ORIGINAL

Skipping of an alternative intron in the *srsf1* 3' untranslated region increases transcript stability

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Abstract : The *srsf1* gene encodes serine/arginine-rich splicing factor 1 (SRSF1) that participates in both constitutive and alternative splicing reactions. This gene possesses two ultraconserved elements in the 3' untranslated region (UTR). Skipping of an alternative intron between the two elements has no effect on the protein-coding sequence, but it generates a premature stop codon (PTC)-containing mRNA isoform, whose degradation is considered to depend on nonsense-mediated mRNA decay (NMD). However, several cell lines (HCT116, RKO, HeLa, and WI38 cells) constitutively expressed significant amounts of the *srsf1* PTC variant. HCT116 cells expressed the PTC variant nearly equivalent to the major isoform that includes the alternative intron in the 3' UTR. Inhibition of NMD by silencing a key effecter UPF1 or by treatment with cycloheximide failed to increase amounts of the PTC variant in HCT116 cells, and the PTC variant was rather more stable than the major isoform in the presence of actinomycin D. Our results suggest that the original stop codon may escape from the NMD surveillance even in skipping of the alternative intron. The *srsf1* gene may produce an alternative splice variant having truncated 3' UTR to relief the microRNA- and/or RNA-binding protein-mediated control of translation or degradation. J. Med. Invest. 58 : 180-187, August, 2011

Keywords **:** *srsf1 gene, alternative splicing, premature stop codon, nonsense-mediated mRNA decay*

INTRODUCTION

In the human genome, >90% of primary transcripts undergo alternative splicing, which is a common mechanism for regulating the transcription of mRNA and increasing protein diversity (1, 2). The family of serine/arginine-rich splicing factor (SRSF) comprising at least 12 RNA-binding proteins (SRSF1 to SRSF12) has diverse roles in RNA processing including control of export, translation, stability, and constitutive and alternative splicing (3). Another interesting feature of the SRSF family is that every member possesses ultraconserved elements longer than 200 base pairs that are absolutely conserved between orthologous regions of the human, rat, and mouse genomes (4, 5). The ultraconserved elements are also alternatively spliced, either as alternative 'poison cassette exons' containing early inflame stop codons or as alternative introns in the 3' untranslated region (UTR) (4, 5).

Received for publication May 16, 2011 ; accepted May 18, 2011.

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SRSF1, originally named as ASF/SF2, is a well-studied member of the SRSF family participating in both constitutive and alternative splicing reactions (6, 7). Several recent studies have disclosed wide-ranging roles of SRSF1 in the regulation of transcription and mRNA metabolism, such as export (8), translation (9), and degradation (10). Since SRSF1 exerts these functions in a concentration-dependent manner, its expression should be kept in an appropriate level. Moderate (two- to threefold) overexpression of SRSF1 is sufficient to transform immortal rodent fibroblasts, rapidly forming sarcoma in nude mice (11). SRSF1 also shows abnormal expression in many tumors (11). Knockdown of SRSF1 resulted in genomic instability, cell-cycle arrest, and apoptosis (12), and mice deficient of SRSF1 showed the defective postnatal heart remodeling due to incorrect gene splicing (13).

The *srsf1* gene possesses two ultraconserved elements in the 3' UTR (4). Skipping of an alternative intron sandwiched between the elements in the 3' UTR generates a premature stop codon (PTC)-containing mRNA isoform whose degradation is considered to depend on nonsense-mediated mRNA decay (NMD) (4). NMD is one of the key RNA surveillance mechanisms to specifically degrade abnormal mRNA with PTC and prevent the harmful translation of truncated proteins from nonsense mutation, frameshift mutation, or aberrantly spliced mRNA (14). Expression of the *srsf1* PTC variant was estimated to be suppressed around 7% of all *srsf1* transcripts (4). However, we found that several cell lines expressed significant amounts of this PTC variant.

In the present study, using a colon cancer cell line (HCT116), we examined the mechanism for expression of the *srsf1* PTC variant and the physiological significance of alternative splicing in the 3' UTR.

