Title: Resistin in obese subcutaneous adipose tissue impairs human skeletal muscle myogenesis by activation of the NFκB pathway

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Abstract

Adipokines have been implicated in the loss of skeletal muscle mass with age and in several chronic disease states, particularly in overweight individuals. The aim of this study was to determine the effects of human obese and lean subcutaneous adipose tissue secretome on myogenesis and metabolism in skeletal muscle cells derived from both young (18-30 yr) and elderly (> 65 yr) individuals. These effects were determined by quantifying myotube thickness and nuclear fusion index along with indices of mitochondrial function, intracellular lipid content and signalling pathways.

Obese subcutaneous adipose tissue secretome, generated by conditioned media, impaired the myogenesis of old myoblast cultures but had no significant effect on young myoblasts. Resistin was identified as an adipokine that is prolifically secreted by obese subcutaneous adipose tissue and this adipokine was chosen for further study. Stimulation of old myoblasts with resistin impaired both myotube thickness and nuclear fusion. Stimulation of young myoblasts with resistin impaired myotube thickness, but had no effect on the number of nuclei incorporated into myotubes. Depletion of resistin from obese adipose tissue secretome restored myogenesis. Inhibition of the classical NFκB pathway protected myoblasts from the detrimental effect of resistin on myogenesis. Resistin also increased myotube respiration, ATP production and proton leak, promoted intramyocellular lipid accumulation and enhanced fatty acid oxidation by myotubes.

In conclusion, resistin derived from human obese subcutaneous adipose tissue impairs myogenesis of human skeletal muscle, particularly older muscle, and alters muscle metabolism in developing myotubes. These findings may have important implications for the maintenance of muscle mass and metabolic control in older people with chronic inflammatory conditions, or older people who are obese or overweight.

Introduction

The loss of skeletal muscle mass with ageing (sarcopenia) is accompanied by an increased systemic inflammatory burden (Himmerich, et al. 2006) and accumulation of adipose tissue (Kyle, et al. 2001), which is known to be a prolific secretor of pro-inflammatory cytokines (Lehr, et al. 2012) termed adipokines. Notably, the prevalence of sarcopenia is greater in obese than in non-obese older individuals (Srikanthan, et al. 2010) and this association has been referred to as sarcopenic obesity (Stenholm, et al. 2008). Sarcopenic obesity is considered to be an important public health concern in the elderly as it confers a higher risk for developing disability in the muscle functional activities of daily living, culminating in reduced quality of life (Baumgartner, et al. 2004). Significantly, sarcopenic obesity is associated with increased levels of systemic pro-inflammatory markers after adjusting for the presence of other pro-inflammatory states such diabetes and cancer (Batsis, et al. 2016).

Cross-sectional studies of the body composition of individuals across the lifespan found that total adipose tissue mass (Schautz, et al. 2012) and visceral adipose tissue (VAT) mass (Yamada, et al. 2014) negatively correlated with skeletal muscle mass. Indeed, the cross-sectional Health, Aging, and Body Composition (Health ABC) study of 3075 men and women aged 70–79 years demonstrated that those with high systemic concentrations of TNFα and IL-6 had a smaller mid-thigh muscle cross-sectional area and decreased grip strength (Visser, et al. 2002). Furthermore, circulatory levels of adiponectin and resistin are increased in old individuals, compared to the young, and are inversely associated with muscle strength (Bucci, et al. 2013). Collectively, these studies indicate that sarcopenia is – in part – an adipokine-driven phenomenon. Indeed, increased adipocyte-derived pro-inflammatory cytokines and lipid metabolites may contribute to decreased regenerative capacity (Akhmedov and

Berdeaux 2013) and myogenesis (Takegahara, et al. 2014) of skeletal muscle. However, although the anti-myogenic and muscle atrophic actions of some adipokines (such as TNFα and IL-6) are well studied in this respect (Adams, et al. 2008; Garcia-Martinez, et al. 1993; Garcia-Martinez, et al. 1994; Haddad, et al. 2005; Tsujinaka, et al. 1995; Tsujinaka, et al. 1996), the functional effects of many other obesity-associated adipokines, including resistin, on human skeletal muscle are not well characterised. Furthermore, very few studies have examined the inflammatory milieu secreted by human subcutaneous adipose tissue (SAT).

Despite the prominent attention that visceral adipose tissue (VAT) has received as a secretor of adipokines, SAT also secretes pro-inflammatory adipokines, albeit to a lesser extent (Blaber, et al. 2012; Pellegrinelli, et al. 2015; Skurk, et al. 2007). Importantly, SAT represents a much greater proportion of total adipose tissue mass than VAT (Isaac, et al. 2011; Rosqvist, et al. 2017; Rossi, et al. 2011), and therefore may be greatly underappreciated as a contributor to the systemic inflammatory burden. To our knowledge, a single study has directly examined the effect of an inflammatory milieu secreted by human SAT adipocytes on primary human myotube morphology (Pellegrinelli et al. 2015). This particular study showed that conditioned medium from SAT adipocytes derived from lean individuals does not alter the MTT or NFI of myotubes in myogenic cultures isolated from a neonate (Pellegrinelli et al. 2015). Interestingly, adipocytes isolated from obese SAT showed an intermediary inflammatory profile and negative effect on MTT between lean SAT and obese VAT. In the same study, conditioned medium from obese VAT adipocytes significantly diminished MTT but not NFI when compared with control and lean SAT. Furthermore, direct co-culture of obese VAT adipocytes with myoblasts derived from a neonate, resulted in significant reduction in the expression of the myogenic transcription factors

MyoD1 and myogenin. However, the study used adipocytes, rather than whole adipose tissue to generate conditioned medium representing the secretome, and thus did not characterise the effects of adipose tissue's complete inflammatory milieu on myogenic cultures. Critically, the stromal vascular fraction of adipose tissue, which includes preadipocytes and macrophages, is a more prolific secretor of pro-inflammatory cytokines than mature adipocytes (Blaber et al. 2012). Furthermore, the myotubes used were neonate not from adult donors. Consequently, adipokine secretion by human adipose tissue – not just that by adipocytes – must be characterised and its effect on human myofibre size and function determined.

