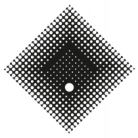

Striated muscle activator of Rho signalling (STARS) is reduced in ageing human skeletal muscle and targeted by miR-628-5p

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Striated muscle activator of Rho signalling (STARS) is reduced in ageing human skeletal muscle and targeted by miR-628-5p

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Abstract

Aim: The striated muscle activator of Rho signalling (STARS) is a muscle-specific actin-binding protein. The STARS signalling pathway is activated by resistance exercise and is anticipated to play a role in signal mechanotransduction. Animal studies have reported a negative regulation of STARS signalling with age, but such regulation has not been investigated in humans.

Methods: Ten young (18–30 years) and 10 older (60–75 years) subjects completed an acute bout of resistance exercise. Gene and protein expression of members of the STARS signalling pathway and miRNA expression of a subset of miRNAs, predicted or known to target members of STARS signalling pathway, were measured in muscle biopsies collected pre-exercise and 2 h post-exercise.

Results: For the first time, we report a significant downregulation of the STARS protein in older subjects. However, there was no effect of age on the magnitude of STARS activation in response to an acute bout of exercise. Finally, we established that miR-628-5p, a miRNA regulated by age and exercise, binds to the STARS 3'UTR to directly downregulate its transcription.

Conclusion: This study describes for the first time the resistance exercise-induced regulation of STARS signalling in skeletal muscle from older humans and identifies a new miRNA involved in the transcriptional control of STARS.

Keywords ageing, exercise, microRNA, skeletal muscle, striated muscle activator of Rho signalling.

Ageing is characterized by a gradual increase in cellular damage and a decline in physical function over time. At the molecular level, multiple pathways are dysregulated with ageing, contributing to the arrest of cell growth and repair (Smith-Vikos & Slack 2012). In mammals, ageing is associated with a decline in skeletal muscle mass and function, referred to as sarcopaenia. It is estimated that human adults lose about 10% of their muscle mass by the age of 50, with a further average reduction of 1% per year until death (Dorrens & Rennie 2003, Phillips 2012). Age-related muscle atrophy is linked to a disruption in skeletal muscle protein turnover (Boirie 2009) and reduced regenerative capacity (Brack & Rando 2007). In addition, the elderly demonstrate impaired phosphorylation of several key proteins involved in skeletal muscle protein synthesis following resistance exercise (Léger *et al.* 2008, Kumar *et al.* 2009, Zacharewicz *et al.* 2014). This phenomenon is referred to as anabolic resistance. Ageing is also associated with impaired

Notch, Wnt (Arthur & Cooley 2012) and myogenic regulatory factor (MRF) (Marsh *et al.* 1997, Dedkov *et al.* 2003) signalling. These pathways are important for efficient myogenesis (Corbu *et al.* 2010, Walker *et al.* 2012) and their downregulation is believed to contribute to inefficient muscle repair and function with age (Shadrach & Wagers 2011). The molecular mechanisms leading to age-related muscle wasting are complex, and our understanding is far from complete. Continued research is required to identify other signalling pathways that are important for the maintenance of muscle size and function and are perturbed in ageing human muscle.

The striated muscle activator of Rho signalling (STARS) protein is an actin-binding protein highly expressed in cardiac, smooth and skeletal muscle (Arai *et al.* 2002, Mahadeva *et al.* 2002, Peng *et al.* 2008, Troidl *et al.* 2009). STARS increases actin polymerization, resulting in the nuclear translocation of the serum response factor (SRF) transcriptional co-activator myocardin-related transcription factor-A (MRTF-A; also known as megakaryoblastic leukaemia 1 protein, MKL1) (Arai *et al.* 2002, Kuwahara *et al.* 2005). This signalling response enhances the activation of SRF gene transcription, which plays a role in maintaining skeletal muscle growth and repair (Olson & Nordheim 2010, Braun & Gautel 2011). STARS is also expressed in the nucleus (Troidl *et al.* 2009, Wallace *et al.* 2011) where it may interact directly with DNA to control gene transcription (Zaleska *et al.* 2015). *Stars* gene expression is decreased in skeletal muscle from aged mice (Sakuma *et al.* 2008) and pigs (Peng *et al.* 2008) when compared to young animals. The nuclear content of MRTF-A, but not total SRF, is lower in skeletal muscle of 24-month-old sarcopaenic mice when compared with 3-month-old mice (Sakuma *et al.* 2008). At present, it is unknown whether the STARS signalling pathway is downregulated in skeletal muscle of older compared with younger humans. Members of the STARS signalling pathway increase following a single bout of resistance exercise as well as training in young healthy subjects (Lamon *et al.* 2009), suggesting that they play a role in skeletal muscle adaptation and function. Molecular signalling responses to exercise are often attenuated in older when compared to younger muscle; however, whether this is the case for STARS signalling has not been investigated.

