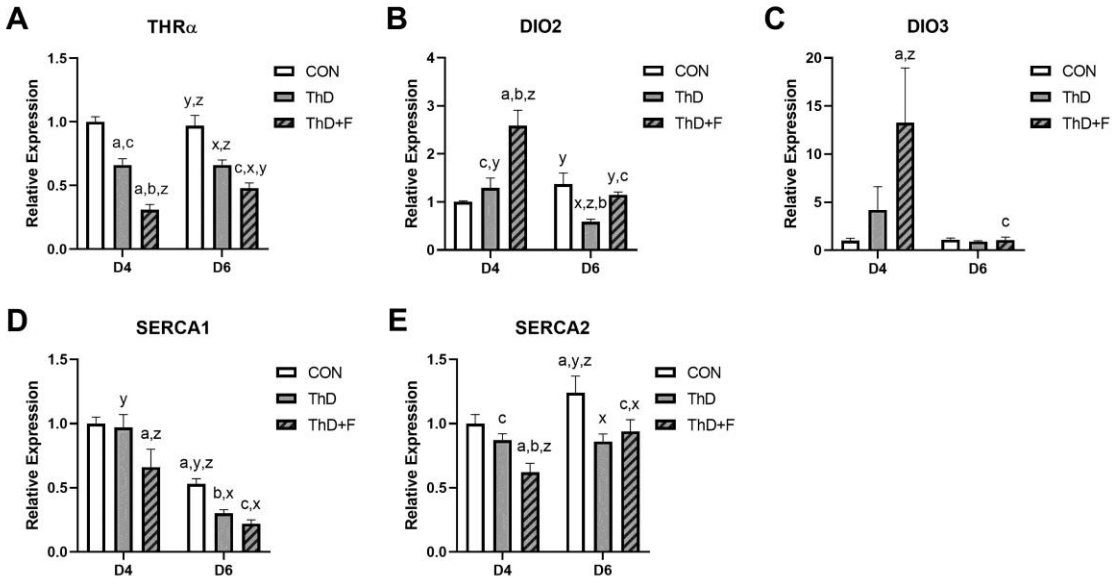

Figure 6. TH metabolism-related gene expression. For each gene graph, the letters a, b, and c represent D4 CON, ThD, and ThD+F, respectively, while x, y, and z represent D6 CON, ThD, and ThD+F, respectively, to signify differences between conditions. **THRα** (**A**): D4 ThD was decreased compared to D4 CON; D4 ThD was greater than D4 ThD+F. D4 ThD+F was decreased compared to D4 CON; D4 ThD+F was also decreased compared to D6 ThD+F. D6 ThD was decreased compared to D6 CON; D6 ThD was greater than D6 ThD+F. D6 ThD+F was decreased from D6 CON. **DIO2** (**B**): D4 ThD was decreased compared to D4 ThD+F; D4 ThD was greater than D6 ThD. D4 ThD+F was greater than D4 CON; D4 ThD+F was greater than D6 ThD+F. D6 ThD was decreased compared to D6 CON; D6 ThD was decreased compared to D6 ThD+F. **DIO3** (**C**): D4 ThD+F was greater

than D4 CON; D4 ThD+F was greater than D6 ThD+F. **SERCA1** (**D**): D4 ThD was greater than D6 ThD. D4 ThD+F was decreased compared to D4 CON; D4 ThD+4 was greater than D6 ThD+F. D6 CON was decreased compared to D4 CON. D6 ThD was decreased compared to D6 CON. D6 ThD+F was decreased compared to D6 CON. **SERCA2** (**E**): D4 ThD was greater than D4 ThD+F. D4 ThD+F was decreased compared to D4 CON; D4 ThD+F was decreased compared to D6 ThD+F. D6 CON was increased compared to D4 CON. D6 ThD was decreased compared to D6 CON. D6 ThD+F was decreased compared to D6 CON. There was an interaction effect for DIO2 and a main effect of time for DIO3, SERCA1, and SERCA2. All reported differences are significant ($p < 0.05$). Data are expressed as mean ± SEM.

Gene Expression Related to Myogenesis

During myogenesis, the regulation of early growth through mature myotubes is controlled by the expression of myogenic regulatory factors (MRF) myogenic factor 5 (Myf5), MyoD1, myogenin (MyoG) and forkhead box O3 (FOXO3) as displayed in Figure 7. There was no change in MyF5 (see Figure 7A) for CON from D4 to D6. Within D4, ThD was lower than CON and ThD+F was greater than ThD. For D6, both ThD and ThD+F were lower than CON. There was also a decrease for ThD+F from D4 to D6. MyoD (see Figure 7B) decreased from D4 to D6 for CON and ThD. Within D4, ThD and ThD+F were lower than CON and ThD+F was lower than ThD. For D6, both ThD and ThD+F were lower than CON and ThD+F was also lower than ThD. There was a decrease in MyoG (see Figure 7C) for CON and ThD from D4 to D6. For D4, ThD+F was lower than both CON and ThD. For D6, ThD+F was lower than both CON and ThD as well. There was no change in FOXO3 (Fig. 7D) from D4 to D6 for all groups. Within D4, ThD+F was lower than both CON and ThD. For D6, ThD+F was lower than CON.

