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Report of Research Results

(a) Title: Role of chronic activation of muscle mTORC1-induced sarcopenia and its effects on muscle regeneration and myogenic differentiation of muscle stem cells

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(c) Summary
Age-related muscle wasting (i.e. sarcopenia) is the progressive loss of muscle strength and functionality that occurs with increasing age. The mTORC1 pathway is a central conserved pathway to organismal ageing, and chronic activation of the mTORC1 pathway in mouse muscle by genetic knockout of its inhibitor TSC1 (TSC1mKO mice) has previously been shown to result in late-onset myopathy and reduced lifespan. While some mTORC1-regulated mechanisms have been implicated in causing sarcopenia in this model, associated molecular mechanisms involved in muscle development and regeneration are incompletely understood. The present study reveals early signs of myopathy in TSC1mKO mouse muscle at 4 mo, with investigation of potential alteration in myogenic differentiation in these mice. Muscle differentiation was investigated by isolation and culture of muscle stem cells (MuSCs) at the same age as well as by polysome analysis of mTORC1-regulated translational targets at 2 mo. MuSCs derived from TSC1mKO muscle were not fewer in number and morphologically showed a similar degree of proliferation and differentiation to those from age-matched controls. Moreover, western blot on muscle protein isolated from mouse even indicated an increase in expression of differentiation markers. By RNA sequencing of polysomal (actively translated) mRNA in TSC1mKO mice (vs control mice), two distinct sets of genes involved in striated muscle differentiation were each significantly up and downregulated, indicating that while total myogenic differentiation may be similar, the translational program regulating the type of MyHC genes expressed may be perturbed in TSC1mKO mouse muscle. This has implications in the fate of differentiated muscle fibres and consequently muscle function in this model, sharing a likeness with muscular dystrophy conditions. Future analyses of muscle fibre types (i.e. actin and myosin isoforms) with ageing may reveal changes in protein expression in muscle that contribute to sarcopenia.

(d) Aim of research
1. Investigate aspects of sarcopenia that are affected by chronic activation of the mTORC1 signalling pathway in muscle at an early stage;
2. Determine whether sarcopenia by chronic mTORC1 activation in muscle affects MuSC differentiation;
3. Determine how mTORC1 regulates the mRNA translation programme involved in myogenic differentiation.

(e) Method of Research & Progression

Animals and in vivo analyses
Wire hang test was used to measure muscle strength in 4 and 12 mo mice. Mice were hanged (by four limbs) from a suspended cage insert and were timed for up to 10 min; time to latency indicates time taken for the mouse to lose grip and fall.
Mice were sacrificed 4 and 12 mo for muscle tissue analyses (histology and western blot), 4 mo for MuSC analysis, and 2 mo for polysome analysis. Mice were housed according to institutional (IACUC) guidelines at National University of Singapore.

In vitro analyses
In vitro muscle fibre strength and size (CSA) were measured as in Levy et al. (2018) at Kings College London (Ochala Lab, UK).
For histological analysis, muscle was frozen in liquid-cooled 2-methylbutane. Muscle was sectioned using a Leica Sakura Cryostat at 15 µm. H&E staining followed standard protocols: hematoxylin was stained for 2 min, ammonia water (0.2%) for 10 s, and acidified eosin for 1 min.
MuSCs were isolated from 4 mo mouse total muscle tissue following published procedures (Liu et al. 2015). Immunostaining followed standard protocols such as in Tsai et al. (2015).

Western blot was used to determine protein expression in muscle tissue and MuSCs by standard techniques. Muscle tissue was homogenised in ice cold RIPA lysis buffer (0.1% SDS) and protein from each sample was normalised following BCA protein assay. 20 µg of protein was separated on 10% polyacrylamide gels, and proteins were transferred to nitrocellulose membranes. Primary antibodies were incubated overnight and secondary antibodies for 2 h. Visualisation was performed using ECL and a Chemidoc imaging system or using X-ray film with autoradiographic exposure.
Polysome profiling was performed using muscle lysate fractionated by sucrose gradient centrifugation at Buck Institute (CA, USA) to separate out polysome (ribosome)-enriched mRNAs ("heavy" fractions). RNA was isolated from polysomal RNA fractions and sent externally for RNA sequencing and bioinformatic analysis.
Graphs were made and statistics were performed using Graphpad Prism software.

