Deane, CD, Hughes, DC, Sculthorpe, N, Lewis, MP, Stewart, CE and Sharples, AP

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Authors:

Colleen S. Deane [b,c≠], David C. Hughes [a,b≠], Nicholas Sculthorpe [d], Mark P. Lewis [b,e,f,g], Claire E. Stewart [a]. & Adam P. Sharples [a,b*].

Affiliations:

[a] Stem Cells, Ageing & Molecular Physiology (SCAMP) Unit, Research Institute for Sport and Exercise Sciences (RISES), School of Sport and Exercise Sciences, Liverpool John Moores University, Tom Reilly Building, Byrom Street, Liverpool, UK.
[b] Muscle Cellular and Molecular Research Group (MCMPRG), Institute of Sport and Physical Activity Research (ISPAR), University of Bedfordshire, UK.
[c] School of Health and Social Care, Bournemouth University, UK.
[d] Institute for Clinical Exercise and Health Science (ICEHS), University of the West of Scotland, Hamilton, UK.
[e] Cellular and Molecular Physiology, Musculoskeletal Biology Research Group, School of Sport, Exercise and Health Science, Loughborough University, Loughborough, UK.
[f] Cranfield Health, Cranfield University, Cranfield, Bedfordshire, UK.
[g] School of Life and Medical Sciences, University College London (UCL), UK.

***Corresponding Author: Dr. Adam P. Sharples BSc. (Hons) MSc. FHEA. PhD.**
Lecturer/Senior Lecturer in Cellular and Molecular Physiology
Managing-Editor (Cellular and Molecular Exercise Physiology)
Stem Cells, Ageing and Molecular Physiology Unit
Research Institute for Sport and Exercise Sciences
School of Sport and Exercise Sciences
Life Sciences Building (rm 1.17)
Byrom Street, Liverpool, UK, L3 3AF
Tel: 07812732670
Twitter: @DrAdamPSharples (Click to Follow) @CelMolExPhysiol (Click to Follow)
Profile (Click here)
Publications (Click here)
Email: a.sharples@ljmu.ac.uk or a.p.sharples@googlemail.com

[≠] Colleen S. Deane and David C. Hughes are considered joint primary authors of this article.

Key words: satellite cell, muscle stem cell, PI3K, Akt, mTOR, myostatin

Running head Title: Testosterone improves impaired hypertrophy

Abstract:

We investigated the ability of testosterone (T) to restore differentiation in multiple population doubled (PD) murine myoblasts, previously shown to have reduced differentiation in monolayer and bioengineered skeletal muscle cultures vs. their parental controls (CON) (Sharples et al. 2011, Sharples et al., 2012). Cells were exposed to low serum conditions in the presence or absence of T (100 nM) ± PI3K inhibitor (LY294002) for 72 hrs and 7 days (early and late muscle differentiation respectively). Morphological analyses were performed to determine myotube number, diameter (μm) and myonuclear accretion as indices of differentiation and myotube hypertrophy. Changes in gene expression for myogenin, mTOR and myostatin were also performed. Myotube diameter in CON and PD cells increased from 17.32 ± 2.56 μm to 21.02 ± 1.89 μm and 14.58 ± 2.66μm to 18.29 ± 3.08μm ($P \leq 0.05$) respectively after 72 hrs of T exposure. The increase was comparable in both PD (+25%) and CON cells (+21%) suggesting a similar intrinsic ability to respond to exogenous T administration. T treatment also significantly increased myonuclear accretion (% of myotubes expressing 5+ Nuclei) in both cell types after 7 days exposure ($P \leq 0.05$). Addition of PI3K inhibitor (LY294002) in the presence of T attenuated these effects in myotube morphology (in both cell types) suggesting a role for the PI3K pathway in T stimulated hypertrophy. Finally, PD myoblasts showed reduced responsiveness to T stimulated mRNA expression of mTOR vs. CON cells and T also reduced myostatin expression in PD myoblasts only. The present study demonstrates testosterone administration improves hypertrophy in myoblasts that basally display impaired differentiation and hypertrophic capacity vs. their parental controls, the action of testosterone in this model was mediated by PI3K/Akt pathway.

1. Introduction

Muscle wasting occurs within many life-threatening diseases such as cancer (termed cancer cachexia) [1], AIDS [2], Sepsis [3], heart failure [4] and ageing (sarcopenia) [5-7]. Individuals that experience muscle loss during these various disease states have a reduced physiological and functional capacity, altered metabolism [8,9] and a reduction in circulating levels of Growth Hormone, Insulin-like-Growth Factor-I (IGF-I) and Testosterone [2]. These accumulative factors manifest themselves in increased frailty and morbidity and therefore reduced quality of life that subsequently leads to earlier mortality [10, 11]. In the instance of cancer cachexia approximately 50% of patients suffer with cachexia (muscle atrophy), which alone accounts for 25% of all cancer deaths [12-14]. The regulation of skeletal muscle mass is reliant on the balance between hypertrophy (e.g. protein synthesis/anabolism) and atrophy (e.g. protein breakdown/catabolism). A potential clinical intervention for promoting a positive net balance in the favour of protein synthesis is Testosterone (T) administration [15, 16]. T replacement therapy has been observed to increase muscle strength and mass in various clinical populations including patients with AIDS, COPD and sarcopenia [12, 17-20]. However a limited number of studies have investigated the role of T in skeletal muscle hypertrophy and atrophy [21-23] at the cellular level.

Skeletal muscle fibre numbers are set *in-utero* i.e. fibres are terminally differentiated or post mitotic and unable to divide. Skeletal muscles regenerative capacity therefore occurs as a result of a specialised cell type, the satellite cell, which resides underneath the basal lamina of the mature fibre and has mitotic potential. With the relevant cues, satellite cells are activated (termed myoblasts), then proliferate or return to quiescence for subsequent regenerative bouts. Activated myoblasts repair the muscle by fusing with the existing fibres, a process known as differentiation [24, 25]. Most recently, our laboratories have highlighted two myoblast models to study reduced differentiation capacity [7, 26, 27]. The first investigated the parental mouse C_2 myoblasts vs. their subclone, the C_2C_{12} cells. Despite their shared origins, we observed differences in morphological and biochemical responses between the C_2 and C_2C_{12} cells. The C_2 cells displayed slower and diminished differentiation profiles compared to the C_2C_{12} cells and were also more susceptible to TNF-α-induced inhibition of differentiation and induction of apoptosis [27]. Because muscle wasting is associated with reduced muscle mass [28, 29] and increased susceptibility to TNF-induced muscle protein degradation [30-32], this comparative model provided us with an excellent representation of muscle atrophy, hypertrophy and adaptability, thus, enabling the determination of potential regulators associated with muscle wasting. The second model utilised C_2C_{12} cells that had undergone multiple population doublings (PD) vs. parental

control cells (CON), which have undergone no doublings relative to the PD cells [7]. These cells also display impaired differentiation in monolayer [7] and in three dimensional culture systems [26] vs. their parental controls. We reported that the PD cells had a significantly reduced number of cells exiting the cell cycle in G1, a prerequisite for fusion, with corresponding decreases in transcript expression of IGF-I, myoD, myogenin and reduced activation of Akt with increases in IGFBP5 mRNA and JNK activation vs. control cells [7]. Interestingly, similar morphology, transcript and signalling processes were also observed in cells isolated from aged human muscle [33-35] and in whole tissue biopsies [36, 37]. Thus, these cells can be used as a representative model to investigate mechanisms of atrophic phenotypes (PD) vs. parental control cells (CON) that display hypertrophic phenotypes [7].