MATERIALS AND METHODS

Cell culture

Human colon cancer cell lines (HCT116 and RKO) were cultured in McCoy's 5A medium supplemented with 5% (v/v) heat-inactivated fetal calf serum and antibiotics at 37°C in 5% CO_2. Dulbecco's modified Eagle medium and Eagle's minimum essential medium were used for cultivation of HeLa and WI38 cells, respectively.

Extraction of RNA and quantitative real-time reverse transcription PCR (qPCR)

Total RNA was isolated using Trizol reagent (Invitrogen). Contaminating DNA was removed with deoxyribonuclease (Ambion, Austin, TX). The quantity was measured by ND-1000 (Nanodrop, Wilmington, DE). cDNA was synthesized from 1 μg of total RNA with a PrimeScript RTase Synthesis kit (Takara, Shiga, Japan) using random and oligo (dT) primers. Target mRNA levels were measured by qPCR using power SYBR green PCR master mix (Applied Biosystems, Foster City, CA). Each PCR reaction was performed using the ABI 7500 real time PCR system (Applied Biosystems) with specific primer sets (Table 1), and data were analyzed using SDS 2.2 software (Applied Biosystems).

Table 1. List of primer sets used.

Targets	Sequences (5'-3')
Constitutive *srsf1* transcripts	AGGGAACAACGATTGCCGCATCTAC (forward)
	ATGTCGCGGATAGCGCCGTATTTGT (reverse)
srsf1 Reference isoform	ACAGATGAAATTGGCAGTATTGACC (forward)
	ATTTTGCCACAATTGCCAAGGTTTA (reverse)
srsf1 PTC isoform	ATAATGGAGGCAATGGTTTGGATTG (forward)
	TAAAAAAATCCACACGAATGCGGTT (reverse)
srsf1 3' UTR	GTGGTTATCTTACCTGGGGAAGTTC (forward)
	TAAAAAAATCCACACGAATGCGGTT (reverse)
upf1	GCTGAAGGAGTCCCAGACTCAA (forward)
	CCCTTTGTACCGCAGGCATATC (reverse)
gapdh	AGCCACATCGCTCAGACAC (forward)
	GCCCAATACGACCAAATCC (reverse)

Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) mRNA (NM_002046.3) was used as an endogenous quantity control. All PCR reactions were performed in the linear amplification range and in triplicate.

For measurement of microRNAs (miRNAs), stem-loop quantitative reverse transcription (RT) was performed with a TaqMan microRNA RT kit (Applied Biosystems), and then quantitative PCR for mature miRNAs was done with TaqMan microRNA assays (Applied Biosystems). The PCR primers for miR-7 (AB assay ID ; 000386), miR-200c (000505), and miR-214 (000517) were purchased from Applied Biosystems. Since we could not obtain any appropriate endogenous quantity control for miRNAs, amounts of miRNAs were expressed their threshold cycles (Ct values). Lower Ct values indicate higher expression levels.

RNA interference

For RNA interference, RNAiMAX (Invitrogen, Carlsbad, CA) was used to transfect cells with Stealth small interference RNA (siRNA) (Invitrogen) targeting *upf1* (NM_002911.3) or a control siRNA. We used two siRNAs targeting different sites of *upf1* mRNA. Sequences of these siRNAs are follows : UPF1 siRNA #1, 5'-GAGACAGUCCUGGAGUGCUACAACU-3' (exon 9-10 junction) ; UPF1 siRNA #2, 5'-UGACAGGAUGCAGAGCGCAUUGAAA-3' (exon 23). HCT116 cells were treated with 20 nM of each siRNA for the indicated times.

Anti-miR-200c (AM17000, ID AM11714), anti-miR-7 (ID AM10047), and a negative control (AM 17010) were purchased from Ambion (Austin, TX). HCT116 cells were transfected with one of these antisenses using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) following the manufacturer's protocol.

Immunoblot analysis

Whole-cell lysates were prepared in RIPA buffer (Thermo Scientific, Rockford, IL) containing a protease and phosphatase inhibitor mixture (Roche Applied Science, Indianapolis, IN). The extracted proteins (10-40 μg per lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidenedifluoride membrane (Bio-Rad, Hercules, CA). The membrane was blocked for 1 h at room temperature with 5% non-fat skim milk (Cell Signaling Technology, Danvers, MA) or 1% Block Ace powder (DS Pharma Biomedical, Osaka, Japan), and then incubated overnight at 4°C with an antibody against SRSF1 (1 : 500 ; Santa Cruz Biotechnology, Santa Cruz, CA), UPF1 (1 : 500 ; Bethyl, Montgomery, TX), or β-actin (1 : 5000 ; Abcam, Cambridge, MA). β-actin was used as a loading control.

