Supplemental Digital Content File

Methods

Immunohistochemistry

Three staining protocols were applied:

Staining 1) Muscle fibre cross-sectional area (CSA) and fibre type;

Staining 2) Satellite cells and fibre type;

Staining 3) Proliferating satellite cells.

For all staining protocols, primary and secondary antibodies were diluted in 1% BSA in TBS (tris-buffered saline, tris-base 0.05 mol/l, sodium chloride 0.154 mol/l, pH 7.4-7.6), and sections were washed 2x5 minutes (staining 1) or 3x5 minutes (staining 2+3) in TBS between each protocol step. After removing sections from the freezer, sections were dried at room temperature and fixed in 4% PFA for 10 minutes (staining 1) or 5 minutes (staining 2+3), and incubated with primary antibodies overnight at 4 °C. The following day the appropriate secondary antibodies were diluted in 1% BSA in TBS and applied for 45 minutes at room temperature.

Primary and secondary antibodies are shown in Supplemental Table S1. In sections for confocal imaging (fibre CSA) nuclei were stained blue with Hoechst dye diluted in TBS (Hoechst 33342; 2.5µg/ml; H1399; Invitrogen), and slides were mounted with cover glasses in mounting medium (Molecular Probes ProLong Gold anti-fade reagent, cat. no. P36930). In sections for widefield microscopy (satellite cells and proliferating cells) DAPI in the mounting medium stained nuclei blue (Blue, Molecular Probes ProLong Gold anti-fade reagent, cat. no. P36931).

Image acquisition and analysis

All image analysis was performed in ImageJ (version 1.51n; National Institute of Health; USA) by an investigator blinded to subject ID, treatment and time point.

Staining 1, Muscle Fibre CSA: Representative images are available in Figure 2C-E. Sections stained for fibre CSA were digitally captured with a confocal laser scanning microscope (20x/0.8NA objective; pinhole = 3µm; LSM710, Carl Zeiss, Oberkochen, Germany). Images (716.8x716.8 µm/2048x2048 pixels) were captured as tiles (160 µm / 457 pixel overlap between tiles), using a semi-automated motorized function in the Zeiss ZEN software. The overlap between tiles was marked in ImageJ (Figure 1C), ensuring no fibres were excluded or analyzed twice, and tiles were randomly selected for analysis of fibre CSA. A detailed description of the method for fibre CSA analysis has furthermore been published elsewhere (1). Briefly, a macro developed for

ImageJ automatically delineated all fibres in a tile, followed by a manual approval step of all fibres included in the analysis (Figure 1D). Tiles were included for the CSA analysis until a minimum of 150 fibres of each fibre type (MHC-I positive/negative) had been analyzed, if possible. Regions with longitudinally cut fibres and fibres on the edges of the biopsy were not included in the fibre CSA analysis.

Staining 2, Satellite cells: Representative images are available in Figure 3A-D. Sections were imaged with a 10x/0.30NA objective on an Olympus BX51 microscope with a 0.5x camera (Olympus DP71, Olympus Deutschland GmbH, Hamburg, Germany) mounted on the microscope (image size 1587x1237µm or 3705x2700 pixels), controlled by the software Cell^F (Olympus Soft Imaging Solutions, GmbH, Münster, Germany). Images were recorded as tiles with manually adjusted overlap (approx. 150-300 pixel overlap) and automatically stitched together in ImageJ (plugins/stitching/deprecated/stitch directory with images (unknown configuration)). All fibres in the biopsies were included for the SC analysis, excluding damaged fibres and regions with longitudinally cut fibres.

Staining 3, Proliferating cells and Satellite Cells: Representative images are available in Figure 3G-J. $Ki67^+$ cells were counted in sections stained for proliferating cells and satellite cells, excluding large bands of connective tissue. The count was performed using the microscope eyepieces and a 20x/0.5NA objective, in the same microscope used for SC analysis. Images of the Ki67 channel were recorded with a 4x/0.1NA objective, and the cross-sectional area of included regions were analyzed manually in ImageJ.

Gene expression

RNA extraction: 100 cryo sections of 10 µm from the embedded muscle tissue were homogenized in 1 mL of TriReagent (Molecular Research Center, Cincinnati, OH, USA) containing five stainless steel balls of 2.3 mm in diameter (BioSpec Products, Bartlesville, Oklahoma, USA), and one silicon-carbide sharp particle of 1 mm (BioSpec Products), by shaking in a FastPrep®-24 instrument (MP Biomedicals, Illkirch, France) at speed level 4 for 15 s. Following homogenization, bromo-chloropropane was added to separate the samples into an aqueous and an organic phase. Following isolation of the aqueous phase, RNA was precipitated using isopropanol. The RNA pellet was then washed in ethanol and subsequently dissolved in 20 µL RNAse-free water. Total RNA concentrations and purity were determined by spectroscopy at 260, 280 and 240 nm. Good RNA integrity was ensured by gel electrophoresis.

Real-time RT-PCR: 500 ng total RNA was converted into cDNA in 20 µL using the OmniScript reverse transcriptase (Qiagen, California, USA) and 1 µM poly-dT (Invitrogen, Naerum, Denmark) according to the manufacture's protocol (Qiagen). For each target mRNA, 0.25 µL cDNA was amplified in a 25 µL SYBR Green polymerase chain reaction (PCR) containing 1 × Quantitect SYBR Green Master Mix (Qiagen) and 100 nM of each primer (for primers see Supplemental Table S2). The amplification was monitored real time using the MX3005P Real-time PCR machine (Stratagene, California, USA). The Ct values were related to a standard curve made with known concentrations of cloned PCR products or DNA oligonucleotides (UltramerTM oligos, Integrated DNA Technologies, Inc., Leuven, Belgium) with a DNA sequence corresponding to the sequence of the expected PCR product. The specificity of the PCR products was confirmed by melting curve analysis after amplification. RPLP0 mRNA was chosen as internal control. To validate this use, another unrelated "constitutive" mRNA, GAPDH, was measured and normalized with RPLP0. No difference was seen for GAPDH, supporting the use of RPLP0 for normalization.

References

1. Karlsen A, Bechshøft RL, Malmgaard-Clausen NM, et al. Lack of muscle fibre hypertrophy, myonuclear addition, and satellite cell pool expansion with resistance training in 83-94-year-old men and women. *Acta Physiol*. 2019;227(1):e13271.