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In vitro mitochondrial and myogenic gene expression is influenced by formoterol in human myotubes

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Abstract

Background Exercise is an effective treatment for establishing and maintaining skeletal muscle health. The interconnected cascade of gene expression pathways related to myogenesis, mitochondrial homeostasis, and thyroid hormone metabolism are critical to skeletal muscle health. This in vitro study was conducted to investigate the effects of exercise mimetic (formoterol) stimulation on human skeletal muscle cell signaling during myogenesis, and to provide insight on potential targets for future studies exploring therapies for skeletal muscle atrophy. Human myoblasts were cultured and differentiated to evaluate the effects of exercise mimetic stimulation on gene expression during mid and late myogenesis.

Results We characterized the expression of 24 genes related to myogenesis, mitochondrial biogenesis, thyroid hormone metabolism, and cellular homeostasis and found that 21 genes were altered in response to formoterol, thus affecting related skeletal muscle pathways. Additionally, formoterol stimulation resulted in a myogenic program that appears to favor prolonged myoblast proliferation and delayed myotube maturation. Robust, yet distinctive effects of exercise mimetic stimulation on gene expression during mid-myogenesis and at terminal differentiation occurred. For instance, MYF5 increased in D6 FORM compared to other groups ($p < 0.001$) while MYOD and MYOG both decreased expression in the FORM groups compared to CON ($p < 0.01$). Secondly, mitochondrial biogenesis genes were stimulated following formoterol administration, namely PGC-1α, PGC-1β, and TFAM ($p < 0.05$). Uniquely in our study, thyroid hormone metabolism related genes were differentially expressed. For instance, DIO2 and DIO3 were both stimulated following formoterol administration ($p < 0.05$).

Conclusions The results of our study support the groundwork for establishing further experiments utilizing exercise signaling as a clinical treatment in models targeting dysfunctional skeletal muscle cell growth.

Keywords Skeletal muscle, Myotube, Myogenesis, Mitochondria, Exercise

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Background

The benefits of cultivating and maintaining healthy skeletal muscle (SKM) throughout life are numerous and potent (Smith et al. 2023; Piercy et al. 2018; Nieman and Pence 2020). Loss of muscle mass, whether initiated by disease (atrophy) or by decades of sedentary lifestyle (sarcopenia), often results in decreased quality of life and increased morbidity (Carter et al. 2015; Wilkinson et al. 2018; Wang et al. 2022). Exercise, especially modes that induce SKM hypertrophy, has been established as an effective treatment for attenuating or reversing SKM loss (Carter et al. 2015; Dungan et al. 2023). However, the interconnected scope of effects that exercise signaling has on multiple molecular pathways within SKM cells remains largely uncharacterized, especially as it relates to aging and disease. Thus, there is importance in further elucidating the exercise-stimulated response of molecules involved in the many physiological processes of human SKM growth, regeneration, repair, reactive oxidative species (ROS) mitigation, hormonal signal transduction, as well as factors related to atrophy.

Myogenesis, a process of SKM regeneration and repair, is the control of genetic networks that activate satellite cells (i.e. myoblasts) and initiate their differentiation into mature myofibers (Pizza and Buckley 2023; Johnson et al. 2023; Chal and Pourquié 2017). This process is regulated by the expression of myogenic regulatory factors (MRFs) such as MYF5, MYOD, MYOG, and MEF2c, which coordinate the process of myoblast proliferation and myotube differentiation. These factors regulate tissue repair and regeneration that is influenced by both disease and exercise (Pizza and Buckley 2023; Johnson et al. 2023; Chal and Pourquié 2017). Specifically, MYOD influences the commitment of myoblasts into the process of differentiating into mature myotubes and MYOG further directs the maturing differentiating process (Johnson et al. 2023). Additionally, SKM can further grow when protein synthesis is upregulated by mechanistic target of rapamycin (mTOR) signaling, a protein kinase that regulates protein synthesis especially in response to exercise-induced mechanical stimulation (Chen et al. 2023; Yoon 2017; Duplanty et al. 2017).

Within the SKM cell, in addition to providing the foundation for healthy mitochondrial function, the maintenance of mitochondrial homeostasis is essential for promoting balanced myofibrillar protein synthesis, new growth, and regeneration (Carter et al. 2015; Zhang et al. 2020; Shen et al. 2018). Healthy mitochondrial function is essential for muscle growth, especially in response to energetic or oxidative demand (e.g. exercise) (Carter et al. 2015; Ferri et al. 2020). Further, the dysfunction of mitochondrial processes (e.g. biogenesis, respiration, mitigation of oxidative stress) within the SKM cell contributes to the etiology of sarcopenia (Carter et al. 2015; Hadjispyrou et al. 2023; Alizadeh Pahlavani et al. 2022). Several important transcription factors contribute to SKM mitochondrial homeostasis and were investigated in this study. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha/beta (PGC-1α and PGC-1β), mitochondrial transcription factor A (TFAM), and nuclear respiratory factor 1/2 (NRF1, NRF2) are regulators of mitochondrial homeostasis and promoters of mitochondrial biogenesis (Yin and Cadenas 2015). As mitochondrial health is related to processes involved in overall cellular and organismal health, both directly and indirectly, it is therefore an important therapeutic target for mitigating SKM dysfunction and preventing sarcopenia (Carter et al. 2015; Long et al. 2023).

Another important, albeit less studied factor for SKM health is the metabolism of thyroid hormone (TH) within the SKM cell. Once transported into the SKM cell, deiodinases 2 and 3 (DIO2 and DIO3, respectively) convert TH to the active form, triiodothyronine (T3) or the inactive form, reverse T3 (rT3), respectively (Bianco et al. 2019). Active T3 stimulates specific TH nuclear receptors (e.g. THRα), aiding in the regulation of cellular metabolism, myogenesis, mitochondrial homeostasis, and calcium homeostasis (Bianco et al. 2019). Thus, the inclusion of TH metabolism in the present study is of importance to highlight the significance of TH action on SKM processes, particularly when an exercise mimetic is introduced into the SKM cell.