Assessment of stability of srsf1 mRNA isoforms in HCT116 cells

We assessed the half-life of *srsf1* mRNA isoforms in the presence of a *de novo* gene transcription inhibitor, actinomycin D, and the rate of each mRNA decay was monitored by qPCR measurement. After HCT116 cells were treated with 5 μg/ml actinomycin D for the indicated times, total RNA was extracted, and *srsf1* mRNA isoform levels were measured by qPCR using the gene-specific primer pairs (Table 1). *gapdh* mRNA levels were measured as an endogenous quantity control. The levels of *srsf1* mRNA isoforms standardized by *gapdh* mRNA levels were plotted as the percentage of mRNA remaining, compared with the levels of the same mRNA at time zero.

RESULTS AND DISCUSSION

Various types of cells express an alternative splice isoform of srsf1 having a short 3' UTR

As shown in Fig. 1a, the *srsf1* gene contains 217- and 188-nucleotide (nt) ultraconserved elements in the 3' UTR (indicated by gray boxes). The major isoform, indicated as 'Reference isoform', encodes full-length SRSF1 and has a long 3' UTR (Fig. 1a). In the 3' UTR, an alternative intron (921 nt) is sandwiched between the two ultraconserved elements, and the *srsf1* gene produces an alternative splice isoform skipping this intron. In association with this alternative splicing event, the introduction of a new exon junction complex (EJC) > 50 nucleotides downstream of the original stop codon marks it as a premature stop codon (PTC) and targets the transcript for NMD. Therefore, we refer here this isoform harboring a short 3' UTR as 'PTC variant' (Fig. 1a).

It was reported that expression of the *srsf1* PTC variant was suppressed around 7% of all *srsf1* transcripts (4). However, several cell lines including colon cancer cells (HCT116 and RKO cells), HeLa cells, and human lung fibroblasts (WI38) expressed significant amounts of the *srsf1* PTC variant, which were detected by RT-PCR (Fig. 1b) and qPCR (Fig. 1c). In particular, HCT116 cells expressed the PTC variant nearly equivalent to the Reference isoform (Fig. 1c).