In the present study we utilised the latter model to investigate T administration on PD cells displaying impaired differentiation. As the PD cells display a reduction in Akt activation [7], the PI3K/Akt/mTOR pathway was investigated in the present study, as it is inextricably involved in protein synthesis [38] and most recently linked to T's regulation of muscle hypertrophy [23].

The **aims** were to: 1) Improve the impaired differentiation and hypertrophy profiles observed previously in PD cells using T administration; 2) Manipulate the role of PI3K/Akt in Ts regulation of differentiation in PD and CON cells. We **hypothesised** that T would induce improved differentiation and hypertrophy in CON cells and would improve the impaired differentiation in the PD cells. Further by inhibiting PI3K, testosterone's ability to restore differentiation action would be negated in both cell types. The overall **objective** of the research was to utilise an *in vitro* model that is representative of impaired differentiation (PD) in order to elucidate the ability of T to improve differentiation and hypertrophy and to investigate its cellular and molecular mechanisms of action.

2. Materials and Methods

2.1 Cell Culture

Mouse C_2C_{12} [39, 40] (ATCC, Rockville, MD, USA) skeletal muscle myoblasts were seeded at 80,000 cells per ml in 2 ml of growth media (GM) per well (Dulbecco's modifed eagle's medium, DMEM (Sigma, Dorset, UK)), 20% fetal bovine serum (FBS) (PAA, Somerset, UK), 1% PenStrep (Invitrogen, Paisley, UK)) onto 0.2% porcine gelatin-coated (Sigma, Dorset, UK) 6-well plates (Fisher Scientific, Loughborough, UK) and grown in a humidified 5% CO_2 atmosphere at 37°C. Population doubled (PD) cells and their parental controls (CON) were developed as detailed in Sharples et al. [7, 26]. Briefly, PD cells underwent an extra 58-60 population doublings vs. their CON cells. Once confluent, the myoblasts were changed from growth media to low serum media/differentiation media (DM; composed of: DMEM, 2% horse serum (HS), 1% Penstrep and 1% L-Glutamine) which promotes the fusion of the myoblasts into multinucleated myotubes. C_2C_{12} myoblasts undergo spontaneous differentiation into myotubes on serum withdrawal, and do not require growth factor addition to stimulate the process [40]. Cells were incubated in DM for 30 minutes at 37°C in a 5% CO_2 atmosphere with this period of equilibration denoted as, 0 hour time point. Cells at the time points of 72 hrs and 7 days (early and late muscle differentiation respectively) were fixed (see below) for subsequent morphology analyses or isolated for reverse transcription quantitative real-time polymerase chain reaction (RT-PCR).

2.2 Cell Treatments

All treatments were administered in DM described above. The treatments comprised of a vehicle control (DMSO at a concentration of 0.01%), Testosterone alone (T) 100 nM (Tocris Bioscience, Bristol, UK), PI3K inhibitor (LY) 5 μM (LY294002- Calbiochem, Middlesex, UK), 100 nM T + 5 μM LY. DMSO was used as the solvent for T reconstitution at the same concentration as the vehicle (0.01%). For results and figure legends the following nomenclature will be used DMSO, T, LY and T + LY respectively. All treatments were added at 0 hrs and existing media was further supplemented at 72 hrs. The inhibitor, LY at a dose of 5 μM has been extensively shown to be highly specific and effective in C_2C_{12} cells in inhibiting PI3K and downstream Akt [41-46].

2.3 RNA Isolation

Plates for each time point (0 hrs, 72 hrs and 7 days) were washed with 1ml/well phosphate buffer saline (PBS) (Fisher Scientific, Loughborough, UK) and extracted for RNA using 250 µl TRIzol™ Reagent (Sigma, Dorset, UK) in each well. Total RNA was extracted by following manufacturer's instructions. RNA concentration and purity were assessed through UV spectroscopy at ODs of 260 and 280 nm, using the Nanodrop spectrophotometer 3000 (Fisher, Rosklide, Denmark). Only samples with a 260:280 ratio of between 1.9 and 2.15 were carried forward for reverse transcription and PCR analysis detailed below.

2.4 Primer Design

Primer sequences (Table 1.) were identified using Gene (NCBI, www.ncbi.nlm.nih.gov/gene) and designed using both web-based OligoPerfect™ Designer (Invitrogen, Carlsbad, CA, USA) and Primer-BLAST (NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast). Sequence homology searches ensured specificity. Three or more GC bases in the last 5 bases at the 3' end of the primer were avoided. Secondary structure interactions (hairpins, self-dimer and cross dimer) within the primer were avoided. All primers ranged between 18 and 23 bp and amplified a product between 173 and 197 bp. GC content was between 36.3 and 55.5% (Table 1). Primers without the requirement of further purification were purchased from Sigma (Suffolk, UK).

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2.5 Reverse Transcription Quantitative Real Time Polymerase Chain Reaction (rt-qRT-PCR)

70 ng of RNA/sample was reverse transcribed and amplified using QuantiFast™ SYBR® Green RT-PCR one-step kit on a Rotogene 3000Q (Qiagen, Crawley, UK) supported by Rotogene software (Hercules, CA, USA). rt-qRT-PCR was performed as follows: 10 min, 50°C (reverse transcription), 5 min 95°C (transcriptase inactivation and initial denaturation), Followed by: 10 secs, 95°C (denaturation), 30 secs, 60°C (annealing and extension) for 40 cycles. Following completion, melting curve analyses were performed to exclude primer-dimer and non-specific amplification (all melt analysis in this study presented single reproducible peaks). All PCR efficiencies were comparable (standard deviation ± 0.03%) across all conditions and genes. Relative mRNA expression was quantified for myogenin, mTOR and myostatin (Table 1.) using the comparative Ct ($^{\Delta\Delta}$ct) method [7, 47] against a

stable reference gene of RP-IIb (combined Ct value for all runs across experimental conditions 16.63 ± 0.38) and calibrator of CON treatment at 0 hrs.

2.6 Morphological Analysis

Following media aspiration and 2 × PBS washes (1 ml/well), cells were fixed by adding 1 ml methanol/acetone (1:1) to 1 ml PBS in each well in a drop-wise manner and incubated for 10 mins RT. Following aspiration, 2 ml methanol and acetone (1:1) were added to each well and incubated for a further 10 mins. Finally PBS (2ml/well) was added after removal of the methanol and acetone mix and plates were stored at 4°C until further analyses. This fixing process allows nuclei to become discernible under light microscopy alone, without the need for additional nuclear staining. A total of 30 fields per condition for each time point were captured with a cell imaging system at x10 magnification (Inverso-TC, CETI, Medline Scientific Limited, Oxon, UK) and analysed using Image J (Java) software (National Institutes of Health, USA). Morphology was assessed by determination of myotube diameter, number of myotubes per view, mean number of nuclei per myotube per field of view, fusion index (cell fusion) and total nuclei counts (changes in total cell number and therefore an indices for proliferation). A myotube was defined as containing 3^{+} nuclei encapsulated within cellular structures, so to avoid counting of single cells undergoing mitosis. Myotube diameter (μm) was determined by measuring the diameter of 3 equidistant points on each myotube (left end, middle, right end) and determining the mean of the 3 values as previously described [48, 49]. An indicator of myonuclear accretion (fusion index) was calculated by dividing myotubes into two classes; myotubes which expressed 3-4 nuclei or myotubes which expressed 5+ nuclei, with the data expressed as percentages.