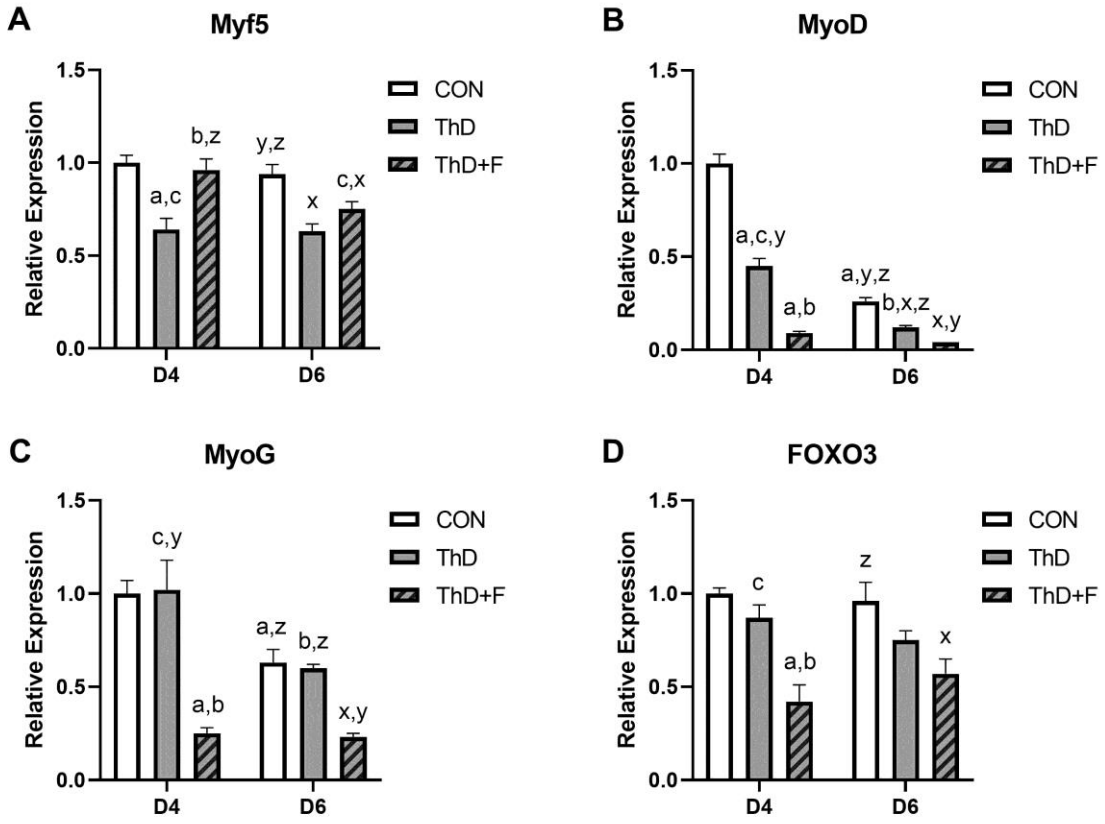

Figure 7. Myogenesis-related gene expression. For each gene graph, the letters a, b, and c represent D4 CON, ThD, and ThD+F, respectively, while x, y, and z represent D6 CON, ThD, and ThD+F, respectively, to signify differences between conditions. **Myf5** (**A**): D4 ThD was decreased compared to D4 CON and D4 ThD+F. D4 ThD+F was greater than D6 ThD+F. D6 ThD was decreased compared to D6 CON. D6 ThD+F was decreased compared to D6 CON. **MyoD** (**B**): D4 ThD was decreased compared to D4 CON and greater than D4 ThD+F; D4 ThD was greater than D6 ThD. D4 ThD+F was decreased compared to D4 CON and D4 ThD. D6 CON was decreased compared to D4 CON. D6 ThD was decreased compared to D6 CON and greater than D6 ThD+F. D6 ThD+F was decreased compared to D6 CON. **MyoG** (**C**) D4 ThD was greater than D4 ThD+F and D6 ThD. D4 ThD+F was decreased compared to D4 CON and D4 ThD. D6 CON was decreased compared to D4 CON. D6 ThD was decreased compared to D4 ThD; D6 ThD was greater than D6 ThD+F. D6 ThD+F was decreased compared to D6 CON and D6 ThD. **FOXO3** (**D**): D4 Thd was greater than D4 ThD+F. D4 ThD+F was decreased compared to CON. D6

ThD+F was decreased compared to D6 CON. There was an interaction effect for MyoD and MyoG. All reported differences are significant ($p < 0.05$). Data are expressed as mean ± SEM.

Gene Expression Related to Mitochondrial Homeostasis

In SKM mitochondria, the regulation of energy metabolism and free radical byproducts are essential roles for the target genes in this study. As the master regulator of mitochondrial biogenesis, peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) is upregulated by exercise and indirectly upregulated by TH. The expression of PGC-1α (see Figure 8A) was greater for ThD+F compared to CON and ThD for D4. Similarly, for D6, ThD+F was greater than CON and ThD. For PGC-1β (see Figure 8B), ThD+F decreased from D4 to D6 but was also greater than CON and ThD within D6. There was no change in TFAM (See Figure 8C) for CON from D4 to D6. There was a decrease for ThD+F from D4 to D6. Within D4, ThD+F was greater than CON and ThD. For D6, both ThD and ThD+F were lower than CON. NRF1 (See Figure 8D) expression decreased from D4 to D6 for both CON and ThD. Within D4, ThD and ThD+F were lower than CON and ThD+F was lower than ThD. For D6, ThD and ThD+F were lower than CON. For Nrf2 (see Figure 8E), CON increased from D4 to D6, while ThD and ThD+F decreased from D4 to D6. Within D6, both ThD and ThD+F were lower than CON. There was no change in SOD2 (see Figure 8F) for CON and ThD from D4 to D6. Within D4, ThD+F was greater than CON and ThD. For D6, ThD and ThD+F were lower than CON.

