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We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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None of the authors as any conflict of interest to disclose.

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ABSTRACT

Introduction/Aims: *Klf10* is a member of the Krüppel-like family of transcription factors which is implicated in mediating muscle structure (fiber size, organization of the sarcomere), muscle metabolic activity (respiratory chain) and passive force. The aim of this study was to further characterize the roles of Klf10 in the contractile properties of skeletal muscle fibers.

Methods: Fifty two single fibers were extracted from female wild-type (WT) and *Klf10* knock-out (KO) oxidative (soleus) and glycolytic (EDL: extensor digitorum longus) skinned muscles. Each fiber was immersed successively in relaxing (R), washing (W) and activating (A) solutions. Calcium was included in the activating solution to induce a maximum contraction of the fiber. The maximum force (F_{max}) was measured and normalized to the cross-sectional area to obtain the maximum stress ($Stress_{max}$). After a steady state in contraction was reached, a quick stretch-release was performed; the force at the maximum stretch ($F_{stretch}$) was measured and the stiffness was assessed.

Results: Deletion of the *Klf10* gene induced changes in the contractile parameters (F_{max}, $Stress_{max}$, Stiffness), which were lower and higher for soleus and EDL fibers compared to littermates, respectively. These measurements also revealed changes in the proportion and resistance of attached cross bridges.

Discussion: Klf10 plays an important role in the homeostasis of the contractile behavior of skeletal muscle fibers in a muscle fiber type specific manner. These findings further implicate important roles for Klf10 in skeletal muscle function and shed new light on understanding the molecular processes regulating the contractility of skeletal muscle fibers.

1 INTRODUCTION

Krüppel-like transcription factor 10 (*Klf10*), also known as TGFβ inducible early gene-1 (*TIEG1*) is a member of the Krüppel-like family of transcription factors that regulates gene expression in multiple cell and tissue types.[1-2] Klf10 is implicated in multiple biological processes and diseases (osteoporosis[3], cancer[4-5] and hypertrophic cardiomyopathy[6]). Recently, *Klf10* has been implicated in Duchenne muscular dystrophy suggesting a role for Klf10 in the regulation of fibrosis.[7]

Loss of *Klf10* expression results in multiple muscle phenotypes[8-9] such as hyperplasia, hypertrophy, a decrease in succinate dehydrogenase, cytochrome c oxidase and menadione activities, ultrastructural muscle disorganization (smaller sarcomeres and absence of I bands) and changes in mitochondrial shape and respiration. All of these phenotypes were only observed in female animals with no significant defects or differences detected between male *Klf10* knock-out (KO) and wild-type (WT) mice. Similar sex specific differences have also been observed in the skeleton on *Klf10* KO mice where only female animals exhibit an osteopenic phenotype.[10] The basis for sex specific differences in bone are attributed to defects in estrogen signaling in *Klf10* KO mice.[3]

Skeletal muscle must be characterized in passive[11] and active[12] conditions to achieve a comprehensive understanding of this tissue. Previous studies have only implemented passive stretch[13] and compressive[9] tests to reveal changes in elasticity of *Klf10* KO muscle fibers. Here we assess the role of Klf10 on the contractile properties of muscle fibers from slow (soleus) and fast extensor digitorum longus (EDL) twitch muscles.

2 METHODS

2.1 Animals

Klf10 KO mice were generated using a neomycin targeting vector to delete approximately 5.5 kb of the *Klf10* locus including the entirety of exons 1 and 2.[14] *Klf10* KO animals utilized in this study were on a congenic C57BL/6 background. WT and *Klf10* KO animals were derived from heterozygous breeding. Female animals at an age of 3 months old were utilized. The protocol was approved by the French ministry of higher education, research and innovation (DUO-4776) and the Picardie regional ethics Animal Care and Use Committee (CREMEAP; APAFIS#2 8905-2021011109249708).

2.2 Transmission electron microscopy (TEM)

Eight soleus and EDL muscles were harvested from 4 WT and 4 *Klf10* KO mice using the published protocol.[15] Transverse ultrathin (90 nm) sections were cut using a Leica UC7 ultramicrotome and were placed on copper grids. Five micrographs of each specimen were randomly captured across the muscle using a JEOL 1400Plus, operating at 80 kV with a magnification of 42 000x. ImageJ 1.46/Java 8 software (NIH, Bethesda, MD, United States) was used with the plugin "Analyze Particles" to determine the myosin cross-sectional area (myoCSA).[16]