Exercise mimetic stimulation of SKM, specifically the β2-adrenergic receptor (B2AR) pathway via formoterol treatment, has been used to increase mitochondrial biogenesis (Miura et al. 2007; Wills et al. 2012; Azevedo Voltarelli et al. 2021), oxidative metabolism (Pearen et al. 2008), and influence the modulation of myoblast differentiation (Kim et al. 2019). The B2AR is upregulated by endogenous hormones (i.e. epinephrine and norepinephrine) in response to exercise in vivo resulting in cardioprotective and SKM physiological improvements (Barisione et al. 2010), warranting in vitro investigations to evaluate B2AR stimulation directly in place of downstream regulators. Individually, many genes related to SKM processes have been investigated using exercise-mimetic stimulation designs, usually in reference to a limited range of pathways. To date, genes investigated using these designs have been mostly studied using animal models. However, gene expression does differ between rodents, humans, and non-human primates, thereby making translations to human physiology based on animal model data confounding. For example, in humans, TH stimulation of PGC-1α occurs indirectly via T3 stimulation of AMPK, but happens directly by T3 in rodents (Barbe et al. 2001). Additionally in the absence

of exercise stimulation, PAX7 expression remains detectable longer while MYOD and MYOG expression is lower in human SKM satellite cells compared to mouse SKM satellite cells, despite differentiation occurring in culture, suggesting differences in the myogenic program between mice and humans and potentially differences in the myogenic response to exercise stimulation (Bareja et al. 2014). Thus, characterization of multiple gene expression pathways relating to exercise-stimulation in human SKM is warranted.

SKM exhibits high levels of plasticity and is influenced by a wide range of epigenetic stimuli. Cross-sectional analysis of SKM tissue signaling in vivo reveals various stages of ongoing myogenesis, thus limiting conclusions made at one discrete time point. Therefore, we developed a purpose-designed in vitro culture model to isolate SKM cells (during mid and late myogenesis) and investigate the influence of exercise-related signaling (via formoterol stimulation) on the expression of 24 genes related to myogenesis, mitochondrial biogenesis, TH metabolism, and cellular homeostasis. Furthermore, we set to determine if formoterol stimulation would be a viable alternative to other exercise mimetics in a SKM in vitro model. We hypothesized that the results of our study will deliver insights on the inner workings of a complex network of gene expression programs and inform on future experiments that can utilize similar methods for investigating the effects of exercise-related signaling on muscle growth and atrophy in SKM disease.

Methods

Cell culture and myotube formation

Primary human SKM myoblasts obtained from a healthy adult male donor (age 23 years; Cook MyoSite, Pittsburgh, PA, USA), stored in a − 80 °C freezer for quality preservation, were cultured in 35 mm 6-well collagen coated plates (Gibco, New York, NY, USA) at 37 °C and 5% CO_2 in growth media (151–500 Sigma-Aldrich) as six sets of technical replicates for each experimental condition. Myoblasts were seeded at a density of 8×10^4 cells per well at passage 4, as calculated using a hemocytometer and Trypan Blue (Gibco, New York, NY, USA), and once cells reached 80–90% confluency determined objectively by a microscope, differentiation medium (151D-250 Sigma-Aldrich) was used to initiate myotube formation and replaced every 48 h. All experimental procedures using human tissue were approved by the Texas Woman's University Institutional Review Board (IRB-FY2021-71).

Exercise mimetic stimulation

Formoterol fumarate dehydrate > 98% HPLC (10 mg, F9552 Sigma-Aldrich), was reconstituted in dimethyl sulfoxide (DMSO) and mixed into differentiation medium at a concentration of 30Nmol/L (Fig. 1). 0.1% DMSO was used as a control by adding a volume equal to formoterol to the differentiation media for all CON groups. Four 6-well plates were concomitantly cultured and represent the following groups: day 4 (mid-myogenesis) without/with formoterol treatment (D4 CON and D4 FORM, respectively) and day 6 (mature myotube formation) without/with formoterol treatment (D6 CON and D6 FORM, respectively). Three hours before scheduled cell extraction (day 4 and day 6), differentiation media was changed via a serological pipette by removing all media from the 6-well plates, washed with PBS, and immediately replaced with either fresh control differentiation media or formoterol-treated differentiation media for the duration of the remaining three-hour incubation period. Figure 2 provides an overview of the methodology used in this study

Gene expression

Cells were extracted at days 4 (mid-myogenesis) and 6 (mature myotubes) of myogenesis. Total RNA was extracted using a Qiagen miRNeasy kit (Qiagen, Germantown, MD, USA). Complementary DNA was synthesized from 1 µg of the resulting total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Real-time quantitative polymerase chain reaction (qPCR) detection was performed for 40 cycles in duplicate using PowerUp SYBR and the QuantStudio RealTime 3 PCR System (Applied Biosystems, Carlsbad, CA, USA)

1. 10mg of Formoterol (Molecular weight: 420.46g) added to 2.38mL of DMSO to equal 10μMol concentration
2. 21.42mL of PBS added to 10 μMol to make a stock of 23.8mL of 1mMol Formoterol
3. 10μL of the 1mMol Formoterol stock was added to 10mL differentiation media to make 1μMol Formoterol
4. 360μL of the 1μMol Formoterol stock was added to 11.64mL differentiation media to make the final 30μMol Formoterol

Fig. 1 Formoterol concentration dilution to make 30μMol Formoterol

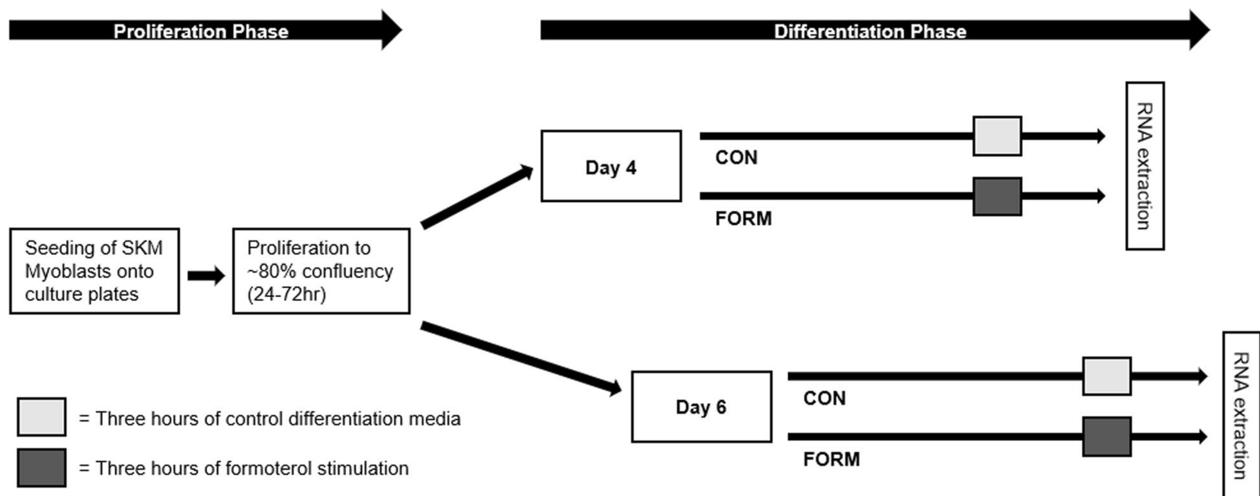

Fig. 2 Overview of study timeline and methodology

(Biosystems et al. 2014). Forward and reverse primers from IDT (Integrated DNA Technologies, Coralville, IA, USA) were used for analyses and are listed in Table 1. Data were analyzed using the comparative Ct (ΔΔCt) method for quantification (Biosystems et al. 2014; Livak and Schmittgen 2001). Ribosomal protein S13 (RPS13) was used as the endogenous control for comparative data of target genes (Rashid et al. 2021)