Striated muscle activator of Rho signalling is a transcriptional target of several transcription factors including MyoD, ERRα, SRF and MEF2 (reviewed in Wallace *et al.* 2012). The recent identification of microRNAs (miRNAs) as additional regulators of gene expression has added another level of complexity in our understanding of transcriptional regulation. miRNAs are now considered as essential regulators of skeletal muscle health (reviewed in Zacharewicz *et al.* 2013). In human skeletal muscle, exercise and age regulate miRNA expression (Zacharewicz *et al.* 2013, 2014). miRNAs mainly act by degrading specific mRNA species to prevent the resulting protein translation (Olsen & Ambros 1999, Lee *et al.* 2004b, Wightman *et al.* 2004, Humphreys *et al.* 2005, Pillai *et al.* 2005, Huili *et al.* 2010), and miRNA activity is largely dependent on its binding capacity to the target mRNA molecule (Brennecke *et al.* 2005, Hu & Bruno 2011). While it has been established that miR-9 (Buller *et al.* 2012), miR-133a (Liu *et al.* 2008), miR-150 (Liu *et al.* 2015), miR-200 (Buller *et al.* 2012), miR-320a (Chen *et al.* 2015) and miR-483-5p (Qiao *et al.* 2011) regulate SRF expression and miR-31 (Katsura *et al.* 2016) and miR-206 (Zhang *et al.* 2015) regulate MRTF-A expression, the miRNA regulation of STARS is unknown.

The aims of this study were to, firstly, measure the basal expression levels of STARS and members of its signalling pathway in skeletal muscle from young and older male subjects. Secondly, we compared the regulation of STARS and members of its signalling pathway in young and older human subjects following a single bout of resistance exercise. Thirdly, we used a high-throughput PCR-based miRNA array platform to determine whether there was an effect of age and exercise on the regulation of the miRNAs known to target SRF and MRTF-A. Finally, we combined bioinformatics prediction modelling with luciferase reporter technology to identify the potential miRNA regulation of STARS.

Methods

Subjects

The subject characteristics and methodology have been published previously (Stefanetti *et al.* 2014). Ten young (18–30) and 10 older (60–75) healthy males participated in the study. The study was approved by the Deakin University Human Research Committee (EC: 2011-043) in accordance to the *Declaration of Helsinki* (2013) (http://www.wma.net/en/30publications/10policies/b3/). Informed consent and inclusion and exclusion criteria have been described elsewhere (Stefanetti *et al.* 2014).

Trial protocol

Dual-energy X-ray absorptiometry scan (DXA), preliminary testing, exercise protocol and muscle biopsy procedure have been described in the study by Stefanetti *et al.* (2014). Briefly, the subjects completed an acute bout of resistance exercise that consisted of

three sets of 14 repetitions of leg extension exercise at 60% of maximal voluntary contraction (1 RM). Muscle biopsies were collected immediately after exercise and 2 h later.

Protein extraction and western blotting

Total protein was extracted using RIPA buffer (Millipore, North Ryde, NSW, Australia) with 1 μL/mL protease inhibitor cocktail (Sigma-Aldrich, Castle Hill, NSW, Australia) and 10 μL/mL Halt Phosphatase Inhibitor Single-Use Cocktail (Thermo Scientific, Rockford, IL, USA). Total protein content was determined using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Electrophoresis was performed using a 4–12% NuPAGE® Novex Bis-Tris Gel (Life Technologies, Mulgrave, Vic., Australia) in NuPAGE® SDS MOPS Running Buffer (Life Technologies). Protein transfer was performed onto PVDF membrane in a Bjerrum buffer containing 50 mM Tris, 17 mM glycine and 10% methanol. The membranes were blocked with 5% BSA in PBS, after which they were incubated at 4 °C with the following primary antibodies diluted 1:1000 in 5% BSA in PBS: STARS (Institute of Medical and Veterinary Science, Adelaide, SA, Australia); SRF (sc-335; Santa Cruz, CA, USA); MRTF-A (sc-32909; Santa Cruz); phospho-SRF-S103 (ab53130; Abcam, Cambridge, MA, USA). Following overnight incubation, the membranes were washed and incubated for 1 h with a goat anti-rabbit IgG antibody labelled with an infrared-fluorescent 800 nm dye (Alexa Fluor® 800; Life Technologies) diluted 1:5000 in PBS containing 50% Odyssey® blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) and 0.01% SDS. After washing, the proteins were exposed on an Odyssey® Infrared Imaging System (LI-COR Biosciences) and individual protein band optical densities were determined using the Odyssey® Infrared Imaging System software. All blots were normalized against the GAPDH protein (G8795; Sigma-Aldrich). C_2C_{12} myotubes overexpressing ADV-STARS (100 MOI) were used as a STARS-positive control (Wallace *et al.* 2016). C_2C_{12} myoblasts (~70% confluent) were treated for 1 h with phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), also known as 12 myristate 13-acetate. TPA stimulates the serum response element, providing a positive control for phosphorylated SRF (Gineitis & Treisman 2001).

RNA extraction and reverse transcription

RNA extraction and reverse transcription procedures have been described in the study by Stefanetti *et al.* (2014). miRNA reverse transcription has been described in the study by Zacharewicz *et al.* (2014).

