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Transgenic mice expressing mutant Pinin exhibit muscular dystrophy, nebulin deficiency and elevated expression of slow-type muscle fiber genes

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ABSTRACT

Pinin (Pnn) is a nuclear speckle-associated SR-like protein. The N-terminal region of the Pnn protein sequence is highly conserved from mammals to insects, but the C-terminal RS domain-containing region is absent in lower species. The N-terminal coiled-coil domain (CCD) is, therefore, of interest not only from a functional point of view, but also from an evolutionarily standpoint. To explore the biological role of the Pnn CCD in a physiological context, we generated transgenic mice overexpressing Pnn mutant in skeletal muscle. We found that overexpression of the CCD reduces endogenous Pnn expression in cultured cell lines as well as in transgenic skeletal muscle fibers. Pnn mutant mice exhibited reduced body mass and impaired muscle function during development. Mutant skeletal muscles show dystrophic histological features with muscle fibers heavily loaded with centrally located myonuclei. Expression profiling and pathway analysis identified over-representation of genes in gene categories associated with muscle contraction, specifically those related to slow type fiber. In addition nebulin (NEB) expression level is repressed in Pnn mutant skeletal muscle. We conclude that Pnn downregulation in skeletal muscle causes a muscular dystrophic phenotype associated with NEB deficiency and the CCD domain is incapable of replacing full length Pnn in terms of functional capacity.

1. Introduction

Pinin (Pnn) was first characterized as a desmosome-associated protein [1,2]. Subsequently it was found co-localized with splicing factors within the nuclear speckles [3]. Using proteomic analysis, a total of 146 known proteins as well as 32 uncharacterized proteins of nuclear speckles were identified [4]. Given the fact that Pnn was identified in the speckle fraction and the ability of Pnn to interact with splicing proteins SRp75, SRm300 and SRp130 via its C-terminal RS domain [5], and with RNPS1 via its N-terminal coiled-coil domain [6], it is plausible that Pnn participates in mRNA splicing regulation. In addition, proteomic analysis of the spliceosome identified Pnn not only in catalytically active complex C [7], but also in the exon junction complex [8], suggesting that Pnn may also take part in mRNA biogenesis via regulation of splicing as well as nuclear export of mRNA.

Skeletal muscle formation is a multistep process from stem cells to myotubes, which encompasses the transition of many muscle-specific splicing factors and alternative splicing of large proteins during myogenesis and development. Deep-sequencing analysis of different human tissue shows that skeletal muscle is one of the tissues with highest number of differentially expressed alternative exons [9]. A prominent example of a shift in alternative splicing in development is cardiac troponin T (cTNT), in which loss of exon 5 occurs from embryo to adult [10]. A number of regulatory RNA binding proteins, including members of the CELF family (CUGBP and ETR3 like), MBNL, hnRNP H and PTB have been shown to regulate muscle-specific alternative splicing events [11]. It is expected that identification of novel muscle-specific transcription regulators will shed light on the underlying mechanism responsible for not only skeletal muscle biogenesis but also the molecular etiology of muscular dystrophy.

The N-terminal region of Pnn is found highly conserved from mammals to *Caenorhabditis elegans*, but the C-terminal RS domain-containing region is absent in lower species. The function and evolutionary origin of N-terminal coiled-coil domain (CCD) is, therefore, of interest. To explore the biological role of the Pnn CCD

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in a physiological context and to find out whether a Pnn mutant without the C-terminal region could replace endogenous Pnn, in this study we generated transgenic mice overexpressing mouse *Pnn* mutant (*Pnn*1-303 sequence based on Drosophila full length *Pnn*, which exclusively contains the CCD) under the control of human skeletal actin (HAS) promoter. The results provide insights into the mechanism underlying Pnn expression regulation and suggest a role for Pnn in skeletal muscle development.

2. Materials and methods

2.1. Cell culture and transfection

The HeLa, U2OS, L6 and C2C12 were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Gibco., Carlsbad, CA) supplemented with 10% fetal bovine serum plus 1% penicillin–streptomycin–glutamine, and cultured at 37 °C in a humidified chamber with 5% CO_2. The transfection was performed using Lipofectamine 2000 (Invitrogen Gibco) according to the manufacturer's instructions.

2.2. Immunofluorescent microscopy

The cells were transfected with expression vectors encoding myc-tagged pnn1-303. Cultured cells or cryosections (8 μm) from mouse skeletal muscles were incubated with mixed primary antibodies and processed for immunostaining as described previously [12]. Primary antibodies used were mouse anti-myc monoclonal antibody (clone 9E10.2) or chicken anti-myc polyclonal antibody (1:500, Bethyl Laboratories, Montgomery, TX) and rabbit anti-Pnn polyclonal antibody (P3a, 1:500).