2.7 Statistical Analyses

Experiments were performed 3 times (n = 3) in triplicate. Data are presented as mean ± S.D. Morphology data at 72 hrs were assessed using a (2×2) mixed two-way factorial ANOVA (GraphPad Software, Inc., San Diego, USA) for interactions between cell type (PD, CON) and treatments (DMSO, T). LY and T+LY treatment were excluded from the aforementioned analysis, as there were no observable myotubes at 72 hrs in these conditions. Morphology data at 7 days were assessed using a (2×4) mixed two-way factorial ANOVA for interactions between cell type (PD, CON) and treatments (DMSO, T, LY and T + LY). Gene expression data were assessed using a (2×2×4) mixed three-way factorial ANOVA for interactions between cell type (PD, CON), time (72 hrs and 7 days) and treatments (DMSO, T, LY and T + LY). Bonferroni post hoc analyses were performed where main effects for treatment or cell type occurred, without a significant interaction between treatment and cell type. If significant interactions were present, independent t-tests were conducted to confirm

statistical significance between variables of interest. Furthermore, paired-sample t-tests were undertaken for variable of interests within cell type and treatment. Myonuclear Accretion (fusion index) measures were analysed using Chi Square to investigate whether myotube categories (3-4 nuclei or 5+ nuclei) differed from one another based on treatment exposure. A P-value of ≤ 0.05 was considered to represent a statistically significant difference.

3. Results

3.1 Exogenous T treatment increases myotube hypertrophy in both CON and PD cells

The administration of exogenous DMSO, T, LY, T+LY to CON and PD cells brought about morphological changes in differentiation (Figs 1 & 2). Firstly, it is important to mention that in support of previous observations showing reductions in differentiation in PD vs. CON cells [7]; a significant difference for mean myotube diameter at 72 hrs was again observed in the present study (CON 17.32 ± 2.56 vs. PD 14.58 ± 2.66, P = 0.01, Fig. 3A, supporting the atrophic phenotype observed previously in PD cells [7,26]. Importantly, in the present study, T administration appeared to increase hypertrophy (myotube diameter) in both cell types. Statistical analyses confirmed the morphological observations in Fig. 1 and 2 and revealed that a T-stimulus significantly increased myotube diameter in both CON and PD cells after 72 hrs (CON+T 21.02 ± 1.89 vs. CON 17.32 ± 2.56; PD+T 18.29 ± 3.08 vs. PD 14.58 ± 2.66, P = 0.01, Fig. 3A) and 7 days (CON+T 22.03 ± 2.65 vs. CON 17.50 ± 2.38; PD+T 20.49 ± 1.99 vs. PD 15.70 ± 1.59, P = 0.01, Fig. 3A) compared with non-treated cells, respectively.

<<<INSERT FIGURE NEAR HERE 1.>>>

Importantly, the magnitude of change between both cell models in response to T administration and compared with their respective baseline controls was not significantly different at either time points, with T increasing myotube diameter in both PD (+25% and +23%) and CON (+21% and +20%) cells to the same extent after 72 hrs (Fig. 3A) and 7 days (Fig. 3C); respectively. Overall, this suggests that cells with a reduced differentiation phenotype have the same capacity to undergo hypertrophy in response to exogenous testosterone administration as CON cells. Furthermore, the addition of T significantly increased myotube number in CON cells (CON+T 2.90 ± 0.72 vs. CON 2.23 ± 0.68, P ≤ 0.05, Fig. 3D) after 7 days exposure only (at 72 hrs although there were mean increases this was not statistically significant (P = N.S.)). There were no significant changes in myotube number in PD cells after 72 hours or 7 days exposure (P = N.S.). This suggests testosterone

increases hypertrophy (myotube diameter) in both cells types; however, T only enhanced myotube number in CON cells at 7 days.

<<<INSERT FIGURE NEAR HERE 2.>>>

Together with the changes in myotube diameter at both 72 hrs and 7 days, T significantly increased the mean number of nuclei per myotube in CON cells (CON 3.77 ± 0.86 vs. CON+T 4.98 ± 1.76, P ≤ 0.001, Fig. 3B) at 72 hrs and at 7 days (CON 3.68 ± 0.67 vs. CON+T 4.91 ± 1.10, P ≤ 0.01, Fig. 3B); again substantiating the morphological observation that hypertrophy, rather than hyperplasia was occurring in response to T administration. Furthermore, in the PD cells, a similar response was observed for nuclei/tube in the presence of T at 72 hrs (PD+T 4.27 ± 1.03 vs. PD 3.33 ± 0.71, P = 0.03, Fig. 3B), and 7 days (PD+T 5.18 ± 1.31 vs. PD 3.84 ± 0.71, P ≤ 0.001 Fig. 3B) exposure compared to non-treated cells. Interestingly, again there were similar magnitude increases in CON and PD cells in the presence of T vs. basal conditions at 72 hrs (24.3% CON vs. 22% PD) and 7 days (25.1% CON vs. 25.9% PD).

<<<INSERT FIGURE NEAR HERE 3.>>>

3.2 PI3K/Akt inhibitor (LY) inhibits testosterone-induced increases in differentiation and hypertrophy for CON and PD cells

The presence of LY inhibitor alone led to a lack of differentiation with no quantifiable myotubes being present in both CON and PD cells at 72 hrs (Fig. 1 & 2). As highlighted above; the addition of T significantly increased differentiation shown by enhanced myotube number in CON cells (CON+T 2.90 ± 0.72 vs. CON 2.23 ± 0.68, P ≤ 0.05, Fig. 3D) after 7 days exposure. The presence of LY co-incubated with T significantly reduced the effect on myotube number (CON+T+LY 2.21 ± 0.56 vs. CON+T 2.90 ± 0.72, P ≤ 0.05, Fig. 3D). LY alone was also able to reduce myotube number vs. T treatment (CON+LY 1.77 ± 0.65 vs. CON+T 2.90 ± 0.72, P ≤ 0.05, Fig 3D) and non-treated controls (2.23 ± 0.68, P ≤ 0.01, Fig 3D). Interestingly, T in the presence of the LY inhibitor was unable to restore differentiation, shown by non-significant differences with LY alone conditions (P = N.S.) and non-treated

CON cells (Fig. 3D). These observations highlight that Ts increase in myotube number in CON cells, as previously observed, is blunted in the presence of the LY inhibitor; returning differentiation back to basal levels. As previously highlighted there were no significant changes in myotube number after T administration in PD cells. However, LY alone was able to reduce myotube number vs. T treatment (PD+LY 1.89 ± 0.80 vs. PD+T 2.35 ± 0.69, P ≤ 0.05, Fig 3D) and non-treated controls (PD 2.26 ± 0.53, P = 0.05, Fig 3D).