(f) Results of Research

1. Chronic activation of the mTORC1 pathway leads to hallmarks of sarcopenia in male mice at 4 mo, including loss of muscle strength, size, and myopathy

Age-related sarcopenia is the loss of muscle strength and function with advancing age (Janssen et al. 2002). The mTORC1 pathway is central to ageing, and genetic upregulation of mTORC1 activity by knockout of TSC1 (TSC1mKO mice) has been shown to lead to late onset myopathy and reduced lifespan (maximal lifespan: ~12 mo) by approximately 50% of that of wild-type mice from the same background (Castets et al. 2013; TSY lab, unpublished data). In the present study, TSC1mKO mice were utilised as an accelerated ageing model, for investigation of the molecular mechanisms of how increased activation of the mTORC1 pathway can lead to sarcopenia.
To confirm that TSC1mKO mice exhibit sarcopenia, mice were tested in vivo in comparison to their control mice (expressing TSC1 gene), analysed at 4 and 12 months of age (mo), considered "young" and "old" TSC1mKO mice, respectively. First, it was confirmed that TSC1 was knocked out in the muscle of TSC1mKO mice (Fig. 1A). At just 4 mo, muscle strength was shown to be reduced in TSC1mKO mice, indicated by a trend in vivo by wire hang assay (Fig. 1B; $P<0.1$) with statistical significance in vitro shown by reduced muscle fibre force (Fig. 1C; $P<0.0001$). Further analysis indicated that whereas individual TSC1mKO muscle fibres were not weaker at this age (Fig. 1D), the size of each myofiber was reduced (Fig. 1E; $P<0.0001$), indicating that a reduction in muscle fibre size contributed to the loss of muscle strength in male TSC1mKO mice. The degree of myopathy was then examined (Fig. 1F). At 4 mo, while control mice showed a healthy muscle phenotype with fibres of even size and angular morphology and peripherally located nuclei, TSC1mKO mouse muscle showed an uneven distribution of rounder muscle fibres with many centrally located nuclei, indicative of ongoing pathological regeneration in the muscle (Guiraud & Davies 2019). This phenotype was exacerbated at 12 mo in the TSC1mKO mice, with an increasing number of central nuclei and presence of potentially infiltrating inflammatory cells, which was not present even in 12-mo control mice. Furthermore, very small angular muscle fibres indicate apoptosis and cell death of the muscle fibres in TSC1mKO samples, which has been previous reported in both TSC1mKO and naturally aged muscle (Tang et al. 2019). Inflammation and apoptosis in TSC1mKO muscle will be determined in subsequent muscle histological analyses. Regarding centralisation of myonuclei, following muscle injury, activated satellite cells (muscle stem cells, MuSCs) move from the periphery of the muscle to fuse with the damaged myofiber; subsequently, the nucleus migrates to the central portion of the myofiber and then back to the periphery following muscle repair (reviewed in Folker & Baylies, 2013). While the exact reason for this migration is not clear, an increased number of centrally positioned nuclei is a hallmark of muscular dystrophies including Duchenne Muscular Dystrophy (Wang et al. 2000). Furthermore, Folker & Baylies speculated that centralised nuclei impede muscle function as a physical barrier to muscle contraction. Therefore, this may partially explain why TSC1mKO mice show co-occurrence of central nuclei with reduced muscle strength and fibre size at 4 mo. Overall, while a late onset myopathy in TSC1mKO mice has been previously reported (Castets et al. 2013), it is apparent in the present study that pathological changes take place in the muscle of TSC1mKO mice from as young as 4 mo.