2.3. Western blot

Proteins were isolated from the various cell lines 2 days post-transfection. Cells were lysed in 2× sample buffer (5% SDS, 0.25 M Tris pH 6.8, 5% β-Mercaptoethanol, 0.01% Bromophenol Blue) and heated to 95 °C for 10 min. Equal amount of samples were separated by SDS–PAGE and immunoblotting was performed as described previously [12]. Primary antibodies used include mouse anti-Pnn, mouse anti-myc and mouse anti-β-actin (1:3000, Sigma, St. Louis, MO). The peroxidase-labeled blots were developed using an ECL kit (Amersham Pharmacia Biotech, Piscataway, N.J.).

2.4. Transgenic mice

To generate Pnn CCD mutant under the control of HSA promoter, human Pnn open reading frames (nucleotides 1-303) were cut by EcoR1 and XhoI from hPnn1-303/pcDNA3.1 and ligated to pCMVtag3B. Subsequently myc-hpnn1-303 fragments were generated by using NotI and Bsp120I and cloned to pre-digested HSA-VP1 vector to form HSA-myc-hPnn1-303 transgene vector. The transgene vectors were purified for microinjection into fertilized eggs recovered from super-ovulated FVB female mice. HSA-myc-hPnn1-303 transgenic vector contains HSA promoter and VP1 enhancer (a SV40 intron sequence), which can direct expression of Pnn1-303 specifically in skeletal muscle. Mice were maintained according to the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care. Animal experiments were approved by the Chang Gung University Institutional Animal Care and Use Committee.

2.5. RNA isolation and microarray analysis

The muscle tissue of tibialis anterior (TA) was isolated from 3-month-old mice. Tissue (50–100 mg) was put into a tube containing 1 ml Trizol, and homogenized with a tissue homogenizer (Precellys 24, Stretton Scientific, UK). Samples were centrifuged to remove the debris and polysaccharides and RNA was extracted according to the instruction manual (MDBio, Taipei, Taiwan). The RNA pellet was air-dried and resuspended in RNase free ddH_2O.

The RNA sample expression level diagnosis was sent to Phalanx Biotech Group (Taiwan), and gene expression chip performed with MOA v2.1 mouse OneArray. The differential expression gene lists were outputted by using Gene Ontology biological process analysis and Gene Set Enrichment analysis of the canonical pathway database.

2.6. Semi-quantitative RT-PCR

The cDNAs were synthesized from 2 μg of TA muscle total RNA using oligo$(dT)_{22}$ and MMLV reverse transcriptase (Epicenter, Madison, WI). PCR were performed using cDNA (100 ng), dNTP (2.5 mM), 10× reaction buffer, the indicated primer sets (10 μM; Supplementary Table 1) and Tag polymerase. PCR program was 28–30 cycles each at 95 °C for 3 min, 55 °C for 45 s, and 72 °C for 90 s.

2.7. Histochemical staining

Tissue cryosections were fixed with 3.7% formaldehyde followed by treatment with 0.5% Triton X-100. Sections were stained with hematoxylin and eosin, dehydrated by ethanol, cleared with xylene and then mounted (Entellan new, Millipore, Billerica, MA) and examined with a light microscope.

Connective tissue was stained using a Masson trichrome stain kit (Sigma, St. Louis, MO). The slides were briefly washed out O.C.T by water, allowed to mordant in Bouin's solution overnight. Samples were stained in Biebrich Scarlet-Acid Fuchsin solution and rinsed in deionized water. Slides were placed in a working phosphotungstic/phosphomolybdic acid solution, followed by aniline blue solution and acetic acid (1%). Finally, slides were rinsed with deionized water, dehydrated with alcohol, cleared in xylene and mounted.

3. Results

3.1. Pnn CCD mutant (Pnn1-303) showed reduced endogenous Pnn expression in various cell lines

Our previous study showed that Pnn is well conserved across mammalian species [2]. However, detailed analysis of the database indicated that there is a great divergence in protein coding regions between vertebrate and non-vertebrate Pnn. Only the coiled-coil domains (CCD, including C1, C2 and C3) is highly conserved from mammals to insects (Fig. 1A), suggesting that it is functionally important. This CCD domain, when overexpressed, could inhibit reporter minigene pre-mRNA splicing and partly block bulk mRNA export [6]. Therefore, we speculated that the CCD of Pnn can function as a dominant negative mutant regulating Pnn expression and function.

To investigate whether the Pnn CCD domain can modulate endogenous Pnn expression and function, based on the Drosophila full length Pnn, we generated a Pnn1-303 construct, which contained a myc-tagged mutant Pnn encoding mouse CCD domain, as a tool to study its impact on Pnn expression in various cell lines. Pnn1-303 exhibited a nuclear distribution with a speckle-like pattern, like that of endogenous Pnn (Fig. 1B(a, d, g, j)). Surprisingly, a