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Alternative splicing induces cytoplasmic localization of RBFOX2 protein in calcific tendinopathy

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ABSTRACT

Background: Calcific tendinopathy (CT) is characterized by deposits of calcium, most commonly found in the shoulder tendons. The exact cause and pathogenesis of CT are not fully understood. This study analyzed the expression pattern of RNA-binding protein fox-1 homolog 2 (RBFOX2), a crucial splicing regulator in tissue differentiation.

Methods: Normal and calcific tendons were compared for *RBFOX2* mRNA level using quantitative reverse-transcription polymerase chain reaction. Intracellular localization of RBFOX2 protein was investigated using immunofluorescence microscopy. Normal and calcific tendon cDNAs were used to clone RBFOX2. Sequencing analysis identified coding sequences of the RBFOX2 isoform.

Results: The intracellular localization of RBFOX2 protein differed with disease status, with RBFOX2 localized in the cytoplasm in calcific tendons and the nucleus in normal tendons. Analysis of the RBFOX2 protein-coding sequence showed that exon 10, responsible for nuclear localization, was absent in calcific tendons. Splicing of RBFOX2 target genes *CHD2* and *MBNL1* was significantly affected by cytoplasmic localization of RBFOX2 in calcific tendons.

Discussion: Given the function of RBFOX2 as a splicing regulator in the nucleus, cytoplasmic localization of RBFOX2 protein in calcific tendons may have affected overall splicing events and altered gene expression. These results provide insights for comprehension of CT pathogenesis.

1. Introduction

Calcium hydroxyapatite crystal deposition in the rotator cuff muscle of calcific tendinopathy (CT) patients leads to formation of calcific tendons that causes dysfunction of the shoulder accompanied by severe pain (Peng et al., 2013). For treatment of CT, nonsteroidal anti-inflammatory drugs are currently being used, although the biological mechanisms of these drugs on calcific tendons remain unclear. The process of calcium deposition in tendons has not yet been precisely defined, preventing development of effective drugs to inhibit CT pathogenesis. For these reasons, studies investigating CT pathogenesis should precede the development of novel drugs to efficiently suppress CT progression.

More than 95% of human genes are affected by alternative splicing, which leads to formation of isoforms with different functions from a single gene. RNA-binding proteins (RBPs) bind to the pre-mRNA of target genes and regulate alternative splicing to form isoforms. RBP malfunctions related to cancer, metabolic disorders, muscle dysfunction, and neuronal disease (Castello et al., 2013; Lukong et al., 2008).

RNA-binding protein fox-1 homolog 2 (RBFOX2) regulates RNA splicing in the nucleus by binding to pre-mRNA. RBFOX2 is crucial for differentiation and maintenance of tissues (Gehman et al., 2012;

Abbreviations: CT, calcific tendinopathy; RBFOX2, RNA-binding protein fox-1 homolog 2; E10, exon 10; qRT-PCR, quantitative reverse transcription polymerase chain reaction; H & E, hematoxylin and eosin; NMHC II-A, myosin II-A heavy chain; TG2, transglutaminase 2; NLS, nuclear-localization signal

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Ponthier et al., 2006). Numerous studies report important functions of RBFOX2 in differentiation of muscle, brain, and heart and its close correlation with neurodegeneration, muscular atrophy, and congenital heart disease (Singh et al., 2018; Verma et al., 2016). During muscle differentiation using mouse myoblast C2C12 cells, RBFOX2 induces myoblast fusion, which is essential for muscle differentiation, by regulating alternative splicing of Mef2d and Rock2 (Singh et al., 2014). CaV1.2, which is responsible for calcium ion influx in muscle, is also regulated by the RBFOX2 alternative splicing process (Tang et al., 2009). RBFOX1 and RBFOX2 are important for muscle maintenance and function. RBFOX1 conditional knockout mice have myofibers with reduced size and altered calcium homeostasis (Pedrotti et al., 2015). The pathogenesis of CT is deposition of calcium in the rotator cuff muscle. However, the correlation between CT pathogenesis and RBFOX family functions is unknown.

