主論文の要旨

SRSF1 suppresses selection of intron-distal 5' splice site of *DOK7* intron 4 to generate functional full-length Dok-7 protein

（SRSF1 は *DOK7* intron 4 の 5'遠位端スプライスサイト選択を抑制し機能性 Dok-7 タンパク産生を促す）

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【Background】

Dok-7 is a non-catalytic cytoplasmic adapter protein. It is abundantly expressed in the skeletal muscle and is essential for formation of the neuromuscular junction. Although the regulatory mechanisms of alternative splicing of *DOK7* have not been dissected in detail, annotation databases of the human genome including RefSeq, Ensemble, and AceView indicate that intron 4 of the human *DOK7* gene harbors two 5' SSs (Figure 1A). Selection of the intron-proximal 5' SS generates a long transcript variant 1 (T-var1) encoding the canonical Dok-7 protein (Dok-7 isoform 1). In contrast, selection of the intron-distal 5' SS generates a frame-shifted transcript variant 2 (T-var2) encoding a truncated Dok-7 protein (Dok-7 isoform 2) lacking two tyrosine residues, which are target motifs of the Src homology 2 (SH2) domain and are indispensable for clustering of acetylcholine receptors (AChR) at the neuromuscular junction (Figure 1A). In the present study, we dissected the underlying mechanisms that regulate selection of the two competing 5' SSs at *DOK7* intron 4.

【Methods】

We employed RT-PCR, siRNA-mediated knockdown. cDNA overexpression, RNA affinity purification, mass spectrometry, tethered function assay, early spliceosome assembly assay, immunoblotting, AChR clustering analysis, MuSK phosphorylation assay and bioinformatic analysis of CLIP-seq and RNA-seq data to dissect the molecular mechanism that regulate selection of the two 5' SSs at *DOK7* intron 4.

【Results】

We initially examined differential selections of two 5'SSs sites of *DOK7* intron 4 by RT-PCR of total RNA extracted from various human tissues and cell lines (Figure 1B and C). We observed alternative selection of the intron-distal 5' SS of *DOK7* intron 4 in the skeletal muscle, brain and, heart (Figure 1B) as well in immortalized human myogenic KD3 cells and HeLa cells (Figure 1C). In contrast, *DOK7* intron 4 was constitutively spliced in the smooth muscle, liver, and spleen (Figure 1B).

We overexpressed Dok-7 isoform 1 and isoform 2 in C2C12 myoblasts. After myotube differentiation we added agrin and observed AChR clusters. We found that overexpression of isoform 1 significantly enhanced AChR clustering, while isoform 2 has a no effect on AChR clustering (Figure 2A, B). Phosphorylation of MuSK was markedly induced by overexpression of isoform 1, whereas induction of MuSK phosphorylation was insufficient with isoform 2 (Figure 2C). These results suggest that selection of the intron-distal 5' SS results in production of a non-functional Dok-7 protein.

We constructed a human *DOK7* minigene (Figure 3A) and found a similar splicing pattern to that we observed with endogenous transcripts (Figure 3A, right panel). The strength of the intron-

proximal 5' SS (SD score -2.646 and MaxEntScan::score5ss 8.59) is higher than that of the intron-distal 5' SS (SD score -3.084, MaxEntScan::score5ss 6.34). We made two mutant constructs, Mut-1 and Mut-2, in which splicing strengths of the intron-distal and intron-proximal 5' SS, respectively, were drastically weakened. RT-PCR analysis showed that the intact 5' SS was exclusively selected in both constructs (Figure 3B). To identify splicing regulatory *cis*-element(s), we scanned the entire exon 4 by substituting 12 blocks with splicing neutral sequence. We found that disruption of Block-12 prominently enhanced selection of the intron-distal 5' SS (Figure 3C, lower panel) suggesting presence of a splicing regulatory *cis*-element in Block-12. To characterize the minimal essential sequences in Block-12, we mutated a segment of six or seven nucleotides in Block-12 (Mut-3, -4, and -5 in Figure 3d). We found altered 5' SS selection in Mut-5 (Figure 3D). Thus, the Mut-5 region in Block-12 close to the 3' end of exon 4 harbors a critical splicing regulatory *cis*-element.

We performed an RNA affinity purification assay using wild-type Block-12 (Wt) biotinylated RNA probe (Figure 4A). Our analysis identified one distinct band of ~30 kDa that was associated with the wild-type Block-12 (Wt), but not for the nucleotide-substituted mutant (Mut-5) or the deletion mutant (ΔMut-5) sequences (Figure 4B). Mass spectrometry analysis of the excised band disclosed that the identified band was SRSF1, which was also confirmed by immunoblotting using antibody against SRSF1 (Figure 4C). We next knocked down endogenous *SRSF1* mRNA in HeLa cells and found that down regulation of SRSF1 increased usage of the intron-distal 5' SS in the minigene transcripts as well as in endogenous *DOK7* transcripts (Figure 4D). We artificially tethered SRSF1 to the *cis*-element using MS2-mediated artificial tethering system. We observed that replacement of the *cis*-element with MS2 hairpin compromised usage of the intron-proximal 5' SS (Figure 4F, lane 2), and that tethering of SRSF1-MS2 specifically restored usage of the intron-proximal 5' SS (Figure 4F, lane 4).