Real-time PCR

Real-time PCR was carried out using a Stratagene MX3000 thermal cycler to measure mRNA levels. mRNA levels of *STARS, SRF, MRTF-A* and *PPIA* were measured using Brilliant® Multiplex QPCR Master Mix (Agilent Technologies, SantaClara, CA, USA) and 5 ng of cDNA. Primer and probe details have been published previously (Lamon *et al.* 2009). The PCR conditions were 1 cycle of 10 min at 95 °C; 40 cycles of 30 s at 95 °C, 60 s at 60 °C. For *PPIA*, a melting curve was included at the end of the PCR cycles. To compensate for variations in input RNA amounts and efficiency of the reverse transcription, data were normalized to the *PPIA* (peptidylprolyl isomerase A, cyclophilin) gene. *PPIA* gene expression levels were stable and did not differ between age, time or a combination of both factors (Fig. S1). Ct values were obtained from the MxPro qPCR software (Agilent Technologies). Ct values were then logarithmically transformed and mean log-transformed Ct values (referred to as arbitrary unit [AU] values) were considered for further analysis.

miRNA arrays and statistical analysis

miRNA expression in the samples was assessed using the TaqMan® Array Human MicroRNA A+B Cards version Set v3.0 (Life Technologies) according to the manufacturer's instructions (Zacharewicz *et al.* 2014), and the data were normalized using the global normalization function. Statistical treatment of the data (split plot ANOVA) has been comprehensively described in the study by Zacharewicz *et al.* (2014).

Identification of miRNAs predicted to target the STARS signalling pathway

The miRNAs predicted to target *STARS* were predicted on the basis of 3'UTR sequence homology using miRWalk (Jin *et al.* 2009). The miRWalk software enables the prediction of miRNA targets by incorporating several known prediction software programs. miRanda, miRDB, miRWalk, RNA22 and Targetscan were chosen within the miRWalk program. A predicted mRNA/miRNA interaction was considered significant when identified by a minimum of four prediction software programs.

Luciferase reporter assay

HEK293 cells (ATCC, Manassas, VA, USA) (1–2×10^5/mL) were seeded in black-walled 96-well plates. Twenty-four hours after seeding, cells were cotransfected with 150 ng pNanoglo2 vector

(Promega, Alexandria, NSW, Australia) containing either: no insertion (empty control); the full-length human *Stars* 3' UTR; the putative miR-628-5p STARS target site (including the predicted seed site with 10 base pairs on either side); or its mutant control (primers listed in Table S1), cloned between SacI and NheI downstream of the Nanoluc luciferase; together with 5 nM miR-628-5p mimics (mirVana™ miRNA mimic; Life technologies), or an irrelevant miRNA control (miR-99b-5p), using Lipofectamine 2000 (Life technologies) following the manufacturer's protocol. Four or 6 h post-transfection, the media was removed and replaced by normal media (DMEM with 10% FBS). Twenty-four hours later, cells were consecutively assayed for Firefly and Nanoluc luciferase expression using the Nano-Glo® Dual-luciferase® Reporter assay kit (Promega) following the manufacturer's protocol.

Statistical methods

All data are reported as mean ± SEM. After normal distribution of the data and homogeneity of variance established, analysis of variance (ANOVA) was used to compare group means. *Post hoc* analysis was conducted where appropriate using the least significant difference (LSD) test. The significance levels were set at $P < 0.05$.

Results

Subjects' demographics

Subjects' physiological characteristics have been described elsewhere (Stefanetti *et al.* 2014).

Regulation of STARS, SRF and MRTF-A mRNA expression with exercise and ageing

Resistance exercise increased *STARS* mRNA expression in young (10.3-fold) and older (6.7-fold) subjects (main effect of exercise, $P < 0.001$) (Fig. 1a). Similarly, *SRF* mRNA expression increased by 1.63-fold and 1.3-fold in young and older subjects respectively (main effect of exercise, $P < 0.05$) (Fig. 1b). There was no significant age x exercise interaction. *MRTF-A* mRNA levels did not vary with age or exercise (Fig. 1c).

Regulation of STARS, SRF and MRTF-A protein expression with exercise and ageing

Exercise had no effect on the protein levels of STARS, MRTF-A, SRF and phospho-SRF measured in young or older subjects 2 h post-exercise (Fig. 2). A

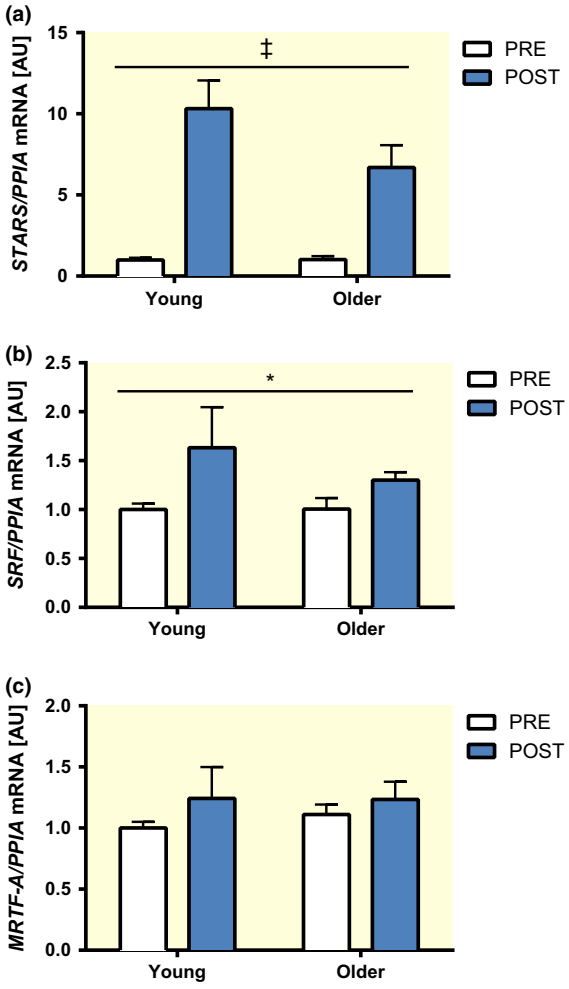

Figure 1 Striated muscle activator of Rho signalling (STARS), serum response factor (SRF) and myocardin-related transcription factor-A (MRTF-A) mRNA expression levels in skeletal muscle from young and older subjects pre- and post-exercise. *Main effect of exercise, $P < 0.05$. ‡Main effect of exercise, $P < 0.001$.

significant decrease in STARS protein expression was observed in older subjects when compared to young subjects (main effect of age, $P < 0.01$) (Fig. 2a). Phospho-SRF protein levels were greater in older subjects than in young subjects (main effect of age, $P < 0.05$) (Fig. 2d). Age had no effect on MRTF-A or total SRF protein expression (Fig. 2b,c).