Following these observations for indices of differentiation we next wished to ascertain the impact of T in the presence of the PI3K inhibitor (LY) on myotube hypertrophy. At 7 days, as previously highlighted above, the addition of T significantly increased myotube diameter in CON and PD cells (Fig. 3C). The presence of LY co-incubated with T significantly reduced the effect on myotube diameter in both CON (CON+T+LY 17.49 ± 2.21 vs. CON+T 22.03 ± 2.65, P ≤ 0.05, Fig. 3C) and PD cells (PD+T+LY 17.10 ± 2.40 vs. PD+T 20.49 ± 1.99, P ≤ 0.05, Fig. 3C). LY alone was also able to reduce myotube diameter vs. T treatment in CON cells (CON+T 22.03 ± 2.65 vs. CON+LY 17.7 ± 2.91, P ≤ 0.05, Fig 3C). Similar observations were observed in PD cells (PD+T 20.49 ± 1.99 vs. PD+LY 15.83 ± 2.53, P ≤ 0.05, Fig 3C). Interestingly, T was unable to restore myotube diameter in the presence of the LY inhibitor shown by non-significant differences with LY alone conditions in CON cells and non-treated CON cells (P = N.S, Fig. 3C). The same observations were mirrored in PD cells where T was unable to restore myotube diameter in the presence of the LY inhibitor shown by non-significant differences with LY alone conditions in PD cells (P = N.S, Fig. 3C). Overall, this shows that T's observed increases in hypertrophy was blunted in the presence of the LY inhibitor returning myotube diameter back to basal levels in both cell types.

3.3 T-treatment enhancement in differentiation and hypertrophy is accompanied by increases in cell fusion

To address the effects of the various treatments on cell fusion, myonuclear accretion was calculated. The administration of T resulted in a greater percentage of myotubes expressing 5+ nuclei for both CON (CON+T 67.9% vs. CON 32.1%, χ_1^2= 32.99, P ≤ 0.05) and PD cells (PD+T 67.2% vs. PD 32.8%, χ_1^2= 20.58, P ≤ 0.05) compared to untreated cells after 7 days exposure (Fig 4A & 4B respectively). Furthermore, the addition of LY with T treatment reduced the percentage of myotubes expressing 5+ nuclei back to similar levels of LY-alone treated cells (CON+T+LY 28.1% vs. CON+LY 15.2%, χ_1^2= 2.53, P = N.S; PD+T+LY 37.5% vs. PD+LY 21.6%, χ_1^2= 3.4, P=N.S, Fig 4A & 4B respectively). In accordance with these observations, there was no significant difference in total nuclei counts for all treatments in either cell type at both time points (P = N.S). There was however a significant interaction between cell type as the PD cells had a significantly higher total nuclei count (P ≤ 0.05)

compared to CON cells at both time points, a finding confirmed previously by Sharples et al. [7], who highlighted a continued proliferation at the expense of exiting the cell cycle in G1 and differentiating.

<<<INSERT FIGURE 4. NEAR HERE>>>

3.4 Expression of myogenin mRNA increases after T-administration in both PD and CON cells

There was a significant difference in myogenin transcript expression levels between CON and PD cells after 72 hrs (CON 191.51 ± 12.84 vs. PD 48.58 ± 8.14, $P \leq 0.001$, Fig. 5A) and 7 days exposure (CON 128.36 ± 27.11 vs. PD 59.22 ± 4.57, $P \leq 0.001$, Fig. 5A). Whilst still remaining significantly different, the expression of myogenin significantly increased in both CON and PD cells when treated with T (CON+T 222.18 ± 36.63 vs. CON 191.51 ± 12.84; PD+T 67.00 ± 5.35 vs. PD 48.58 ± 8.14, $P \leq 0.05$, Fig. 5A) after 72 hrs. The increase in myogenin mRNA expression also continued at 7 days in PD treated cells (PD + T 74.15 ± 8.13 vs. PD 59.22 ± 4.57, $P \leq 0.001$, Fig. 5A), yet there was no difference in myogenin mRNA expression at this time point between non-treated and T-treated CON cells. However, absolute levels of myogenin transcript expression were basally significantly higher (shown above) in CON treated cells vs. PD treated (CON+T 131.46 ± 24.53 vs. PD+T 67.00 ± 5.35, $P = 0.001$) cells at 7 days. The presence of LY, significantly reduced myogenin expression after 72 hrs exposure in CON cells (CON+LY 134.38 ± 16.13 vs. CON 191.51 ± 16.00, $P \leq 0.05$ Fig. 5A), with no reductions being observed in PD cells at the same time point. Furthermore, LY alone had no effect on myogenin mRNA expression after 7 days culture in either cell type (P = N.S Fig. 5A).

<<<INSERT FIGURE NEAR HERE 5.>>>

T in the presence of LY inhibitor significantly attenuated myogenin expression after 72 hrs when compared with T alone in both cell types (CON+T 222.18 ± 36.63 vs. CON+T+ LY 146.09 ± 24.58, $P \leq 0.05$; PD+T 67.00 ± 5.35 vs. PD+T+LY 56.04 ± 11.50, $P \leq 0.05$, Fig. 5A). At 7 days, there were no significant changes for any treatment conditions in CON cells (P = N.S for all comparisons) for myogenin expression. However in PD cells, T further enhanced

myogenin expression significantly, even when administered in the presence of LY (PD 59.22 ± 4.57 vs. PD+T+LY 69.93 ± 3.98, P ≤ 0.05; PD+LY 57.04 ± 9.22 vs. PD+T+LY 69.93 ± 3.98, P ≤ 0.05 Fig. 5A). Thus, T caused an increase in myogenin expression in both cells types, whether LY was present or absent. This may suggest at the molecular level at least, that T elevates myogenin expression, independently of PI3K/Akt, but appears to be not sufficient enough to rescue morphological differentiation/hypertrophy in PD cells.

3.5 Testosterone increases mTOR expression in the presence of PI3K inhibitor in CON cells

The expression of mTOR did not change after 72 hrs in either cell type (CON 0.97 ± 0.08 vs. PD 0.97 ± 0.12, Fig. 5B). After 7 days exposure, T administration alone significantly increased mTOR mRNA expression compared to CON non-treated cells (CON+T 121.08 ± 15.87 vs. CON 111.95 ± 19.36, P ≤ 0.05 Fig. 5B). Furthermore, the treatment of T + LY significantly increased mTOR mRNA compared to LY alone (CON+T +LY 135.14 ± 16.08 vs. CON+LY 112.70 ± 13.62, P ≤ 0.001, Fig. 5B) and untreated controls (CON+T +LY 135.14 ± 16.08 vs. CON 111.95 ± 19.36, P ≤ 0.05). This highlights T administration increases in mTOR expression even in the presence of LY. In the PD cells, all treatments were without effect on mTOR expression at 7 days exposure (Fig. 5B). However, at 7 days under basal conditions, CON cells displayed significantly higher mTOR expression than PD cells (CON 111.95 ± 19.36 vs. PD 1.01 ± 0.10, P ≤ 0.05, Fig. 5B).

3.6 T-stimulus reduces negative muscle mass regulator myostatin in PD cells only

Myostatin transcript expression was slightly elevated in PD cells after 72 hrs compared to CON, although these elevations were not statistically significant (CON 5.93 ± 1.87 vs. PD 7.63 ± 2.58, Fig. 5C). However, at 7 days, statistically significant observations were observed for increases in myostatin mRNA expression in PD cells compared to CON cells (PD 12.93 ± 4.69 vs. CON 4.57 ± 1.20, P ≤ 0.001 Fig. 5C). This novel finding suggests that under basal conditions, myostatin may block differentiation in these myoblasts and thus contribute towards reduced myotube hypertrophy. In CON cells, T treatments, LY alone or co-incubations of T+LY at 72 hrs did not alter myostatin mRNA expression (Fig. 5C). The presence of LY alone was the only treatment to significantly reduce myostatin mRNA expression after 72 hrs exposure to PD cells (PD+LY 4.27 ± 2.47 vs. PD 7.63 ± 2.58, P ≤ 0.01 Fig. 5C).