In this study, we found preferential cytoplasmic localization of RBFOX2 proteins in calcific tendons in contrast to nuclear localization in normal tendons. In addition, we identified the RBFOX2 coding sequence in calcific tendons. These results indicated that the functions of RBFOX2 protein were altered in calcific tendons, suggesting that these proteins are closely modulated in CT pathogenesis.

2. Materials and methods

2.1. Human specimens

The validity of all matched human normal and calcific tendons was confirmed via patient clinical diagnosis. Tendon tissues of CT were collected from 7 patients undergoing arthroscopic calcific deposit removal and subsequent capsular release. Chief complaints were severe shoulder pain and stiffness. Because of shoulder stiffness, we performed arthroscopic capsular release of contracted joint capsules. During arthroscopic removal of calcific deposits and capsular release, 3 × 3 mm specimens were collected at two sites: tendons with calcific deposits and subscapularis tendons. Using an arthroscopic punch, tissue samples were harvested from calcific tendons during calcium deposit removal in the subacromial space and from subscapularis normal tendons during capsular release of the glenohumeral joint. All human tissue specimens were confirmed to be free from other diseases, and patients were notified in advance that specimens would be used for CT research. The Institutional Review Board (IRB) of The Catholic University of Korea approved the use of human tissues (IRB number DC18TESI0087). Information on human tissue specimens used is shown in Table 1.

2.2. Cell culture and transfection

Human epithelial cervix carcinoma (HeLa) cells (ATCC, Manassas, VA, USA) were maintained using Dulbecco's modified Eagle medium (Corning, Corning, NY, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin (HyClone; GE Healthcare, Logan, UT, USA) at 37 °C under 5% CO_2 conditions. All transfection experiments used jetPEI reagent (Polyplus, Illkirch-Graffenstaden, France) according to the manufacturer's instructions.

2.3. Plasmids

An expression construct encoding human Myc-RBFOX2-FL in the pCS3 + MT vector was obtained from normal tendon cDNA. An expression construct encoding human Myc-RBFOX2-ΔE10 in the pCS3 + MT vector was obtained from calcific tendon cDNA. PCR primers were 5′- GCG CAG ATC TGG ATG GAG AAA AAG AAA ATG GTA ACT CAG G -3′ and 5′- GGC CCT CGA GTC ACG TCA CTT CAG TAG GGG −3′. Underlining shows adapter sequences and *Bgl*II and *Xho*I restriction enzyme sites.

2.4. RNA preparation and PCR

Total RNA was isolated from calcific tendons and calcific tendon-adjacent normal tendons using eCube tissue RNA mini kits (Philekorea, Seoul, Korea). RT-PCR used M-MLV reverse transcriptase (Promega, Madison, WI, USA) with random hexamers. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using Prime Q-Master mix (GENETBIO, Seoul, Korea) in an AriaMx real-time PCR system (Agilent Technologies, Santa Clara, CA, USA). PCR primers were 5′- TAC GCT TCG GCC CAG TTT GCT C -3′ and 5′- ACG GTC TGA GCG CTC GTG TCC G -3′ for RBFOX1, 5′- CTT TTA CTA CCA TCC CAT TTC C -3′ and 5′- GGT AGA TTT ACT CTC TGA AT -3′ for RBFOX2, 5′- CCA GGC TCC GAG GCC AGC ACA C -3′ and 5′- TGT AGG GTC GGA GGG GTG GAG -3′ for RBFOX3 5′- ACC TGC TCA ACC TCA ACC TG -3′ and 5′- TGT TGA TAA CTG GCT CCA CG -3′ for TG2, and 5′- TCA CCC ACA CTG TGC CCA TCT ACG A -3′ and 5′- CAG CGG AAC CGC TCA TTG CCA ATG G -3′ for β-actin. The specificity of PCR products was confirmed by gel electrophoresis. PCR used Taq PCR Master Mix (Bioneer, Seoul, Korea) and PCR primers 5′- TTC CCT TAC CCT ACT GCA GC -3′ and 5′- GCA GGC TGT GCA TAT CTG TAG -3′ for RBFOX2 exon 10 region, 5′- ATG GAT TCA TTC CCA AAC CA -3′ and 5′- CAG GAA GGG ATT TCT CCA CA -3′ for CHD2 exon 16 region, 5′- TCC AAT ACC AAC AGG CTC TAG -3′ and 5′- ATG TTG TTG CTG CGG ACA CAG -3′ for MBNL1 exon 8 region. Band intensity was analyzed by ImageJ software.