2. Chronic activation of the mTORC1 pathway in muscle of 4 mo mice does not impede MuSC proliferation or differentiation

Muscle regeneration can be categorised into overlapping phases of MuSC physiology: activation, proliferation, differentiation, and self-renewal/return to quiescence. Upon stimulation to activate (e.g.

following muscle injury), MuSCs exit quiescence, proliferate, and differentiate into mature myofibers. Quiescent MuSCs express the marker gene Pax7, whereas terminally differentiated myofibers express MyHC, with myogenin and MyoD as intermediate markers. Ageing involves a reduction in MuSC self-renewal (loss of Pax7), resulting in depletion of the MuSC quiescent pool, and gain markers of differentiation (e.g. myogenin) (Chakkalakal et al. 2012). Therefore, it is thought that muscle regeneration capacity is lost with sarcopenia.

The present study investigated proliferation and differentiation of MuSCs isolated from TSC1mKO muscle. First, MuSCs were isolated from TSC1mKO mice in comparison to age-matched control mice by FACS. 60,000 MuSCs were plated and stimulated to proliferate for 4 days (Fig. 2A). There were no observable changes in the rate of MuSC proliferation, indicated by a similar number of MuSCs at P4. TSC1mKO mice at this age did not show a reduced MuSC number, and in fact showed a (non-statistical) increase in the number of MuSCs isolated at 4 mo (Fig. 2B). Furthermore, the same TSC1mKO mouse-derived satellite cells were stimulated to differentiate by growth factor (bFGF) removal and resulted in a similar degree of differentiation as control mouse muscle. This was supported by western blot analysis, revealing that terminal differentiation marker MyHC protein expression was similar between control and TSC1mKO mice following differentiation to muscle fibres (Fig. 2C). TSC1 is knocked out in differentiated myofibres and mature myotubes by expression of muscle creatine kinase at these stages of differentiation (Bruning et al. 1998; Musaro & Rosenthal 1999). The C2C12 line was used as a positive control, whereby MyHC protein was expressed following 7 days of differentiation, and TSC1 protein increased in expression during differentiation indicating increasing inhibition of mTORC1 during differentiation. This is concurrent with the literature indicating that mTOR (mTORC1 component) negatively regulates differentiation in proliferating myoblasts (Wilson et al. 2016). However, in TSC1mKO mouse MuSCs, TSC1 was also expressed to a similar degree in at D3, indicating that myofibers at this stage may not express creatine kinase and/or may not be terminally differentiated. Overall, 4-mo TSC1mKO mouse muscle-derived MuSCs did not show loss of function and may not be distinct from to age-matched controls at either early (MuSC proliferation and commitment) or late (terminal differentiation) stages of gene expression.

To rule out changes to MuSCs acquired in the proliferative phase, 5,000 MuSCs from each mouse were seeded into chamber slides and immediately stimulated to activate (Fig. 3). Morphologically, TSC1mKO MuSCs appeared to be less differentiated following 9 days of activation compared to those of control mice (Fig. 3A). By this method, myofibers should have formed by day 5 post-activation (Liu et al. 2015), although myofibers did not form as well as following MuSC proliferation (see Fig. 2A). To determine the degree of differentiated myofiber formation, MyHC protein expression was analysed by immunohistochemistry (Fig. 3B). At day 9 post-activation, there was a reduced degree of staining of myonuclei with MyHC in TSC1mKO mice compared to control mice, whereas TSC1mKO mice showed a higher degree of myofiber staining with MyHC. Muscle differentiation involves myoblast fusion to form elongated multinucleated mature myofibres. Compared to control mice, which had larger elongated myonuclei indicating a post-fusion state, TSC1mKO mice showed multiple smaller myonuclei colocalised with MyHC-stained fibres, indicating a more advanced timepoint in differentiation (Chal & Pourquie 2017). Notably, this method of MuSC activation produced technical difficulties regarding terminal differentiation, making quantification a challenge. Current protocols will need to be further optimised if this method is to be used to produce reliable quantifiable data with robust conclusions. To determine changes to the whole mouse muscle, total muscle protein from TSC1mKO mice was compared to age-matched control mice (2 mo) (Fig. 3C). TSC1mKO mice showed an increase in differentiation markers MyHC as well as myogenin. This indicates that TSC1mKO mice may not have a defect in differentiation and may in fact show enhanced myogenic differentiation.