Table 1 qPCR primer sequences

Target gene	Forward primer (5′ to 3′)	Reverse primer (3′ to 5′)
AMP-activated protein kinase (AMPK)	TGTCCAGGGCTTGTTCTATTC	CCTAAAGAGAGGGCCACATAAA
Autophagy related 5 (ATG5)	CCATTCCTTCCAAGCTAGTG	GCAGTCAAGAGACAGGTAATC
Beta 2 adrenergic receptor (B2AR)	CCTGCTGACCAAGAATAAGG	GCAGGTCTCATTGGCATAG
Deiodinase 2 (DIO2)	GGCCCAAGTCATTCCTAATC	CACCTACTTTGGGAGGAAATC
Deiodinase 3 (DIO3)	CCTCAAAGCAGCACCTAAA	CCAATGCCTCTCAAGCTATC
Estrogen related receptor alpha (ERRα)	GCTCCTCCTCTCATCATTTG	TGGAGTCTGCTTGGAGTTA
Forkhead box O3 (FOXO3)	CCACCCTTGGCCTCTAAATAA	GGTAACAGGTATCAGGTTCTGG
Glucose transporter 4 (GLUT4)	CTGGGCTTCTTCATCTTCAC	GTTCTCATCTGGCCCTAAATAC
Glutathione synthetase (GSS)	GTAAGCTGCTCTGAGGTAAAG	CTCCTACCACTCAGTCCTATC
Myocyte enhancer factor 2 (MEF2c)	GCTGAAGAAGGAGATTTGTTTG	CTCTCTCGTCCCTGAAATTATG
Mechanistic target of rapamycin (mTOR) (Wei et al. 2019)	GGACTACAGGGAGAAGAAGA	CATCAGAGTCAAGTGGTCATAG
Myogenic factor 5 (MYF5)	GCTTCTAGTTCCAGGCTTATC	GCCTTCTTCTTCCTGTGTATTA
Myogenic differentiation 1 (MYOD) (Rienzo et al. 2019)	CACAACGGACGACTTCTATG	GTGCTCTTCGGGTTTCAG
Myogenin (MYOG)	CCCTGAATTGAGAGAGAAGAAG	CGGATGGCAGCTTTACAA
Nuclear respiratory factor 2 (NRF2)	GTGAGAACACACCAGAGAAAG	TCAACAACAGGGAGGTTAATG
Nuclear respiratory factor 1 (NRF1)	GATGGCACTGTCTCACTTATC	GTCATCTCACCTCCCTGTAA
Peroxisome proliferator-activated receptor gamma coactivator1-alpha (PGC-1α)	TCTCTCTCTCTCTCTCTCT	CATGGGTGTCAGGATTAAGG
Peroxisome proliferator-activated receptor gamma coactivator1-beta (PGC-1β)	GCTCCAAGTGTGTGTCTATC	CACAGTCATTTCGCCTCTT
PGC-1α isoform 4 (PGC-1α4)	CAACCTTGTCCCTGGTTTAT	ATTCTCACTTGCCATCATTCT
Ribosomal protein S13 (RPS13)	GCATCTTGAGAGGAACAGAAA	AGGACTCGCTTGGTCTTAT
Sarco/endoplasmic reticulum Ca^{2+} ATPase isoform 1 (SERCA1)	GTTCATCCGCTACCTCATTT	GTCACCAAGTTCACCCATAG
Sarco/endoplasmic reticulum Ca^{2+} ATPase isoform 2a (SERCA2)	CTACCTGGAACCTGCAATAC	CAACCGAACACCCTTACAT
Superoxide dismutase 2 (SOD2)	TTTCTTTCTCCTGCCTGATG	CCTCTCTCACCACTCCTATT
Transcription factor A, mitochondrial (TFAM)	CTCAAACTCCTGGCATCAA	GGTGGCTCACACCTATAATC
Thyroid hormone receptor alpha (THRα)	GTCACTCTCTGCCTTTAACC	CTCCTGACTCTTCTCGATCT

and the experimental group data was normalized to D4 CON values.

Statistical analyses

Data are presented as mean ± standard error of the mean (SEM) and statistical significance was set at $p < 0.05$. Statistical significance of the differences between the means was evaluated using one-way analysis of variance test (ANOVA), with Tukey's HSD post-test. Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 25.0 (IBM Corp, Armonk, NY, USA).

Results

Formoterol influences SKM gene expression related to myogenesis

MYF5 [$F(3, 20) = 36.99$, $p < 0.001$] was increased for D6 FORM (1.50 ± 0.06) compared to D4 CON (1.00 ± 0.09, $p < 0.001$), D4 FORM (0.85 ± 0.01, $p < 0.001$), and D6 CON (0.75 ± 0.03, $p < 0.001$). D6 CON was significantly lower than D4 CON ($p = 0.02$; Fig. 3a). We found similar results for both MYOD [$F(3, 20) = 76.09$, $p < 0.001$] and MYOG [$F(3, 20) = 36.74$, $p < 0.001$] as expression was lower for D4 FORM (0.43 ± 0.01 and 0.28 ± 0.01, respectively) compared to D4 CON (1.00 ± 0.03 and 1.00 ± 0.10, respectively; $p < 0.001$). Additionally, for both day 6 groups MYOD and MYOG was reduced compared to D4 CON ($p < 0.001$), and D6 FORM (0.52 ± 0.01 and 0.33 ± 0.03, respectively) was lower compared to D6 CON (0.67 ± 0.05, $p < 0.01$ and 0.56 ± 0.02, $p = 0.03$, respectively; Figs. 3b, c). MEF2c [$F(3, 20) = 5.23$, $p < 0.01$] was decreased for D6 FORM (0.63 ± 0.03) compared to D6 CON (1.16 ± 0.07, $p < 0.01$), further indicating less commitment to the maturing of myotubes (Fig. 3d). Further, PGC-1α4 [$F(3, 20) = 3.57$, $p = 0.03$] expression was significantly greater for D6 FORM (1.69 ± 0.06) compared to D4 and D6 CON (1.00 ± 0.24 and 1.00 ± 0.16, respectively; $p = 0.04$; Fig. 3e). The expression of mTOR [$F(3, 20) = 6.18$, $p < 0.01$] was significantly decreased for D6 CON (0.46 ± 0.19) compared to D4 CON (1.00 ± 0.07, $p < 0.01$), however, this day 6 decrease in mTOR was