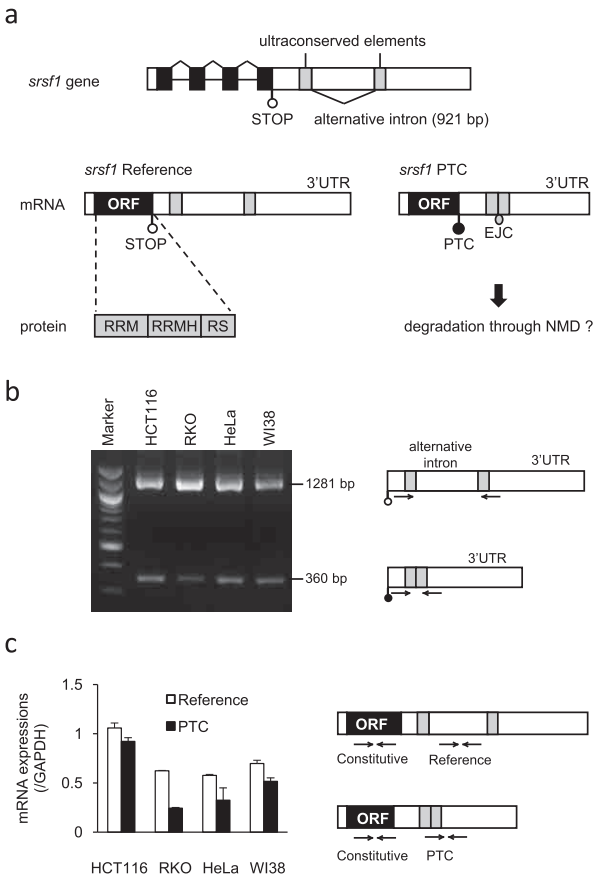

Fig. 1. Expression of *srsf1* mRNA isoforms.
(a) The *srsf1* gene contains two ultraconserved elements in the 3' UTR (indicated by gray boxes) and generates the major mRNA isoform (*srsf1* Reference) and a premature stop codon (PTC)-containing isoform (*srsf1* PTC) lacking an alternative intron between the two elements. A newly added exon junction complex (EJC) associated with this skipping marks the original stop codon (STOP) as PTC. The PTC variant is believed to be decomposed by NMD. (b) Total RNA was prepared from HCT116, RKO, HeLa, and WI38 cells. The alternative intron-containing Reference isoform and PTC isoform in these cells were detected using the specific primer sets. Forward and reverse primers are indicated by arrows in the right panel (primer sequences are shown in Table 1). (c) Levels of all transcripts (Constitutive), Reference isoform, and PTC isoform were also measured by qPCR using primer sets shown in the right panel. The sequences of those primers are listed in Table 1. Expression levels of Reference and PTC isoforms are shown in the left panel. Values are means± SD, n=3.

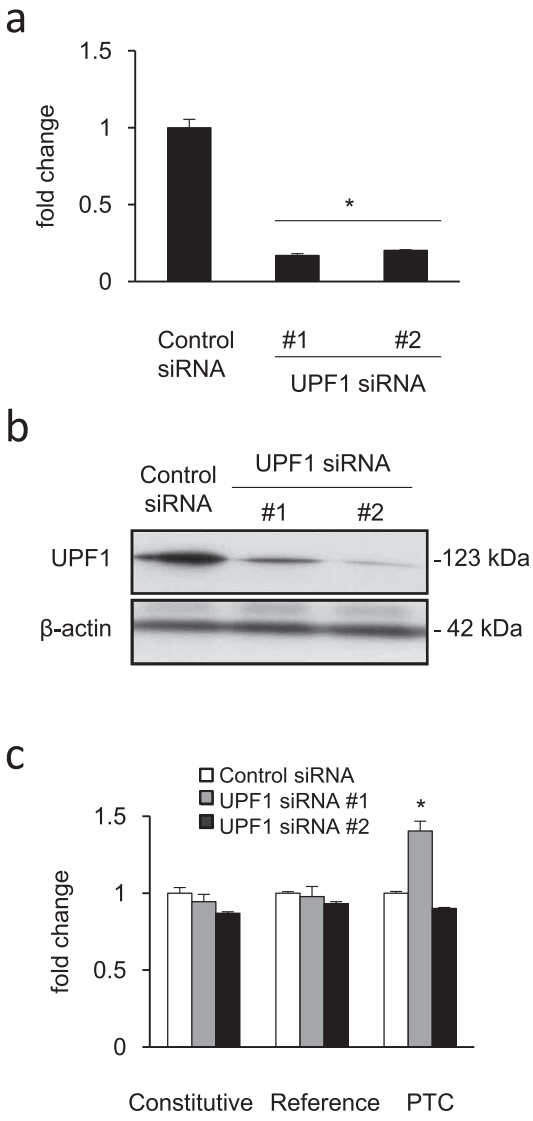

Fig. 2. Effects of UPF1 siRNAs on *srsf1* mRNA isoform expression.
After HCT116 cells were treated with 20 nM of UPF1 siRNA #1, UPF1 siRNA #2, or control siRNA for 72 h, total RNA was extracted and *upf1* mRNA levels were measured by qPCR using *gapdh* mRNA as an endogenous quantity control. Values are means± SD, n=3. *Significantly decreased compared with control siRNA-treated cells ($P<0.05$ by ANOVA and Scheffé's test). (b) Whole-cell proteins were prepared from HCT116 cells after treatment with 20 nM of UPF1 siRNA #1, UPF1 siRNA #2, or control siRNA for 72 h. Amounts of UPF1 were measured by Western blot analysis using β-actin as a loading control. (c) Total RNA was extracted from HCT116 cells treated with 20 nM of UPF1 siRNA #1, UPF1 siRNA #2, or control siRNA for 72 h, and amounts of all *srsf1* transcripts (Constitutive), Reference isoform, and PTC isoform in these cells were assayed by qPCR using the primer sets shown in Fig. 1c and *gapdh* mRNA as an endogenous quantity control. Values are means± SD, n=3. *Significantly different compared with control siRNA-treated cells ($P<0.05$ by ANOVA and Scheffé's test).