2.3 Skinned muscle preparation

For this analysis, a second group of mice was utilized for TEM experiments. Soleus muscles were dissected, from the right hindlimbs of 8 WT mice and 8 *Klf10* KO mice, and permeabilized at 4°C using a relaxing solution where different percentages of glycerol (12.5, 25 and 50%)

were added.[13] Fiber bundles were stored in 50% glycerol relaxing solution at -20°C for 2 weeks.[17]

2.4 Active mechanical tests

Single muscle fibers were isolated from each *Klf10* KO (N_{soleus} = 11, N_{EDL} = 16) and WT (N_{soleus} = 11, N_{EDL} = 14) skinned muscles. First, the fibers were placed in a small bath filled with the relaxing solution at 20°C. The extremities were connected to a force transducer (5 mN) and to a motor (1400A - Aurora Scientific). The fiber length, diameter (D) and the distance between the successive sarcomeres were measured using a camera (UI-1220LE). The fibers were placed with an initial[13] sarcomere length of 2.5 µm and immersed in a bath containing a washing solution free of calcium for 8 s. To activate the actin-myosin cross bridges, fibers were then immersed in another bath containing an active solution of calcium (pCa 4.5) resulting in muscle fiber contraction and the measurement of the maximum force (F_{max}).[18-19] This value was measured as an average of data during 1 s when the force had reached a steady state plateau. The maximum stress ($Stress_{max}$) was measured by dividing the maximum force F_{max} by the cross-sectional area of the fiber (CSA). After 31 s of immersion in the calcium solution, a quick stretch-release cycle was performed with an amplitude ($\varepsilon_{stretch}$) of 0.2 % of the fiber length during 1 s, and an edge at a speed of 1 fiber length s^{-1}.[20] The force ($F_{stretch}$) at the peak of the stretch was determined and the stiffness was calculated as follows:

$$Stiffness = \frac{F_{stretch} - F_{max}}{\varepsilon_{stretch} \cdot CSA}$$

2.5 Statistical analysis

The SystatTM V11 (Systat Software Inc., CA, USA) was used and two-sample Mann-Whitney tests were utilized to compare all the parameters (myoCSA, D, stiffness, stress) as a function of genotype. Results were considered significant for $P < 0.05$.

3 RESULTS

The muscle fiber active force (Figure 1) revealed lower and higher forces for *Klf10* KO soleus and *Klf10* KO EDL muscle, compared to WT littermates, respectively. TEM acquisitions revealed a reduction in the mean number of myosin filaments per region of interest (Figure 2A and 2B) within the *Klf10* KO muscles ($N_{Soleus} = 90$, $N_{EDL} = 103$) compared to the WT muscles ($N_{Soleus} = 117$, $N_{EDL} = 112$). TEM analysis of the myosin area (Figures 2C and 2D) also revealed a significant increase ($P < 0.001$) in cross-sectional area (myoCSA) in *Klf10* KO soleus and EDL compared to WT littermates. It can be noted that no significant differences in the mean $\pm$ SEM muscle fiber diameter (D) for the EDL ($D_{WT} = 44 \pm 6$ µm, $D_{Klf10\_KO} = 44 \pm 1$ µm) or the soleus ($D_{WT} = 46 \pm 3$ µm, $D_{Klf10\_KO} = 39 \pm 1$ µm, $P = 0.065$) as a function of genotype were detected.

The maximum normalized active force ($Stress_{max}$) was significantly ($P = 0.001$) lower for the *Klf10* KO soleus fibers compared to the WT littermates (Figure 3A). Opposite results were observed for the EDL with *Klf10* KO soleus fibers showing significantly higher ($P = 0.008$) values compared to the WT littermates (Figure 3A). For the WT genotype, no significant difference was noted for the maximum stress between soleus and EDL fibers.

Results of stiffness measurements were similar to those for maximum stress, with significantly lower ($P = 0.008$) and higher ($P = 0.004$) values for the *Klf10* KO soleus fibers and *Klf10* KO EDL fibers, respectively, compared to the WT littermates (Figure 3B). No significant differences in stiffness were observed between WT soleus and WT EDL fibers.

4 DISCUSSION

This study has elucidated the impact of *Klf10* expression on parameters related to muscle fiber contraction for oxidative and glycolytic muscles. Previously, we demonstrated that sarcomeric length is shorter in skeletal muscles of *Klf10* KO mice[13]. In spite of this phenotype, we conclude that the *Klf10* KO sarcomere is functioning normally based on the data reported here.

Thus, the decrease in the maximum stress for *Klf10* KO oxidative fibers compared to WT fibers may be indicative of a smaller proportion of attached cross bridges or a weaker adhesion between the myosin head and the actin molecule during the isometric contraction.[21] This possibility is supported by the TEM results showing a decreased density of myosin in soleus *Klf10* KO fibers.