2.5. Hematoxylin and eosin staining

Normal and calcific tendons were fixed in 4% paraformaldehyde for at least 24 h, followed by cryoprotection with 30% sucrose in phosphate-buffered saline. Tissues embedded in Tissue-Tek O.C.T. Compound (Sakura® Finetek, Torrance, CA, USA) were sectioned at 12 μm. Cryosections were stained with hematoxylin and eosin (H & E) and evaluated under a light microscope (Nikon, Tokyo, Japan).

2.6. Immunofluorescence microscopy

Paraformaldehyde-fixed normal and calcific tendons were embedded in O.C.T. compound and cut into 12-μm sections. HeLa cells were seeded at 20,000 cells per well in 4-well chamber slides (Lap-Tek, NY, USA). HeLa cells with *RBFOX2*-expression plasmids were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline. Prepared samples were stained with rabbit anti-RBFOX1 (Biosynthesis Inc., TX, USA), mouse anti-RBFOX2 (Bethyl

Table 1
Calcific tendinopathy patient samples information.

Sample	Sex	Age	Tissues	Experiment
1	M	49	Normal and calcific tendons	qRT-PCR
2	F	55	Normal and calcific tendons	qRT-PCR
3	F	72	Normal and calcific tendons	qRT-PCR
4	F	65	Normal and calcific tendons	H & E staining, Immunofluorescence
5	F	72	Normal and calcific tendons	Immunofluorescence
6	F	74	Normal and calcific tendons	Immunofluorescence
7	F	55	Normal and calcific tendons	PCR