3. Muscle differentiation may be translationally regulated by mTORC1

mTORC1 plays a role in muscle differentiation and has been linked with regulation of muscle size. For example, myostatin (mTOR [mTORC1 component] inhibitor) expression has been shown to inhibit differentiation-inducing genes, including myogenin and myoD (Trendelenburg et al. 2009), whereas deletion of myostatin expression in mice results in increased muscle mass (McPherron et al. 1997). This indicates that mTORC1 positively regulates differentiation, although how mTORC1 regulation of myogenic differentiation is affected by ageing and whether this contributes to myopathy with chronic mTORC1 activation has not been elucidated.

mTORC1 has a primary role in muscle protein synthesis, and it is thought that mTORC1 regulates a specific translational program resulting in different proteins being synthesised at differential efficiencies (reviewed in Roux & Topisirovic 2018). To investigate mTORC1 regulation of protein

synthesis, polysome profiling was performed on RNA from 2-mo mouse muscle. Of the gene ontology (GO) categories that showed significant differences, Striated Muscle Cell Differentiation was both up (gene ratio: 23/806; p-adjust = 0.010) and downregulated (gene ratio: 23/817; p-adjust = 0.026). While it is not yet clear whether total muscle differentiation is reduced, there is likely to be a perturbation, which may be due to changes in muscle protein expression. For example, the polysome analysis indicates that expression of the differentiation marker myogenin (Myog) was shown to be translationally downregulated in TSC1mKO mouse muscle (Table 1), indicating that differentiation may be reduced with chronic mTORC1 activation, although myogenin expression was actually upregulated at the protein level (Fig. 3C). Furthermore, the data indicates that TSC1mKO mouse muscle increases in its expression of muscle developmental genes. For example, cardiac muscle alpha-actinin (Actc1) is predominantly expressed during early foetal skeletal muscle development but is downregulated in adult skeletal muscle (Ilkovski et al. 2005). Moreover, Mef2c has been implicated in both skeletal muscle development and muscle regeneration (Taylor and Hughes 2017). During muscle development and repair, muscle expresses developmental genes indicating regeneration, which is observed in muscular dystrophies (Guiraud & Davies 2019). Moreover, muscular dystrophy models show similar features to TSC1mKO mice in the present study, with no loss of MuSC number, proliferation, or differentiation, but show incomplete myofiber maturation due to increased expression of the developmental isoform of MyHC (Ribeiro et al. 2019). Future work will focus on changes in the mTORC1-regulated translational programme with ageing, including changes in expression of specific myosin heavy chain proteins, which govern muscle fibre type and function.

(g) Future Areas to Take Note of, and Going Forward

Important findings from the present study indicate that TSC1mKO mice exhibit characteristics of sarcopenia even from a young age of 4 mo. Notably, there is increased muscle regeneration in TSC1mKO mice, indicating muscle fibre damage and repair. Further analysis will be performed to determine qualities associated with sarcopenia with ageing, including apoptosis, fibrosis, and inflammation, with corresponding changes in mTORC1-regulated muscle signalling in TSC1mKO muscle.

Regarding MuSC differentiation, it was not apparent whether activation of MuSCs resulted in terminal myogenic differentiation, since TSC1 was still expressed at all stages in TSC1mKO muscle-derived MuSCs. Furthermore, we were not able to reliably detect satellite cell marker Pax7 or markers of muscle differentiation myogenin or MyoD, by either western blot or immunohistochemical staining of isolated or differentiated MuSCs, respectively. Therefore, future analyses will only focus on MuSCs prior to differentiation, and any changes that may occur with ageing, such as epigenetic marks of senescence (Garcia-Prat et al. 2016). Since isolation of MuSCs requires one whole mouse per analytical sample and MuSCs cannot be stored alive in their freshly isolated state for subsequent culture, it is intuitively more economical to perform multiple analyses per mouse. For example, the number of MuSCs can be quantified in muscle by Pax7 immunohistochemistry in either muscle sections or by fibre isolation and culture (Rion et al. Skelet Muscle 2019). Furthermore, MuSCs can be analysed and stained in situ together with the myofiber by the whole fibre isolation method. Therefore, to make better use of materials and overcome technical concerns, future analyses of MuSC function will be performed by these two methods.