The weaker stiffness observed for the soleus *Klf10* KO compared to WT fibers is consistent with reduced contractile properties. Indeed, the stretch applied after the active steady state reflects the resistance of the attached cross bridges[12] which are weaker in soleus *Klf10* KO and associated to the reduced density of myosin.

In a previous study,[8] we have demonstrated that the *Klf10* KO soleus and EDL muscles exhibit hypertrophy with an increase in the wet weight of *Klf10* KO soleus (7.8 ± 0.6 mg) compared to WT soleus (6.3 ± 0.7 mg) and *Klf10* KO EDL (8.3 ± 0.4 mg) compared to WT EDL (6.8 ± 0.4 mg). This may be one causative factor underlying the force measurement differences reported here. Another potential explanation would be differences in myosin content or myosin isoforms which will need to be investigated in future studies. Although we have analyzed myofiber structures using various methods, it is difficult to compare the diameter of intrinsic structure (myosin) and the diameter of the entire muscle fiber. We have previously demonstrated that the

Klf10 KO sarcomeres are shorter than WT sarcomeres with a shorter H band where the myosin is located. It could therefore be assumed that the myosin may be compressed and this phenomenon may induce an increase of the CSA.

The increase in the contractile properties for *Klf10* KO EDL fibers compared to littermate controls could be due to the homeostasis of the muscle in an effort to maintain the contractile properties.

To conclude, we have demonstrated that Klf10 plays a crucial role in the homeostasis of the contractile behavior of skeletal muscle fibers and functions in a muscle type specific manner. In future studies, eccentric contraction force could be measured with repetitive stimulations to provide evidence for a greater role of Klf10 in muscle physiological forces. It is of interest to determine if differences in calcium sensing, extra cellular matrix composition and excitation-contraction coupling exist in *Klf10* KO muscles and if such differences contribute to deficits in the force measurements described here.

Abbreviations:

A, activating solution; CSA, cross-sectional area; D, diameter; EDL, extensor digitorum longus; F_{max}, maximum force; $F_{stretch}$, stretch force; Klf, Krüppel-like factor; KO, knock-out; myo, myosin; NIH, National Institute of Health; P, P-value; pCa, -log(10) of the calcium concentration; R, relaxing solution; SEM, Standard Error of Mean; $Stress_{max}$, maximum stress; TEM, transmission electron microscopy; TIEG1, TGFβ inducible early gene-1; W, washing solution; WT, wild-type.

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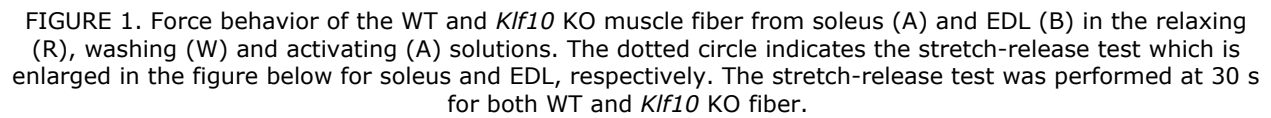
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Figure Legends

FIGURE 1. Force behavior of the WT and *Klf10* KO muscle fiber from soleus (A) and EDL (B) in the relaxing (R), washing (W) and activating (A) solutions. The dotted circle indicates the stretch-release test which is enlarged in the figure below for soleus and EDL, respectively. The stretch-release test was performed at 30 s for both WT and *Klf10* KO fiber.

FIGURE 2. Region of interest (1500x1500 pixels, pixel size: 0.22 nm) from transmission electron microscopy of transversal section from soleus (A) and EDL (B) muscles for both genotypes (WT, *Klf10* KO). The scatterplots depict that the individual data points between WT and *Klf10* KO mice segregate, more obviously for EDL than soleus. Cross-sectional area of myosin (myoCSA) was measured in the region of interest in four soleus (C) and four EDL (D) muscles for both genotypes (WT, *Klf10* KO). Myosin area is significantly different between WT and *Klf10* KO fibers for both soleus and EDL muscles ($P < 0.001$): in soleus muscle (myoCSA$_{WT}$ = 136.8 ± 1.4 nm², N= 466; myoCSA$_{Klf10\_KO}$ = 155.3 ± 1.3 nm², N = 360) and in EDL muscles (myoCSA$_{WT}$ = 141.4 ± 0.8 nm², N = 448; myoCSA$_{Klf10\_KO}$ = 174.0 ± 1.4 nm², N = 412). All data is presented as Mean ± Standard Error of Mean and "N" is the number of myosin filaments used in the analysis.