Age and exercise regulation of miRNAs targeting members of the STARS pathway

The miRNA array analysis returned a total of 26 miRNAs that were significant for one or more of the contrasts studied, indicating that the expression levels of these miRNAs varied with age, exercise or a combination of both factors (previously reported in Zacharewicz *et al.* 2014). Of the miRNAs known to directly

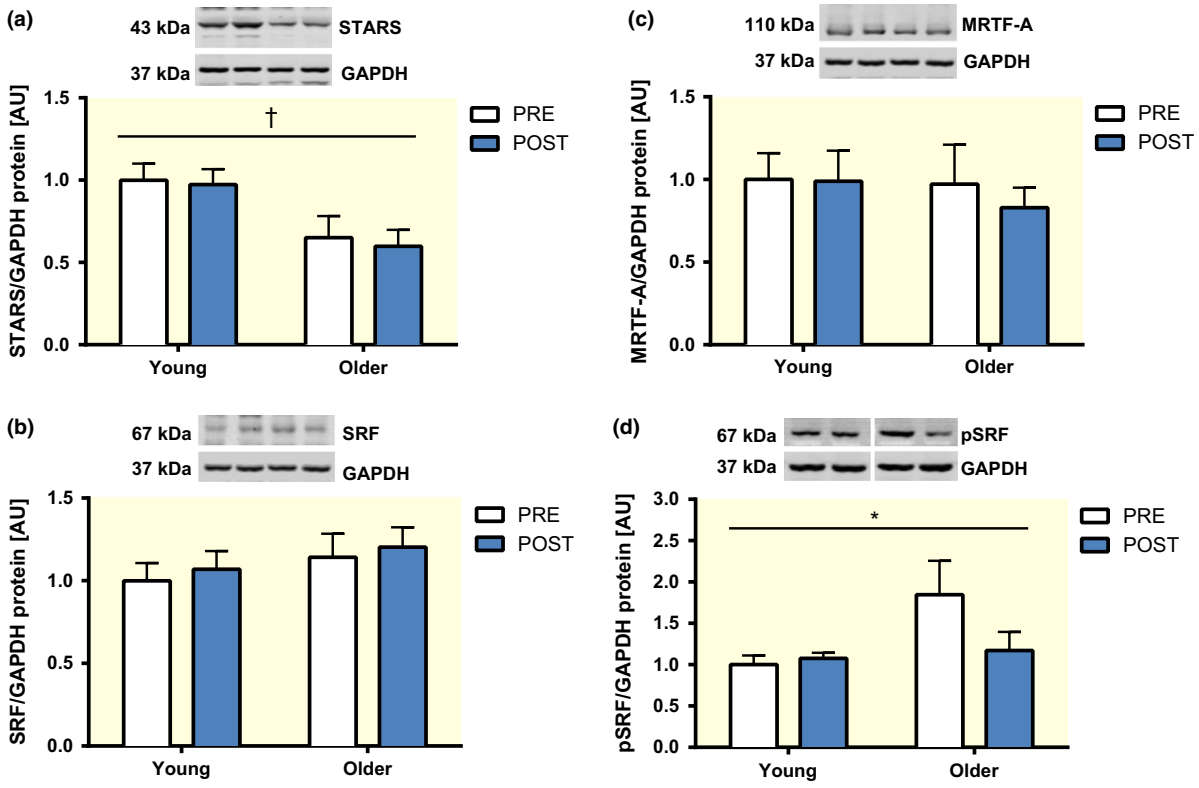

Figure 2 Striated muscle activator of Rho signalling (STARS), serum response factor (SRF) and myocardin-related transcription factor-A (MRTF-A) and phospho-SRF protein expression levels in skeletal muscle from young and older subjects pre- and post-exercise. Exercise had no effect on any of the proteins measured. *Main effect of age, $P < 0.05$. †Main effect of age, $P < 0.01$. Note that the reported statistical significance of any comparisons is based on analyses of the transformed data, but the reported means ± SEMs represent the original (untransformed) scale. Note that non-contiguous gel lanes are demarcated by white spaces; no adjustment to digital images does not alter the information contained therein.

target SRF or MRTF-A, miR-320a and miR-483-5p were regulated with age in our model (Fig. 3). No change was observed for miR-9, miR-133a, miR-150 and miR-206 (Fig. S2), and miR-31 and miR-200 levels were expressed below the threshold for detection. Both miR-320a and miR-483-5p expression levels were higher in older than in young subjects (main effect of age, $P < 0.01$ and $P < 0.001$ respectively) (Fig. 3a,c). A significant negative correlation ($P = 0.04$; $r = 0.37$) was observed between *Srf* mRNA and miR-483-5p, but not miR-320a, levels when all subjects (pre- and post-exercise) were analysed (Fig. 3b,d).