Polysome profiling of TSC1mKO mouse muscle produced novel results, confirming and contributing to the knowledge of mTORC1 regulation of translation. From this analysis, translational targets that may contribute to the sarcopenic phenotype will be analysed at both the mRNA and protein levels by RT-qPCR and western blot, respectively, and quantified across ageing in TSC1mKO mice. Importantly, since mTORC1 regulates mRNA translation through phosphorylation targets S6K1 and 4E-BP1, genetic mouse models with reduced mTORC1 activity through each of these specific targets will provide important insight into how mTORC1 regulates protein synthesis and how this contributes to sarcopenia.

(h) Means of Official Announcement of Research Results

Regarding the official announcement of research results, selected novel publication-quality results from the awarded project will be submitted together with related data acquired during my PhD study. We aim to submit the manuscript to the journal in Summer 2020. MSIWF will be acknowledged for their generous support of our work.

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Figures

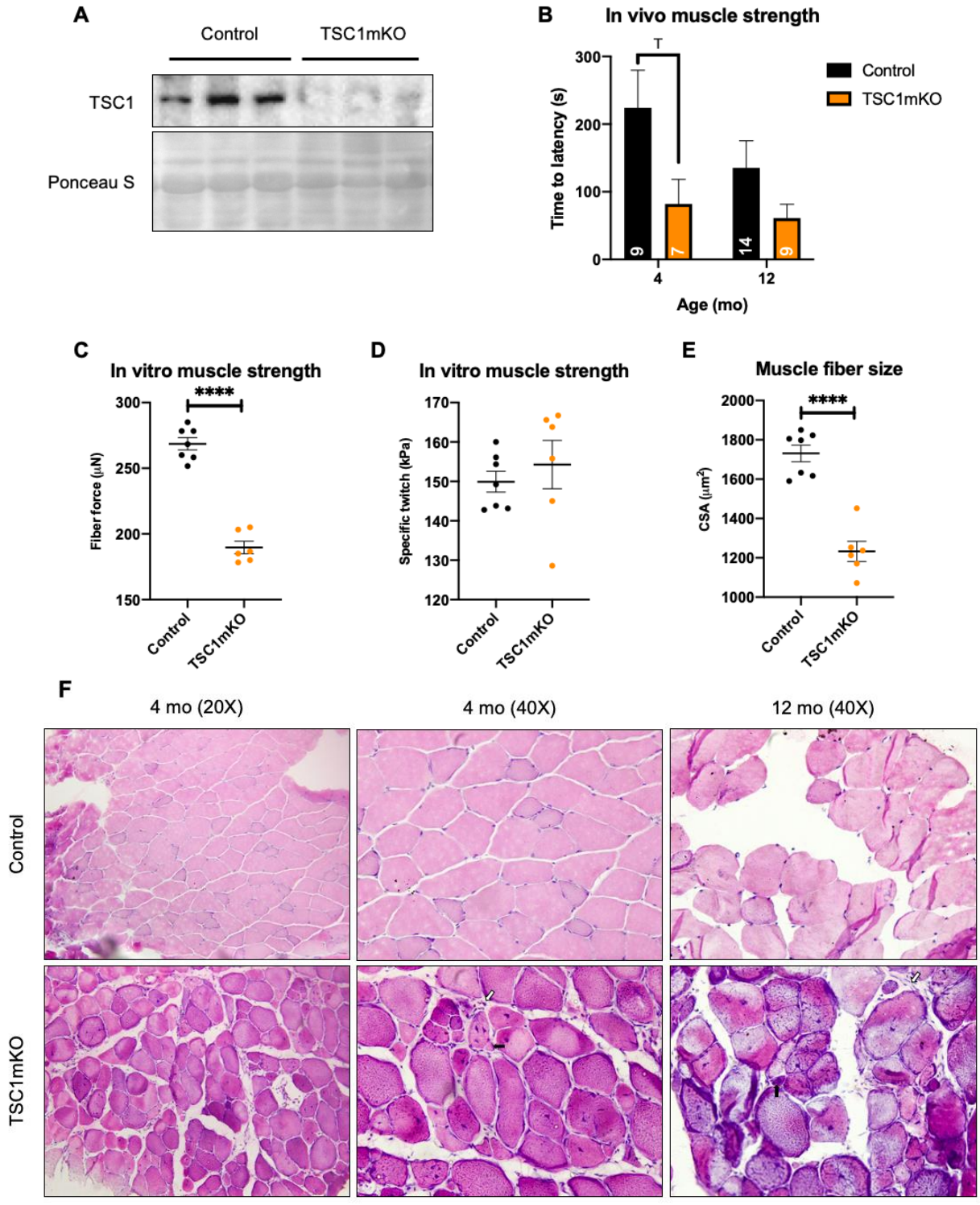

Figure 1. (A) TSC1 protein expression in quadriceps muscle (male, 12 mo); ponceau S was used as a loading control. (B) In vivo muscle strength as measured by the wire hang test; T, P<0.1; n values are indicated on bars. Average muscle fiber force in vitro, shown as absolute force (C) and specific force per fibre (D). (E) Average in vitro muscle fibre size (cross-sectional area, CSA). Values are shown as mean ± SEM. (F) In situ H&E staining of muscle sections at 4 and 12 months of age (mo). Black arrows, angular fibres (potentially apoptotic myofibers); white arrows, infiltrating cells (potentially inflammatory cells).