At 7 days, there were no changes in myostatin mRNA expression in CON cells (CON 4.57 ± 1.20; CON+T 5.99 ± 1.40; CON+LY 4.32 ± 1.65; CON+T+LY 4.84 ± 1.01, Fig. 5C). However in the PD cells, T treatment significantly impaired elevations in myostatin expression compared to PD non-treated cells (PD + T 9.73 ± 2.56 vs. PD 12.93 ± 4.69, P ≤ 0.05 Fig. 5C).

The reduction in myostatin transcript expression was significantly larger in magnitude with the addition of T with LY (-36%) in PD cells at the 7 day time point (PD + T + LY 3.47 ± 0.83 vs. PD 12.93 ± 4.69, P ≤ 0.01 Fig. 5C), yet similar effects were not observed in CON cells. However, the presence of LY alone also brought about reductions in myostatin expression (PD+LY 4.98 ± 2.25 vs. PD 12.93 ± 4.69, P ≤ 0.001 Fig. 5C) and the reductions were similar to T+LY (P = N.S). Overall, suggesting both LY and testosterone alone reduce myostatin expression in PD cells only. It therefore appears that T potentially reduces myostatin expression in atrophied PD cells only suggesting a potential mechanism that may explain the increase in differentiation/hypertrophy seen in this cell type, whereas in CON cells, the increases with T are independent of myostatin expression.

4. Discussion

The present study supports a role for testosterone in enhancing myogenic differentiation and myotube hypertrophy [50-55] but more importantly, shows the capacity to improve hypertrophy and myonuclear accretion in a previously established model displaying impaired differentiation and hypertrophy similar to that of atrophic phenotypes such as ageing [7, 26]. Furthermore, testosterone treatment enhanced hypertrophy shown by increases in myotube diameter, nuclei per myotube and a larger number of myotubes with 5 or more nuclei in both control (CON) and multiple population doubling (PD) cells. Interestingly, testosterone improved hypertrophy (myotube diameter) to the same magnitude in both cell types at 72 hrs and 7 day time points. Thus, despite PD cells having undergone multiple population doublings vs. their controls, and therefore displaying a reduced basal differentiation capacity as previously described in Sharples et al. [7, 26]; testosterone was able to exert similar magnitude increases in myotube hypertrophy. Despite this interesting observation, testosterone was unable to fully restore differentiation (myotube number) to the level observed in untreated control cells at 7 days; perhaps suggesting testosterones predominant role in hypertrophy rather than differentiation in this model. Furthermore, in line with previous findings, the current study showed that PD cells had reduced myogenin expression vs. CON cells [7, 26], with the present study also supporting previous morphological and biochemical (reduced CK activity) findings [7, 26], showing myotube diameter was reduced in the PD cells. This further consolidates the use of these cells as a representative model to investigate cellular and molecular mechanisms of atrophic (PD) vs. hypertrophic phenotypes (CON).

Importantly, testosterone mediated increases in myotube hypertrophy were potentiated via the PI3K/Akt pathway, where inhibition of this pathway in the presence of testosterone rendered the hormone unable to exert its potent influence on myotube

hypertrophy in both cell types. This study supports previous work where the PI3K/Akt pathway has been implicated in testosterones action in L6 myoblasts [22] and human skeletal myoblasts [56] where the same PI3K inhibitor (LY294002) also reduced testosterone induced hypertrophy. Kovacheva and colleagues [57] have also previously reported that Akt signalling downstream of PI3K was restored by testosterone administration in elderly mice. The present study further supports that testosterones action in myotube hypertrophy is mediated via PI3K/Akt pathway, especially where reductions in Akt activation have been previously reported in this model (i.e. lower in PD vs. CON) [7]. Overall, these observations further suggest that the manipulation of this pathway by testosterone may be imperative for therapeutic restoration of muscle atrophy [38].

Interestingly, exogenous testosterone improved myogenin expression in both CON and PD cells types, whether the PI3K inhibitor (LY294002) was present or absent, suggesting testosterone-mediated activation of myogenin independently of the PI3K/Akt signalling pathway. Testosterone has previously been shown to increase myogenin in C_2C_{12} cells [50, 51]. Indeed, community-dwelling older men treated with grades doses of testosterone show increases in muscle fibre size accompanied by increased myogenin expression [55]. Despite this, testosterone induced increases in myogenin observed in the presence of PI3K inhibitor were unable to restore the differentiation/hypertrophy impinged by the PI3K inhibitor alone. Therefore, suggesting that myogenin was not the main mechanism of action in testosterone-induced increases in hypertrophy. Overall however, absolute levels of myogenin were severely reduced in PD vs. CON cells with testosterone unable to rescue absolute myogenin expression levels in the cells that display atrophic phenotypes (PD's) to those observed in untreated control cells. Thus, perhaps highlighting myogenins more pertinent role in differentiation (not hypertrophy) as testosterone was unable to restore absolute levels of myogenin and corresponding myotube numbers in PD cells vs. untreated control levels. Although, as discussed above, testosterone was able to improve myotube diameter i.e. hypertrophy.

In addition to this, testosterone increased mTOR expression in CON cells at 7 days, previously testosterone has been shown to increase mTOR phosphorylation in both L6 rat [22] and in C_2C_{12} [23] myoblasts. Indeed, mTOR mRNA expression in CON cells was increased in the presence of testosterone plus PI3K inhibitor (LY), perhaps suggesting an important role for testosterone in increasing mTOR independently of PI3K/Akt. Furthermore, White and colleagues [23] also observed increases in mTOR phosphorylation with incremental doses of T in C_2C_{12} cells, independently of Akt activation. However, mTOR expression was not increased in PD cells in the presence of testosterone alone or in combination with the PI3K inhibitor suggesting impaired mTOR transcript expression in

response to exogenous testosterone in the atrophic, reduced differentiation phenotype PD vs. control cells. Previously, in our labs we have observed reduced IGF-I transcript expression with corresponding reductions in Akt phosphorylation in PD myoblasts [7]. As Akt is upstream of mTOR this suggests Akt may be involved the reduction of mTOR observed between CON and PD myoblasts basally at 7 days. However, activity of mTOR was not directly assessed in the present study and warrants further investigation.

In agreement with the present study where mTOR expression in CON cells was increased in the presence of testosterone plus PI3K inhibitor (LY) (albeit mRNA expression and not protein activity), other studies have shown that mTOR activation can occur via signals independent of canonical IGF-I/Akt, via a pathway involving phospholipase D, phosphatidic acid and a downstream regulator Rheb (ras homologue enriched brain) [58-61]. Redd 2 may also be important in inhibiting mTOR via the tuberous sclerosis 1 (TSC1) and 2 (TSC2) complex [62]. As mTOR mRNA expression is increased in CON vs. PD cells, testosterone increases mTOR expression in CON cells which is blunted in PD cells at 7 days, and mTOR transcript expression is still increased with testosterone even the presence of PI3K/Akt inhibitor it begs the question as to the role of testosterone in regulating phospholipase D, phosphatidic acid, Rheb and Redd2 and their subsequent interaction with mTOR. Especially in light of recent data where following mechanical overload Redd2 is reduced to enable mTOR to initiate p70S6K activity, which is involved in protein synthesis and hypertrophy, a process that is impaired in the elderly [63].