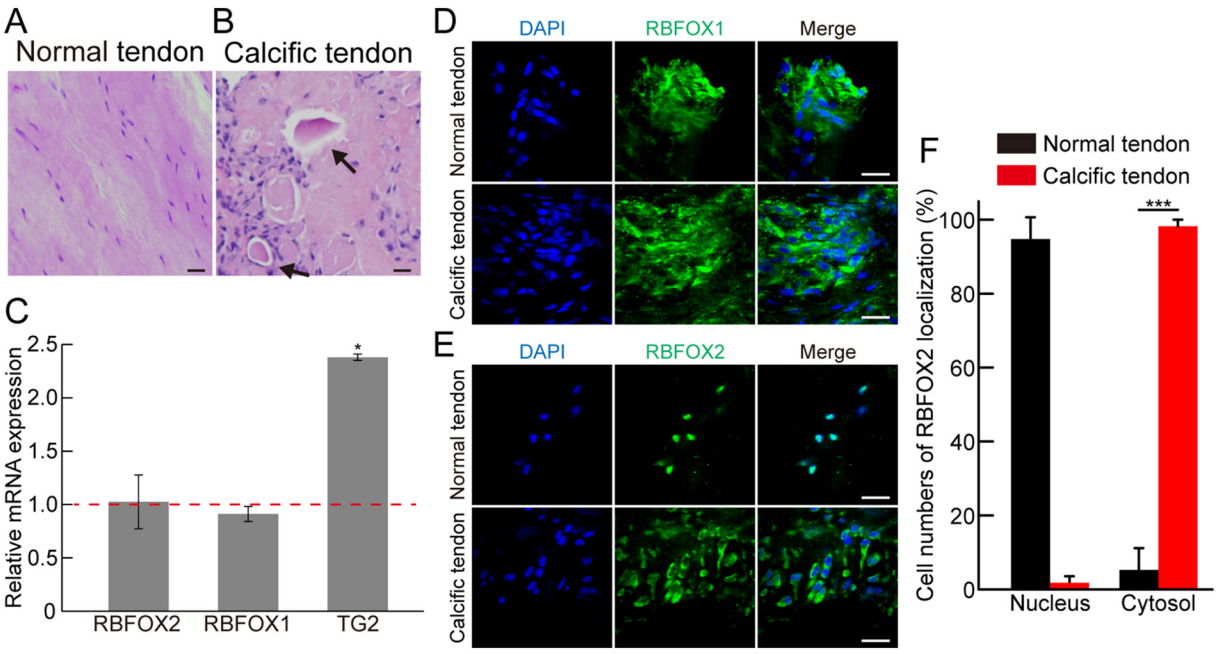

Fig. 1. Localization of RBFOX2 protein in normal and calcific tendons. (A) H & E staining of normal and calcific tendons. Normal tendons with long, thin nuclei in flattened fibroblasts and abundant intervening collagen. (B) Calcific tendons with calcific deposition (arrow) with background of rotator cuff tendon fibrocartilaginous metaplasia. (C) Tissue mRNAs analyzed by qRT-PCR with *β-actin* mRNA as control, $n = 3$. (D) Immunofluorescence of RBFOX1 (green) and DAPI for nuclei (blue) in normal and calcific tendons of sample 4. (E) Immunofluorescence of RBFOX2 (green) and DAPI for nuclei (blue) in normal and calcific tendons of sample 4. Scale bars, 20 μm. (F) Results are mean ± standard error percentage of RBFOX2 protein localization based on quantification of ~70 cells from each of three tissues from samples 4, 5, and 6. $*p < .05$, $***p < .001$ versus normal tendons. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Laboratories, TX, USA), mouse anti-RBFOX3 (anti-NeuN, Millipore, MA, USA), mouse anti-Myc (Thermo Fisher Scientific), or rabbit anti-non-muscle myosin II-A heavy chain (NMHC II-A; Covance, Durham, NC, USA). Secondary antibodies were goat antibodies (Thermo Fisher Scientific) conjugated to Alexa Fluor 488 for mouse IgG and Alexa Fluor 532 for rabbit IgG. Nuclear DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA). Images were captured by an LSM 510 live configuration Vario Two VRGB confocal laser-scanning microscope (Carl Zeiss, Oberkochen, Germany).

2.7. RNA-seq library preparation and splicing analysis

For RNA-seq library preparation, we followed the Illumina TruSeq RNA Sample Preparation Kit v2 manual. At least 50 million, 100-bp-long paired-end reads were mapped to the GRCh38/hg38 version of the human genome per replicate using STAR2.4.2a using the default parameters. For the splicing patterns of RBFOX2, CHD2, and MBNL1 genes, Mixture of isoforms (MISO) (with default parameters) was applied to the aligned RNA-seq data to examine the inclusion levels of alternative exon of RBFOX2, CHD2, and MBNL1 genes between normal and calcific tendons, which estimates expression at alternative splice-event level by computing Percent Spliced In (PSI) values. The alternative splicing events were examined with a minimum read coverage filter of 10 reads.

2.8. Statistical analysis

Unpaired two-tailed Student's *t*-test was used for statistical analysis of data. *P* values lower than 0.05 were considered statistically significant.

3. Results

3.1. Subcellular localization of RBFOX2 protein in calcific tendons

Microscopic findings of normal tendons (sample 4) showed bundles of thick collagenous fibers with mature tenocytes with long, thin nuclei (Fig. 1A). Calcific tendons showed large and small calcific deposits with some chondrocytes, tenocytes, and mononuclear inflammatory cells (Fig. 1B). These findings were diagnosed as calcific tendinopathy.

