Online Supplementary Information

Methods

Patient selection criteria and ethical approval

Patients with a diagnosis of heart, renal or liver failure, a systemic inflammatory or metabolic disorder or a moderate/severe exacerbation (i.e. requiring antibiotics, oral steroids, or hospitalisation) in the preceding 4 weeks were excluded. All subjects gave written informed consent and the protocol was approved by the Royal Brompton & Harefield NHS Trust Research Ethics Committee (Studies 06/Q0404/35 and 06/Q0410/54).

Physiological assessment

Lung volumes measured using plethysmography, carbon monoxide transfer factor assessed using the single breath technique (CompactLab, Jaeger, Germany) and post-bronchodilator spirometry were measured according to ATS/ERS guidelines [1]. Blood gas tensions were measured in arterialised capillary earlobe blood. Fat-free mass index (FFMI) was calculated using bioelectrical impedance (Bodystat 1500, Bodystat, UK) as described previously [2].

Quadriceps strength was determined by measuring supine isometric maximal voluntary contraction (MVC) of the leg ipsilateral to the dominant hand, using an adaptation of the technique of Edwards *et al*[3] and correcting for weight (the main independent predictor of MVC in patients and controls), and by measuring the unpotentiated twitch quadriceps force (TwQ) as described by Polkey *et al*[4]. Quadriceps endurance was measured non-volitionally as the time taken to decay to 80% of initial force (T80) using the method of Swallow *et al* [5]

Exercise performance was assessed 5 minutes post-bronchodilator with the 6 minute walking test (6MW), performed according to ATS 2002 guidelines[6] and results corrected using

accepted reference equations[7]. Physical activity was measured in a subset of individuals (13 controls and 28 patients) using a tri-axial accelerometer (Dynaport Activity Monitor; McRoberts BV, Netherlands) worn for 12h per day, for 2 days during normal activities. Locomotion time, standing, sitting and lying time were recorded as described by Pitta *et al* [8] and average movement time for the two days was calculated (locomotion time as a % of 12h). Percutaneous needle biopsy of the *vastus lateralis* in the anterior mid-thigh of the leg that strength was tested was performed under local anaesthesia using the Bergstrom technique[9].

mRNA quantification

Real time quantitative PCR (RT-PCR): RNA was extracted from muscle biopsies using the Qiagen RNeasy® kit (Qiagen, UK), the concentration of RNA was quantified using a spectrophotometer (Nanodrop (ND1000, Wilmington USA) and first strand cDNA generated using Superscript® II Reverse Transcriptase (Invitrogen). The qPCR analysis was carried out in duplicate on each cDNA sample for every target gene and for the reference genes RPLPO using a 10 µl reaction of SYBR® Green Quantitative RT-PCR Kit (Sigma Aldrich, UK) and the primer pair (2pmol/µl) in 96 well plates (MicroAmp, Fast optical 96 well reaction plate (0.1 ml) (Applied Biosystems, UK.), covered by an optical plate cover (MicroAmp, Optical adhesive film (PCR compatible), Applied Biosystems, UK.). The qPCR reactions were run on the 7500 Fast Real-Time PCR System (Applied Biosystems, UK.), with the following cycle program: 95 ºC for 10 minutes, then 40 cycles of 95 ºC for 15 seconds, 64°C for 30 seconds, 72°C for 30 seconds. The annealing temperature was optimised for each individual pair of primers. The PCR products were run on a 2% agarose gel to confirm the size of the correct base pair size. Q-PCR data for each gene was normalised to the value for RPLPO from the

same sample as previously described [10]. The Primer Sequences used are as follows: RPLPO Forward TCTACAACCCTGAAGTGCTTGATATC, RPLPO Reverse GCAGACAGACACT GGCAACATT, SRF [11], MRTF [11], IGF-1 [12], HDAC4[13], MHC type I [14] and type IIa [15] primers have previously been described.

MicroRNA quantification

MicroRNA expression was analysed in trizol extracted RNA using the Ncode^{TM} SYBR green miRNA-qRT-PCR kit (Invitrogen) according to the manufactures instructions except that the final RT prduct was diluted to 200μl. Forward primers specific for each miRNA were obtained from Invitrogen and the reverse primer was present in the kit. The qPCR reactions were run on the 7500 Fast Real-Time PCR System (Applied Biosystems, UK.), with the following cycle program: 95 ºC for 10 minutes, then 40 cycles of 95 ºC for 15 seconds, 60°C for 60 seconds. The PCR products were run on a 2% agarose gel to confirm the size of the correct base pair size. Q-PCR data for each gene was normalised to the value for 5S RNA from the same sample as previously described [10].