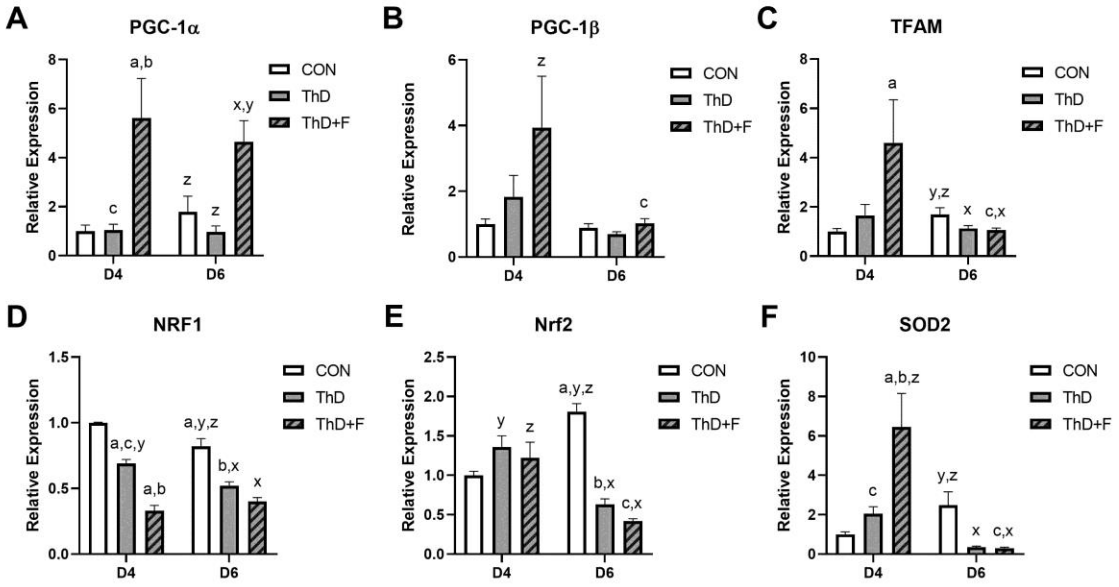

Figure 8. Mitochondrial-related gene expression. For each gene graph, the letters a, b, and c represent D4 CON, ThD, and ThD+F, respectively, while x, y, and z represent D6 CON, ThD, and ThD+F, respectively, to signify differences between conditions. **PGC-1α** (**A**): D4 ThD was decreased compared to D4 ThD+F. D4 ThD+F was increased compared to D4 CON; D6 CON was decreased compared to D6 ThD+F. D6 ThD was decreased compared to D6 ThD+F. **PGC-1β** (**B**): D4 ThD+F was greater than D6 ThD+F. **TFAM** (**C**): D4 ThD+F was greater than D4 CON; D4 ThD+F was greater than D6 ThD+F. D6 ThD was decreased compared to D6 CON. D6 ThD+F was decreased compared to D6 CON. **NRF1** (**D**): D4 ThD was decreased compared to D4 CON; D4 ThD was greater than D4 ThD+F; D4 ThD was greater than D6 ThD. ThD+F was decreased compared to D4 CON. D6 CON was decreased compared to D4 CON. D6 ThD was decreased compared to D6 CON. D6 ThD+F was decreased compared to D6 CON. **Nrf2** (**E**): D4 ThD was greater than D6 ThD. D4 ThD+F was greater than D6 ThD+F. D6 CON was greater than D4 CON. D6 ThD was decreased compared to D6 CON. D6 ThD+F was decreased compared to D6 CON. **SOD2** (**F**): D4 THD was decreased compared to D4 ThD+F. D4 ThD+F was greater than D4 CON; D4 ThD+F was greater than D6 ThD+F. D6 ThD was decreased compared to D6 CON. D6 ThD+F was decreased compared to D6 CON. There was an interaction effect for TFAM, NRF1, Nrf2, and SOD2 and a main effect of time for PGC-1β. All reported differences are significant ($p < 0.05$). Data are expressed as mean ± SEM.

Gene Expression Related to Homeostatic Cell Function

Gene targets related several cellular homeostatic roles were examined in response to low availability of TH and formoterol treatment. There was an increase for AMPK (see Figure 9A) for CON from D4 to D6, but ThD and ThD+F decreased from D4 to D6. Within D4, ThD+F was lower than CON. For D6, Both ThD and ThD+F were lower than CON and ThD+F was lower than ThD. B2AR expression (see Figure 9B) increased for CON from D4 to D6. There were no changes during D4. Within D6, both ThD and ThD+F were lower than CON with ThD+F being lower than ThD. There was no change in GSS (see Figure 9C) for CON from D4 to D6, but ThD+F decreased from D4 to D6. For D6, ThD+F was lower than ThD. There was an increase for ATG5 (see Figure 9D) for CON from D4 to D6 while ThD+F decreased from D4 to D6. Within D4, ThD+F was greater than CON. For D6, both ThD and ThD+F was lower than CON. GLUT4 (see Figure 9E) expression did not change for CON from D4 to D6 while ThD+F decreased from D4 to D6. Within D4, ThD+F was greater than CON. For D6, there were no differences between conditions. There was no change for any condition from D4 to D6 for ERRα (see Figure 9F). Within D4, there were no differences between groups. For D6, the expression of ERRα was lower in ThD+F than both CON and ThD.