PTC-containing srsf1splice variant is resistant to NMD

Using HCT116 cells, we tested whether the alternative splicing event could be escaped from the NMD surveillance. We introduced siRNAs targeting two different sites of *upf1* mRNA that encodes a key effecter molecule of the NMD machinery, and examined whether NMD actively decomposed the PTC variant. Both UPF1 siRNAs #1 and #2 effectively down-regulated expression of *upf1* mRNA (Fig. 2a) and UPF1 protein (Fig. 2b). UPF1 siRNA #1 significantly increased the PTC variant, while UPF1 siRNA #2, which was more effective for reduction of UPF1 levels (Fig. 2b), failed to increase the PTC isoform levels (Fig. 2c). Next, NMD was

indirectly inhibited by treatment with 100 μg/ml cycloheximide, while any concomitant increase in the PTC variant levels was not observed (Fig. 3). These results suggest that the *srsf1* PTC variant is likely to escape from the NMD system.

Splicing and EJC components are involved in the mammalian NMD mechanism. EJCs are pointed out as the "marks" used to discriminate premature from normal termination. Several lines of evidence indicate that mammalian NMD is triggered during what has been called "the pioneer round of translation". During this initial round, EJCs are eliminated by translating ribosomes, and mRNAs with no remaining EJCs are free to undergo multiple rounds of translation (14). In the case of PTC located more than 50-54 nt upstream of the final EJC, the EJC will remain associated with the transcript, and a series of enzymes including UPF1/Rent1 helicase is recruited to decompose mRNA having PTC (14). Although the alternative splicing in the *srsf1* 3' UTR has no effect on the protein-coding sequence, it is conceivable that the introduction of a new EJC>50 nt downstream of the original stop codon marks the stop codon as premature and targets the transcript for NMD. However, recent data suggest that NMD can occur in the absence of downstream EJCs (14). A positional effect of PTC in NMD triggering is generally observed in all organisms : the closer the PTCs are to the 3' end of the mRNA, the lesser the sensitivity of the transcript to NMD. Moreover, it has been suggested that NMD is triggered as a consequence of two antagonistic signals that can act on the translation termination event : UPF1 promotes NMD activation and cytoplasmic poly(A)-binding protein 1 (PABPC1) promotes a normal termination event, acting as an NMD repressor (14). If PABPC1 is favorably located to interact with the termination complex, it impairs the association of UPF1, repressing NMD triggering. Thus, multiple mechanisms are likely to be involved in PTC definition. Our results suggest that the original stop codon, marked as PTC by the downstream EJC, is likely not to become a target for NMD.

