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SR proteins regulate V_6 exon splicing of CD44 pre-mRNA

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CD44 pre-mRNA includes 20 exons, of which exons 1-5 (C_1-C_5) and exons 16-20 (C_6-C_{10}) are constant exons, whereas exons 6-15 (V_1-V_{10}) are variant exons. V_6-exon-containing isoforms have been known to be implicated in tumor cell invasion and metastasis. In the present study, we performed a SR protein screen for CD44 V_6 splicing using overexpression and lentivirus-mediated shRNA treatment. Using a CD44 V_6 minigene, we demonstrate that increased SRSF3 and SRSF4 expression do not affect V_6 splicing, but increased expression of SRSF1, SRSF6 and SRSF9 significantly inhibit V_6 splicing. In addition, using a constitutive exon-specific primer set, we could not detect alterations of CD44 splicing after SR protein-targeting shRNA treatment. However, using a V_6 specific primer, we identified that reduced SRSF2 expression significantly reduced the V_6 isoform, but increased $V_{6\text{-}10}$ and $V_{6,8\text{-}10}$ isoforms. Our results indicate that SR proteins are important regulatory proteins for CD44 V_6 splicing. [BMB Reports 2016; 49(11): 612-616]

INTRODUCTION

In pre-mRNA splicing, introns are removed, and exons are ligated together to produce mRNA (1). Alternative splicing that produces multiple proteins from a gene plays an important role in gene expression. Alternative splicing occurs in ~95% of human genes. Thus it regulates various biological processes including cell cycle, energy transfer. Genetic diseases and cancers that are caused by abnormal RNA splicing (2-4). Both cis- and trans-acting elements are known to regulate alternative splicing. Cis-acting elements are RNA sequences that are located in exons or introns and function as splicing enhancers or inhibitors. Trans-acting elements are proteins or protein-RNA complexes that regulate alternative splicing. The best known trans-acting elements are heterogeneous nuclear ribonucleoprotein (hnRNP) and Serine-Arginine rich (SR) proteins (5, 6). The splicing-processing machinery, also known as the spliceosome, is composed of several proteins and U-small-nuclear RNP particles (snRNPs).

The CD44 receptor directs intracellular signaling in cell growth motility through mediating adhesion and communications of cells with adjacent cells or the extracellular matrix. CD44 is a cell adhesion membrane glycoprotein. Ligands for CD44 determine CD44 functions. While growth factors modulate the growth-promotion function of CD44, hyaluronic acid mediates the tumor suppressor function of CD44 (7). Constant exons 1-5 (C_1-C_5), constant exons 16-20 (C_6-C_{10}) and various exons 6-15 (V_1-V_{10}) are included in CD44 pre-mRNA (8). The presence of variant exons leads to production of a large number of mRNA isoforms that encode proteins with different ligand-binding properties and diverse post-transcriptional modifications (9-11). Variant exons 6-15 are included or excluded to different extents to generate a great number of splicing variants. CD44 protein sizes ranged from 85 kDa (CD44s) to 250 kDa (CD44 V_3-V_{10}). V_6 exon-containing isoforms play important roles in tumor cell invasion and metastasis. The V_6 exon has been shown to be highly expressed in tumors compared with normal tissues. A CD44 V_6 exon-containing isoform can make a complex with a tyrosine kinase receptor, Met, and hepatocyte growth factor, HGF, and then activate Met-dependent Ras signaling by the association of ezrin radixin-moesin (ERM) to CD44 on its cytoplasmic tail (12, 13).

SR proteins are a protein family that includes 13 members - SRSF1-12 and tra2β SR proteins include a RNA recognition motif (RRM) domain and a RS domain (5). SR proteins play important roles in alternative and constitutive splicing. In constitutive splicing, SR proteins are known to promote the binding of U1 snRNP to 5′ splice-site and the binding of U2 snRNP binding to a branch-point in spliceosome assembly (14, 15). In alternative splicing, SR proteins are shown to antagonize hnRNP functions (16). SR proteins could promote exon inclusion or skipping through interactions with exons or introns. In addition to playing different roles in RNA splicing, SR proteins also function in transcription elongation, RNA

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stability, mRNA transport and mRNA translation (17). In the present study, we performed a SR protein screen for CD44 V_6 splicing using overexpression and lentivirus-mediated shRNA treatment. Using a CD44 V_6 minigene, we demonstrate that SRSF3 and SRSF4 do not affect V_6 splicing. SRSF1, SRSF6 and SRSF6 significantly inhibits V_6 splicing.