The aim of this study was therefore to determine the effects of lean and obese SAT conditioned media secretome, and in particular the adipokine resistin, concentrations of which are known to be increased in the serum of obese individuals (Jonas, et al. 2017; Philp, et al. 2017), on human skeletal muscle myogenesis using muscle cell cultures derived from both old and young individuals.

Materials and Methods

Skeletal muscle biopsy and myogenic culture isolation

Three young healthy subjects (2 males and 1 female; age 24.4 ± 1.5 yr; BMI 22.6 ± 2.2 kg/m^2) and four elderly healthy individuals (2 males and 2 females; age 70.5 ± 2.8; BMI 21.8 ± 1.3 kg/m^2) were recruited and gave written informed consent. All participants were physically active (at least 150 minutes of self-reported moderate intensity activity per week). Participants were free from cardiovascular, metabolic, neuromuscular or other diseases that might affect muscle growth and metabolism during screening. The study was approved by the University of Nottingham Medical School Ethics Committee (G11092014SoLS) and was conducted in accordance with the guidelines of the Declaration of Helsinki. A vastus lateralis muscle biopsy was obtained from each subject and the satellite cell population extracted as previously described (O'Leary, et al. 2017). Our isolation technique has consistently generated cultures in our laboratories that produce desmin positive multinucleated myotubes that are negative for the fibroblast marker TE7 (O'Leary et al. 2017). Additionally, commercially available primary human myoblasts (Thermo Fisher cat. No. A12555), isolated from a female aged 21 yr were used for the mechanistic studies presented in Figures 3-6. They were cultured in the same media and conditions as the cultures that we isolated in-house.

Generation of adipose conditioned medium secretome

Following ethical approval (UK National Research Ethics Committee 16/SS/0172), SAT was obtained intraoperatively from n = 13 lean (BMI<25, age 68.1 ± 3.3 years) and n = 22 non-lean (BMI>25, age 69.5 ± 1.8 years) older individuals undergoing elective total joint replacement surgery at either the Royal Orthopaedic Hospital

(Birmingham, UK) or Russell's Hall Hospital (Dudley,UK). SAT was incubated in myotube differentiation medium at a ratio of 1 g tissue to 10 mL medium for 24 h at 37 °C, 21 % O_2 and 5 % CO_2. Larger samples were divided into segments of ~ 1 g to ensure that the surface area of adipose tissue exposed to medium remained approximately constant. At 24 h the adipose conditioned medium (ACM) was removed, aliquoted into 5 mL sample containers and stored at - 80 °C. For experimental use, the ACM was diluted 1:2 with differentiation medium, to ensure a sufficient nutrient composition to sustain myogenic differentiation.

Immunofluorescence staining

Myotubes were differentiated for 8 d in the presence of ACM secretome or recombinant resistin protein. The details of cytokine concentrations, as well as the timing and duration of such stimulations, are described in the relevant results section and Figure legend. Media were renewed every 2 d. The culture medium was removed and the cells fixed with 2% formaldehyde in PBS for 30 min. Following permeabilization in 100% methanol for 10 min, wells were blocked with 5% goat serum in PBS for 30 min. The primary antibody was diluted (Desmin, 1:1000, Dako) in 1% BSA/PBS and 150 µL was added per well for 1 hour. Wells were subsequently incubated with 150 µL/well secondary antibody (Goat anti-Mouse IgG (H+L), Alexa Fluor® 488 conjugated, Thermo Fisher) for 1 h in the dark. Each well was washed with PBS and 150 µL/well DAPI/PBS (1:5000, Cell Signalling Technology) was added for 5 min in the dark. Wells were further washed with PBS, a drop of mountant added to each well (ProLong Diamond Antifade, Thermo Fisher) and a coverslip applied.

2.4 Quantification of myotube thickness and nuclear fusion index

24-well plates of immunuofluoresence (IF) stained myotubes were imaged on an epifluorescence/brightfield microscope (Leica DMI6000). Triplicate wells were stimulated for each biological replicate and for each treatment condition. Multiple images were taken in each well for the quantification of myotube thickness (MTT) and nuclear fusion index (NFI). For quantification of MTT, 15 images per well were obtained using a 63x objective, the first image being obtained at a fixed starting point and subsequent images selected by moving to the next field of view in a predefined pattern. For assessment of NFI, 5 images per well were obtained in the same fashion, using a 20x objective. Image analysis was carried out by a blinded researcher, using Image J software. A myotube was defined as a desmin positive structure, containing 3 or more nuclei. The MTT of each myotube was calculated by taking the average of 5 measurements obtained along its length. The NFI was defined as the number of nuclei clearly incorporated into myotubes expressed as a proportion of the total visible nuclei in each field of view.

Immunoblotting

Protein extraction, SDS-PAGE and immunoblotting were performed as previously described (O'Leary et al. 2017). Primary antibodies for NFκB p65 (RRID:AB_10828935) (Cell Signalling Technology #6956), phosphorylated (Ser^{536}) NFκB p65 (RRID:AB_331284) (Cell Signalling Technology #3033) and resistin (RRID:AB_326017) (polyclonal rabbit IgG, Thermo Fisher PA1-1049) were used. Anti-mouse (RRID:AB_772210) (NA931V, GE Healthcare) and anti-rabbit (RRID:AB_772206) (NA934, GE Healthcare) HRP-linked secondary antibodies were diluted 1:5000 in TBS-T, and blots were developed using ECL-plus (GE Healthcare,

Amersham Biosciences, Amersham, UK) according to the manufacturer's instructions. Bands were visualised on the ChemiDoc MP imaging system (Bio-Rad, UK).

Immunoprecipitation of resistin from adipose conditioned media

70 µL Protein A Sepharose® beads (Abcam, ab193256) were incubated with 1 µg anti-resistin primary antibody (polyclonal rabbit IgG, Thermo Fisher PA1-1049) or 1 µg rabbit IgG isotype control (Sigma Aldrich, 12-370). The antibody-bead mixture was incubated for 4 h at 4 °C on a shaker. The beads were centrifuged at 3,000 g for 2 min at 4 °C and the supernatant was discarded. Beads were then washed twice with PBS, 5 mL ACM added to each bead-antibody conjugate and the ACM-bead-antibody mixture incubated for 24 h at 4 °C with rotary agitation. The mixture was centrifuged at 3,000 g for 2 min at 4 °C and the supernatant (ACM) was retained and stored at -80 °C. The antibody-bead conjugates were washed in PBS as before. The antigen-antibody complexes were eluted from the sepharose beads by the addition of 50 µL 2x Laemmli sample loading buffer. The elutes were incubated at 50 °C for 10 min and stored at – 80 °C in advance of their use in immunoblotting for the detection of resistin.