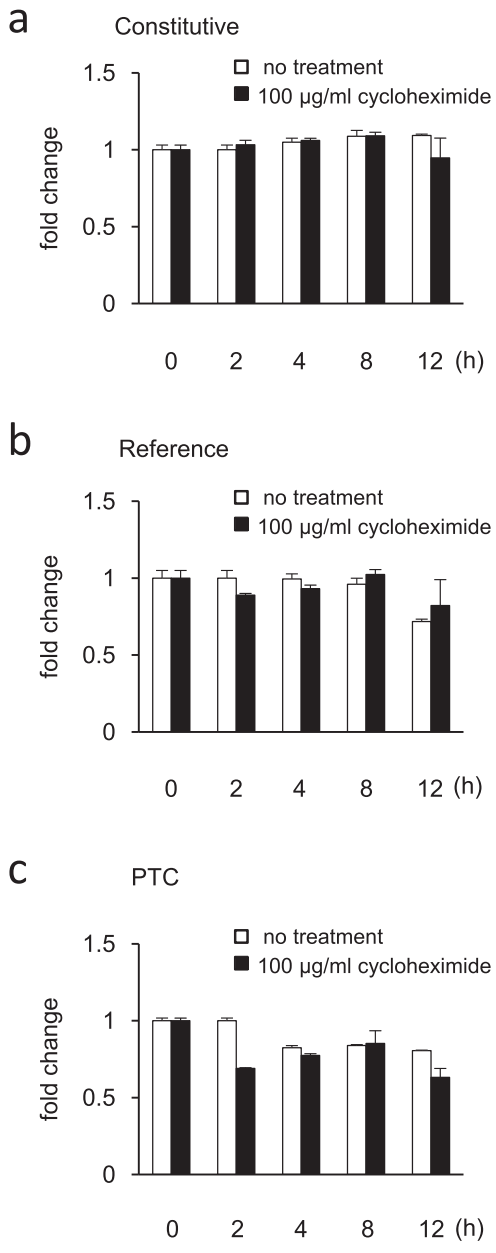

Fig. 3. Effects of cycloheximide on *srsf1* mRNA isoform expression.
After HCT116 cells were untreated or treated with 100 μg/ml cycloheximide for the indicated h, total RNA was extracted, and amounts of all *srsf1* transcripts (Constitutive), Reference isoform, and PTC isoform in these cells were assayed by qPCR as described in the legend to Fig. 2. Values are means ± SD, n=3.

PTC-containing srsf1 mRNA isoform is more stable than a major mRNA isoform

The *srsf1* 3' UTR encodes several elements targeted by miRNAs and RNA-binding proteins, both of which can regulate translation and stability. To understand the physiological significance of the PTC-containing *srsf1* isoform harboring a short 3' UTR, we first compared stability between the Reference and PTC isoforms.

The stability of *srsf1* Reference and PTC variant isoforms was estimated by measuring these mRNA levels after treatment with 5 μg/ml actinomycin D for the indicated times by qPCR using *gapdh* mRNA an endogenous quantity control. As shown in Fig. 4, the PTC variant was more stable than the Reference isoform having a long 3' UTR. Thus, the skipping of the alternative intron in the srsf1 3' UTR rather increased the transcript stability.

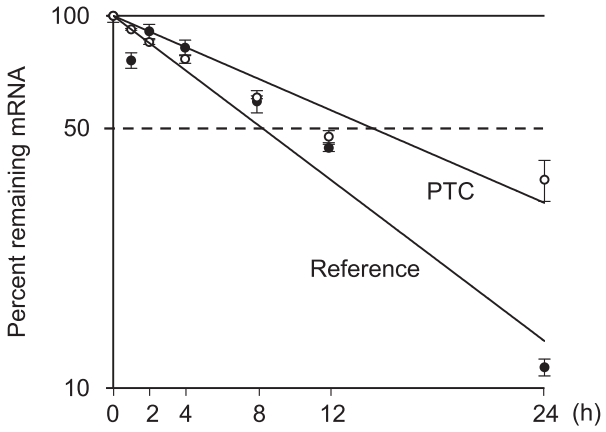

Fig. 4. Comparison of stability between *srsf1* Reference and *srsf1* PTC isoforms. HCT116 cells were treated with 5 μg/ml actinomycin D for the times indicated. Amounts of *srsf1* Reference and *srsf1* PTC isoforms remained in these cells were measured by qPCR using *gapdh* mRNA as an endogenous quantity control. Using a semilogarithmic scale, the half-lives were calculated as the time required for each mRNA decrease to 50% of its initial abundance (discontinuous horizontal line). The half lives of *srsf1* Reference and *srsf1* PTC isoforms were determined to be around 8 and 15 h, respectively. The results represent from duplicate experiments.

Alternative intron in the srsf1 3' UTR contains target sequences for miRNAs

Next, we investigated possible interactions between the alternative intron in the *srsf1* 3'UTR and miRNAs responsible for the decreased stability of the major (Reference) mRNA isoform harboring a long 3' UTR. The sequence of the 921 nt region and putative miRNA target sites identified using TargetScanHuman 5.1 (http : //www.targetscan.org/) are shown in Fig. 5. TargetScanHuman 5.1 software picked up target sites for miR-200c, miR-7, and miR-214. Each target motif is underlined. miRNAs either inhibit translation of target mRNAs or facilitate deadenylation and subsequent degradation, and the level of miRNA-mediated repression depends on the ratio of a particular mRNA target relative to miRNA (15, 16).

Based on the above information, we measured expression levels of these 3 miRNAs by qPCR.