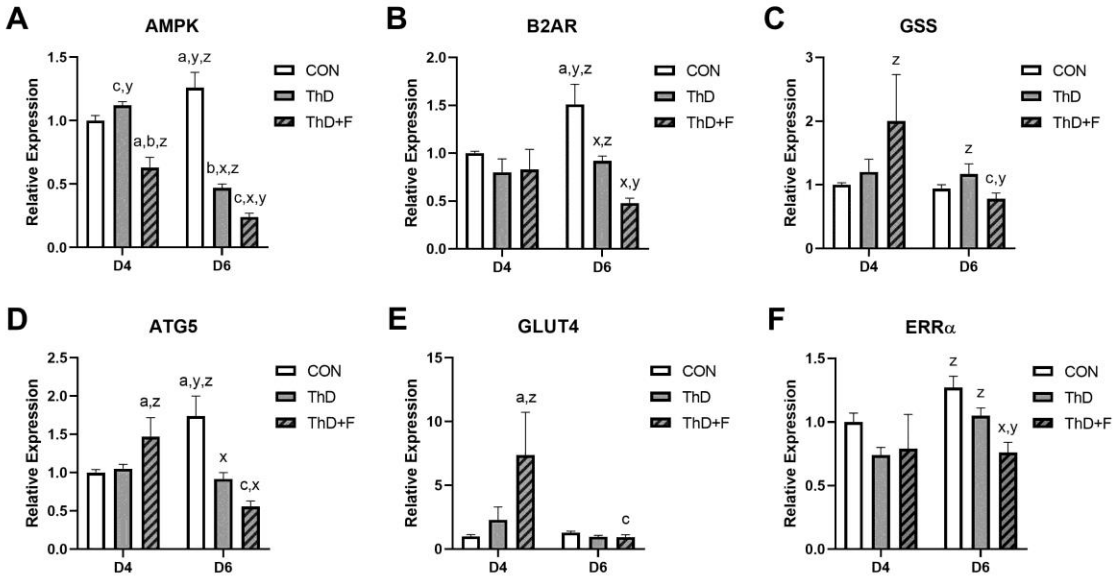

Figure 9. Cell homeostasis-related gene expression. For each gene graph, the letters a, b, and c represent D4 CON, ThD, and ThD+F, respectively, while x, y, and z represent D6 CON, ThD, and ThD+F, respectively, to signify differences between conditions. **AMPK** (**A**): D4 Thd was greater than D4 ThD+F; D4 ThD was greater than D6 ThD. D4 ThD+F was decreased compared to D4 CON; D4 ThD+F was greater than D6 ThD+F. D6 CON was greater than D4 CON. D6 ThD was decreased compared to D6 CON; D6 Thd was greater than D6 ThD+F. D6 ThD+F was decreased compared to D6 CON. **B2AR** (**B**): D6 CON was greater than D4 CON. D6 ThD was decreased compared to D6 CON; D6 Thd was greater than D6 ThD+F. D6 ThD+F was decreased compared to D6 CON. **GSS** (**C**): D4 ThD+F was greater than D6 ThD+F. D6 ThD was greater than D6 ThD+F. **ATG5** (**D**): D4 ThD+F was greater than D4 CON; D4 Thd+F was greater than D6 ThD+F. D6 CON was greater than D4 CON. D6 ThD was decreased compared to D6 CON. D6 ThD+F was decreased compared to D6 CON. **GLUT4** (**E**): D4 ThD+F was greater than D4 CON; D4 ThD+F was greater than D6 ThD+F. **ERRα** (**F**): D6 ThD was greater than D6 ThD+F; D6 ThD+F was decreased compared to D6 CON. There was an interaction effect for AMPK, B2AR, and ATG5. All reported differences are significant ($p < 0.05$). Data are expressed as mean ± SEM.

CHAPTER V

IMPLICATIONS, RECOMMENDATIONS, AND CONCLUSIONS

The *in vitro* model of low TH availability utilized in our study parallels an *in vivo* environment whereby the conditions of hypothyroidism elicit deleterious effects within the SKM cell. In the present study, we report pronounced and interconnected effects on the expression of genes related to SKM physiology resulting from low availability of TH. Additionally, treatment with the exercise mimetic, formoterol, resulted in a multitude of varying effects that provide profound insights that will inform future studies on the potential benefits (or dangers) of exercise in hypothyroid populations. Previous studies conducted in our lab led to characterization of a SKM cell culture model for using formoterol as an exercise mimetic treatment during phases of myogenesis (Duplanty et al., in preparation for submission; hereafter referred to as "FORM Study"). The FORM Study enabled us to interpret the effects of formoterol on healthy SKM cells and the resultant expression of similar categories of target genes as used in this study.

In consideration of the complexity of the present study, it is important to put into context that these investigations take place during and at the end of the myogenic process, where the metabolic needs for myocyte growth and maturity may act in direct opposition to the metabolic effects of formoterol-induced stimulation of the exercise-

related cell signaling. This phenomenon is further compounded by the pathophysiological condition of low availability of TH, which has known negative effects on normal cell metabolic capabilities. Overall, these results have emerged to tell a very interesting story and paint a vivid and, yet, abstract picture of the potential mechanisms affecting gene expression programs relating to hypothyroid muscle physiology. The following subsections will address the results of our study organized by genes related to the specific categories as previously outlined in Table 2, followed by a summary of the interconnected and overlapping meaning of these categorical results when combined as a whole.