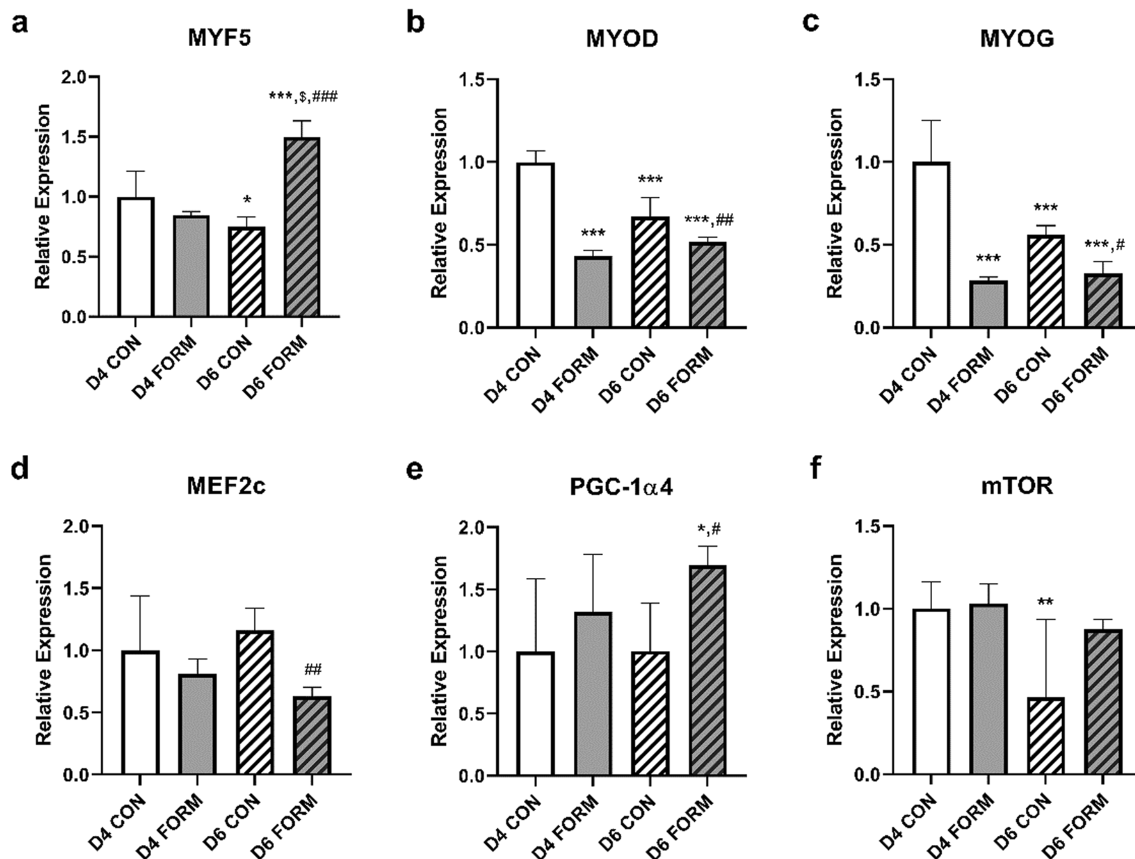

Fig. 3 SKM myogenesis related gene expression. **a**. Difference in gene expression between all groups for MYF5. **b**. Difference in gene expression between all groups for MYOD. **c**. Difference in gene expression between all groups for MYOG. **d**. Difference in gene expression between all groups for MEF2c. **e**. Difference in gene expression between all groups for PGC-1α4. **f**. Difference in gene expression between all groups for mTOR. Data are presented as mean ± SEM. * = significance compared to D4 CON, $ = significance compared to D4 FORM, # = significance compared to D6 CON; p values: *< 0.05, **< 0.01, ***< 0.001

notably absent in the D6 FORM group (0.87 ± 0.02, $p = 0.83$; Fig. 3f).

Formoterol stimulates expression of genes related to SKM mitochondrial biogenesis

We analyzed several genes related to the production of essential proteins for mitochondrial biogenesis. PGC-1α [F(3, 20) = 12.78, $p < 0.001$] was increased from D4 CON (1.00 ± 0.15) to D6 CON (2.08 ± 0.33, $p = 0.02$; Fig. 4a). Formoterol treatment caused a robust day 4 and 6 increase of PGC-1α (2.76 ± 0.28 and 2.79 ± 0.11, respectively) compared to D4 CON ($p < 0.001$; Fig. 4a). PGC-1β [F(3, 20) = 17.55, $p < 0.001$] was increased for both day 4 and day 6 FORM groups (2.39 ± 0.21 and 2.16 ± 0.10, respectively) compared to D4 CON (1.00 ± 0.21; $p < 0.001$ and $p < 0.01$, respectively), and D6 FORM was significantly greater than D6 CON (0.98 ± 0.16, $p < 0.01$; Fig. 4b). For TFAM [F(3, 20) = 4.92, $p = 0.01$], D6 FORM (1.59 ± 0.06) was greater than both D4 and D6 CON groups (1.00 ± 0.14, $p = 0.02$ and 1.05 ± 0.15, $p = 0.03$, respectively; Fig. 4c). NRF1 [F(3, 20) = 16.186, $p < 0.001$] was decreased for all groups compared to D4 CON (Fig. 4d). NRF2 [F(3, 20) = 76.675, $p < 0.001$] increased at D6 CON (2.71 ± 0.11) and D6 FORM (1.39 ± 0.06) compared to D4 CON (1.00 ± 0.12; $p < 0.001$ and $p = 0.03$, respectively), however, D6 FORM was found to be significantly lower than D6 CON ($p < 0.001$; Fig. 4e). Superoxide dismutase 2 (SOD2; [F(3, 20) = 22.14, $p < 0.001$]) was increased for D6 CON (4.55 ± 0.64) compared to D4 CON (1.00 ± 0.16; $p < 0.001$), and D6 FORM (2.00 ± 0.17) was found to be significantly lower than D6 CON ($p < 0.001$), indicating a lesser presence of the superoxide anion at D6 with formoterol stimulation (Fig. 4f).