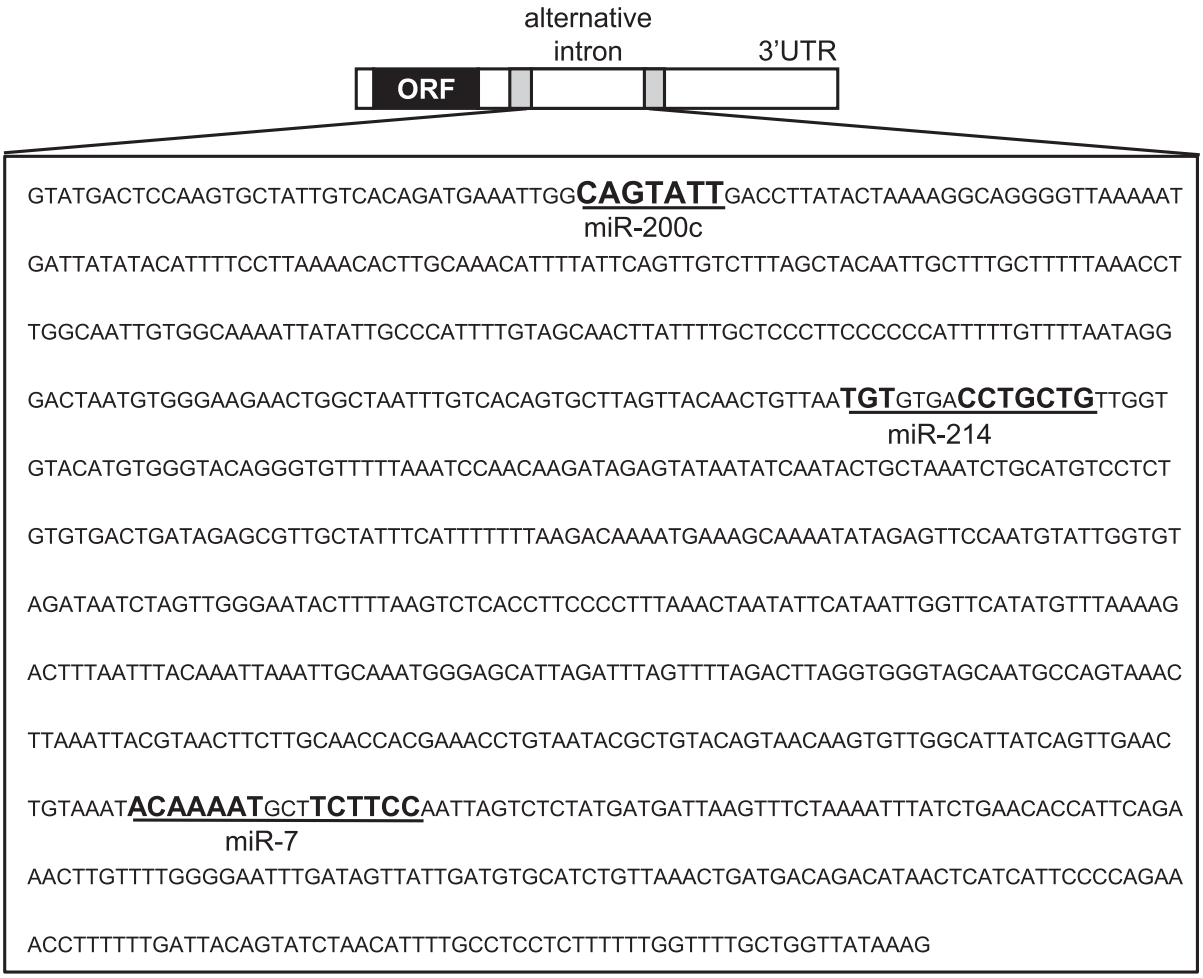

Fig. 5. Expression of miRNAs targeting an alternative intron in the *srsf1* 3' UTR.
Nucleotide sequence of the alternative intron in the *srsf1* 3' UTR. Putative target sites for miRNAs are identified using TargetScanHuman 5.1 (http : //www.targetscan.org/) and underlined. Conserved nucleotides are indicated in bold.