In addition, using a constitutive exon-specific primer set, we could not detect alterations of CD44 splicing after SR protein-targeting shRNA treatment. Using a V_6-specific primer, we identified that reduced SRSF2 expression significantly reduced the V_6 isoform, but increased $V_{6\text{-}10}$ and $V_{6,8\text{-}10}$ isoforms. Our results indicate that SR proteins are important regulatory proteins for CD44 V_6 splicing.

RESULTS

SRSF3 and SRSF4 did not affect V_6 exon splicing of CD44 pre-mRNA

In order to identify the SR proteins that affect V_6 exon splicing of CD44 pre-mRNA, we used a MCF7 stable cell line that expresses the pFlare-V_6 plasmid (18). As previously described, in the pFlare-V_6 plasmid, V_6 exon and its flanking introns are inserted between β-globin exon 1 and the GFP exon (Fig. 1A). GFP is expressed when V_6 is skipped, and then RFP is expressed when V_6 is included. To detect V_6 exon splicing, we used a primer set that base pairs with β-globin and the GFP exon (Fig. 1A). Consistent with the previous results (18), the V_6 included isoform was dominantly expressed, whereas the V_6 excluded isoform was expressed at a much less significant level (lane 1, Fig. 1B). It was also consistent with the previously published conclusion (18) that SRSF2 significantly promotes a V_6 skipped isoform and inhibits V_6 inclusion. Next, we tested the function of SRSF3 and SRSF4 on V_6 splicing. Although V_6 exon and flanking introns include a number of potential binding sites for SRSF3 and SRSF4, Fig. 1B shows that neither SRSF3 nor SRSF4 affected V_6 splicing. Thus we conclude that SRSF3 and SRSF4 are not regulatory factors for V_6 exon splicing of CD44 pre-mRNA.

SRSF1, SRSF6, and SRSF9 inhibit V_6 exon splicing

We further asked whether other SR proteins regulate V_6 exon splicing. We also noticed that V_6 exon and flanking introns contain significant numbers of potential binding sequences for SRSF1, SRSF6, and SRSF9. These sequences suggest that the proteins may regulate V_6 exon splicing. Therefore, we expressed SRSF1 or SRSF6 or SRSF9 in the pFlare-V_6 cell line. Fig. 2 shows that, by contrast to SRSF3 and SRSF4, treatment of these proteins induced the V_6 skipped isoform at significant

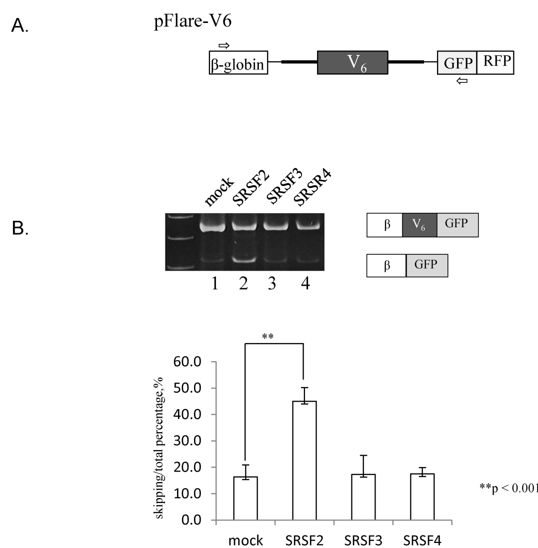

Fig. 1. SR proteins regulate V_6 exon splicing of CD44 pre-mRNA. (A) pFlare-V_6 minigene is shown. V_6 exon is shown with a black box, β-globin and GFP/RFP exons are shown with white/gray boxes. Introns that flank V_6 are shown with thicker lines, introns of β-globin and GFP are shown with thinner lines. (B) RT-PCR analysis using RNAs from pFlare-V_6 MCF7 cells treated with pcDNA3.1＋ or SRSF2 or SRSF3 or SRSF4. Quantitation results by Image J from three independent experiments are shown at the bottom. The significant change was evaluated by Student's *t-test*. The error bars represent the standard deviation of the repeats.