Assessment of protein levels:

Biopsy samples were homogenised under liquid N_2 and the homogenate resuspended in lysis buffer (Tris pH 7.4 (50mM), NaCl (250mM), EDTA (5mM), 1% Nonidet P40 (Roche Applied Science)) supplemented with protease and phosphatase inhibitor cocktails (Sigma). To determine the levels of Akt and phospho-Akt the protein supernatants (1mg/ml) were analysed by fluorescent bead array using Akt and phospho-Akt specific beads (Invitogen) on a Luminex 100 analyzer instrument (Luminex Corp.) according to the manufacturer's recommendation

Western blotting

Western blotting was performed as previously described [16] and blots were probed with anti-HDAC-4 (Abcam) diluted 1:1000 and the secondary antibody was anti-rabbit HRP (Dako) 1:3000. Bands were visualised by chemiluminescence and quantified by densitometry. Each value was normalised to the total protein transferred quantified by Ponceau Red staining of the blot.

Determination of MRTF activity by luciferase assay

C2C12 myobalsts were cultured as described in [17] and transfected with lipofectamine as described in [18]. For measurement of luciferase activity cells were cultured in 24 well plates and each transfection contained 0.4µg of plasmid comprised of 0.2µg pmiR-luciferase vector, 0.0125 µg, pRLTK 0.0625 µg MRTF-A or pcDNA3 and a further 0.125µg of pcDNA3. Twenty four hours after transfection the cells were harvested and firefly and renilla luciferase activities were measured as described previously[18]. The MRTF plasmids were kindly provided by Prof E. Olson and the miR-luciferase vectors were provided by Dr J. Chen and Dr D. Srivastava.

Immunofluorescence

Serial muscle sections (10 µm) with fibres predominantly in transverse section were cut at –20°C and thaw mounted on slides and stored at –80°C until analysis. For staining, sections were air-dried for thirty minutes at room temperature, rehydrated in Phosphate-Buffered Saline (PBS) supplemented with 0.5% Triton X-100 (5 mins) then washed in PBS (5 mins). For determination of fibre type and size the sections were

incubated for one hour at room temperature with a mix of primary antibodies in PBS with 0.05% Tween 20 (PBST) (A4.840; mouse (IgM) anti human MHCI [diluted 1:40], N2.261; mouse (IgG_1) anti human MHCIIA [diluted 1:40], both from Developmental Studies Hybridoma Bank (DSHB), University of Iowa, USA and L-9393; rabbit anti-laminin [diluted 1:50] (Sigma, Zwijndrecht, the Netherlands). After washing the slides for five minutes in PBST and five minutes in PBS twice, the sections were incubated with secondary antibody mix in PBST (A-21426; AlexaFluor555 goat anti-mouse IgM, dilution 1:500, A-21121; AlexaFluor488 goat anti-mouse IgG1, dilution 1:200, A-11069; AlexaFluor350 goat anti-rabbit IgG, dilution 1:130) in the dark in a humidification box for one hour at room temperature. All secondary antibodies were from Molecular Probes, Invitrogen, Breda, the Netherlands. The slides were washed (five minutes in PBST, five minutes in PBS twice) and Faramount aqueous mounting medium (Dako, USA), a coverslip applied and stored in the dark at 4°C.

Image capture and fiber classification

Epifluorescence signal was recorded using a Nikon Eclipse 800 microscope with a DXM 1200 camera (Nikon Instruments Europe BV, the Netherlands) under a x10 objective using three filters: Texas Red (395 to 410 nm), FITC (490 to 505nm) and DAPI UV (395 to 410 nm). Four to nine images of fibres in transverse section were captured for each section to incorporate as many fibres as possible. Fibres were classified as type I (red), IIa (IIa and IIa/IIx, green), IIx (no staining) and hybrid I/IIa (red and green staining) and the laminin fibre border was used to measure fibre CSA using Lucia 4.81 software (Laboratory Imaging, Czech Republic). Apparent type IIa/IIx fibres, i.e. only

weakly/moderately-stained for type IIa myosin, were classified as type IIa as they were not distinguishable from pure type IIa fibres stained less strongly for a technical reason. A minimum of 100 muscle fibres were analysed for each subject. If it was not possible to capture 100 fibres in transverse section from one biopsy specimen, another biopsy from the same subject was sectioned and stained to provide additional data.

Calculation of fibre data

For each individual, type I, I/IIa, IIa and IIx proportions and median fibre CSA for each fibre type was recorded. The fibre proportions for type IIa and IIx fibres were pooled, and the CSA each type IIa and IIx fibre was pooled to calculate a median CSA for type II fibres. From this, a subject's overall type I fibre CSA and type II fibre CSA in 100 fibres was calculated.