HCT116 cells contained relatively abundant miR-200c with Ct values of 26.35± 0.01 (mean± SD, n= 3) and miR-7 (Ct values, 28.62± 0.07), while only a small amount of miR-214 (Ct values, 33.18± 0.01) was expressed in these cells. We therefore focused on miR-200c and miR-7 and introduced an anti-miR-200c or anti-miR-7. As shown in Fig. 6, anti-miR-200c could reduce miR-200c levels to below 30%

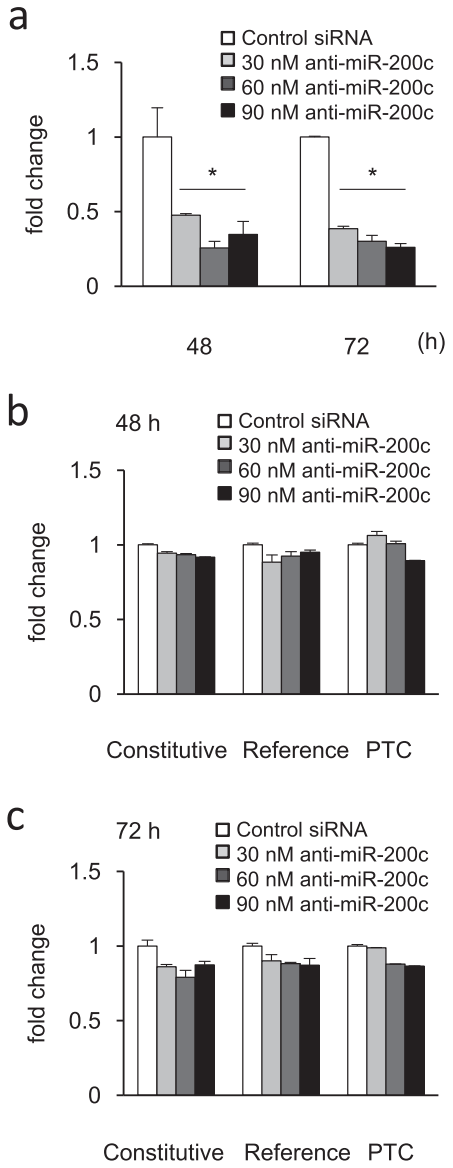

Fig. 6. Effects of miR-200c silencing on *srsf1* isoform expression.
HCT116 cells were treated with different concentrations of anti-miR-200c or control siRNA for 48 h or 72 h. Total RNA were extracted, and *srsf1* mRNA levels were measured by qPCR using *gapdh* mRNA as an endogenous quantity control. Values are means± SD, n=3. *Significantly different compared with control siRNA-treated cells ($P< 0.05$ by ANOVA and Scheffé's test). (b) (c) Amounts of all *srsf1* transcripts (Constitutive), Reference isoform, and PTC isoform in HCT116 cells treated with different concentrations of anti-miR-200c or control siRNA for 48 h (b) or 72 h (c) were assayed by qPCR using *gapdh* mRNA as an endogenous quantity control. Values are means± SD, n=3.

(Fig. 6a), while treatment with 30-90 nM of anti-miR-200c for 48 h (Fig. 6b) or 72 h (Fig. 6c) did not increase the major isoform (Reference) having a long 3' UTR. We also examined the effect of anti-miR-7, but it was not effective to inhibit miR-7 (data not shown). These results indicate that miR-200c may not be involved in the regulation of the stability of the major *srsf1* mRNA isoform, and suggest the possible involvement of RNA-binding proteins as well as the other miRNAs in the stability. Further studies are necessary to address this issue.

The regulation of mRNA stability is one of the important steps for controlling gene expression at the posttranscriptional level. The 3' UTR encodes several elements targeted by miRNAs and RNA-binding proteins, both of which play crucial roles in the regulation of translation and stability. It is possible to speculate that the *srsf1* gene may produce an alternative splice variant having the truncated 3' UTR to relief miRNA-mediated translational repression or degradation. SRSF1 is overexpressed in many tumors (12), and proliferating cells express mRNAs with shortened 3' UTRs and fewer miRNA target sites (17). These reports together with our finding suggest an important role of the *srsf1* splice isoform with a short 3' UTR in tumor growth.

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