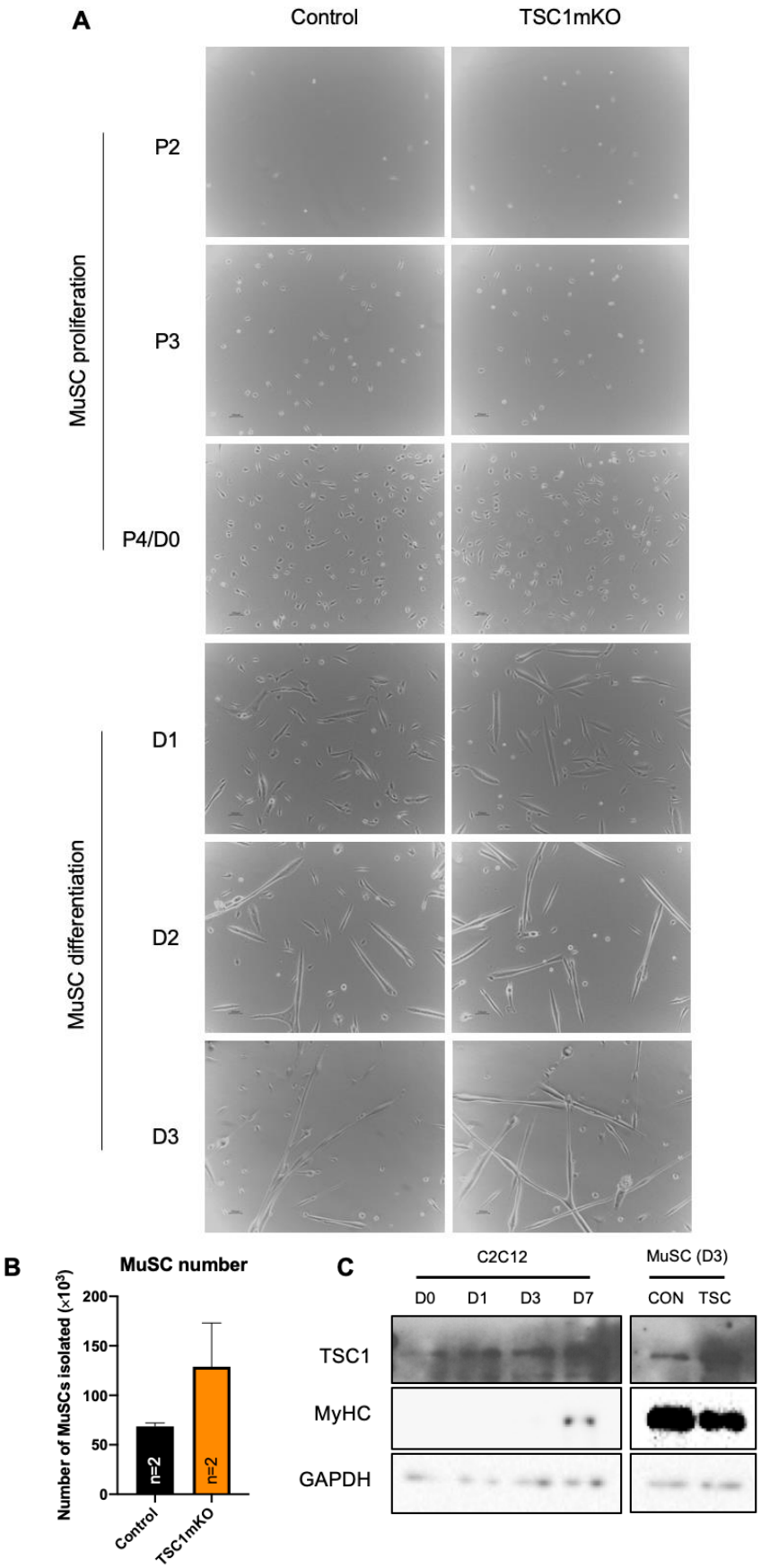

Figure 2. Muscle stem cell (MuSC) proliferation (4 days; P2-P4 shown) and muscle stem cell differentiation (3 days; D1-D3 shown). (A) Phase contrast images, 10X magnification. (B) Number of MuSCs isolated from 4-mo mice. (C) Western blot of myogenic differentiation markers. MuSCs are shown at D3 (after 3 days of differentiation); differentiating myoblasts (C2C12) are included as a positive control of myogenic differentiation (MyHC expression at D7). GAPDH was used as a loading control.

