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Variable cardiac α-actin (*Actc1*) expression in early adult skeletal muscle correlates with promoter methylation

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ABSTRACT

Different genes encode the α-actin isoforms that are predominantly expressed in heart and skeletal muscle. Mutations in the skeletal muscle α-actin gene (*ACTA1*) cause muscle diseases that are mostly lethal in the early postnatal period. We previously demonstrated that the disease phenotype of *ACTA1* mouse models could be rescued by transgenic over-expression of cardiac α-actin (ACTC1). ACTC1 is the predominant striated α-actin isoform in the heart but is also expressed in developing skeletal muscle. To develop a translatable therapy, we investigated the genetic regulation of *Actc1* expression. Using strains from The Collaborative Cross (CC) genetic resource, we found that *Actc1* varies in expression by up to 24-fold in skeletal muscle. We defined significant expression quantitative trait loci (eQTL) associated with early adult *Actc1* expression in soleus and heart. eQTL in both heart and soleus mapped to the *Actc1* locus and replicate an eQTL mapped for *Actc1* in BXD heart and quadriceps. We built on this previous work by analysing genes within the eQTL peak regions to prioritise likely candidates for modifying *Actc1* expression. Additionally we interrogated the CC founder haplotype contributions to enable prioritisation of genetic variants for functional analyses. Methylation around the *Actc1* transcriptional start site in early adult skeletal muscle negatively correlated with *Actc1* expression in a strain-dependent manner, while other marks of regulatory potential (histone modification and chromatin accessibility) were unaltered. This study provides novel insights into the complex genetic regulation of *Actc1* expression in early adult skeletal muscles.

1. Introduction

Cardiac α-actin (Actc1) is the most abundant striated α-actin isoform in the mature heart and the predominant striated α-actin in foetal skeletal muscle [1,2]. After birth, Actc1 protein expression in skeletal muscle is down-regulated to negligible levels, but may be expressed in diseased mature skeletal muscle (such as in muscular dystrophy) in regenerating myofibres [3,4]. Modulation of *Actc1* expression could potentially have a therapeutic benefit for certain striated muscle diseases. For example, reduced levels of ACTC1 protein are detected in failing heart [5].

ACTC1 also represents a therapeutic target for disease caused by skeletal muscle α-actin (*ACTA1*) mutations. *ACTA1* mutations usually produce a severe skeletal muscle phenotype with reduced movement at birth, and death within the first year of life [6]. There is currently no cure for *ACTA1* disease and present treatments such as mechanically assisted ventilation and feeding, only manage symptoms (reviewed in [7]). We previously showed that patients with recessive *ACTA1* mutations had a complete absence of ACTA1 protein but retained expression of ACTC1 in their skeletal muscles into adolescence. These patients had a less severe phenotype leading us to propose that upregulation of ACTC1 could be a viable therapy for *ACTA1* patients [8]. In mouse

Abbreviations: CC, the Collaborative Cross; EDL, extensor digitorum longus; TA, tibialis anterior; SNP, single nucleotide polymorphism; LOD, $\log_{10}$ odds; ChIP, chromatin immunoprecipitation; ChART, chromatin accessibility by real time PCR; UTR, untranslated region; Chr, chromosome; AU_{log2}, arbitrary units $\log_2$; NOD, NOD/LtJ; B6, C57BL/6J; PWK, PWK/PhJ; TSS, transcriptional start site; GWAS, genome wide association studies

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models of *ACTA1* disease, we showed that transgenic over-expression of ACTC1 in postnatal skeletal muscle could effectively compensate for absent [9] or mutant Acta1 [10]. Therefore, identifying genetic elements controlling endogenous *Actc1* expression would be important for possible future treatments for either cardiac or skeletal muscle diseases.

In order to inform future treatments for human *ACTA1* disease, we investigated expression levels of *Actc1* in skeletal muscles from young adult mice. To do so, we utilised the Collaborative Cross (CC), a powerful mouse genetics resource [11] we co-developed, to map expression quantitative trait loci (eQTL) for *Actc1*.

Mapping an eQTL involves correlating variation in gene expression levels with genetic differences, usually single nucleotide polymorphisms (SNPs) [12]. Genes or regulatory regions causative for the eQTL can then possibly be manipulated for modulation of gene expression, including for therapeutic purposes. eQTL are most conveniently determined by genetic mapping in a reference population of recombinant inbred mice. One of the most widely used mammalian recombinant inbred panels is the BXD resource, derived from the parental strains C57BL/6J and DBA/2J (e.g. [13–19].).

Conventional recombinant inbred lines derived from two parental strains lack genetic diversity because they involve only two closely-related founder genomes [20]. This level of genetic diversity effectively produces blind spots that can hamper genetic mapping [21]. Recently, Wang and colleagues argued that one way to overcome this potentially problematic linkage disequilibrium is to validate results in human cohorts and alternative mouse resources such as the CC [22].

The CC was created from eight genetically diverse founder strains (A/J, C57BL/6J, 129S1/SvImJ, NOD/ShiLtJ, NZO/HiLtJ, CAST/EiJ, PWK/PhJ and WSB/EiJ) [21]. Collectively, these founder strains capture over 90% of common genetic variation in the mouse species [20]. The genetic map for CC strains is denser than previous recombinant inbred panels, with strains typed at 143,259 SNP markers using the GigaMUGA array (GeneSeek). The increased genetic diversity, coupled with the high-density SNP profiles for each of the CC strains, makes this a powerful resource for mapping complex traits [23].