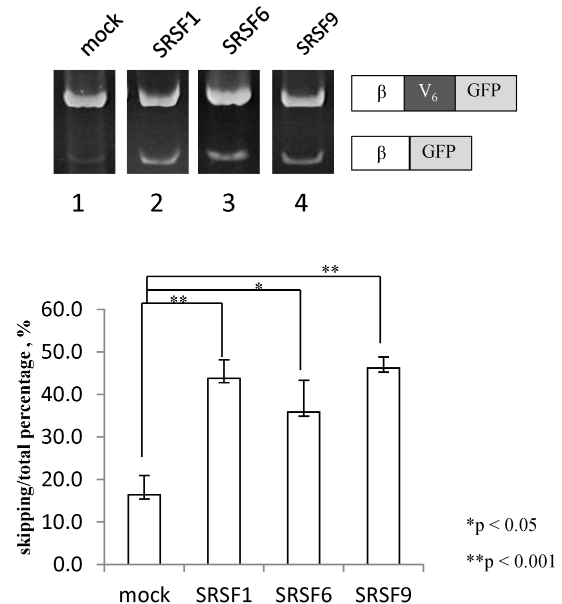

Fig. 2. SRSF1, SRSF6, and SRSF9 inhibit V_6 exon splicing. RT-PCR analysis using RNAs from pFlare-V_6 MCF7 cells treated with pcDNA3.1＋ or SRSF1 or SRSF6 or SRSF9. Quantitation results by Image J from three or more independent experiments are shown at the bottom. The significant change was evaluated by Student's *t-test*. Results are expressed as percentages of ratio skipping V_6 /total. The error bars represent the standard deviation of the repeats.

level (∼44%, ∼36% and ∼46% independently). Therefore, we conclude that SRSF1, SRSF6, and SRSF9 inhibit V_6 exon splicing.

Using a primer set that base pairs with constitutive exons could not detect the induction of the various exon-included isoforms obtained by reducing SR protein expression

We next wondered whether reduced expression of SR proteins could induce alteration of endogenous CD44 splicing. To address this question, we treated MCF7 cells using lentivirus-mediated shRNA and then extracted the RNA from cells. The standard primers that base pair with constitutive C_5 and C_6 exons were used to detect both the isoform that includes only constitutive exons (C) and the isoforms that include any variant isoform (V) (lower panel, Fig. 3). Consistent with the previously reported results (19, 20), RT-PCR results for CD44 splicing using these primers show that the C isoform was predominantly detected, whereas V isoforms were not detected (lane 1, Fig. 3). Moreover, non-silencing (NS) shRNA treatment did not induce any alteration of CD44 splicing (lane 2), suggesting that the NS shRNA can be used as a negative control. The results using shRNAs that target different SR proteins suggest that reduced expression of SRSF1, SRSF2, SRSF3, SRSF4, and SRSF9 did not induce production of any V isoform (lanes 3-7). The results are consistent with the conclusion that SRSF3 and SRSF4 did not affect CD44 splicing, but not with the conclusion that SRSF1, SRSF2, and SRSF9 regulate V_6 exon splicing.