Myostatin is a negative regulator of muscle mass in many species [64-67]. There is evidence indicating that myostatin inhibits satellite cell activation, proliferation, and differentiation [55, 68, 69], possibly mediated through perturbation of Akt and mTOR signalling [49]. Therefore reductions in myostatin, as previously observed with testosterone administration [69], can enhance muscle mass regulation in muscle wasting diseases such as cachexia and sarcopenia [70-72]. Interestingly, in the current study myostatin mRNA expression was reduced in myoblasts that have basally impaired hypertrophy (PD cells) when administered with testosterone with corresponding increases in myotube hypertrophy. The reduction in myostatin mRNA expression at 7 days with T treatment was accompanied by increases in myogenin mRNA expression in PD cells, which is supported recent literature highlighting myostatin's role for inhibiting myogenic differentiation [49, 73, 74]. Despite this, testosterone did not return absolute levels of myostatin expression to the low levels observed in the control cells. These observations suggest that although testosterone may reduce myostatin expression, it is not entirely responsible for the improved hypertrophy observed in the atrophic phenotype cells. Furthermore, the PI3K inhibitor (LY) alone also

substantially reduced myostatin expression where there is a distinct lack of differentiation and hypertrophy. This is in contrast to recent studies where there was a reduction in the activity of Akt observed in the presence of exogenous myostatin in human myoblasts [49]. Therefore, if myostatin was reduced, PI3K/Akt activity may be expected to increase. Although inhibition of PI3K in the present study makes it difficult to compare with the aforementioned study, this phenomenon requires further investigation in the current model.

It is important to note the potential effect of T breakdown which may occur within the cell culture media as the steroidgenic enzymes 5-α reductase and aromatase are critical in converting T to dihydrotestosterone (DHT) and estradiol respectively [75, 76]. Therefore, knowing if T is the active steroid compound stimulating hypertrophy in the current model is a limiting factor. Future studies may address the impact of other steroid hormones such as DHT in rescuing impaired differentiation and hypertrophy via the PI3K/Akt pathway.

5. Conclusion

In the present study exogenous testosterone was able to increase hypertrophy in myoblasts with reduced differentiation potential (PD cells) to a similar magnitude as the control cells. Testosterone induced myotube hypertrophy was mediated via the PI3K/Akt pathway, where inhibition of this pathway in the presence of testosterone rendered the hormone unable to exhibit its potent influence on myotube hypertrophy in phenotypically atrophic (PD) and hypertrophic control (CON) cells Testosterone-induced hypertrophy was also accompanied by increased myonuclear accretionin both cell types, with corresponding increases in myogenin expression. Furthermore, blunted mTOR expression was observed in response to exogenous testosterone administration in atrophic myotubes (PD) vs. control (CON) myotubes. Myostatin expression was reduced in the presence of testosterone in atrophic cells only. Overall, administration of testosterone shows strong potential to enhance hypertrophy in a previously atrophic cell type via the PI3K/Akt pathway.

Acknowledgements

The laboratory work was carried out within the Stem Cells, Ageing & Molecular Physiology (SCAMP) Unit, Research Institute for Sport and Exercise Sciences (RISES), School of Sport and Exercise Sciences, Liverpool John Moores University, Tom Reilly Building, Byrom Street, Liverpool, UK and the MCMPRG, Bedford, UK. The manuscript was prepared within SCAMP, RISES, LJMU.

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Table 1. Primer sequences for genes of interest.

Gene	Primer Sequence (5'-3')	Ref. Sequence Number	Amplicon Length (bp)	GC% Content
Myostatin	**F**: TACTCCGAATAGAAGCCATAA	NM_010834	194	36.3
	R: GTAGCGTGATAACGTCATC			45
mTOR	**F:** CACTCCACTATCCTGTTACCT	NM_020009	190	47.6
	R: GAGATCCTTGGCACACCT			55.5
Myogenin	**F:**CCAACTGAGATTGTCTGTC	NM_031189	173	47.3
	R: GGTGTTAGCCTTATGTGAAT			40
RP-IIβ	**F:**GGTCAGAAGGGAACTTGTGG TAT	NM_153798.1	197	50
	R:GCATCATTAAATGGAGTAGC GTC			44.4

Figure 1.

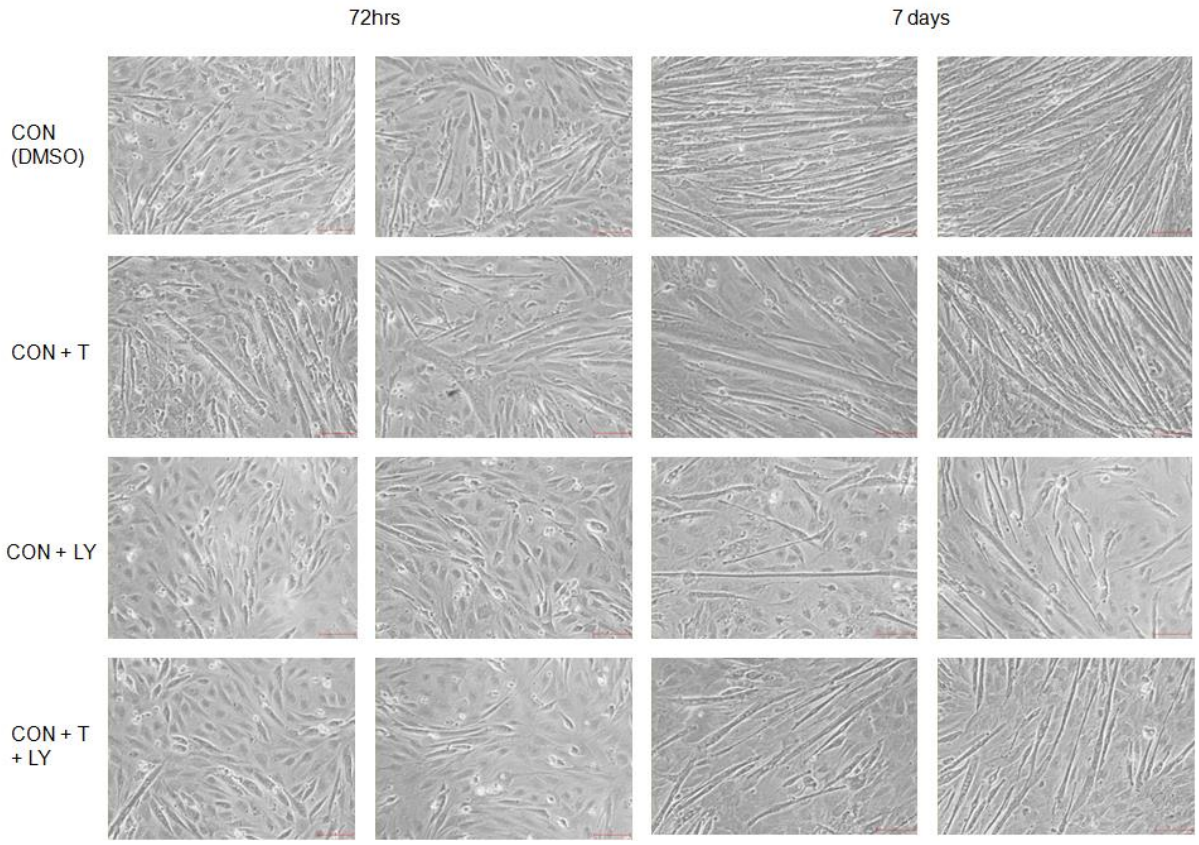

Fig. 1. Representative light microscope images (10× magnification) of treatments (DMSO, T, LY, T + LY) exposed to the CON cell type after 72 h and 7 days.

Figure 2.