Identification of new miRNAs potentially targeting STARS

As there has been no validation of a miRNA that regulates STARS, we performed a miRNA prediction analysis using miRwalk on the subset of 26 miRNAs that were significantly regulated in our model (Zacharewicz *et al.* 2014). Of these, miR-520g-3p, miR-539-5p and miR-628-5p were predicted to target the human *STARS* transcript by four prediction software programs included in the miRwalk software. Figure 4 displays the expression levels of miR-520g-3p, miR-539-5p and miR-628-5p in young and older subjects 2 h after exercise. MiR-520g-3p was significantly reduced post-exercise in young subjects only ($P < 0.05$) (Fig. 4a). There was a main effect of age for miR-539-5p, which expression was greater in older subjects ($P < 0.05$) (Fig. 4b). Finally, there was a significant interaction effect for miR-628-5p expression levels ($P < 0.05$), which were reduced post-exercise in young subjects and lower at rest in the older subjects ($P < 0.05$ and $P < 0.01$ respectively) (Fig. 4c).

Luciferase reporter assay

To confirm whether miR-628-5p, miR-520g-3p or miR-539-5p was able to directly bind and regulate the *STARS* 3'UTR, HEK293 cells were cotransfected with a reporter plasmid containing the full-length *STARS*

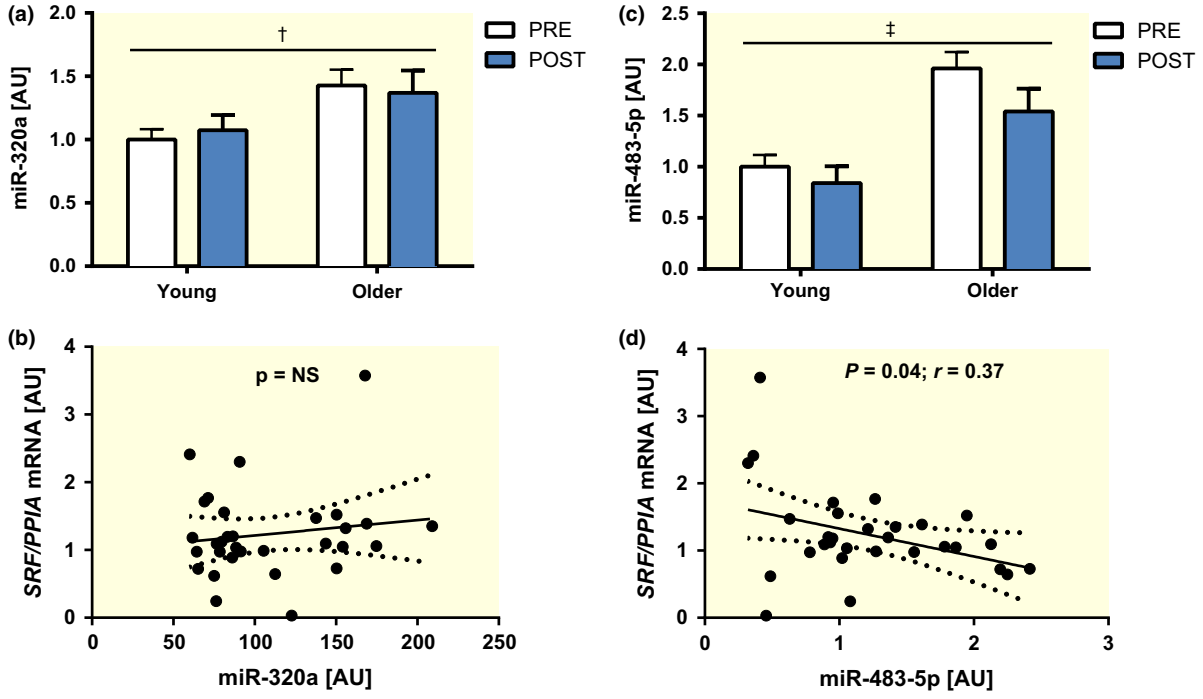

Figure 3 MiR-320a and miR-483-5p expression and linear correlations with SRF mRNA levels in skeletal muscle from young and older subjects pre- and post-exercise. †Main effect of age, $P < 0.01$. ‡Main effect of age, $P < 0.001$. NS, not statistically significant.

3'UTR as well as either a miR-628-5p, miR-520g-3p or miR-539-5p mimic, an irrelevant miRNA that did not have a predicted binding site on the *STARS* 3'UTR or no mimic at all. Twenty-four hours after the cotransfection of the reporter plasmid and mimics, there was 30% reduction in Nanoluc luciferase activity ($P < 0.05$) (Fig. 5a) in the cells containing the miR-628-5p mimic when compared to cells transfected with an irrelevant miRNA. When completing the reporter assay using the miR-520g-3p or miR-539-5p mimics, there was no reduction in the luminescence levels demonstrating that these miRNAs did not bind to the *STARS* 3'UTR (data not shown). We then sought to investigate the miR-628-5p putative binding site on the *STARS* 3'UTR. HEK293 cells were again cotransfected with the miR-628-5p mimic or an irrelevant miRNA; however, this time we used a reporter plasmid that contained a shorter version of the *STARS* 3'UTR. This shorter version contained only the putative miR-628-5p binding site as well as 10 base pairs either side of the site. As an additional control, cells were also transfected with this shorter *STARS* 3'UTR reporter plasmid; however, the miR-628-5p binding site was mutated. Twenty-four hours after the cotransfection of reporter plasmid and the mimics, there was a 32% reduction in Nanoluc luciferase activity ($P < 0.001$) (Fig. 5b) in the cells containing the miR-628-5p mimic when compared to cells transfected with an irrelevant miRNA. No differences were observed when the cells were transfected with the *STARS* 3'UTR reporter containing the mutated miR-628-5p binding site.