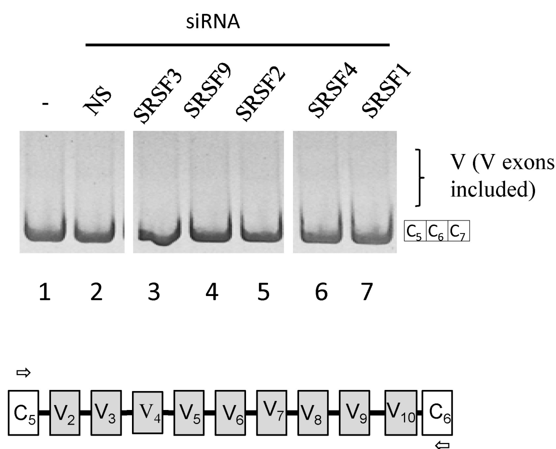

Fig. 3. Using a primer set that base pairs with constitutive exons could not detect the induction of the various exon-included isoforms obtained by reducing SR protein expression. (Upper panel) RT-PCR analysis using RNAs from cells treated with shRNA viruses that target SRSF3, SRSF9, SRSF2, SRSF4 or SRSF1. Non-silencing shRNA was used as a control. The identities of spliced products are shown at right. (Lower panel) primers used in RT-PCR analysis are shown with arrows.

Using V_6 exon-specific primer could detect the induction of various exon-included isoforms by reduced SRSF2

As the primers could not detect various isoform containing isoforms, we determined to use one primer that base pairs with V_6 exon and the other primer that base pairs with the C_6 exon (lower panel, Fig. 4). The primers could detect $V_{6\text{-}10}$ exon combinations, but not V_2-V_5. The Fig. 4 results show that the isoform that includes only V_6 isoform among $V_{6\text{-}10}$ was dominantly detected (V_6, lane 1). In addition, an isoform that includes V_6, V_7, V_8, V_9 and V_{10} ($V_{6\text{-}10}$) and an isoform that includes V_6, V_8, V_9 and V_{10} ($V_{6,8\text{-}10}$) were produced in less significant levels. We next asked whether reduced expression of SR proteins affects the expression of these CD44 isoforms. Fig. 4 results demonstrate that reduced expression of SRSF3 and SRSF1 caused a decrease of $V_{6\text{-}10}$ and $V_{6,8\text{-}10}$ isoforms (lanes 2 and 6). Moreover, reduced SRSF9 and SRSF4 expression did not induce a significant change of CD44 isoforms. Most significantly, reduced expression of SRSF2 induced decreased expression of V_6 but increased expression of both $V_{6\text{-}10}$ and $V_{6,8\text{-}10}$ expression. Our results suggest that SRSF2 is a key player in CD44 V_6 splicing.

DISCUSSION

CD44 pre-mRNA splicing is one of the most complicated splicing events in human genes. The CD44 pre-mRNA includes 10 constant exons, exons 1-5 (C_1-C_5) and 16-20 (C_6-C_{10}), and 10 various exons, exons 6-15 (V_1-V_{10}) (18-20). Here, we studied the function of SR proteins on V_6 exon splicing of CD44 pre-mRNA. First, in the overexpression of SR proteins into the pFlare-V_6- minigene-harboring MCF7 stable cell line, we demonstrated that SRSF1, SRSF6, and SRSF9 (but not SRSF3 and SRSF4) inhibit V_6 exon splicing. Next, we

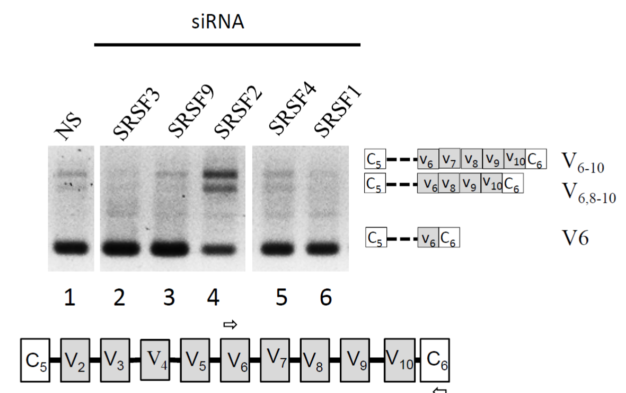

Fig. 4. Using V_6 exon-specific primer could detect the induction of various exon-included isoforms by reduced SRSF2. (Upper panel) RT-PCR analysis using RNAs from SR protein-targeting shRNA viruses treated cells. The identities of the spliced products are shown at right. (Lower panel) primers used in the RT-PCR analysis are shown with arrows.