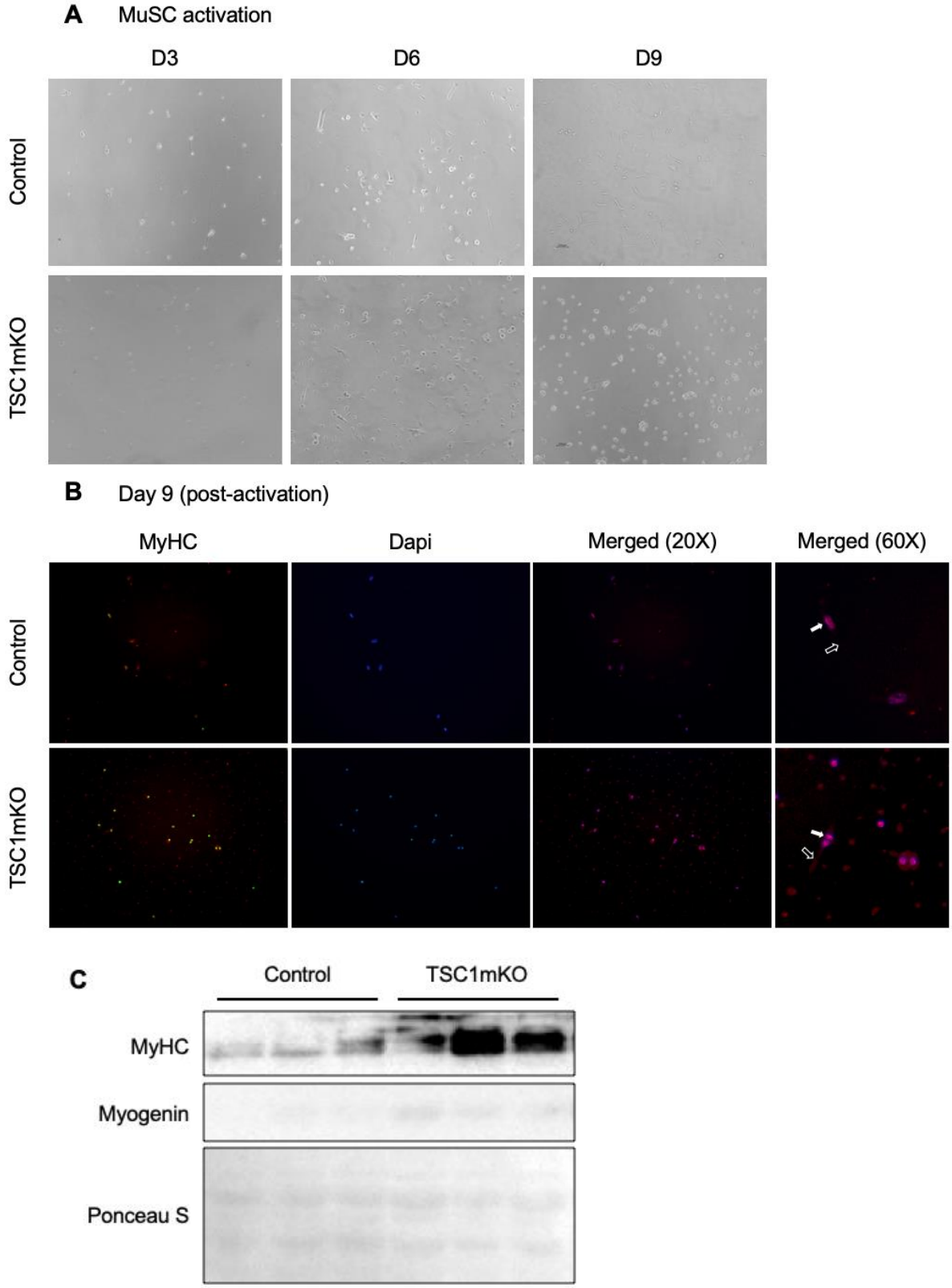

Figure 3. MuSC activation. (A) Phase contrast of MuSCs after 3, 6 and 9 days of activation; 20X magnification. (B) Immunofluorescence of myosin heavy chain (MyHC) at day 9 following activation. Red, MyHC; blue, Dapi (nuclei). Closed arrow, myonuclei; open arrow, differentiated muscle fibre. (C) Western blot of differentiation markers in total muscle isolated from 2 mo mice. Ponceau S is used as a loading control.

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Table 1. Differentially regulated genes in TSC1mKO vs Control mice under the GO category: Striated Muscle Cell Differentiation. Top 20 genes with the greatest fold change were selected for presentation. UP, upregulated genes; DOWN, downregulated genes.

UP			DOWN		
Gene	log2FoldChange	padj	Gene	log2FoldChange	padj
Actc1	1.75001	3.71E-10	Tsc1	-1.6202981	1.70E-08
Rgs4	1.710472	1.39E-08	Ccl8	-1.5265886	0.00063788
Sox6	1.30173608	0.00059466	Gpx1	-1.3000765	0.00038662