Discussion

The STARS signalling pathway plays an essential role in skeletal muscle adaptation to mechanical stimuli (Lamon *et al.* 2014). Previous studies have reported a reduction in the mRNA and protein levels of members of the STARS/SRF signalling axis with ageing, but the effects of age on the regulation of the STARS pathway in human skeletal muscle have not been investigated. We report for the first time that STARS protein, but not mRNA, levels are significantly decreased in human skeletal muscle from older when compared to the young subjects. A reduction in *Stars* gene expression was previously reported in skeletal muscle from aged mice (Sakuma *et al.* 2008) and pigs (Peng *et al.* 2008). This discrepancy between gene and protein expression suggests the existence of alternative post-transcriptional regulatory processes that may include regulation via miRNAs. Although its function in skeletal muscle has not been fully elucidated, STARS is anticipated to be necessary for muscle cell development and repair. Overexpressing the STARS protein in porcine smooth muscle cells and in the A10 rat

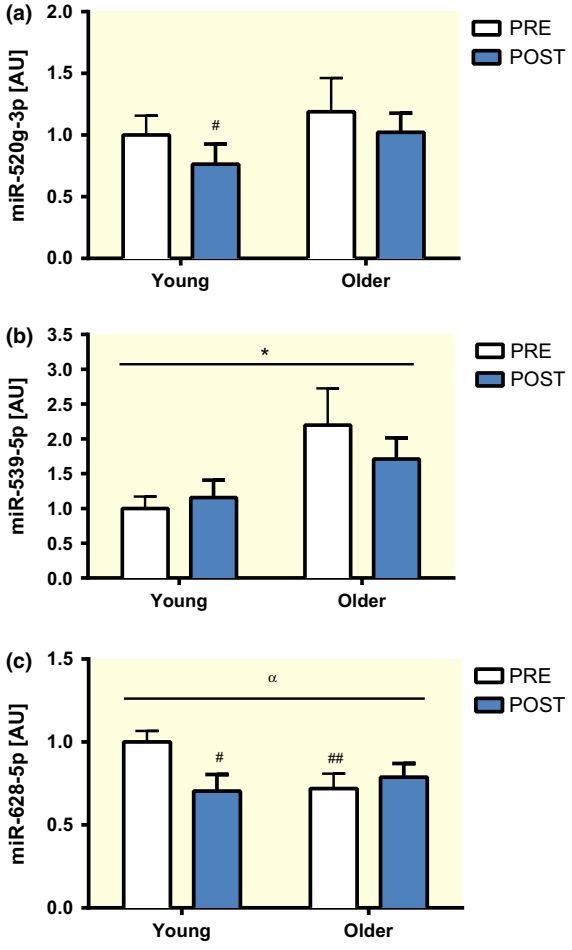

Figure 4 miR-520g-3p, miR-539-5p and miR-628-5p expression levels in skeletal muscle from young and older subjects pre- and post-exercise. *Main effect of age, $P < 0.05$. $^{\alpha}$ Main interaction effect, $P < 0.05$. $^{\#}$significantly different from PRE, $P < 0.05$. $^{\#\#}$significantly different from Young, $P < 0.01$.

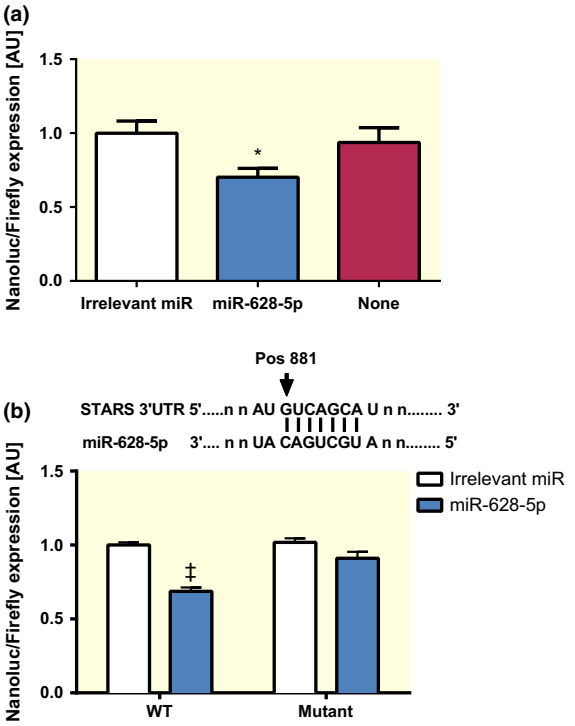

Figure 5 Luciferase reporter assay. (a) Nanoluc luciferase activity was assessed in HEK293 cells following cotransfection with 150 ng pNanoglo2 vector containing the full-length STARS 3'UTR and either 5 nM miR-628-5p mimic, 5 nM of an irrelevant miRNA (negative control) or no miR. Experiment was conducted once using $n = 6$ wells per group. *Significantly different from irrelevant miR ($P < 0.05$). (b) Nanoluc luciferase activity was assessed in HEK293 cells following cotransfection with 150 ng pNanoglo2 vector containing either the putative miR-628-5p binding site of STARS 3'UTR (CGUUUCAUUACaugucagcauAUAACGAGUCACCG CGGG) or its mutant control (CGUUUCAUUACcuucguuuua AUAACGAGUCACCGCGGG) and either 5 nM miR-628-5p mimic or 5 nM of an irrelevant miRNA (negative control). The putative binding site between miR-628-5p and the STARS 3'UTR is schematically represented above the graph. ‡Significantly different from irrelevant miR ($P < 0.001$). Experiments were repeated three times using $n = 6$ wells per group.