The *RBFOX2* gene is expressed in various tissues. To investigate the correlation between CT pathogenesis and RBFOX2 protein, we compared *RBFOX2* mRNA levels in calcific and normal tendons from samples 1, 2, and 3 using qRT-PCR. Consistent with previous studies reporting higher transcription levels of tissue transglutaminase 2 (TG2) in calcific tendons relative to normal tendons, our results showed elevated *TG2* mRNA expression in calcific tendons compared to normal tendons. No significant difference was seen in *RBFOX2* mRNA level between calcific and normal tendons. Results were similar for mRNA for *RBFOX1*, which is in the same family as RBFOX2 (Oliva et al., 2011) (Fig. 1C).

We investigated staining patterns of RBFOX protein family members in calcific and normal tendons of samples 4, 5, and 6. We examined protein-expression patterns by immunofluorescence microscopy using anti-RBFOX. RBFOX1 protein was predominantly localized in the cytoplasm in both normal and calcific tendons (Fig. 1D). RBFOX2 proteins are splicing regulators inside the nucleus, and RBFOX2 proteins exhibited nuclear localization in normal tendons (Ponthier et al., 2006) (Figs. 1E, F, and Supplementary Fig. 1). Unlike in normal tendons, the RBFOX2 protein in calcific tendons exhibited cytoplasmic localization. We hypothesized that RBFOX2 protein expressed in calcific tendons was likely to exhibit different characteristics from RBFOX2 protein, which localizes in the nucleus in normal tendons, where it regulates pre-mRNA splicing. We were unable to detect RBFOX3 expression at both the mRNA and protein levels (data not shown). Thus, *RBFOX2* mRNA