analyzed the SR proteins function by reducing their expression levels through shRNA treatment. We found that, by using a primer set that base pairs with the constitutive exons of CD44 pre-mRNA, the changes of alternative splicing by SR proteins were not detectable. However, Using the primer that base pairs with the V_6 exon, we show that SRSF2-targeting shRNA decreased the V_6 isoform significantly, and also increased $V_{6\text{-}10}$ and $V_{6,8\text{-}10}$ isoforms. Our results indicate that CD44 V_6 splicing is regulated by SR proteins.

SR proteins have been known to function through binding to the enhancer to promote spliceosome assembly (21-23). Recently it was also reported that SR proteins can either promote or inhibit exon inclusion (24-26). In addition, using tethered SR proteins, it was demonstrated that splicing activation and repression by SR proteins depends on the location of their binding (27). Our results demonstrate that although all of the SR proteins we analyzed could potentially interact with V_6 exon and flanking introns, only some of them could inhibit V_6 splicing. Furthermore, the locations of SR protein binding did not affect their functions. The results can be explained that various potential binding locations of SR proteins on V_6 exon and flanking introns could possibly function through combinatory or synergistically. How these combination or synergistic effects regulate alternative splicing has not been well understood. One of our most striking results is that reduced SRSF2 expression could induce various V_6 exon-containing isoforms. Further study is need to determine the protein functions of these mRNA isoforms.

Our results indicate that the SR proteins, whose over-expression showed inhibitory effects on V_6 splicing, did not demonstrate significant effects as their expressions were reduced. This kind of quantitative differences have been reported in other studies (19, 20, 28). Another possibility is the assay systems in our experiments: While overexpression experiments were performed using a minigene-harboring stable cell line, shRNA treatments were performed by analyzing endogenous CD44 splicing. Another reason for differing results between the assay systems could be attributed to varying primer sequences used for analyzing V_6 splicing of CD44 pre-mRNA. The results indicate that various exons in CD44 pre-mRNA should be detected using a primer that base pairs with itself.

MATERIALS AND METHODS

Cell culture

MCF7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone) supplemented with antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin) and 10% of Fetal Bovine Serum (FBS; Hyclone) under humidified 5% CO2 conditions at 37°C. The stable pFlare-V_6 stable cell was obtained as previously described (18). Different SR proteins plasmids were transfected into the stable cells using polyethyleneimine (PEI) Reagent according to the manufacturer's protocol.

RT-PCR

Total RNAs were extracted using RiboEx reagent (GeneAll) following manufacturer's protocol. RT-PCR was conducted as previously described (19, 20). For the endogenous CD44 pre-mRNA splicing, RT-PCR was conducted as previously described (29). A specific primer, CD44RT (5′-ATG CAA ACT GCA AGA ATC-3′) was used for reverse transcription. The following primers were used to detect CD44 splicing: primers for detection of pFlare-V_6 stable cells [pFlareV_6 Fwd (5′-GGA AGA GTT GGT GGT GAG G-3′), pFlareV_6 Rev (5′-GGT GCA GAT GAA CTT CAG G-3′)], endogenous CD44 splicing [For (5′-AAG ACA TCT ACC CCA GCA AC-3′), Exon C_7 Rev (5′-TTT GCT CCA CCT TCT TGA CTC C-3′)], V_6 splicing [Fwd (5′-TCC AGG CAA CTC CTA GTA GT-3′), Exon C_7 Rev (5′-TTT GCT CCA CCT TCT TGA CTC C-3′)]. The endogenous RT-PCR products were confirmed by sequencing.

shRNA treatment

The shRNA lentivirus was prepared using different SR protein shRNA plasmid as previous described (19, 20). Knockdown of SR proteins was performed by treating cells with the virus for 72 h.

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