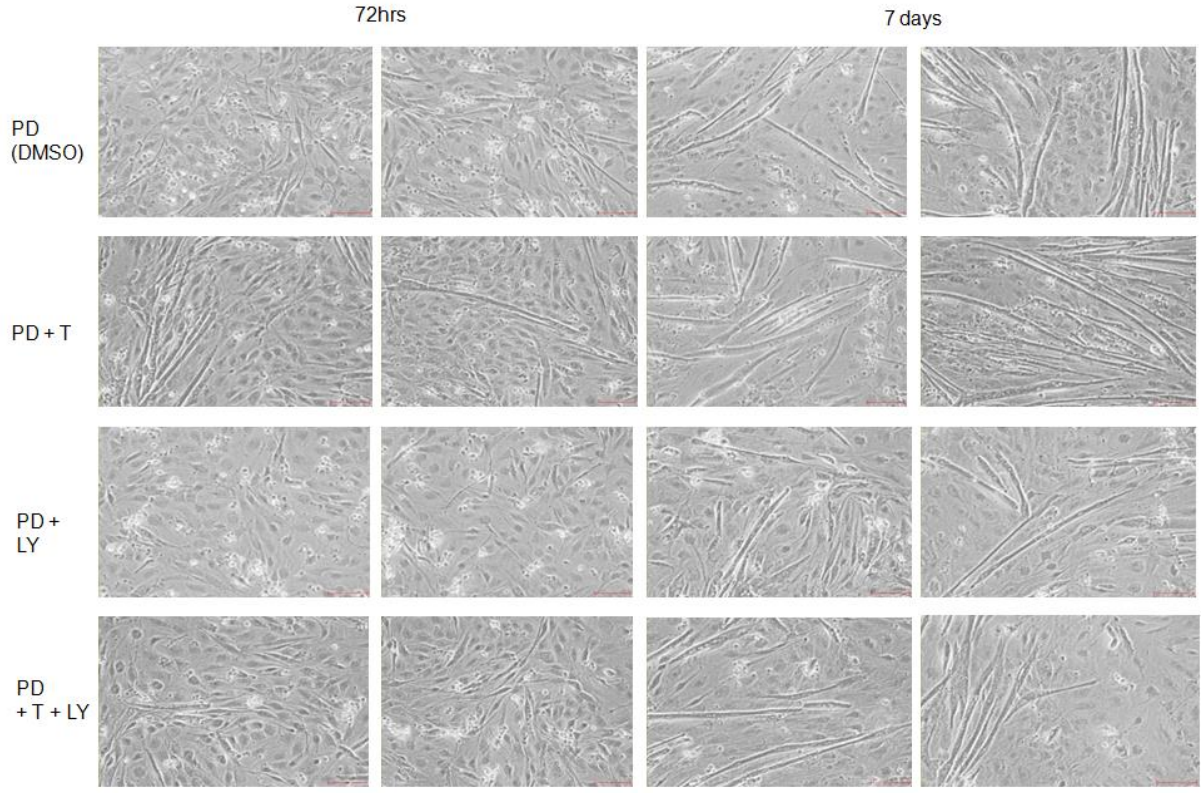

Fig. 2. Representative light microscope images (10× magnification) of treatments (DMSO, T, LY, T + LY) to population doubled cells after 72 h and 7 days.

Figure 3.

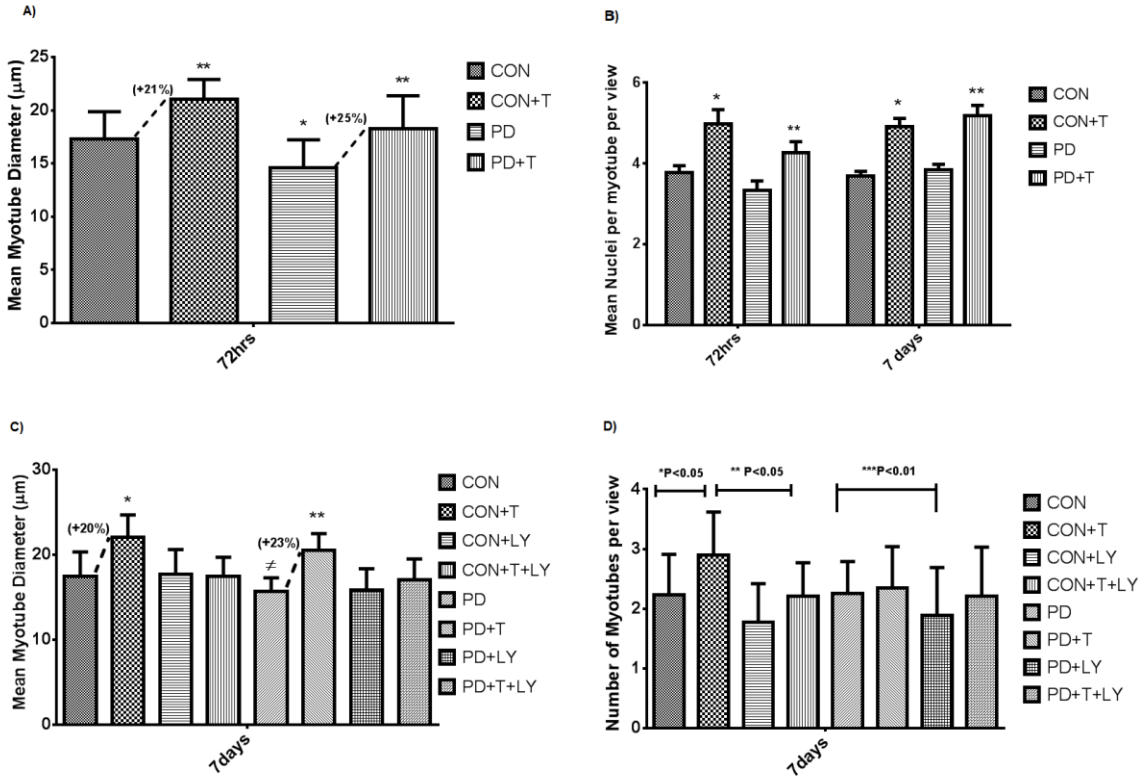

Fig. 3. The effect of T-stimulus on CON and PD cells on various morphology variables after 72 h and 7 days exposure. (A) The effect of T-stimulus on mean myotube diameter for CON and PD cells after 72 h exposure. *Reduced mean myotube diameter between CON and PD cells (P≤0.01). **T-stimulus significantly increased mean myotube diameter in CON cells vs. non-treated CON and PD (P ≤0.01). Dash line represents magnitude change in mean myotube diameter with T-stimulus, similar in magnitude for both CON (+21%) and PD (+25%) cells. A similar pattern was observed at 7 days (CON +20%; PD +23%). (B) The changes in mean nuclei per myotube at 72 h and 7 days with and without exogenous T administration. *T significantly increased mean nuclei per myotube in CON (+24%; P ≤ 0.001) cells after 72 h and 7 days (+25%; P≤0.001) exposure vs. non-treated cells. **T-treatment in PD cells increased mean nuclei per myotube after 72 h (+22%; P = 0.033) and 7 days exposure (+25%; P≤0.001). (C) Myotube number is attenuated when LY is co-incubated with T at 7 days. *The presence of LY significantly reduced the effect of T on myotube diameter (P ≤ 0.05) in CON cells. **A similar effect was observed in PD cells (P ≤ 0.05). / = Myotube diameter significantly reduced in PD vs. CON cells, similar to that observed at 72 h (see Fig. 2A) (P ≤ 0.05). Dash line represents magnitude change in mean myotube diameter with T-stimulus, similar in both CON (+20%) and PD (+23%) cells. It appears that T's action on myotube hypertrophy requires the activity of the PI3K/Akt pathway in both cell types. (D) The effect of T and LY treatment on myotube number in CON and PD cells at 7 days. *T-treated CON cells significantly exhibited a greater number of myotubes compared to non-treated cells (P≤0.05). **The addition of LY alone and in the presence of T significantly reduced the number of myotubes compared to T-treated CON cells (P≤0.05). ***In PD cells, LY alone significantly reduced myotube number (P≤0.01) at 7 days, with all other treatments showed no significant effects.