vascular smooth muscle cell line (Ounzain *et al.* 2008) enhanced proliferation and overexpressing STARS accelerated C_2C_{12} cell differentiation (Wallace *et al.* 2016). The elderly display a reduction in satellite cell (myoblast) number and activation (Corbu *et al.* 2010, Walker *et al.* 2012) leading to impaired regenerative capacity. Reduced activity of STARS might therefore contribute to the age-related muscle atrophy process by impeding muscle cell regeneration in the elderly.

We did not observe an age-related decrease in *SRF* mRNA or in the total and phosphorylated forms of the SRF protein. *Srf* mRNA levels were also similar in the gastrocnemius muscle of young (3 months) when compared to old (15 month) mice (Lahoute *et al.* 2008). However, in whole muscle homogenates, total SRF protein levels were reduced in old (Lahoute *et al.* 2008) and sarcopaenic (24 month) mice (Sakuma *et al.* 2008) when compared with young mice. In contrast, analysis of the nuclear extracts following muscle protein fractionation revealed that the protein levels of the SRF transcriptional coactivator MRTF-A were lower in sarcopaenic mice when compared with young mice, with no difference in SRF (Sakuma *et al.* 2008). Nuclear SRF represents the active, phosphorylated form of SRF. Combined with our observed increase in nuclear pSRF in human muscle from older subjects, these results suggest that ageing skeletal muscle maintains its ability to regulate gene transcription via SRF. The reason and mechanism for elevated basal levels of

phosphorylated SRF in muscle from older subjects is not known. SRF is phosphorylated and activated by numerous kinases, including, but not limited to, casein kinase, CaM kinase IV and MAPKAP kinase 2 (Manak *et al.* 1990, Misra *et al.* 1991, Janknecht *et al.* 1992, Marais *et al.* 1992, Heidenreich *et al.* 1999). In addition to MRTF-A, several factors co-activate SRF transcription, such as inositol polyphosphate multikinase (IPMK) (Kim *et al.* 2013), Elk-1 (Janknecht & Nordheim 1992), cardiogenic homeodomain factor Nkx-2.5 (Chen & Schwartz 1996), SRF accessory protein (SAP-1) (Dalton & Treisman 1992), p65/NF-kappaB (Franzoso *et al.* 1996) and GATA binding protein 4 (GATA-4) (Belaguli *et al.* 2000). In the absence of MRTF-A, these other coactivators may play a compensatory role in assisting with SRF gene transcription. Due to limited sample availability, we have not determined whether these kinases or alternative coactivators were upregulated in our model. Elevated phosphorylated SRF levels in the older subjects may reflect an attempt to maintain muscle size, cell survival and muscle repair (Miano 2010). In relation to the exercise intervention, a high basal SRF phosphorylation level in older subjects might be reflective of an already maximal activation of the SRF protein at rest, and therefore reduce the capacity for further activation following an anabolic stress, such as resistance exercise.

The regulation of members of the STARS pathway in skeletal muscle is sensitive to the mechanical stress induced by resistance exercise in untrained younger subjects (MacNeil *et al.* 2010, Lamon *et al.* 2013, Vissing *et al.* 2013), but its regulation in older subjects has not been investigated. Here, we observed an increase in the mRNA levels of members of the STARS signalling pathway 2 h after an acute bout of resistance exercise that was independent of age. Our results revealed no changes in the protein levels of the members of the STARS signalling pathway in response to exercise. Any potential increase may possibly occur later in the post-exercise recovery period. Indeed, two recent studies from our group investigated the regulation of the STARS signalling pathway following acute exercise in trained and untrained young subjects (Lamon *et al.* 2013, Vissing *et al.* 2013). In line with the results of the present study, we only reported minor changes in protein expression within the 3 h following acute exercise (Vissing *et al.* 2013). The early induction of STARS in response to resistance exercise is believed to trigger intracellular signals responsible for muscle adaptation to exercise; however this has not yet been experimentally validated. While the precise mechanisms involved are unknown, it has been suggested (Arai *et al.* 2002, Lamon *et al.* 2013, 2014) that this adaptive response may protect against contraction-induced muscle damage, in addition to helping regeneration and repair, two processes that are commonly disrupted in elderly subjects (Brack & Rando 2007). Reduced basal levels of the STARS protein in older subjects may therefore reflect a reduced capacity of STARS to protect the muscle following exercise.