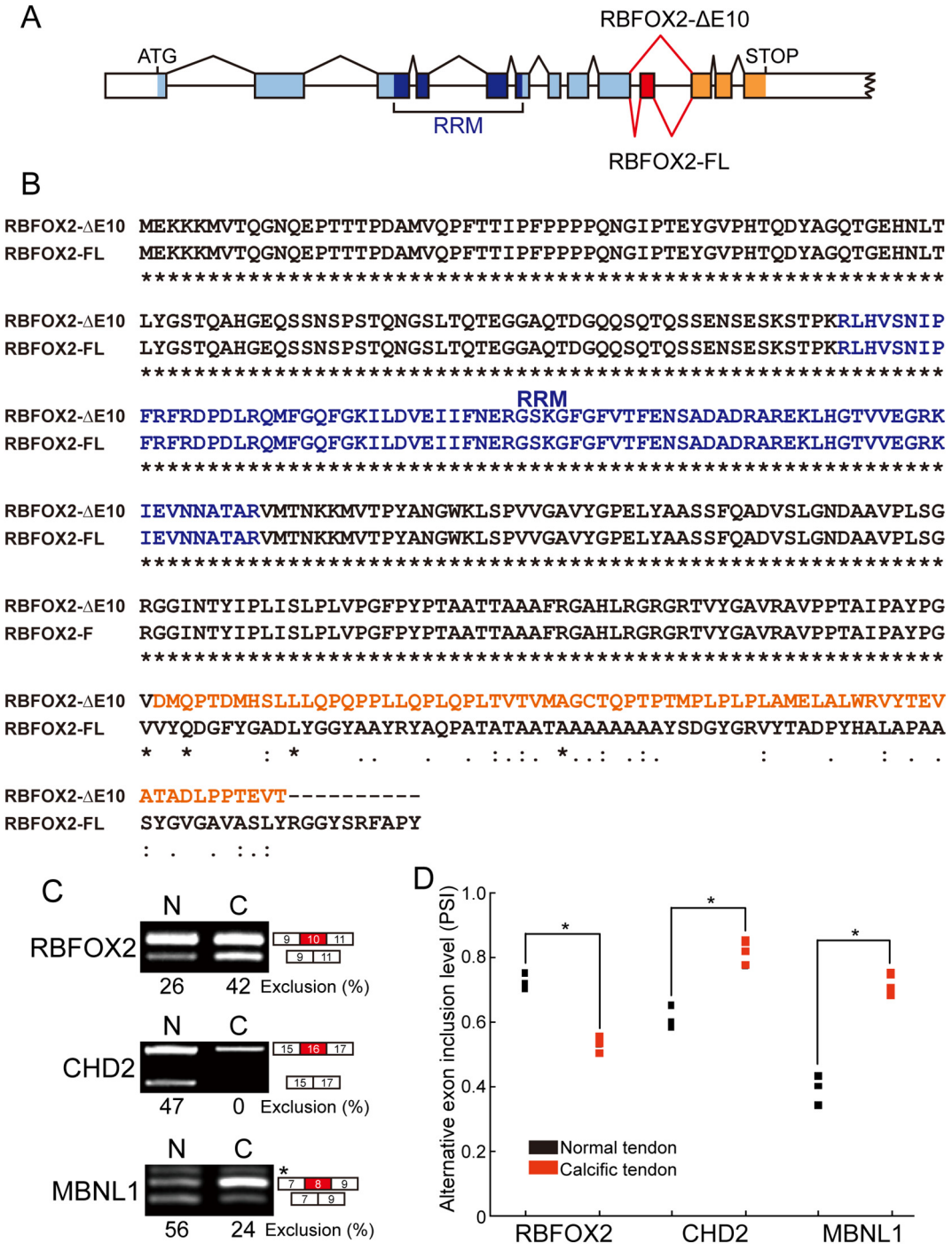

Fig. 2. Amino acid sequences of RBFOX2 isoforms. (A) Diagram of RBFOX2-FL and RBFOX2-ΔE10 cDNA structures. Red bar, site of E10 cassette. (B) Amino acid sequence alignment of RBFOX2-FL and RBFOX2-ΔE10. Blue amino acids, RRM; orange, frameshift region. (C) RT-PCR analysis of normal and calcific tendons. Percentages of splicing isoforms calculated by dividing band intensity by number of base pairs. (D) PSI of alternative exon in matched tissues from the RNA-sequencing dataset. n = 3 *$p < .05$ versus normal tendons. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

levels were similar between normal and calcific tendons, with changes in RBFOX2 intracellular localization in normal and calcific tendons.

3.2. Identification of a calcific tendon RBFOX2 isoform

RBFOX2 has variants produced by alternative splicing. The major isoform is located in the nucleus, whereas others are located in the cytoplasm (Nakahata and Kawamoto, 2005). To investigate reasons for the differential intracellular localization of RBFOX2 proteins between normal and calcific tendons, we identified the RBFOX2 isoform expressed in calcific tendons. RNA extracted from calcific tendons of sample 7 was used to generate cDNA for *RBFOX2* cloning by PCR, followed by sequencing to identify the coding sequence of the RBFOX2 isoform (RBFOX2-ΔE10) protein. The RBFOX2-ΔE10 protein expressed in calcific tendons was missing the 40-nucleotide exon 10 (E10), resulting in a frameshift downstream of E10 (Fig. 2A). The C-terminal sequence of RBFOX2 controls intracellular localization, with the RFAPY amino acid sequence acting as the nuclear-localization signal (NLS)