Gene Expression Related to TH Metabolism

In SKM, TH signal transduction exerts widespread subcellular downstream effects by directly influencing target molecules and multiple mechanisms of gene expression machinery. In the present study, the expression of genes related to TH metabolism was obviously affected by low TH availability in culture. In the FORM Study, THRα, the primary nuclear receptor in SKM, which is located in promotor regions of specific gene targets of TH, was found to be decreased by formoterol treatment at both D4 and D6, indicating potential interference of normal TH metabolism from this treatment. In the present study, the expression of THRα was reduced by ThD and further decreased by ThD+F. These results suggest that either low TH or FORM treatment can decrease THRα expression, but the combination of ThD+F results in the

lowest level of expression for this receptor. This further indicates, as will be continuously referred to as a pattern in the present study, a conflict of metabolism by opposing stimulation during myogenesis, further leading into impairments to any other processes that needs meaningful levels of energy in the cell. As such, expression of THRα is imperative for downstream signaling of other TH target genes like GLUT4, SERCA1 and SERCA2, whereby insufficient stimulation by TH leads to low expression of downstream targets.

Intracellular TH activated by DIO2 stimulates nuclear transcripts and subcellular molecules leading to myogenesis, autophagy, metabolism, and function (Dentice et al., 2010; Salvatore et al., 2014; Sinha et al., 2015). Of importance to note, during the cascade of TH metabolism there is apparent overlap with intended targets streaming from exercise stimulation (Iemitsu et al., 2003; Lesmana et al., 2016a). Exercise also upregulates DIO2 expression and, thus, increases production of T_3; generating downstream effects on sarcoplasmic and nuclear targets (Bocco et al., 2016). In the FORM Study, treatment with Formoterol increased DIO2 expression on both D4 and D6 suggesting that exercise is a potent stimulator of DIO2. This is an important finding as DIO2 activity peaks Day 1 of myogenesis and then decreases thereafter (Grozovsky et al., 2009). We found a similar increase in DIO2 expression in this study at D4 ThD+F; however, at D6 ThD+F this effect was ablated suggesting that the exercise signal was diminished by the lack of TH and exercise stress at D6. These findings suggest a

diminished production of intracellular T_3, propagated by insufficient DIO2 expression, which may disrupt normal cellular responses to exercise. Subsequently, DIO3 acts in opposition to DIO2 and provides counterbalance via conversion of T_4 to rT_3. This relation has nullifying effects on TH with implications during disease, stress, or basal maintenance of satellite cell pools (Dentice et al., 2014).

In the present study, there was a rise in DIO3 at D4 with THD+F but not D6 with THD+F, indicating once again a lower response to exercise signaling. It is hypothesized, that DIO3 expression is elevated post-exercise (as found in the FORM Study) as a conservation mechanism that may allow for a shift in bioenergetic allocation towards reestablishment of cellular homeostasis, rather than immediate commitment to myogenesis or protein synthesis, which is energy intensive. It has been reported that, for individuals with hypothyroidism, DIO3 generates excess rT_3, curtailing TH-related gene transcription rather than providing metabolic "down time" for the cell (Simonides, Mucahey, & Redout, 2008). Similarly, this mechanism seemed to be ablated due to low TH availability in the present study, and as will be discussed further, cellular homeostasis may be dysregulated by increased reactive oxygen species (ROS) and markers of dysregulated homeostasis- overall, pointing to multiple avenues of metabolic demand and failure of this preventative mechanism.

During exercise, SKM contraction requires calcium homeostasis, which is modulated by several thyroid-related mechanisms. Hypothyroidism affects SKM

contractility, which has been reported to contribute to negative muscular symptoms (Duyff et al., 2000; Guerin et al., 2019, in submission). Stimulation of THRα exerts a SERCA1-mediated effect predominantly in fast twitch, glycolytic fibers in response to elevated levels of T_3. Conversely, low levels of T_3 result in higher SERCA2 expression that is found in slow twitch, oxidative fibers (Simonides, Thelen, van der Linden, Muller, & van Hardeveld, 2001). In the present study, SERCA1 decreased in response to both ThD conditions at D6, with formoterol causing the most decrease. This response matches the reported trend in decline of DIO2, and can be related to decreased glycolytic phenotype. Paired with this, we found that SERCA2 was decreased in response to low TH, but this decline was ameliorated by presence of formoterol at D6 as compared to D4 ThD+F. This alludes to a potential metabolic "shift" to an aerobic phenotype, as mirrored in our results related to mitochondrial effects, however, as we will discuss later, the further downstream the effects, the more dysregulated these compensatory mechanisms appear to become. In a study by Simonides et al. (2001), they concluded that the contribution of SERCA activity was responsible for up to 50% of energy use by SKM during contraction and thus in hypothyroidism, the move towards type I phenotype is a factor associated with metabolic impairment.

Gene Expression Related to Myogenesis

From the onset of myogenesis to terminal maturation of myotubes, the expression of myogenic regulatory factors (MRF) responds within their respective

timeline and then subside over the course of the process as displayed in Figure 10.