SRF localisation

For analysis of SRF localisation the sections were prepared as above and incubated with rabbit anti-SRF (Santa Cruz, 1:100) as the primary antibody washed as above and stained with anti rabbit AlexaFluor-488 (Invitrogen) 1:250. Washing and analysis were carried out as described in [16] with the first wash supplemented with DAPI. Images were captured as described in [16].

Results

To determine whether MRTFs were able to activate expression of miR-1 C2C12 cells we determined the activity of the miR-1-1 and miR-1-2 promoters in the presence and absence of MRTF-A and MRTF-B. This analysis showed that the activity of both promoters was increased by both MRTFs (Fig. 6D). Furthermore this activity required SRF as it was dependent on the presence of a CArG box in the promoter (Fig. 6D).

Supplementary Table 1: p values for Pearson's coefficient correlation matrix shown in figure 5

	parameter	Log miR-1	Log miR-499	Log miR-181	Log miR-145	Log miR-206	Log miR-133	Log miR-208
Physiological parameters (Fig 5A)	BMI	>0.5	>0.5	>0.5	>0.5	>0.5	>0.5	>0.5
	Lo time (min)	>0.5	>0.5	>0.5	0.179	**0.029**	**0.027**	0.142
	MI (m/s2)	>0.5	>0.5	0.289	0.080	**0.023**	0.057	**0.049**
	Mt (%)	>0.5	>0.5	0.477	0.225	**0.033**	0.071	0.099
	6MW	**0.026**	0.061	>0.5	>0.5	0.300	>0.5	0.305
	FEV1 (% pred)	**0.022**	0.171	>0.5	>0.5	0.235	0.470	0.061
	FFMI (kg/m^2)	**0.025**	**0.012**	0.074	0.088	>0.5	0.356	0.121
	SGRQ	**0.034**	0.112	0.410	>0.5	0.227	0.413	0.290
	Pack yr	**0.007**	**0.027**	0.114	0.488	>0.5	>0.5	>0.5
Muscle parameters (Fig 5B)	Ty 2X (%)	0.144	0.064	0.250	>0.5	>0.5	>0.5	>0.5
	Ty 2A (%)	0.155	0.227	>0.5	0.285	0.265	0.333	>0.5
	CSA Ty1 (%)	0.451	0.360	>0.5	>0.5	0.183	>0.5	>0.5
	T80	>0.5	>0.5	>0.5	0.488	>0.5	0.250	>0.5
	CSA Ty 2A (%)	>0.5	>0.5	>0.5	0.297	>0.5	0.118	>0.5
	TwQ	0.333	0.430	>0.5	>0.5	>0.5	>0.5	>0.5
	CSA 2X (%)	0.412	>0.5	>0.5	0.110	0.250	0.221	0.270
	MVC/BMI	**0.049**	0.071	>0.5	>0.5	>0.5	>0.5	>0.5
	Ty 1 (%)	**0.029**	0.063	>0.5	0.475	0.331	0.365	0.405

Supplementary references

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Supplementary Figure 1: Immunofluorescent assessment of Fibre type proportion and area

10μm cryosections of *vastus lateralis* from patients and controls were fixed and stained as described in Methods. Representative images are presented from a control and a patient. Type I fibres are stained red, type IIA fibres are stained green and type IIX fibres are unstained. Laminin is stained in blue.

Supplementary Figure 2: Expression of MHCI and MHCIIa in patients with COPD

MHCI (A) and MHCIIa (D) mRNA was quantified in muscle biopsy samples from the *vastus lateralis* of patients with COPD and age matched controls as described in Methods. The value for each test gene was normalised to the amount of RPLPO in the same sample. Levels of MHCI mRNA were correlated with smoking history (B, $r=-0.58$, $p<0.001$) as measured by pack year history) and with FEV_1 measured as % predicted (C, $r=0.69$, $p<0.001$). Neither of these correlations reached $p<0.05$ in patients alone.

Supplementary Figure 3: Expression of MHCI and MHCIIA are associated with endurance

Messenger RNA levels determined as described in Fig. 1 for MHCI (A) were directly correlated with endurance measured by 6 minute walk distance measured as % predicted ($r=0.65$, $p<0.001$) whereas those for MHCIIA (B) were inversely correlated with endurance ($r=-0.56$, $p<0.001$) (empty diamonds; patients, filled squares; controls). The association of MHCI with endurance did not reach a significance of $p<0.05$ in the patients alone but the association of MHCIIA was significant when considered alone ($r=-0.62$, $p<0.001$)

Supplementary Figure 4: Hierarchical clustering of physiological and muscle characteristics to determine order of presentation