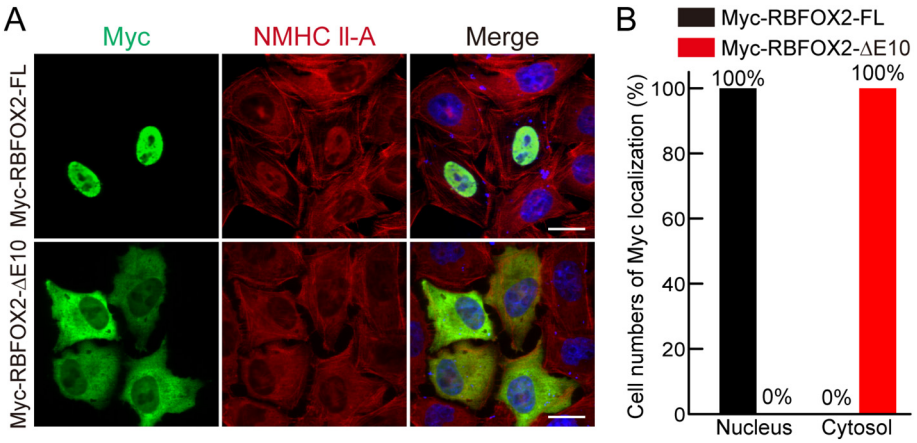

Fig. 3. Subcellular localization of RBFOX2 isoforms. (A) Immunofluorescence of Myc (green), NMHC II-A (red), and DAPI for nuclei (blue). HeLa cells were transfected with Myc-tagged RBFOX2-FL and RBFOX2-ΔE10 expression constructs. Scale bars, 20 μm. (B) Direct visual confirmation determined isoform-specific intracellular localization of exogenous RBFOX2 protein. Results are quantification of ~50 cells from three independent slides, expressed as mean ± standard error percentage of signal. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Kuroyanagi, 2009). Due to the frameshift caused by E10 absence, the RBFOX2-ΔE10 harbors a PTEVT amino acid sequence at the C-terminus. This sequence is known to relocate isoform proteins predominantly to the cytoplasm (Wenzel et al., 2016) (Fig. 2B). To examine the expression level of RBFOX2-ΔE10 mRNA, we performed PCR using cDNA from normal and calcific tendons of sample 7, followed by agarose gel electrophoresis. RBFOX2-ΔE10 increased in calcific tendons compared with normal tendons (Fig. 2C). The exon exclusions of CHD2 and MBNL1, which are target genes for RBFOX2, decreased in sample 7 calcific tendons compared with normal tendons (Misra et al., 2015) (Fig. 2C).

The differences in the alternative exon inclusion levels of RBFOX2, CHD2, and MBNL1 genes in normal and calcific tendons were consistently found in RNA-sequencing results (Fig. 2D). We identified the sequence of the RBFOX2-ΔE10 isoform in calcific tendons and hypothesized that the function of RBFOX2 as an alternative splicing regulator was altered in calcific tendons by localization in cytoplasm.

3.3. *RBFOX2-ΔE10 localizes predominantly in the cytoplasm*

For comparative purposes, we used the RBFOX2 isoform RBFOX2-FL and identified that the RBFOX2-FL and RBFOX2-ΔE10 isoforms differed in the presence of the 40-nucleotide E10 cassette. To investigate intracellular localization of RBFOX2-FL and RBFOX2-ΔE10 proteins, HeLa cells were transfected with plasmids encoding N-terminally Myc-tagged RBFOX2-ΔE10 or RBFOX2-FL for 36 h. Intracellular locations of the exogenously expressed RBFOX2 proteins were detected by immunofluorescence using anti-Myc. Strong signals from the RBFOX2-FL protein with the NLS at its C-terminus were detected in the nucleus. The RBFOX2-ΔE10 protein expressed in calcific tendons was predominantly detected in the cytoplasm (Figs. 3A, B). Our results indicated that altered RBFOX2 localization in normal and calcific tendons was due to expression of an RBFOX2-ΔE10 isoform with an E10 deletion in calcific tendons, resulting in cytoplasmic localization rather than the nuclear localization observed in normal tendons.

4. Discussion

RBFOX2 proteins are crucial for differentiation and maintenance of various tissues. We analyzed the characteristics of these proteins in calcific tendons to understand mechanisms associated with CT pathogenesis. Unlike in normal tendons, RBFOX2 proteins in calcific tendons were predominantly located in the cytoplasm. Our findings showed this to be a consequence of expressing an RBFOX2-ΔE10 isoform lacking an NLS, resulting in cytoplasmic localization.