A limitation when recruiting community-dwelling older subjects to participate in an exercise trial is that they tend to be physically active and therefore not necessarily representative of the average elderly population. We have previously reported that the older subject cohort used in this study was not considered sarcopaenic (Zacharewicz *et al.* 2014). However, at present, this study is the only one that has measured and compared the regulation of the STARS signalling pathway, at rest and following resistance exercise, using an appropriately powered cohort of young and older subjects. Discrepancies in observations made in young and sarcopaenic mice (Sakuma *et al.* 2008) vs. young and older men in the present study may be imputable to species differences or to the fact that our subjects were not sufficiently sarcopaenic. Therefore, our findings might not be representative of a more sedentary and/or sarcopaenic human population.

miRNAs are important regulators of skeletal muscle health (Zacharewicz *et al.* 2013) that regulate gene transcription by directly degrading mRNAs (Huili *et al.* 2010), inhibiting protein translation (Lee *et al.* 2004a, Wightman *et al.* 2004, Humphreys *et al.* 2005, Pillai *et al.* 2005) and in some cases stabilizing mRNAs (Vasudevan & Steitz 2007, Vasudevan *et al.* 2007, Ørom *et al.* 2008). The conditions and level of specificity with which miRNAs interact with their respective targets remain mostly unclear. Within the scope of this study, we investigated the regulation of the miRNAs known to target members of the STARS signalling pathway. The expression levels of miR-320a and miR-483-5p, two miRNAs negatively regulating SRF transcription (Qiao *et al.* 2011, Chen *et al.* 2015), increased with age. MiR-483-5p is embedded in the intron of the insulin-like growth factor 2 (IGF2) gene and inhibits SRF mRNA and protein expression by directly targeting the SRF transcript in endothelial cells (Qiao *et al.* 2011). While SRF increased with exercise in both subject groups, we observed a concomitant decrease in miR-483-5p levels, which resulted in significant negative linear correlation. As miR-483-5p directly binds to the SRF 3'UTR resulting in its suppression (Qiao *et al.* 2011), the resistance exercise-induced downregulation of miR-483-5p may release its inhibition on SRF, therefore contributing to the upregulation of the latter. Whether increased STARS expression and attenuated miR-483-5p expression has an additive effect on SRF transcription would

be of interest to determine. Similarly, miR-320a can also target the SRF transcript resulting in inhibition of cell proliferation and promoting apoptosis in human-derived endothelium cells (Chen *et al.* 2015). When compared to healthy age-matched controls, miR-320a is upregulated in several conditions associated with skeletal muscle degeneration and atrophy, including limb girdle muscular dystrophy type 2A, Miyoshi myopathy, Nemaline myopathy, inclusion body myositis and polymyocytosis (Eisenberg *et al.* 2007). MiR-320a is also known to play a role in energy metabolism. It directly inhibits the rate-limiting step of glycolysis by targeting the enzyme phosphofructokinase and its expression was reduced in C_2C_{12} myotubes and other biological systems when subjected to oxidative stress (Tang *et al.* 2012). We have previously suggested that STARS regulates energy metabolism in the muscle fibre and have shown that STARS shifts the muscle cell to a more oxidative phenotype (Wallace & Russell 2013). Whether this process involves miR-320a is unknown.

Of the three miRNAs predicted to target STARS, miR-628-5p interacted with its putative binding sequence on the *STARS* 3'UTR and repressed *STARS* transcription *in vitro*. While mainly studied in cancer models, it was suggested that miR-628-5p promotes the expression of FOXO3a, a regulator of skeletal muscle atrophy, in leukaemic progenitor cells in response to IL-3 treatment (Favreau & Sathyanarayana 2012), although no regulation of FOXO3a gene or protein was observed in this cohort (Stefanetti *et al.* 2015). The role and regulation of miR-628-5p in skeletal muscle is relatively unknown. One study has reported that miR-628-5p expression in foetal skeletal muscle in human facioscapulohumeral muscular dystrophy (FSHD) is upregulated at 14 weeks of foetal development but down regulated at 22 weeks, when compared with age-matched controls (Portilho *et al.* 2015). Whether miR-628-5p has an acute effect on the regulation of *STARS* mRNA post-exercise remains to be determined. Clearly more studies are required to establish the precise role of miR-628-5p in skeletal muscle growth, repair and function over the lifespan.

In conclusion, we demonstrated that although the resting levels of the STARS protein are lower in skeletal muscle of older when compared to young subjects, this did not affect the resistance exercise-induced upregulation of *STARS* and *SRF* mRNA seen in both age groups. These findings did not translate to the SRF protein, which in addition displayed higher phosphorylation levels in older than in young subjects. Finally, we identified for the first time that miR-628-5p is a resistance exercise responsive miRNA that directly binds the STARS 3'UTR to reduce its transcription *in vitro*.

Conflict of interest

The authors have no conflict of interest to declare.

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Physiological relevance

Striated muscle activator of Rho signalling (STARS) is a molecular pathway that is essential to skeletal muscle adaptation and function via the regulation of SRF signalling. Here, we show for the first time that STARS signalling is downregulated in older when compared to younger human muscle, although STARS regulation following an acute exercise bout is not age dependent. In addition, we identify that miR-628-5p is a resistance exercise responsive miRNA that directly binds the STARS 3'UTR to reduce its transcription *in vitro*.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. PPIA gene expression levels in skeletal muscle from young and older subjects pre- and post-exercise.

Figure S2. MiR-9, miR-133a, miR-150 and miR-206 expression levels in skeletal muscle from young and older subjects pre- and post-exercise.

Figure S3. Predicted binding sites for miR-539-5p-5p, miR-520g-3p and miR-628-5p on human *STARS* 3'UTR.

Table S1. Additional primers used for the Luciferase reporter assay.