Formoterol affects intracellular SKM activity of genes related to thyroid hormone metabolism

Formoterol stimulation resulted in the reduced expression of THRα [F(3, 20) = 6.92, $p < 0.01$] for both day 4 and day 6 myogenesis (0.57 ± 0.02 and 0.54 ± 0.05, respectively) compared to D4 CON (1.00 ± 0.15; $p < 0.01$; Fig. 5a). Despite a decreased signal for THRα expression, DIO2 [F(3, 20) = 95.601, $p < 0.001$]

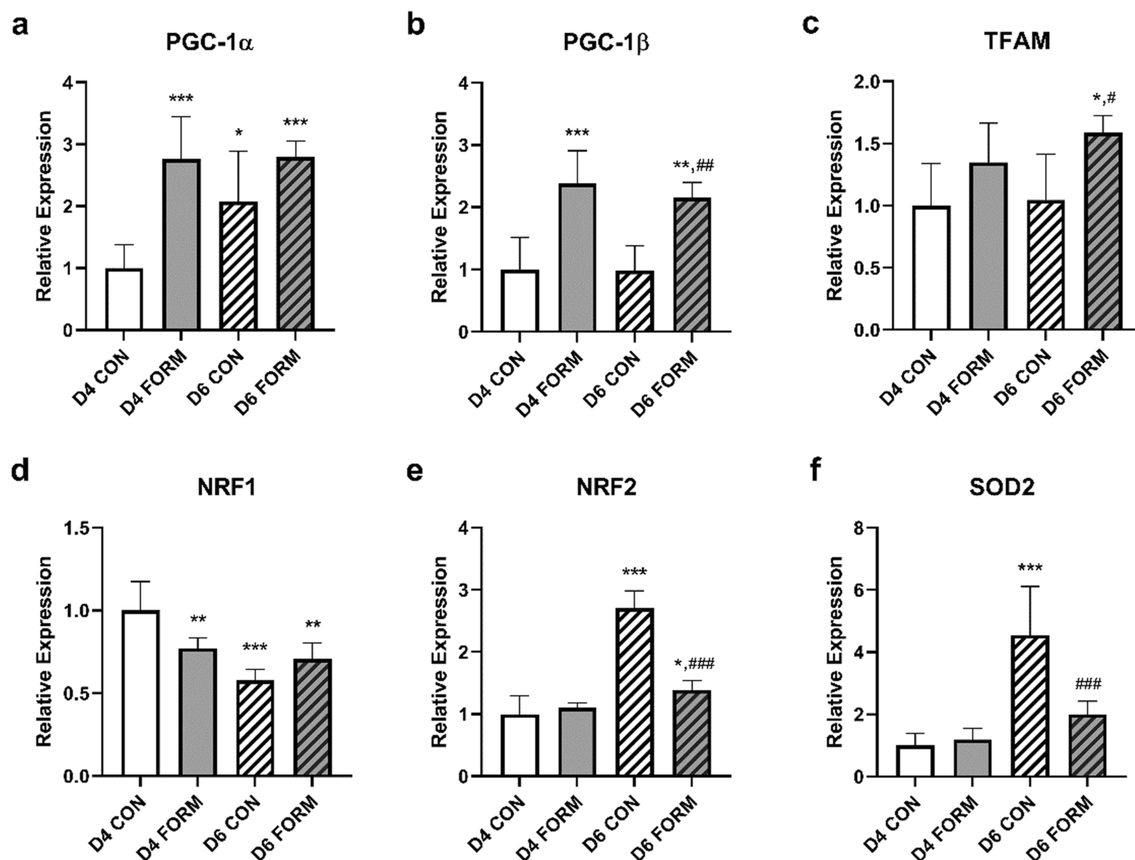

Fig. 4 SKM mitochondria related gene expression. **a**. Difference in gene expression between all groups for PGC-1α. **b**. Difference in gene expression between all groups for PGC-1ß. **c**. Difference in gene expression between all groups for TFAM. **d**. Difference in gene expression between all groups for NRF1. **e**. Difference in gene expression between all groups for NRF2. **f**. Difference in gene expression between all groups for SOD2. Data are presented as mean ± SEM. * = significance compared to D4 CON, # = significance compared to D6 CON; p values: *< 0.05, **< 0.01, ***< 0.001

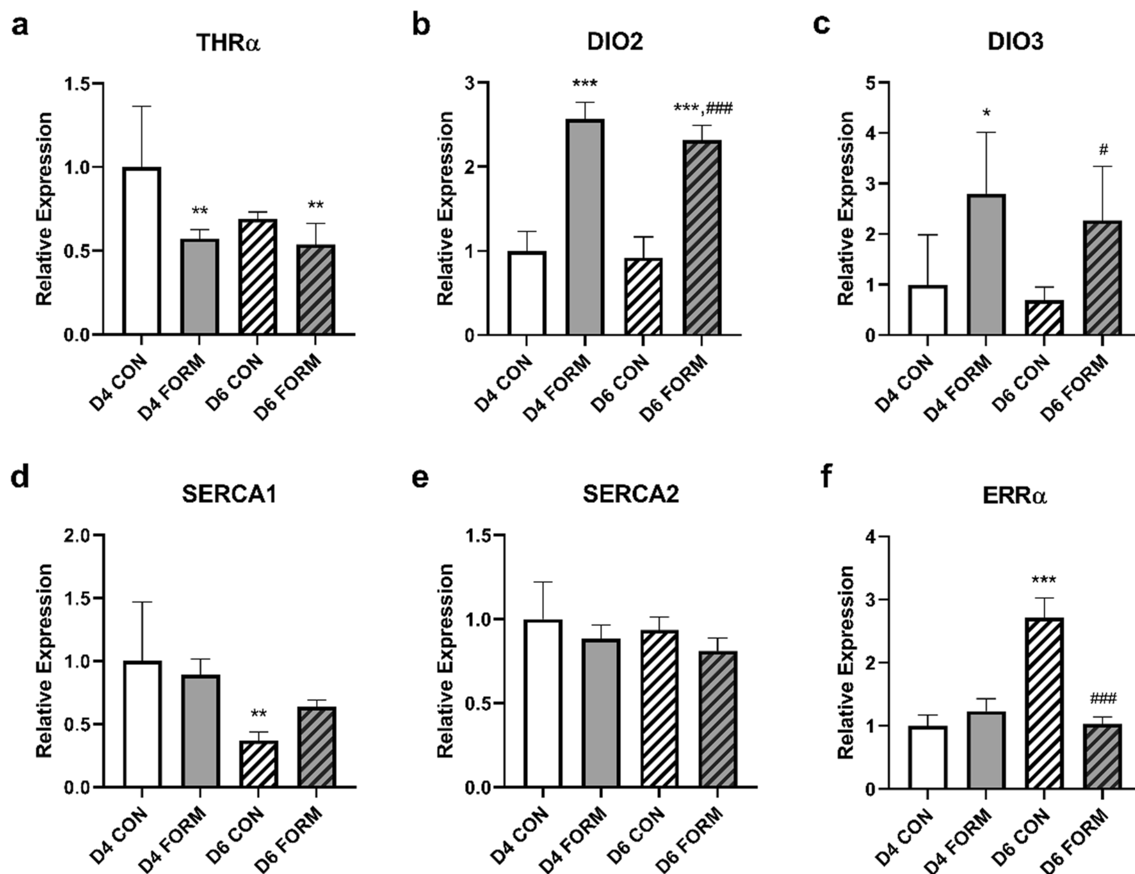

Fig. 5 SKM thyroid hormone metabolism related gene expression. **a**. Difference in gene expression between all groups for THRα. **b**. Difference in gene expression between all groups for DIO2. **c**. Difference in gene expression between all groups for DIO3. **d**. Difference in gene expression between all groups for SERCA1. **e**. Difference in gene expression between all groups for SERCA2. **f**. Difference in gene expression between all groups for ERRα. Data are presented as mean ± SEM. * = significance compared to D4 CON, # = significance compared to D6 CON; p values: *< 0.05, **< 0.01, ***< 0.001