Mammals express three RBFOX family members: RBFOX1, RBFOX2, and RBFOX3. RBFOX1 is expressed in the heart, neurons, and skeletal muscles; RBFOX3 expression is concentrated only in neurons, and RBFOX2 is expressed in a wide array of tissues (Jin et al., 2003; Kim et al., 2009; Yeo et al., 2009). The major isoform of the RBFOX family proteins contains an NLS sequence at the C-terminus that ensures preferential localization to the nucleus; however, in some isoforms, splicing causes a frameshift in the coding sequence, altering the C-terminus sequence and resulting in predominantly cytoplasmic localization (Dredge and Jensen, 2011; Kuroyanagi, 2009; Lee et al., 2009). The RBFOX2-ΔE10 isoform expressed in calcific tendons results from an E10 deletion that causes a frameshift affecting the C-terminus containing the NLS and resulting in cytoplasmic localization. Our study confirmed this result by immunofluorescence microscopy of HeLa cells overexpressing the RBFOX2-ΔE10 isoform, showing its predominant cytoplasmic localization.

The cytoplasmic localization of RBFOX1 and RBFOX2 is linked to their biological functions. When neurons from *RBFOX1*-knockdown mice are induced to overexpress the cytoplasmic RBFOX1 isoform, exogenous RBFOX1 proteins combine with mRNAs in the cytoplasm, enhancing the stability of target mRNAs (Lee et al., 2016). Another study showed that, upon cell stress induction with high salt conditions, RBFOX2 proteins formed stress-response granules in the cytoplasm, combining with the mRNA of genes involved in cell cycle progression and controlling the stability of *retinoblastoma-1* mRNA (Park et al., 2017). Hypoplastic left heart-syndrome patients have cytoplasmic RBFOX2 with a partially deleted C-terminus caused by a nonsense mutation, which decreases RBFOX2 target mRNAs (Verma et al., 2016). We hypothesized that, because of the cytoplasmic localization, RBFOX2 could not regulate alternative splicing, which affected its target genes; CHD2 and MBNL1 showed an increase in the inclusion form (Fig. 2C). In normal tendons, RBFOX2 proteins may be involved in regulating pre-mRNA splicing in the nucleus. In contrast, RBFOX2 isoforms expressed in calcific tendons likely have different functions based on their cytoplasmic localization.

A study using normal mouse mammary epithelial cells showed RBFOX2 protein predominantly localized in the cytoplasm, with TGF-β inducing E10 deletion of RBFOX2 by alternative splicing (Wenzel et al., 2016). TGF-β is involved in calcification in muscle. In patients with heterotopic ossification (HO), TGF-β is activated in calcium deposition lesions. Furthermore, neutralizing TGF-β suppresses calcification in the Achilles tendons of an HO induced mouse model (Wang et al., 2018). Calcific tendon tissue has highly activated inflammation; therefore, TGF-β may be activated, leading to E10 absence of RBFOX2. We are currently investigating the underlying mechanism of cytoplasmic localization of RBFOX2 in calcific tendons.

Although our human calcific tendon specimens had the exon 10-missing isoform of RBFOX2 and cytoplasmic localization, we could not confirm our results as a representative phenomenon of calcific tendons. Thus, investigating more human calcific tendon specimens is necessary to understand the underlying mechanism of RBFOX2 localization.

Our results identified a frameshift variant of *RBFOX2* missing E10 that lacked a nuclear-localization signal and was highly expressed in calcific tendons. Further analyses confirmed nuclear localization of RBFOX2 in normal tendons, thereby identifying the isoform in calcific tendons and providing critical insights into a factor associated with CT pathogenesis.

Conflict of interests

The authors, their immediate families, and any research foundation with which they are affiliated have not received any financial payments or other benefits from any commercial entity related to the subject of this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexmp.2019.104264.

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