To examine the effect of SRSF1 on the assembly of U1 snRNP to either of these 5' SSs, we made 3 x MS2 attached probes; Probe-1 (containing the intron-distal 5' SS with the SRSF1-binding site) and probe-2 (containing the intron-distal 5' SS with a disrupted SRSF1-binding site) (Figure 5A) and then added splicing-competent HeLa nuclear extract to form early spliceosome complex. We found that association of U1 snRNP to probe-1, but not to probe-2, was markedly reduced compared to the control probe (Figure 5B, lanes 2 and 3). We also made two additional probes; Probe-3 (containing the intron-proximal 5' SS with the SRSF1-binding site) and probe-4 (containing the intron-proximal 5' SS with a disrupted SRSF1-binding site) (Figure 5A). The intron-distal 5' SS was mutated in both probes-3 and -4. The assembly of U1 snRNP on the intron-proximal 5' SS was not affected by the disruption of the SRSF1-binding site (Figure 5C), which was in contrast to the effect on the intron-distal 5' SS (Figure 5B).

To examine whether what we observed with *DOK7* intron 4 is applicable to other genes in the human genome, we analyzed RNA-seq of *SRSF1*-knocked down HeLa cells (GSE26463) and CLIP-seq of SRSF1 in HeLa cells (GSE71096), which were deposited in the GEO database. Splicing analysis of RNA-seq with MISO detected 1445 and 427 alternative 5' splicing events, in which the

intron-distal 5' SS and the intron-proximal 5' SS were selected by knockdown of *SRSF1*, respectively (Figure 5D). SRSF1-regulated alternative splicing of *DOK7* intron 4 is similar to the 1445 alternative 5' splicing events, in which the intron-distal 5' SS was selected by SRSF1 knockdown. We analyzed the distribution of SRSF1-CLIP tags around these alternative 5' SSs. We found that SRSF1 clustered immediately upstream to the intron-distal 5' SS in 1445 exons (double-headed arrow in Figure 5D, upper left panel), which was selected by *SRSF1*-knockdown. In contrast, no noticeable SRSF1 cluster was observed around the intron-proximal 5' SS in these 1445 exons (Figure 5D, upper right panel). Thus, the functional significance of SRSF1-binding on these 427 genes remains to be determined. To summarize, binding of SRSF1 immediately upstream to the intron-distal 5' SS suppresses selection of the intron-distal 5' SS in many human genes (Figure 5E).

【Discussion】

We analyzed the regulatory mechanisms of alternative selection of two 5' SSs at intron 4 of *DOK7*, which encodes an indispensable adaptor protein for enhancing AChR clustering at the neuromuscular junction. The truncated Dok-7 isoform 2 marginally enhanced MuSK phosphorylation (Figure 2C), but significantly diminished the AChR clustering activity (Figure 2A, B). Preservation of the PH domain and part of the PTB domain in Dok-7 isoform 2 (Figure 1A) may account for minimal induction of MuSK phosphorylation (Figure 2C). Lack of the tyrosine residues, however, is likely to have abolished AChR clustering activity of the truncated Dok-7 isoform (Figure 2A, B).

We have identified SRSF1 as a regulator of selection of two 5' SSs of *DOK7* intron 4. Binding of SRSF1 immediately upstream to the two competing 5' SSs suppresses selection of the intron-distal 5' SS. In contrast to our finding, a previous study showed that artificial tethering of SRSF1 between two competing 5' SSs facilitates use of the intron-proximal 5' SS. Thus, SRSF1 may exert the opposing effects on alternative 5' SS selection in a position-specific manner. We showed by an integrated global analysis of CLIP-seq of SRSF1 and RNA-seq of *SRSF1*-knocked down cells that binding of SRSF1 immediately upstream to two competing 5' SSs generally suppresses the intron-distal 5' SS (double-headed arrow in Figure 5D).

We found that SRSF1 inhibits binding of U1 snRNP to the intron-distal 5' SS to suppress selection of the intron-distal 5' SS of *DOK7* intron 4. In contrast to our study, a previous report shows that SRSF1 directly interacts with U1 snRNP to make an early spliceosome complex and facilitates splicing. We conclude that alternative selection of the two 5' SSs of *DOK7* intron 4 is finely tuned by (i) binding of SRSF1 to their immediate upstream position, (ii) the distance between the SRSF1-binding site and 5' SS, and (iii) the strength of splicing signals of the two 5' SSs. Similar splicing regulations are likely to be operational in many other human genes.