Multiplex immunoassay

Cytokine and chemokine concentrations were quantified in ACM secretome by multiplex magnetic bead-based immunoassay (Luminex® Screening Assay, R&D Systems) according to the manufacturer’s instructions. 50 µL of a 1x antibody magnetic bead stock (Adiponectin, Serpin E1, Aggrecan, Amphiregulin, CCL11, CCL2, CCL3, CCL20, Chemerin, CXCL10, Dkk1, Galectin-1, gp120, IL-1β, IL-10, IL-15, IL-7, visfatin, TNFα, Galectin-3BP, Lipocalin-2, CCL4, FABP4, LIF, Leptin, IL-6, Resistin) was added to each well of a flat bottom black plate. 50 µL of undiluted sample

or standard were added in duplicate to the plate. The plate was then sealed and incubated for 2 h on an orbital rotator. The plate was washed three times with a magnetic plate washer (Bio-Plex Pro™ Wash Station, Bio-Rad) using the wash buffer provided. 50 µL of a biotinylated antibody cocktail was added to each well; the plate was resealed and incubated for 1 h on the orbital rotator. The wash steps were repeated as before and 50 µL of the provided streptavidin-PE was added to the wells. The plate was incubated on the orbital rotator for 30 min and the wash steps repeated for a final time. Finally, the beads were resuspended in 200 µL wash buffer and the analytes were quantified by the Luminex® 200 multiplex analyser (Luminex® Corporation).

Statistical analysis

Data analysis was carried out using IBM SPSS Statistics 21. All data are presented as means ± SEM of biological replicates. The normality of data was established by a Shapiro-Wilk test, whereas Levene's test was used to establish equality of variances. For parametric data involving two treatment conditions unpaired t tests were used. Non-parametric data were analysed by Mann-Whitney U tests. Where data involving more than two treatment conditions were normally distributed, comparison was performed by a one-way or two-way analysis of variance (ANOVA) with post-hoc Bonferroni correction. Where such data were non-parametric, differences between conditions were analysed by Mann–Whitney U test with post-hoc Holm's sequential Bonferroni correction. A p value of < 0.05 was considered statistically significant. Details of the statistical tests used for each data set can be found in the relevant figure legend.

Results

Quantification of adipokine in the secretome of subcutaneous adipose tissue from lean and non-lean individuals

We initially profiled the concentrations of 22 adipokines in SAT conditioned media (ACM) collected from a cohort of normal weight (BMI<25, n=13) and overweight/obese (BMI>25, n=22) older individuals by multiplex magnetic bead-based immunoassays (Table 1).

Comparing the ACM from the 2 groups, there was no significant difference in the concentration of the prominent adipokines leptin and adiponectin, the concentration of well-known pro-inflammatory cytokines IL-1β and IL-6, or in the concentration of the anti-inflammatory cytokine IL-10. However, the mean concentration of resistin was significantly greater ($p<0.05$) in the ACM from overweight/obese older individuals (1778 ± 109 pg/mL) compared to the ACM collected from normal weight older individuals (1207 ± 225 pg/mL). Furthermore, the median concentration of serpin E1 was significantly greater ($p<0.05$) in the ACM from overweight/obese (median = 10565, IQR = 3420-13450 pg/mL) compared to the ACM from normal weight older individuals (median = 4156, IQR = 1337-6761 pg/mL). Of note, there was also a trend for the median concentration of fatty acid binding protein 4 (FABP4) to be lower in overweight/obese ACM compared to the normal weight, although this did not reach significance (Table 1).

Obese subcutaneous adipose tissue secretome impairs human myogenesis of old, but not young, muscle cells

Having determined the concentrations of adipokines secreted from lean and non-lean SAT, we then sought to determine the effect of the ACM secretome derived from the

SAT of normal weight (NW, BMI < 25) and obese (OB, BMI > 30) individuals on myotube formation. Subconfluent myoblasts from young and old lean, healthy subjects (n = 3 per group) were switched to unconditioned differentiation medium, NW ACM or OB ACM. Each young biological replicate was stimulated together with one from the old experimental group such that both young and old replicates were stimulated with the same NW and OB ACM sample. Media were renewed every 2 d. At 8 d, myotubes were fixed, IF stained for desmin and DAPI and imaged on an epifluorescence microscope (Fig. 1A).

Myotubes from elderly subjects that were stimulated with OB ACM were significantly thinner (30% ± 5%, p = 0.009) than their NW ACM counterparts (Fig. 1B). The NFI of elderly myogenic cultures was also diminished (42 ± 6%, p = 0.0003) by OB ACM compared to NW ACM (Fig. 1C). Young myotubes were not significantly affected by stimulation with the same ACM samples, although a trend (p = 0.09) of reduced NFI was observed when incubated with OB ACM (Fig. 1B, 1C).

The adipokine resistin impairs human myogenesis

Since the adipokine resistin was significantly elevated in the ACM of overweight/obese individuals, we next examined the effect of stimulating myoblasts with recombinant resistin during their differentiation to myotubes. Subconfluent myoblasts from young (n = 3) and elderly (n = 3) subjects were switched to differentiation media or differentiation media containing recombinant resistin (5 ng/mL). Media were renewed every 2 d. At 8 d, cultures were fixed, IF stained for desmin and with DAPI, imaged on an epifluorescence microscope and MTT and NFI were quantified as previously described. Resistin significantly reduced MTT in both young (18 ± 5 %, p < 0.05) and

old (24 ± 6 %, $p < 0.05$) myogenic cultures (Fig. 2A). NFI was significantly diminished in old cultures only (25 ± 13 %, $p < 0.001$) (Fig. 2B).