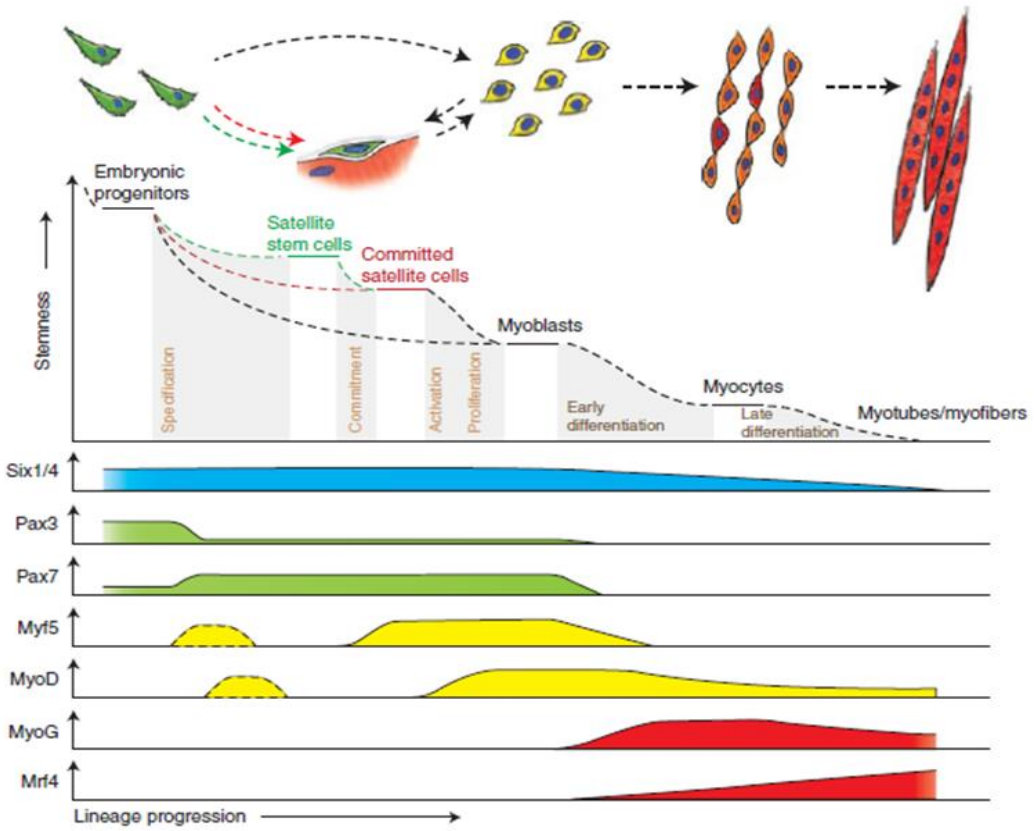

Figure 10. Molecular regulation of myogenesis (Bentzinger, Wang & Rudnicki, 2012).

Several genes related to myogenesis were investigated in the present study and provide evidence that our model is indeed experimenting during the phases of mid-myogenesis (D4) and at terminal myogenesis (D6) whereby SKM cells have reached their predicted stage of differentiation into mature myotubes. This is supported by our findings in the CON groups that follow the typical trajectory indicative of the myogenic program. In low TH conditions, impairment of the myogenic process has been found to

cause delays in terminal differentiation and leads to phenotypical changes-relating back to our findings on SERCA1 and SERCA2 (Schiaffino & Reggiani, 2011). Interestingly, exercise stimulation via formoterol, consistent with our previous investigations, resulted in a marked decrease in both MyoD and MyoG expression during D4 and D6. This is connected to our working hypothesis (in addition to our reported TH metabolic derangements) that the energetic needs of the cell may be funneled away from the calorically expensive process of myogenesis, whether this is driven by hormesis or simply signal dysfunction remains to be elucidated.

In the present study, low availability of TH had no effect on MyoG, but did reduce the expression of both Myf5 and MyoD for D4 and D6. Similar to our previous findings, the ThD+F group resulted in the lowest expression of MyoG and MyoD for both time points. In the absence of an exercise stimulus, the ThD group's response for MyoG was unexpected since it is a downstream target of T_3 via THRα (Downes, Griggs, Atkins, Olson, & Muscat, 1993) and was hypothesized to be decreased compared to CON as was found with MyoD.

As an important precursor to myogenesis and autophagy, FOXO3, drives the myogenic pathway by its stimulation of DIO2 and MyoD expression (Bloise et al., 2018; Dentice et al., 2010). Prior to myogenesis, DIO3 maintains satellite cells until its signal is blocked by FOXO3 (Dentice et al., 2014) leading to stimulation of DIO2 expression, T_3 activation, and stimulation of THRα and T_3 responsive gene targets (Dentice et al., 2010;

Salvatore et al., 2014). Dentice et al. (2010) described this as FOXO3/DIO2 pathway thereby tying autophagy to TH activation and myogenesis. In the present study, D4 and D6 ThD+F were lower than the CON suggesting that formoterol ablated the signal to upregulate the expression of FOXO3, thereby repressing downstream targets of FOXO3 such as DIO2 and MyoD.

Gene Expression Related to Mitochondrial Homeostasis

In SKM metabolism, the regulation of mitochondrial biogenesis and oxidative phosphorylation (OXPHOS) are important factors in cellular and whole-body metabolism. As stated previously, the ATPase activity of SERCA, especially SERCA1, is the main consumer of energy during contraction primarily in glycolytic fibers and SERCA2 is responsible for upregulating preference of OXPHOS metabolism. As previously reported by Duplanty et al. (2018) and further replicated from the FORM Study and the ThD+F condition of the present study, formoterol is a potent stimulator of PGC-1α gene expression. As can be assumed, a formoterol-induced increase in the expression of PGC-1α, should relate to enhanced aerobic metabolism, as reported by Duplanty et al. (2018) where maximal oxygen consumption rate and mitochondrial biogenesis was increased. However, just as the snack machine that takes your money but fails to deliver the snack, our results show an increase in PGC-1α expression for the ThD+F group, but without an associated increase in the expression of genes related to enhanced aerobic metabolism and mitochondrial improvements, such as SERCA2, NRF1, and Nrf2, and TFAM. In SKM

hypothyroidism, there is a very real potential for ROS damage to cellular mechanisms caused by mitochondrial dysfunction.