Xbp1	1.20396388	8.65E-05	Myog	-1.2236532	0.02534211
Homer1	1.17692239	0.00141998	Thra	-1.1930714	6.00E-05
Myh10	1.09619487	0.01724036	Comp	-1.1007852	6.95E-06
Bcl2	1.00366838	0.03644444	Neu2	-1.0686547	0.00692761
Mef2c	0.96277185	0.00748782	Neurl2	-1.0667748	0.0052497
Ripor2	0.91041907	0.01647678	Ryr1	-1.0486207	1.52E-05
Tomm70a	0.85278117	0.00628421	Kdm6b	-1.0022037	0.00328539
Cavin4	0.84822132	0.00860869	Pi16	-0.9859323	0.00328514
Trim32	0.84279409	0.03584535	Cav3	-0.9834997	0.00099819
Sgcd	0.81950198	0.02432827	Dyrk1b	-0.9522865	0.00172872
Gsk3b	0.8101092	0.00925265	Casq1	-0.9343199	4.57E-05
Zmpste24	0.80828502	0.01044318	Obsl1	-0.9107112	0.02224448
Nr3c1	0.78562929	0.02260687	Tgfb1	-0.8817313	0.0201099
Afg3l2	0.78340802	0.00744074	Wfikkn2	-0.8749492	0.00601906
Akap6	0.77949089	0.02015528	Myom3	-0.8315154	0.04827479
Cfl2	0.73817496	0.01190489	Ddx39b	-0.8083547	0.0143408
Fhod3	0.69824187	0.0281283	Gsk3a	-0.7728	0.012572

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