Depletion of resistin from obese subcutaneous adipose tissue secretome improves myogenesis

The effect of resistin on myogenesis was then validated by depletion of resistin from OB ACM secretome by immunoprecipitation. Firstly, in order to confirm the success of the immunoprecipitation, antibody conjugates were lysed and analysed by Western blotting for the detection of bound resistin (Fig. 3A). Resistin was detected in the resistin antibody conjugates lysates but not in the IgG control conjugate lysates (Fig. 3A). Secondly, the OB ACM was analysed before and after resistin immunoprecipitation for the concentration of resistin by ELISA, employing a different anti-resistin antibody than the immunoprecipitation procedure. Resistin concentrations in OB ACM were diminished following resistin immunoprecipitation (Fig. 3B).

To examine the effect of resistin-depleted OB ACM secretome on myogenesis we utilised commercially available primary human myoblasts, which we first validated as responding in a similar way to our in-house cultures (Supplementary Figure 1). Myoblasts were then switched to differentiation media containing either normal OB ACM, or resistin-depleted OB ACM. Media was renewed every 2 days as previously performed and myotubes fixed and stained at 8 days for the quantification of MTT and NFI. Compared to normal OB ACM, myoblasts cultured with the resistin-depleted OB ACM exhibited increased MTT of 53 ± 13 % ($p < 0.05$; Fig. 3C) and increased NFI of 60 ± 16 % ($p < 0.05$; Fig. 3D).

Resistin inhibits myogenesis by activation of the classical NFκB pathway

Classical NFκB pathway signalling is a negative regulator of myogenesis (Bakkar, et al. 2008; Lu, et al. 2012). Furthermore, in multiple cells types, resistin has been shown to activate NFκB signalling (Calabro, et al. 2011; Zhou, et al. 2013; Zuniga, et al. 2017). Therefore, we next investigated whether the resistin-mediated effects on myogenesis were *via* NFκB activation.

Myogenic cultures differentiated for 48 h in the presence of 5 ng/mL recombinant resistin displayed a significant increase in serine^{536} phosphorylation of p65 (p-p65); such phosphorylation was inhibited by the presence of the IKKβ inhibitor 5-(p-Fluorophenyl)-2-ureido]thiophene-3-carboxamide (TPCA-1) (Fig. 4A, 4B). Having established that resistin activates the classical NFκB signalling pathway during myogenesis, the ability of TPCA-1 to rescue myotubes from the anti-myogenic actions of resistin was explored. As before, 8 d resistin stimulation of differentiating myogenic cultures significantly diminished MTT and NFI, a phenomenon that was completely reversed by co-incubation with TPCA-1 (Fig. 4C, 4D).

Discussion

In this study, we describe for the first time the adverse myogenic effects of the obese human SAT secretome, generated using conditioned media. The stromal vascular fraction of adipose tissue is a more prolific secretor of pro-inflammatory cytokines than mature adipocytes (Blaber et al. 2012) and thus our experimental model may be a more physiologically relevant model of adipose tissue adipokine secretion than models which rely upon adipokine secretion by adipocytes alone (Pellegrinelli et al. 2015). Furthermore, since SAT represents a much larger proportion of total adipose tissue mass than VAT (Isaac et al. 2011; Rosqvist et al. 2017; Rossi et al. 2011), its contribution to the systemic inflammatory burden to which skeletal muscle is exposed is likely to be significant. Indeed, myotubes from elderly subjects cultured with obese SAT secretome were 30% thinner and had a 40% reduction in the number of nuclei incorporated into myotubes, compared to those cultured with normal weight SAT conditioned media. Previous work by Pellegrinelli et al. demonstrated that the lean subcutaneous adipocyte inflammatory secretome does not have a detrimental effect on myotube formation in neonatal myogenic cultures, but that the obese VAT adipocyte secretome does inhibit myotube formation in such cultures (Pellegrinelli et al. 2015). Interestingly, adipocytes isolated from obese SAT showed an intermediary negative effect on MTT between lean SAT and obese VAT (Pellegrinelli et al. 2015). Our work builds on these observations by the inclusion of both lean and obese SAT inflammatory milieu, using whole adipose tissue rather than adipocytes to generate conditioned medium and by demonstrating an anti-myogenic effect of OB ACM on elderly (but not young) adult human myogenic cultures. Our results suggest that younger skeletal muscle may be intrinsically more resilient to inflammatory cytokines secreted from SAT.

353 In considering potential secretory factors that could be - at least in part - responsible
354 for the effect of the obese SAT secretome on myogenesis, the concentration of resistin
355 was found to be significantly elevated in the SAT conditioned media secretome
356 collected from non-lean (BMI > 25) older individuals, compared to that collected from
357 lean (BMI < 25) older individuals. Resistin is a pro-inflammatory adipokine that is
358 produced predominantly by monocytes and macrophages in humans, with a smaller
359 proportion being produced by adipocytes (Savage, et al. 2001). Given the importance
360 of adipose tissue M1 macrophage accumulation in ageing and obesity (Cancello, et
361 al. 2005; Fujisaka, et al. 2009; Lumeng, et al. 2007; Weisberg, et al. 2003), adipose
362 tissue secretion of resistin may be of significant consequence in sarcopenia. However,
363 few studies have previously described the effect of resistin on human skeletal muscle
364 and sarcopenia. Plasma resistin concentrations have been reported to have an inverse
365 relationship with quadriceps torque in old (69-81 yr), but not in young (18-30 yr),
366 subjects (Bucci et al. 2013). A recent study has described an inverse relationship
367 between abdominal skeletal muscle density and systemic resistin concentrations (Van
368 Hollebeke, et al. 2018); such increases in skeletal muscle density are thought to
369 indicate improved muscle quality and have been associated with increased muscle
370 strength (Goodpaster, et al. 2001). Furthermore, C2C12 mouse myoblast proliferation
371 is increased by the transfection of a human resistin eukaryotic expression vector, and
372 such transfection reduces the expression of desmin and results in thinner myotubes
373 (Sheng, et al. 2013). We thus identified resistin as warranting further exploration of its
374 myogenic effects.
375 Here, we demonstrate that stimulation of developing myotubes with recombinant
376 resistin, at a concentration reported physiologically in older humans (Philp et al. 2017),
377 has a substantial detrimental effect on the formation of such myotubes. Notably,

myogenic cultures from both young and old subjects were thinner following resistin stimulation, but only old myotubes displayed a reduction in their NFI. Similarly, obese SAT conditioned media that contained more resistin had a greater detrimental effect on both myotube thickness and NFI of elderly myotubes compared to young myotubes. This might indicate that there are age-related differences in the *ex vivo* myogenic capacity of myoblasts under inflammatory conditions, with young muscle being more resistant to pathological levels of adipokines such as resistin than older muscle. The mechanisms underlying the differential responses of young and elderly myotubes to OB ACM were not explored in this study, yet plausible avenues of enquiry exist. Primary human myogenic cultures are known to retain some of the characteristics of their donors (McAinch, et al. 2006; Mott, et al. 2000; Thompson, et al. 1996). Furthermore, aged skeletal muscle displays increased classical NFκB pathway activity (Buford and Manini 2010; Tilstra, et al. 2011). It is possible altered cytokine receptor expression levels leave elderly myogenic cultures more susceptible to the detrimental effects of OB ACM on culture differentiation. However, we are unaware of any comprehensive profile of cytokine receptor gene or protein expression comparing young and old human skeletal muscle.