Figure 4.

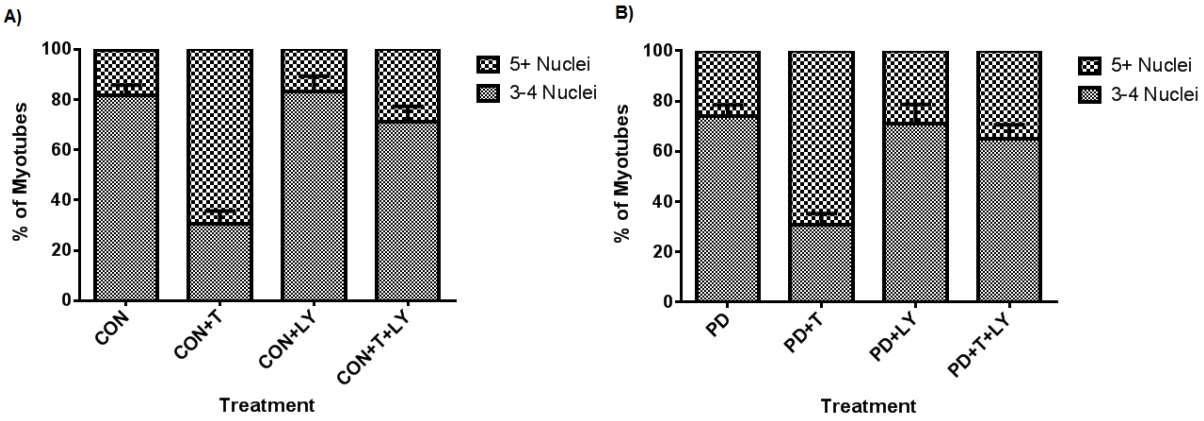

Fig. 4. The effect of T and LY treatment on myonuclear accretion in CON (A) and PD (B) cells after 7 days exposure. T-treatment significantly increased the percentage of myotubes expressing 5+ nuclei in both cell types ($P \leq 0.05$). LY alone reduced the percentage of 5+ nucleated myotubes back to baseline levels in both cell types. T in the presence of PI3K inhibitor (LY) was unable to to restore the number of 5+ nucleated myotubes to the levels induced by T alone.

Figure 5.

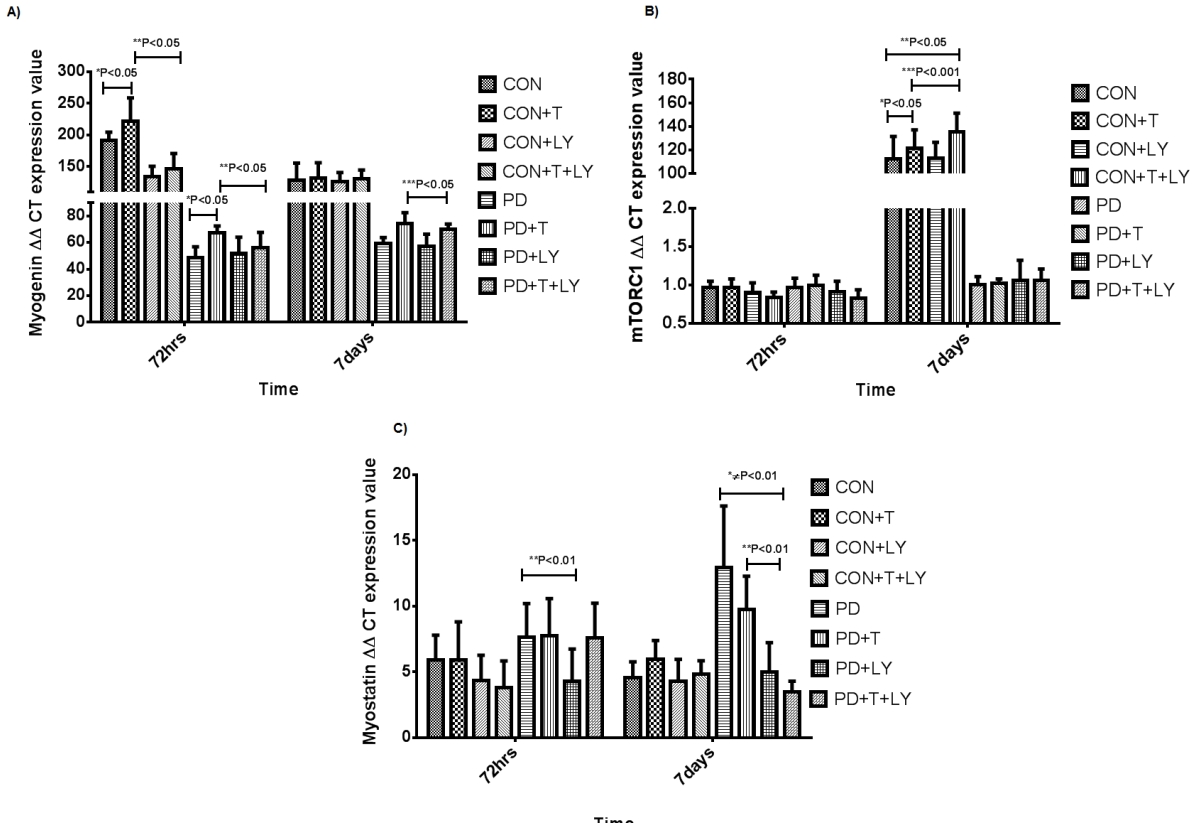

Fig. 5. The effect of T-stimulus on mRNA expression of myogenin (A), mTOR (B) and myostatin (C) in CON and PD cells. (A) The effect of T-stimulus on myogenin mRNA expression levels after 72 h exposure. *T-stimulus significantly increased myogenin expression in CON and PD cells compared to non-treated cells ($P \leq 0.05$). **The presence of LY with T treatment significantly attenuated T's effect in CON cells ($P \leq 0.05$). ***T exposure continued to increase myogenin mRNA in PD cells after 7 days culture, even in the presence of LY ($P \leq 0.05$). B) Changes in mTOR mRNA expression with the various treatments to CON and PD cells after 7 days culture. *CON + T and **CON + T + LY significantly increased mTOR expression vs. CON untreated cells ($P \leq 0.05$). ***There was a significant difference in mTOR expression between CON + LY and CON + T + LY ($P \leq 0.001$). Additionally there was a significant difference in mTOR expression between untreated CON and PD cells in 7 days culture. There were no changes in PD cells with the same treatments at either time points. (C) The effect of T-stimulus on myostatin mRNA expression after 7 days exposure. *T significantly reduced myostatin expression in the PD treated cells after 7 days ($P \leq 0.01$). **LY alone significantly reduced myostatin levels ($P \leq 0.01$) at 72 h and 7 days. / = Plus with the addition of T to LY, this reduction was heightened ($P \leq 0.01$) compared to non-treated PD cells._Basal PD cells had significantly elevated myostatin mRNA levels at 7 days vs. parental controls ($P \leq 0.001$).