Based on our findings, ROS production may have been elevated, as genes associated with ROS mitigation (SOD2, GSS) were increased at D4 in the ThD+F group. The downstream function of these antioxidant-associated genes are involved with managing ROS created from OXPHOS and without this mechanism, damage from ROS could lead to global dysfunction (Baghaiee, Teixeira, & Tartibian, 2016; Qian et al., 2015). Moreover, by D6 SOD2 and GSS were decreased for ThD+F, which can be either interpreted as correlating to a concomitant decrease in ROS production, or a failing of the mitochondrial antioxidant system in general, which in the case of the present study's findings, may be explained by the associated decreases in OXPHOS-related gene expression for the ThD+F group. Similarly, another PPAR isoform, PGC-1β, which has redundancies with PGC-1α, followed the same pattern as the other genes—elevated at D4 then an ablation of the signal by D6 in the ThD+F condition. Taken as a whole, low TH availability when paired with exercise pathway stimulation has the potential to either induce or exacerbate mitochondrial dysregulation.

Gene Expression Related to Cellular Homeostasis

This final category of genes represents the general management of internal environment in myocytes as affected by exercise. Thus far, this study has presented evidence of impairment in TH metabolism, myogenesis, and mitochondria in low TH and

exercise conditions. The next key regulator of cellular metabolism casting a multitude of downstream effects is AMPK. Interestingly, in the previous FORM Study, the expression of AMPK was not affected by exercise stimulation. However, in this study, there were decreases in AMPK at D4 ThD+F and D6 ThD and ThD+F conditions suggesting formoterol ablated the signal to increase AMPK expression thereby reducing the expression of downstream targets like FOXO3 and PGC-1α.

Throughout this discussion, we have examined mechanisms located within the nucleus and sarcoplasm of the myocyte. The stimulation of B2AR via exercise occurs at the sarcolemma and activates an intracellular pathway leading to the expression of TH related genes, PGC-1α and its transcripts, and ROS antioxidants. At D6, the expression of B2AR was decreased for ThD+F that implies that it may have become desensitized to the stimulation of formoterol, which is a B2AR agonist. This may explain many cases in which formoterol induced an increase in expression at D4, but by D6, the signal was lost. Interestingly, the ThD condition was also decreased at D6 for B2AR, which is a phenomenon similar to hypothyroidism *in vivo* (Bloise et al., 2018).

In the FORM Study, the expression of AMPK was not affected by exercise stimulation. However, in the present study, AMPK was decreased at D4 for ThD+F and both ThD and ThD+F at D6, which could explain some of the other responses related to metabolism such as ERRα and mitochondrial-related function in general.

Intracellular recycling and remodeling is imperative for healthy myocyte growth and health and is maintained in balance by genes such as AMPK, FOXO3, and ATG5. In a study by Lesmana et al. (2016b), rodent SKM myocytes were dysregulated via ROS exposure and then treated with TH in order to increase the autophagy of damaged cells, as reported by effects on autophagy-related genes. In the previous FORM Study, the exercise stimulation on ATG5, a marker of autophagy, did not change between conditions. However, in the present study, at D6, both of the TH depleted conditions had a lower expression of ATG5, compared to CON, with ThD+F being even lower than ThD. Although PGC-1α is known to increase expression of ATG5 and FOXO3 expression (Lesmana et al., 2016b), we did not find a concomitant increase in expression as was found for PGC-1α at D6, demonstrating a potential downstream negative interaction of low TH and formoterol.

Individuals with hypothyroidism develop metabolic conditions like diabetes due to changes in energy metabolism. A key gene positively affected by exercise and TH affecting energy transport into the myoctye is GLUT4. The inability to transport glucose into the cell leads to elevated blood glucose and hyperinsulinemia as seen in hypothyroidism (Garvey, Maianu, Hancock, Golichowski, & Baron, 1992; Torrance et al., 1997). As discussed earlier, GLUT4 expression and translocation are responsive to TH concentration in the cell and therefore low TH will reduce the expression of GLUT4

(Torrance et al., 1997). However, the only observed change in the present study for GLUT4 was at D4 for the ThD+F condition, which limits interpretation.

Finally, the orphan gene with no known ligands, ERRα, is another nuclear receptor whose roles include transcription of mitochondrial biogenesis and upregulation of OXPHOS mostly through its co-activation with PGC-1α (Huss, Torra, Staels, Giguere, & Kelly, 2004) and ERRα has been implicated in SKM growth and regeneration after injury (Perry, Dufour, Tam, B'chir, & Giguere, 2014). In the present study, ERRα expression was decreased at D6 for ThD+F, which is consistent with the formoterol condition in the FORM Study, indicating that TH depletion has no effect on this gene.

Study Images

In this study, images were taken to represent each condition at D4 and D6 as displayed in Figure 11. Visually, there is a difference between the growth from D4 to D6 between the CON, ThD, and ThD+F conditions. The number of myotubes appears to be greater in the D4 CON and D6 CON images compared to D4 and D6 ThD and D4 and D6 ThD+F. The D6 CON image displays more densely packed myotubes compared to D6 ThD and D6 ThD+F, whereby there is space for expansion. These images depict the well-reported delay in development of myocytes grown in TH depleted media (Schiaffano & Reggiani, 2011).