Given these findings, it is highly significant that depletion of resistin from obese SAT secretome completely abrogated the anti-myogenic action of obese SAT conditioned media secretome. However, it is important to note that there are likely to be additional factors within obese SAT conditioned media, which we did not assess, that could have contributed to the considerable declines in both myotube thickness and NFI we observed in elderly myotube cultures.

It is clear from the literature that resistin activates NFκB signaling. Such activation has been demonstrated in the HepG2 cells (Zhou et al. 2013), human coronary artery

endothelial cells (Calabro et al. 2011) and in human macrophages (Zuniga et al. 2017). Importantly, genetic approaches have now established the classical NFκB pathway as a negative regulator of myogenesis. Myogenesis has been shown to be enhanced in $p65^{-/-}$ myoblasts (Bakkar et al. 2008), whilst the IKKβ inhibitor IV has been shown to enhance the myogenic differentiation of primary murine cultures from wild-type mice (Lu et al. 2012). Furthermore, NFκB activation in the satellite cells of aged mice inhibited skeletal muscle regeneration in response to cryoinjury (Oh, et al. 2016).

In myogenic cultures, classical NFκB pathway activity is diminished at 48 h post-differentiation (Bakkar et al. 2008). Importantly, we observed that the addition of recombinant resistin to our myogenic cultures resulted in persistent p65 phosphorylation (indicative of NFκB activation) at 48 h, a phenomenon that was reversed by the addition of the IKK2 inhibitor TPCA-1. Importantly, TPCA-1 rescued the differentiation of our myogenic cultures in the presence of recombinant resistin, suggesting therefore that resistin impaired myogenesis *via* activation of the classical NFκB pathway.

In summary, our studies describe a detrimental effect of obese SAT conditioned media secretome on primary human myogenesis and identify resistin as the adipokine that - at least in part - mediates this effect. Furthermore, we demonstrate that resistin exerts its anti-myogenic effects by causing persistent activation of the classical NFκB pathway. These findings may have important implications for the maintenance of muscle mass in older people who are obese or overweight, or those with chronic conditions such as osteoarthritis (Philp et al. 2017) and type 2 diabetes (Gharibeh, et al. 2010), which are associated with increased levels of resistin in the circulation.

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Author Contributions: MOL conceived, designed, performed the experiments, analysed the data and wrote the article. KT, GW and SJ conceived and designed the experiments and wrote the article. AB and ED conceived and designed the experiments.

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Figure 1. Obese subcutaneous adipose conditioned medium inhibits myotube formation in differentiating human myoblasts.

Subconfluent myoblasts were switched to unconditioned differentiation medium or differentiation medium that had previously been conditioned with adipose tissue from normal weight (NW ACM, n = 3; BMI<25 kg/m^2) or obese individuals (OB ACM, n = 3; BMI>30 kg/m^2). Each young (18-30 yr) biological replicate was paired with one from the old (> 65 yr) experimental group, with both being stimulated with the same ACM samples. Media were renewed every 2 d. At 8 d, myotubes were fixed, immunofluorescence stained for desmin and with DAPI and imaged on an epifluorescence microscope. (**A**) Representative images at 20x magnification. (**B**) Myotube thickness data represent the mean ± SEM of n = 3 biological replicates. Each biological replicate comprises 150 total measurements taken at 63x magnification from 30 myotubes per treatment condition. (**C**) Nuclear fusion index data are expressed as mean ± SEM values of n = 3 biological replicates. Each biological replicate comprises 15 images taken at 20x magnification. **p < 0.01, ***p < 0.001 by Mann-Whitney U test with post-hoc Holm's sequential Bonferroni adjustment.

Figure 2. Recombinant resistin impairs myotube formation in myotubes derived from young and elderly subjects.

Subconfluent myoblasts from young and elderly subjects were switched to differentiation media (with or without 5 ng/mL recombinant resistin). Media were renewed every 2 d. At 8 d, myotubes were fixed, immunofluorescence stained for desmin and with DAPI and imaged on an epifluorescence microscope. (**A**) Myotube thickness data represent the mean ± SEM of n = 3 biological replicates. Each biological replicate comprises 150 total measurements taken at 63x magnification from 30

myotubes per treatment condition. (**B)** Nuclear fusion index data are expressed as mean ± SEM values of n = 3 biological replicates. Each biological replicate comprises 15 images taken at 20x magnification. *p < 0.05, ***p < 0.001 by unpaired t test.

Figure 3. Immunoprecipitation of resistin from obese subcutaneous adipose conditioned medium secretome (OB ACM) improves myogenesis.

Resistin was immunoprecipitated from OB ACM using resistin antibody-agarose bead conjugates (OB ACM – resistin IP). IgG isotype antibody control-agarose bead conjugates were used on the same samples as a control (OB ACM). (**A**) Resistin protein is detected by immunoblotting of resistin-antibody lysates but not IgG control lysates following immunopreciptation. (**B**) Depletion of resistin in OB ACM following resistin immunoprecipitation as determined by ELISA. (**C**) Subconfluent, commercially available primary human skeletal myoblasts from a female aged 21 yr were switched to either OB ACM differentiation media (OB ACM, n =4) or to resistin-depleted OB ACM differentiation media (OB ACM Resistin IP, n = 4). Media were renewed every 2 d. At 8 d, myotubes were fixed, immunofluorescence stained for desmin and with DAPI and imaged on an epifluorescence microscope. Nuclear fusion index data are expressed as mean ± SEM values of n = 3 independent experiments. Each independent experiment comprises 15 images taken at 20x magnification. **D)** Myotube thickness data represent the mean ± SEM of n = 3 independent experiments. Each independent experiment comprises 150 total measurements taken at 63x magnification from 30 myotubes per treatment condition. *p < 0.05 vs OB ACM by unpaired t test.