Wang et al. recently used the BXD resource to map a significant eQTL for *Actc1* in heart, where Actc1 is highly expressed [22]. In this study, we have built on the work of Wang and colleagues by confirming the *Actc1* eQTL mapped in the heart with an alternative mouse resource, the CC. We also demonstrate highly variable levels of *Actc1* expression between different CC mouse strains at six weeks of age both in a fast myofibre- and slow myofibre-predominant skeletal muscle, and provide evidence of the expression control mechanisms.

2. Materials and methods

2.1. Mice

All mouse breeding and experimental procedures were approved by the Animal Ethics Committees at the Animal Resources Centre and The University of Western Australia. Mice were housed under specific pathogen-free conditions and were provided with tap water and standard rodent food ad libitum. All cages were kept under the same conditions, maintained at 22 °C constant temperature, with a 15:9-h light/dark cycle. CC strains were generously provided by Geniad Pty Ltd. [23].

2.2. RNA preparation

For microarray analysis, extensor digitorum longus (EDL), soleus and heart muscles were collected from 6-week old male mice ($n \geq 3$ per strain), from 48, 48 and 55 strains respectively. The soleus and EDL muscles were selected as they are commonly used to represent skeletal muscle with a significant proportion of slow twitch (type I) myofibres (soleus), or fast twitch (type II) myofibres (EDL) [24]. Tissues were dissected immediately post euthanasia, snap frozen in liquid nitrogen, and stored at − 80 °C until processing. RNA was extracted using an RNAeasy Fibrous Tissue kit (Qiagen). RNA quality was determined using an Agilent Bioanalyzer with an RNA integrity number of 7 used as the minimum cut-off value [25]. Individual RNA samples from the same tissue and strain were evenly pooled based on amount of RNA.

For qPCR analysis, soleus, EDL, tibialis anterior (TA), quadriceps and heart samples were dissected from 6 and 30 week old C57BL/6J and NOD/ShiLtJ male mice (hereafter referred to as B6 and NOD respectively). RNA and protein were extracted simultaneously using TRIzol reagent (Thermo Fisher Scientific) and RNA integrity was confirmed by gel electrophoresis.

2.3. Microarray hybridizations and data analysis

Whole genome expression profiling of 25,698 transcripts was performed using the MouseRef-8 v2 BeadChip (Illumina, USA). The BeadChip was scanned with a Bead Array Reader and raw data were exported using Illumina Genome Studio software. All microarray expression data were log2-transformed and normalised with Robust Spline Normalization (RSN), using LUMI package in R. EDL samples were processed in two batches, hence a non-parametric batch effects removal was performed using COMBAT R module prior to combining all samples. The results for two *Actc1* probes, ILMN_2598916 and ILMN_2767216, were extracted. Probe sequences were interrogated using the Wellcome Trust Sanger Institute Mouse Genomes Project (http://www.sanger.ac.uk/) to ensure complementarity to all founder genomes. The ILMN_2767216 probe did not overlap any genomic variants present in founder strains. However, the ILMN_2598916 probe overlapped with three SNPs (rs13469211, rs13469210 and rs28306385) found in Cast/EiJ and NOD, Cast/EiJ and NOD, and Cast/EiJ and PWK/PhJ founder genomes respectively. Thus, only the ILMN_2767216 probe was used for analyses. All data have been made publically available at (http://130.95.9.22/webqtl.html). Data can be accessed by selecting 'GeneMine' under the 'Group' heading and then selecting either 'Soleus, 6wk, male, Illumina', 'EDL, 6 wk, male, Illumina', or 'Heart, 6 wk, male, Illumina' under the 'Database' heading.

2.4. Quantitative PCR

The SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific) was used to synthesise cDNA from total RNA using random hexamers according to the manufacturer's protocol. The Rotor-Gene SYBR Green PCR Kit (Qiagen) was used to compose 10 μL reactions containing 1 μL cDNA and 0.8 μM each of forward and reverse primers specific to the transcripts of interest (*Actc1, Tbp, Actb, Eef2*; Supplementary Table 1). Thermal cycling was performed on the Rotor-Gene Q real-time PCR cycler and data analysis was performed with the Rotor Gene Q series software (Qiagen). *Actc1* transcript abundance was normalised to the geometric mean of two endogenous control genes (*Tbp* and *Eef2*) using the delta Ct method [26]. To satisfy assumptions of normality, data were log transformed prior to statistical analysis [26].

2.5. Actc1 protein quantitation

Quantification of *Actc1* protein was performed on EDL ($n = 3$) and soleus ($n = 2$) muscles from 6-week old male B6, HAX2_EF (a CC strain; henceforth HAX2) and NOD mice. Multiple reaction monitoring (MRM)-mass spectrometry on EDL muscle was performed by Proteomics International as per previous methodology [27]. Western blot analysis was conducted using a specific Actc1 mouse monoclonal antibody (clone Ac1–20.4.2, Sigma-Aldrich) [9] with Gapdh (clone GAPDH-71.1, Sigma Aldrich) used as an internal standard for loading. Immunostaining of frozen skeletal muscle sections also utilised the specific Actc1 mouse monoclonal antibody, along with a Zenon® Alexa Fluor® 488 mouse IgG_1 labelling kit (Thermo Fisher Scientific) [9]. Muscle spindle fibres are specialised clusters of striated fibres surrounded by a connective tissue capsule [28] which stain positively for