expression was increased by formoterol treatment for day 4 and day 6 (2.56 ± 0.08 and 2.31 ± 0.07, respectively) as compared to D4 CON (1.00 ± 0.10, $p < 0.001$), with D6 FORM also being significantly greater than D6 CON (0.92 ± 0.10, $p < 0.001$; Fig. 5b). DIO3 [F(3, 20) = 6.66, $p < 0.01$] was greater at D4 FORM and D6 FORM (2.80 ± 0.50 and 2.27 ± 0.44, respectively) compared to D4 CON (1.00 ± 0.40, $p = 0.02$) and D6 CON (0.69 ± 0.11, $p = 0.04$), respectively (Fig. 5c). SERCA 1 [F(3, 20) = 7.79, $p < 0.001$] and 2 [F(3, 20) = 2.27, $p = 0.11$] expression remained unchanged by formoterol stimulation; however, SERCA1 expression in D6 CON (0.37 ± 0.03) was decreased compared to D4 CON (1.00 ± 0.19, $p < 0.01$; Fig. 5d, e). Expression of ERRα [F(3, 20) = 89.26, $p < 0.001$] was increased for D6 CON (2.71 ± 0.13) compared to D4 CON (1.00 ± 0.07, $p < 0.001$). Additionally, D6 FORM (1.03 ± 0.05) was significantly lower compared to D6 CON ($p < 0.001$), but not different from D4 CON ($p = 0.99$; Fig. 5f).

Formoterol increases SKM β2-adrenergic receptor gene expression and influences genes related to cellular homeostasis

To identify the effects of exercise stimulation on cellular homeostasis during SKM myogenesis, several genes related to bioenergetics, cell atrophy, ROS mitigation, as well as B2AR were targeted. We found that formoterol stimulation increased B2AR [F(3, 20) = 3.91, $p = 0.02$] expression at day 6 of myogenesis (D6 CON; 1.31 ± 0.02) compared to D4 CON (1.00 ± 0.08, $p = 0.02$; Fig. 6a). AMPK [F(3, 20) = 0.30, $p = 0.83$] and the autophagy-related marker (ATG5; [F(3, 20) = 1.73, $p = 0.19$]) (Wang et al. 2022) were unchanged for all groups (Fig. 6b, c). The glucose transporter (GLUT4; [F(3, 20) = 7.88, $p < 0.01$]) (Wang et al. 2020) was increased for both D4 FORM and D6 FORM (2.38 ± 0.34 and 1.95 ± 0.24, respectively) compared to D4 CON (1.00 ± 0.27, $p < 0.01$) and D6 CON (0.92 ± 0.14, $p = 0.04$), respectively (Fig. 6d). Glutathione synthetase (GSS; [F(3, 20) = 7.53, $p < 0.01$]), a precursor

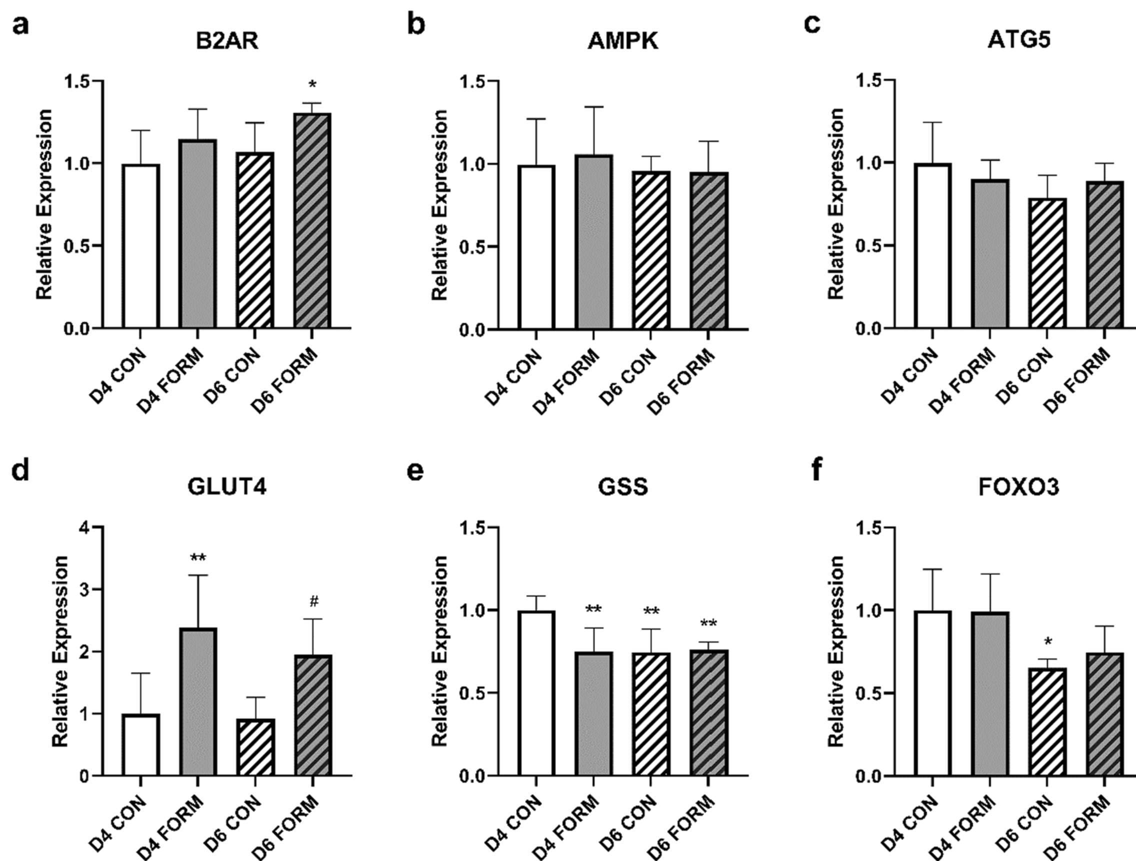

Fig. 6 SKM cellular homeostasis related gene expression. **a**. Difference in gene expression between all groups for β2AR. **b**. Difference in gene expression between all groups for AMPK. **c**. Difference in gene expression between all groups for ATG5. **d**. Difference in gene expression between all groups for GLUT4. **e**. Difference in gene expression between all groups for GSS. **f**. Difference in gene expression between all groups for FOXO3. Data are presented as mean ± SEM. * = significance compared to D4 CON, # = significance compared to D6 CON; *p* values: *< 0.05, **< 0.01

to glutathione, was decreased for all treatment groups compared to D4 CON ($p < 0.01$; Fig. 6e). Also related to autophagy, forkhead box (FOXO3; [$F(3, 20) = 5.29$, $p < 0.01$]) was decreased for the D6 CON group (0.65 ± 0.02) compared to D4 CON only (1.00 ± 0.10, $p = 0.02$; Fig. 6f).