Figure 4. Resistin exerts its anti-myogenic effects via activation of the classical NFκB pathway.

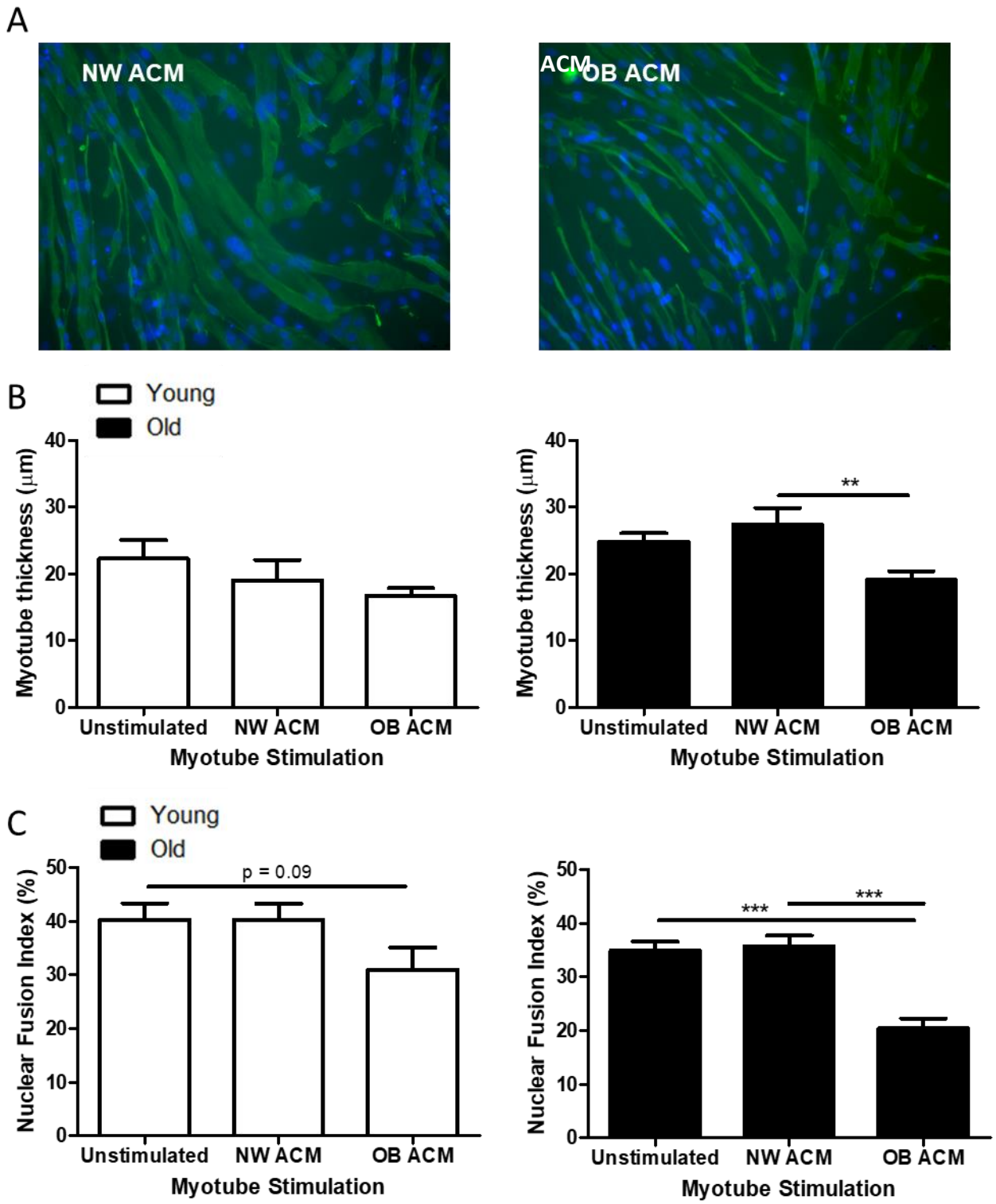
(**A, B**) Subconfluent primary human skeletal myoblasts from a female aged 21 yr were switched to differentiation media (with or without 5 ng/mL recombinant resistin ± 40 nM TPCA-1) for 48 h. Phospho-p65 (Ser536) and total p65 were detected by immunoblotting. US = unstimulated, R = resistin, T = TPCA-1, RT = resistin + TPCA-1. Data are expressed as mean ± SEM values of n = 3 independent experiments. **p < 0.01 by one-way ANOVA with post-hoc Bonferroni correction. (**C, D**) Subconfluent primary human skeletal myoblasts from a female aged 21 yr were switched to differentiation media (with or without 5 ng/mL recombinant resistin ± 40 nM TPCA-1). Media were renewed every 2 d. At 8 d, myotubes were fixed, immunofluorescence stained for desmin and with DAPI and imaged on an epifluorescence microscope. Myotube thickness data represents the mean ± SEM of n = 3 independent experiments. Each independent experiment comprises 150 total measurements taken at 63x magnification from 30 myotubes per treatment condition. Nuclear fusion index data are expressed as mean ± SEM values of n = 3 independent experiments. Each independent experiment comprises 15 images taken at 20x magnification. **p < 0.01, ***p < 0.001 vs unstimulated control by Mann-Whitney U test with post-hoc Holm's sequential Bonferroni adjustment.