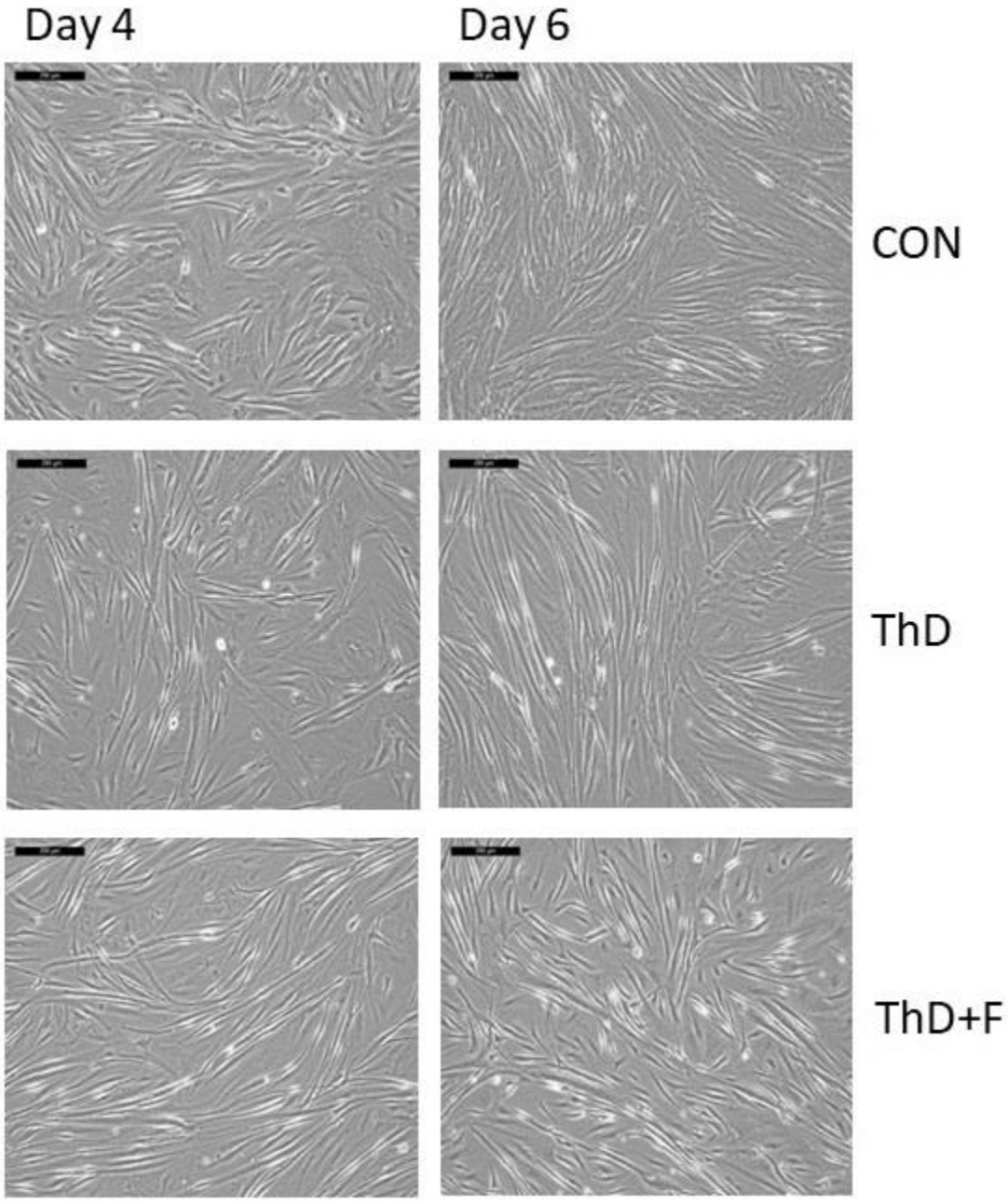

Figure 11. Pictoral representation of myotube growth for all groups.

Limitations

There are limitations to generalization from an *in* vitro model. First, it is unknown exactly how similar the treatment with either low TH availability or Formoterol is to hypothyroidism and exercise. While we can measure the concentration of TH in the circulation of an individual, it is not possible to determine the amount of TH that is entering cells and thus why this model of low TH availability may actually be closer to *in vivo* conditions if little TH is entering into cells with defective transporters. Similarly, formoterol has been shown to upregulate genes stimulated by the exercise pathway; however, being able to directly correlate the amount of formoterol to an exercise dose is not possible. It does present information that allows association between exercise stimulation and effects on cellular mechanisms, however. Lastly, there is an increase in Type I error rate due to the analysis of multiple comparisons and therefore interpretation should be carefully considered.

Conclusions

Hypothyroidism is a prevalent health issue primarily affecting women. Although it is a metabolic condition, there are currently no exercise guidelines addressing comorbidities and reported SKM symptoms like pain, fatigue, weakness, tightness, and cramps. The present study utilized an *in vitro* cell culture model of low availability of TH and the exercise mimetic, formoterol, to examine the effects of low TH status and exercise on SKM. We found specific changes in the expression of genes related to TH

metabolism, myogenesis, mitochondrial homeostasis, and cellular homeostasis. Both low availability of TH and exercise stimulation reduced the expression of genes investigated in this study. Low TH alone did not always inhibit cell signaling mechanisms, however, the combination of low TH and exercise may have exacerbated the cellular stress leading to dysregulated patterns of expression. Hypothyroidism, as represented in this study by low availability of TH, is known to impair TH cellular targets, however, this is the first time and the global effects of a low TH environment have been investigated. Based on this study, more *in vivo* research addressing hypothyroidism and exercise is warranted, as there may be negative consequences to implementation of an exercise program in those with hypothyroidism.

Future Studies

This study informs on future directions for hypothyroidism and exercise research. It is advantageous to utilize an *in vitro* SKM cell model prior to human participant biopsy research to understand potential issues with harvesting cells from individuals with hypothyroidism. Further, *in* vivo studies involving individuals with hypothyroidism and exercise performance should be conducted to more fully comprehend and appreciate their unique responses to exercise stress.

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