Discussion

In this study, we utilized an in vitro SKM cell culture model to investigate the influence of exercise-related signaling on the expression of 24 genes related to myogenesis, mitochondrial biogenesis, thyroid hormone metabolism, and cellular homeostasis. Our study is novel in that multiple SKM gene expression pathways were concomitantly analyzed and interpreted together, not only for terminal stage myotubes, but also during mid-myogenesis.

Skeletal muscle cells naturally cycle through periods of both protein synthesis and protein breakdown, all of which occur during the various stages of myogenesis throughout life. Imbalances to these processes result in increased proteolysis, dysregulated mitigation of ROS, mitochondrial dysfunction, and eventual loss of SKM mass and function (Wilkinson et al. 2018; Dungan et al. 2023). Exercise has many beneficial effects on muscle health and is often prescribed as a treatment for preventing or attenuating sarcopenia and pathological atrophy (Carter et al. 2015; Dungan et al. 2023). However, the cross-pathway mapping of exercise-stimulated gene expression has not been fully elucidated, especially in human tissue.

Formoterol is a long-acting B2AR agonist that has been effectively used as an exercise mimetic with in vivo and in vitro animal models, stimulating many pathways within SKM tissue (Bianco et al. 2019; Miura et al. 2007; Duplanty et al. 2018). Specifically, in rodent models, formoterol increases PGC-1α, mitochondrial respiration, morphology, and biogenesis (Bianco et al. 2019; Miura et al. 2007; Wills et al. 2012). In rhesus macaques, formoterol treatment increases PGC-1α expression, mitochondrial DNA quantity, and improves mitochondrial respiration of unhealthy myoblasts (Duplanty et al. 2018).

Stimulation of B2AR signaling has been implicated as a potential therapeutic target for skeletal muscle wasting disorders, due to this pathway's influence on regulating SKM protein synthesis and degradation (Koopman et al. 2009 Nov; Onslev et al. 2019). While the use of pharmaceutical therapy utilizing B2AR agonists is currently limited for people experiencing muscular atrophy or disease, it is clear that the B2AR pathway plays a significant role in the regulation of SKM growth (Lynch and Ryall 2008; Hostrup et al. 2018), especially in the context of exercise signaling. Additionally, the B2AR pathway is an upstream signaling cascade that is activated by both endurance and resistance exercise and stimulated by epinephrine and norepinephrine. This makes the pathway ideal for in vitro exercise stimulation, rather than stimulating individual downstream molecules with mimetics such as AICAR, which only affects APMK (Višnjić et al. 2021). In this study, we demonstrated that formoterol upregulated B2AR expression and subsequent downstream targets in human skeletal muscle cells, such as PGC-1α, consistent with previous studies, indicating formoterol as an appropriate exercise mimetic and alternative to other commonly used exercise mimetics for in vitro models. Hence, formoterol treatment appeared to upregulate mitochondrial biogenic activity overall during myogenesis, similar to exercise stimulation.

The mechanisms of myogenic regulation have been well characterized (Johnson et al. 2023; Chal and Pourquié 2017). However, this regulation has not been well studied in human myotubes throughout the various stages of myogenesis, especially in the context of exercise stimulation. In this study, our exercise mimetic model induced a myogenic program that appears to prolong myoblast proliferation and delay maturation of myotubes, as evidenced by the changes in MYF5, MYOD, and MYOG for both D4 FORM and D6 FORM. The decreases in MRF expressions as a result of formoterol in this study is unique, as increases in MYOD and MYOG gene expression following exercise have been previously reported (Angleri et al. 2022). Additionally, an increase in MYF5 suggests a shift from differentiation to proliferation of myoblasts during late-stage myogenesis. Based on these results, the effects of exercise signaling on myogenesis may be influenced by the timing of the stimulus in relation to what stage the myotubes are in within the myogenic program (i.e. mid- or late-stage myogenesis). Thus, exercise stimuli could interfere with the regeneration or repair of SKM tissue by delaying or prolonging myogenesis. These results may be meaningful in the context of SKM growth and regeneration, as it indicates the timing of additional exercise stimuli should be strategically considered due to its ability to influence the timeline of myotube formation and maturity.

To investigate muscle growth during myogenesis, we analyzed the gene expression for two important factors that regulate protein synthesis in response to exercise signaling, including PGC-1α4 [an isoform of PGC-1α, which is a promoter of resistance exercise-induced hypertrophy (Moberg et al. 2021)] and the protein kinase mTOR. We found that formoterol treatment may promote an increase in protein synthesis, particularly in mature myotubes, despite delayed signaling for myotube formation, as indicated by an increase in PGC-1β and the rescue of mTOR during late-stage myogenesis. These results could indicate greater cellular energetic demands directed towards myotube formation and differentiation in the CON groups, whereas formoterol may have triggered processes more geared towards protein synthesis and myoblast proliferation. These results are consistent with previous literature examining the influence of resistance and concurrent exercise on protein synthesis targets (Moberg et al. 2021), but further investigation should be conducted to examine the specific cellular demands throughout the myogenic cycle and what effects exercise stimulation may have on these demands.