Table 1. The Inflammatory Secretory Profile of Normal weight (Lean) and overweight/obese (non-Lean) Subcutaneous Adipose Conditioned Medium

	Lean (BMI < 25) (Mean ± SEM, pg/mL)	Non-Lean (BMI > 25) (Mean ± SEM, pg/mL)	P-value (Lean vs. non-lean)
Adiponectin	28716 ± 4524	28841 ± 3779	0.18
Aggrecan	578, 559-606	530, 468-567	0.07¶
Amphiregulin	532 ± 137	703 ± 67	0.22
Chemerin-1	2655 ± 673	3450 ± 583	0.44
Eotaxin	95 ± 41	61 ± 12	0.81
FABP4	$38x10^4$, $27x10^4$-$81x10^4$	$27 x10^4$, $23 x10^4$-$39 x10^4$	0.06¶
Galectin-1	$5.3 x10^4 \pm 0.6 x10^4$	$5.2 x10^4 \pm 0.3 x10^4$	0.85
GP130	29001 ± 10480	31824 ± 7342	0.83
IL-10	2.25 ± 0.56	2.59 ± 0.39	0.62
IL-15	1.81 ± 0.52	2.32 ± 0.37	0.45
IL-1β	12.02 ± 1.59	11.94 ± 1	0.96
IL-6	507, 436-1044	1528, 719-2889	0.12¶
IL-7	3.02 ± 0.27	3.04 ± 0.29	0.96
Leptin	11335 ± 2592	12210 ± 2467	0.83
MCP-1	2372 ± 924	1540 ± 406	0.34
MIP1a	363 ± 54	303 ± 32	0.33
MIP1b	101 ± 37	125 ± 24	0.58
MIP3a	85 ± 25	164 ± 51	0.97
Resistin	1207 ± 225	1778 ± 109	0.01
Serpin E1	4156, 1337-6761	10565, 3420-13450	0.02¶
TNFα	10.43 ± 1.57	10.43 ± 1.17	0.99
Visfatin	114, 1007-1827	917, 2051-2417	0.91¶

Adipokine concentrations were determined by multiplex magnetic bead-based cytokine assays in ACM from lean (BMI < 25, n = 13) and non-lean (BMI > 25, n = 22) subjects. Data are presented as mean ± SEM where normally distributed and as median, 25^{th} percentile-75^{th} percentile where not normally distributed (marked ¶). FABP4= Fatty acid binding protein 4, GP130=glycoprotein 130, MCP-1=Monocyte chemoattractant protein 1, MIP1a=Macrophage inflammatory protein 1a, MIP1b=Macrophage inflammatory protein 1b, MIP3a=Macrophage inflammatory protein 3a. IL=Interleukin; TNFα=Tumor Necrosis Factor alpha.