FIGURE 3. Boxplots of Stress$_{max}$ values (A) and Stiffness (B) for soleus and EDL muscles as a function of genotype. **$P < 0.01$, ***$P < 0.001$

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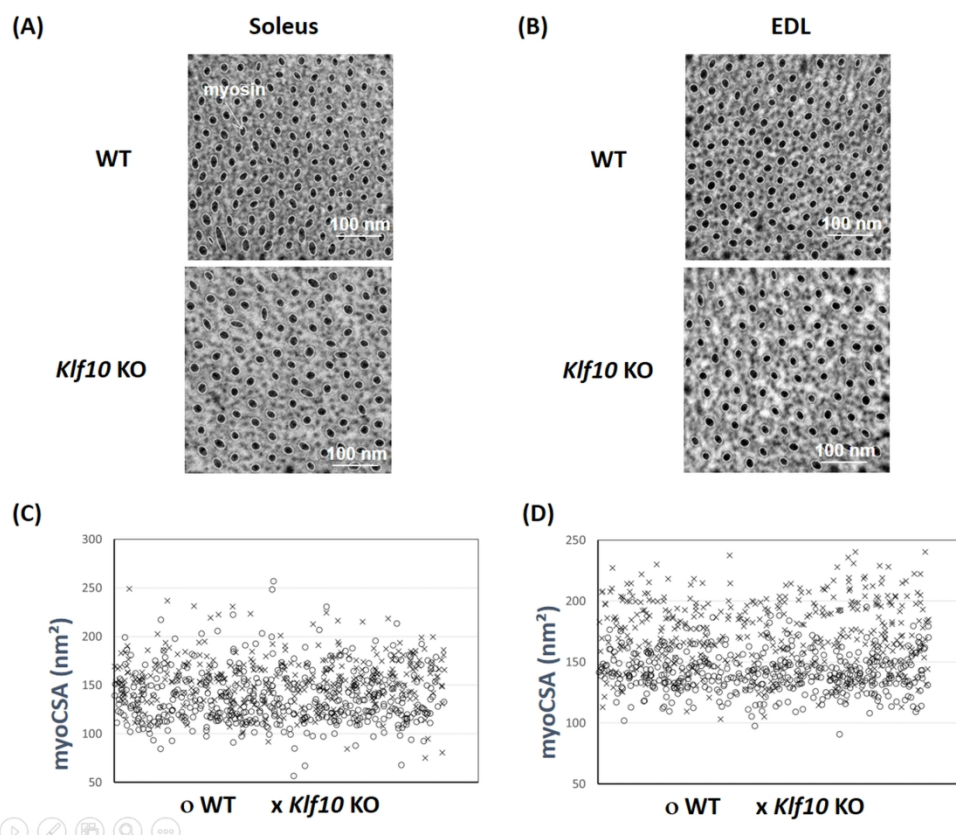
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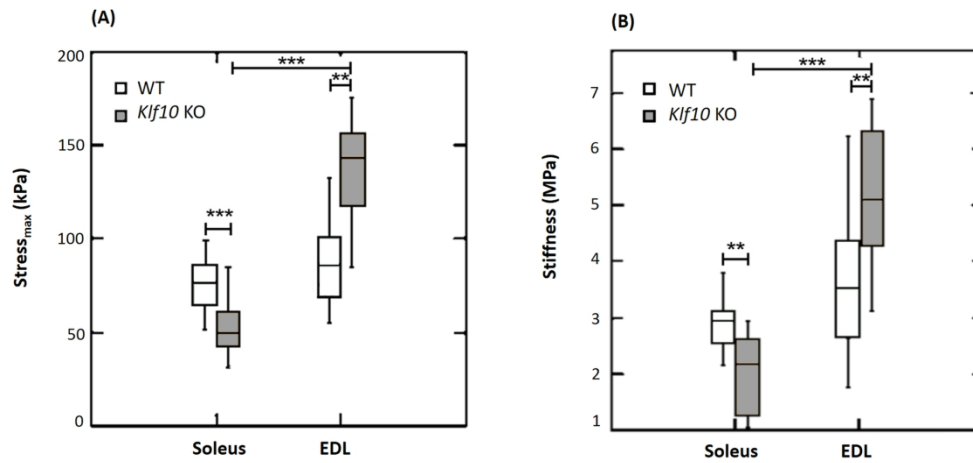
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(A)

(B)

FIGURE 3. Boxplots of Stress$_{max}$ values (A) and Stiffness (B) for soleus and EDL muscles as a function of genotype. **P <0.01, ***P <0.001

209x99mm (300 x 300 DPI)