Autophagy is a normal cellular process that removes damaged organelles while recycling functional cellular components which fuse with proximal healthy cells and is an important process during cell differentiation (Fortini et al. 2016). The regulation of autophagy in healthy cells aims to optimize tissue function as a whole and prevent the accumulation of dysfunctional cells and cellular components, performing as a quality control mechanism. ATG5, a regulator of autophagy, is upregulated throughout the myogenic process and is responsible for myotube fusion (Fortini et al. 2016). Alternatively, exercise upregulates ATG5 in vivo (Wang et al. 2022), implicating autophagy to be a primary contributing mechanism of myogenesis and tissue repair. Regulation of the autophagy system declines linearly with age (Barbosa et al. 2019), but is augmented by chronic exercise training, highlighting the importance of regular exercise in regulating cellular processes throughout the lifespan (Escobar et al. 2019). Interestingly, we found no changes in ATG5 between conditions in this study. However, we found differential responses for ROS mitigation. Skeletal muscle is a major site of ROS generation as a result of energy production and muscle contraction (Nyberg et al. 2014). The accumulation of ROS is detrimental to organismal and tissue health and function with profound tissue dysregulation and inflammation evident as a result (Nyberg et al. 2014). Efficient mechanisms are in place to minimize the accumulation of ROS and prevent damage to DNA, as found in aging muscle (Nyberg et al. 2014). We found reduced GSS expression for all groups compared to D4 CON, and elevated SOD2, a mitochondrial

related antioxidant enzyme, for both D6 groups, resulting in potential ROS mitigation, consistent with previous literature (Powers et al. 2023). Future research should examine the effect of formoterol on ROS mitigation directly in human skeletal muscle cells to confirm this hypothesis.

Recent evidence suggests peripheral effects of thyroid hormone signaling within SKM are substantial and play crucial roles in metabolism, function, and growth (Stefano et al. 2021). For example, within SKM, intracellular calcium is managed by SERCA1/2 which, in turn, is upregulated by T3 and exercise (Tøien et al. 2021). Analyzing the expression of THRα and the deiodinases DIO2 and DIO3 is of particular interest in helping to characterize SKM homeostasis due to their regulatory roles in mitochondrial biogenesis and myogenesis (Bloise et al. 2018). Our results indicate robust activity of TH metabolism in response to exercise mimetic stimulation within SKM. In particular, the increases in DIO2 found in the FORM groups, which indicate increased activation of T4 into T3 within SKM, lead to stimulation of nuclear targets specific to metabolism and myogenesis (Long et al. 2023). To further investigate TH metabolism in connection to mitochondrial homeostasis, we analyzed estrogen-related receptor-α (ERRα), a nuclear receptor that works in tangent with TH receptors, and is co-activated by PGC-1α to regulate mitochondrial processes such as biogenesis, mitophagy, fission, and oxidative phosphorylation (Tripathi et al. 2020). Interestingly, ERRα was elevated at D6 CON but not D6 FORM, despite significant increases in PGC-1α for both groups. We can therefore hypothesize that mitochondrial biogenesis still occurred in the absence of ERRα stimulation, as increases for both PGC-1α and TFAM in the D6 FORM group, were also observed, but further analyses should confirm this hypothesis. One explanation for elevated ERRα in the D6 CON group may be related to the increased NRF2 and SOD2 expression found for this condition. This likely indicates an increased need for oxidative phosphorylation and mitigation of ROS, which may have resulted from less mitochondrial biogenesis than the FORM group. As a result, our group is further analyzing the effects on TH-related genes within a model of hypothyroidism to elucidate the dysregulation this condition may incur in SKM cells and if exercise stimulation can alter these effects.

While this study provides important insights into the use of formoterol as an exercise mimetic in vitro in SKM cells, limitations do exist. First, the small sample size of the experiment impacted the variability of the results and could have influenced statistical analyses that would otherwise have been detected in a larger sample size. Second, only gene expression was analyzed within this study. This study could have benefited from secondary analyses to strengthen hypotheses made from the results, such as Western blots, ROS analyses, and oxygen consumption rate (i.e. Seahorse). Additionally, we were unable to perform replicates of the experiment in different SKM cell lines and female samples as well. Future studies could address this.

Conclusions

Formoterol stimulated the gene expression for SKM pathways related to mitochondrial biogenesis, thyroid metabolism, and cellular homeostasis. Additionally, formoterol resulted in a myogenic program that appears to favor prolonged myoblast proliferation, delayed myotube maturation, and increased protein synthesis. Formoterol also stimulated the conversion of T4 to active T3, subsequently contributing to increased mitochondrial biogenesis and ROS mitigation. The results in this study provide the groundwork for mechanistic studies that require controlling for the vast array of effects that external stimuli (e.g. age, pathology, nutrition, medication) can have on the wide range of physiological processes in human SKM. We simultaneously examined the overlapping and interconnected functions of the many different and complex intracellular components that regulate SKM growth and physiology, which should be a key feature of future research in this topic. RNAseq is a promising analysis tool to examine overlapping and interconnected genetic pathways that would benefit the field of exercise physiology. Current and future research is being aimed at discovering the specific exercise strategies, nutritional interventions, and medical practices that will most effectively support the new growth of SKM for those who are experiencing progressive loss of skeletal muscle mass. In many cases, sarcopenia and atrophy that is pathologic in origin (e.g. alcoholic myopathy, disuse atrophy, and cancer cachexia) may be treated, in significant part, by targeted nutrition and exercise prescription.

Abbreviations

AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
ATG5	Autophagy related 5
B2AR	β2-Adrenergic receptor
DIO2	Deiodinase 2
DIO3	Deiodinase 3
DMSO	Dimethyl sulfoxide
ERRα	Estrogen related receptor alpha
FOXO3	Forkhead box O3
GLUT4	Glucose transporter 4
GSS	Glutathione synthetase
MEF2c	Myocyte enhancer factor 2
MRFs	Myogenic regulatory factors
mTOR	Mechanistic target of rapamycin
MYF5	Myogenic factor 5
MYOD	Myogenic differentiation 1
MYOG	Myogenin

NRF1	Nuclear respiratory factor 1
NRF2	Nuclear respiratory factor 2
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PGC-1α4	PGC-1α isoform 4
PGC-1β	Peroxisome proliferator-activated receptor gamma coactivator 1-beta
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxidative species
RPS13	Ribosomal protein S13
rT3	Reverse T3
SEM	Standard error of the mean
SERCA1	Sarco/endoplasmic reticulum Ca^{2+} ATPase isoform 1
SERCA1	Sarco/endoplasmic reticulum Ca^{2+} ATPase isoform 2
SKM	Skeletal muscle
SOD2	Superoxide dismutase 2
T3	Triiodothyronine
TFAM	Mitochondrial transcription factor A
TH	Thyroid hormone
THR	Thyroid hormone receptor alpha

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Author contributions
A.A.D designed and managed the study. E.L.Z, A.A.D., R.A.G. G.D.G., M.F.B., M.L.S., and B.R.R performed experiments, analyzed the data, and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
All experimental procedures using human tissue were approved by the Texas Woman's University Institutional Review Board (IRB-FY2021-71). During the course of this research, the Helsinki Declaration was strictly adhered to. Participants gave informed consent according to ethical guidelines, received information on study purpose, procedures, risks, confidentiality, and right to withdraw. Participation was voluntary and all data collected were kept confidential and anonymous in compliance with the guidelines.

Consent for publication
Written consent to publish this information was obtained.

Competing interests
No author states to have any conflicts of interest.

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