671 Figure 1



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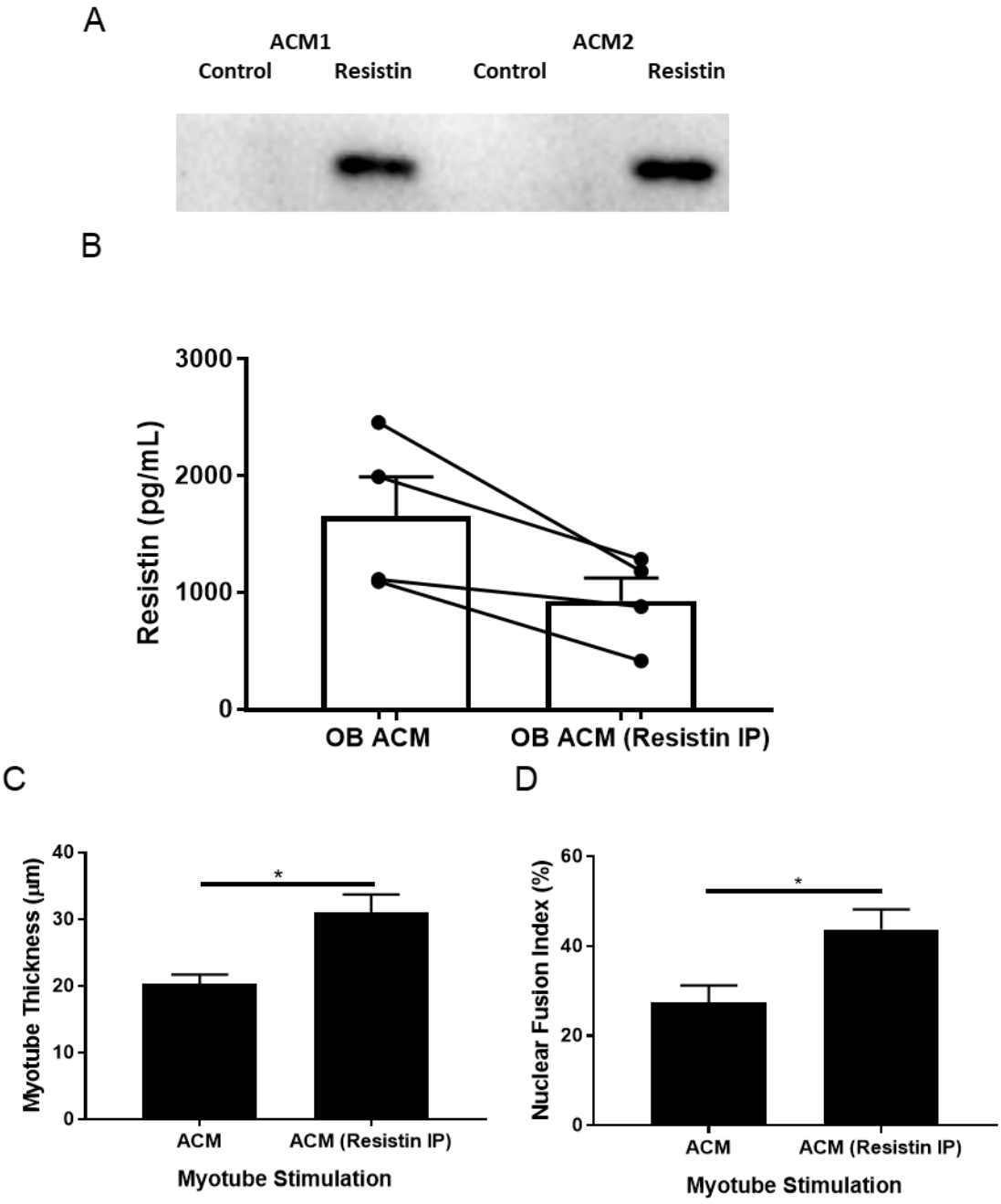
A
ACM1
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Control
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Control
Resistin
B
Resistin (pg/mL)
3000
2000
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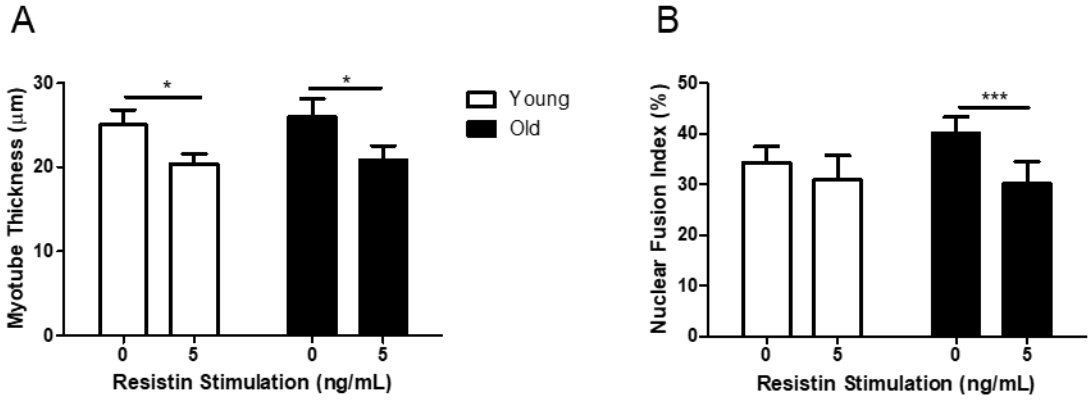
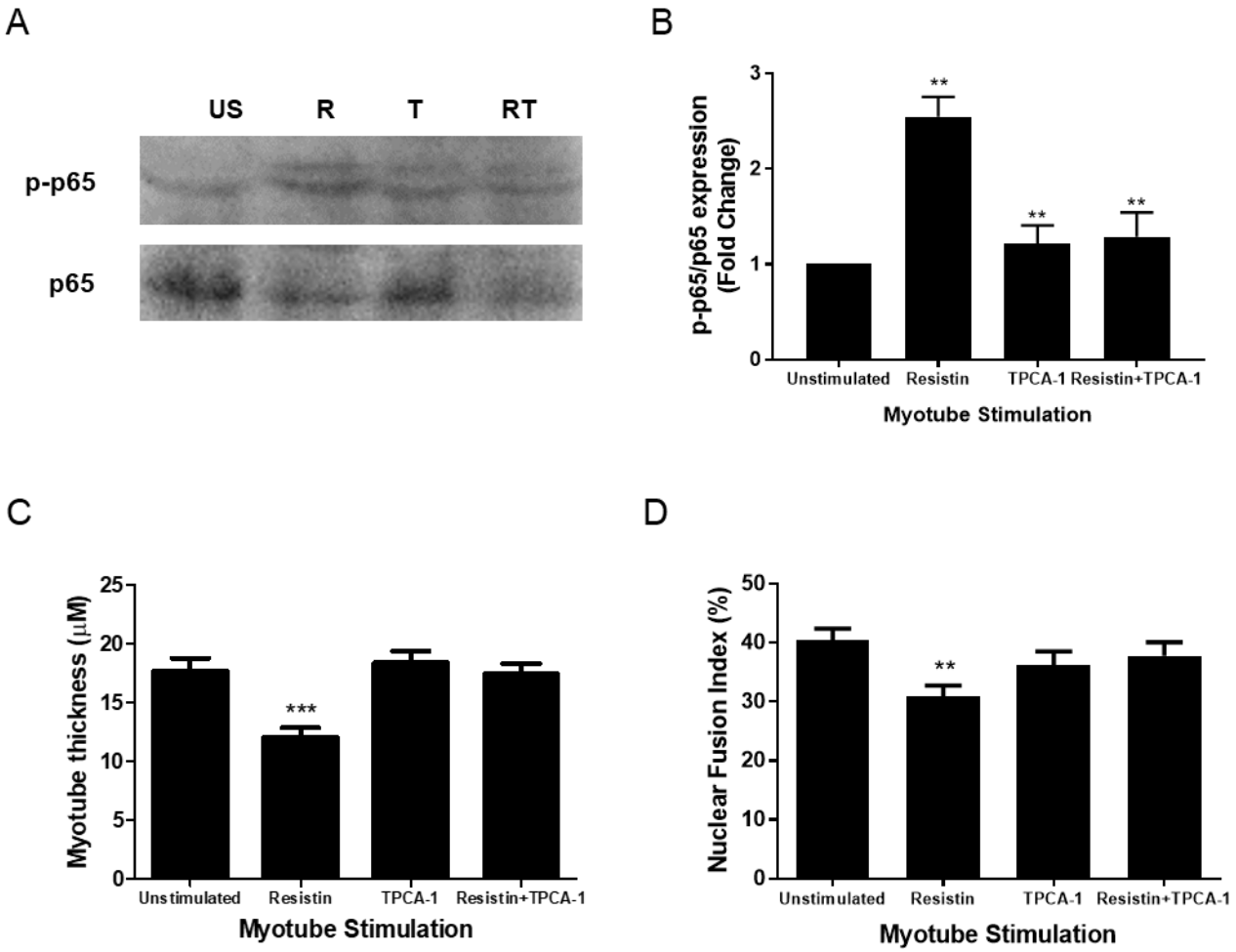
OB ACM
OB ACM (Resistin IP)
C
D
Myotube Thickness (µm)
40
30
20
10
0
*
ACM
ACM (Resistin IP)
Myotube Stimulation
Nuclear Fusion Index (%)
60
40
20
0
*
ACM
ACM (Resistin IP)
Myotube Stimulation

675


A
B
Myotube Thickness (µm)
30
20
10
0
*
*
Young
Old
0
5
0
5
Resistin Stimulation (ng/mL)
Nuclear Fusion Index (%)
50
40
30
20
10
0
***
0
5
0
5
Resistin Stimulation (ng/mL)

676


A
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p-p65
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p-p65/p65 expression (Fold Change)
3
2
1
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Unstimulated
Resistin
TPCA-1
Resistin+TPCA-1
Myotube Stimulation
C
Myotube thickness (μM)
25
20
15
10
5
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Unstimulated
Resistin
TPCA-1
Resistin+TPCA-1
Myotube Stimulation
D
Nuclear Fusion Index (%)
50
40
30
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Unstimulated
Resistin
TPCA-1
Resistin+TPCA-1
Myotube Stimulation

677