ORIGINAL

# Truncated serine/arginine-rich splicing factor 3 accelerates cell growth through up-regulating c-Jun expression

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**Abstract : Serine/arginine-rich splicing factor 3 (SRSF3), a member of the SRSF family, plays a wide-ranging role in gene expression. The human *SRSF3* gene generates a major mRNA isoform encoding a functional, full-length protein and a PTC-containing isoform (*SRSF3-PTC*). The latter is expected to be degraded through the nonsense-mediated mRNA decay system. However, it was reported that *SRSF3-PTC* mRNA was produced under stressful conditions and translated into a truncated SRSF3 protein (SRSF3-TR). To disclose unknown functions of SRSF3-TR, we established Flp-In-293 cells stably expressing SRSF3-TR. The SRSF3-TR-expressing cells increased mRNA and protein levels of positive regulators for G1 to S phase transition (cyclin D1, cyclin D3, CDC25A, and E2F1) and accelerated their growth. c-Jun is required for progression through the G1 phase, the mechanism by which involves transcriptional control of the *cyclin D1* gene. We also found that the *JUN* promoter activity was significantly increased in the Flp-In-293 cells stably expressing SRSF3-TR, compared with mock-transfected control cells. The SRSF3-TR-expressing cells increased c-Jun and Sp-1 levels, which are important for the positive autoregulation and basal transcription of *JUN*, respectively. Our results suggest that stress-inducible SRSF3-TR may participate in the acceleration of cell growth through facilitating c-Jun-mediated G1 progression under stressful conditions. J. Med. Invest. 60 : 228-235, August, 2013**

***Keywords*** **:** *truncated SRSF3 protein, oxidative stress, cell growth, c-Jun*

## INTRODUCTION

The serine/arginine-rich splicing factor (SRSF) family comprises at least 12 conserved and structurally related RNA-binding proteins (SRSF1 to SRSF12) (1). SRSF3 (also known as SRp20) binds *cis*-acting RNA elements through an N-terminal RNA recognition motif (RRM), and a C-terminal region enriched in Ser-Arg dipeptides (SR domain) mediates interactions with other proteins for regulation of both constitutive and alternative splicing of pre-mRNA (2). In addition, several lines of recent

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evidence suggest that SR protein family also regulates RNA elongation (3), transport (4), and translation (5), suggesting a wide-ranging role in gene expression. In addition, SRSF3 is overexpressed in human ovarian cancer, and its knockdown results in apoptosis of ovarian cancer cells (6). Recently, we have shown that reduction of SRSF3 causes G1 cell cycle arrest and apoptosis in colon cancer cells (7). These findings suggest that SRSF3 may be one of the essential factors for regulation of cell fate.

The human *SRSF3* gene is composed of 7 exons and generates two mRNA isoforms. Like other SRSF family member genes, the human *SRSF3* gene contains an element of extreme sequence conservation, termed "ultraconserved element" (UCE). *SRSF3* contains a 577-bp UCE that includes entire exon 4, and this exon 4 has multiple premature termination codons (PTCs). A major *SRSF3* mRNA isoform encoding a functional full-length SRSF3 protein (SRSF3-FL) excludes this exon 4. Alternative splicing of *SRSF3* pre-mRNA produces another mRNA isoform consisting of entire 7 exons. This PTC-containing *SRSF3* isoform (*SRSF3-PTC*) is considered to be degraded through the nonsense-mediated mRNA decay (NMD) system, a surveillance mechanism that decomposes PTC-containing mRNAs. However, it was reported that *SRSF3-PTC* mRNA was translated into a truncated SRSF3 protein (SRSF3-TR) partially lacking RS domain in the murine B-cell lymphoma K46 cells (8). We also found that colon cancer cells (HCT116) induced SRSF3-TR in response to sodium arsenite (unpublished observations). At present, however, the biological function of SRSF3-TR is unknown.

In this study, we established Flp-In-293 cells stably expressing SRSF3-TR, and showed that SRSF3-TR facilitated cell growth through up-regulating c-Jun expression.

## MATERIALS AND METHODS

### *Cell culture and cell counting*

Flp-In-293 cells (Invitrogen, Carlsbad, CA) were maintained in high-glucose Dulbecco's modified Eagle's medium (Nacalai tesque, Kyoto, Japan) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% $CO_2$ in air. Numbers of growing cells were counted manually using a hematocytometer.

### *Plasmid construction*

Using a cDNA library prepared from a human colon cancer cell line (HCT116), the human *SRSF3-PTC* mRNA (Ensembl Transcript ID : ENST00000477442) were amplified by PCR using a primer set : 5'-AAAAAAGGATCCATGCATCGTGATTCCTGTCCATTG-3' (forward, *BamHI* site is underlined) and 5'-AAAAAAGATATCCTATTTCCTTTCATTTGACCTAGA-3' (reverse, *EcoRV* site is underlined). The amplified product was cloned into the mammalian expression vector pCMV-3Tag (pCMV-3Tag/SRSF3-TR ; Stratagene, La Jolla, CA). FLAG was appended to the $NH_2$ termini of SRSF3. Subsequently, PCR was performed using the pCMV-3Tag/SRSF3-TR plasmid as the template and the following primer set : 5'-AAAAAAGGTACCGCCACCATGGATTACAAGGA-3' (forward, *KpnI* site is underlined) and the same reverse primer used for amplification of *SRSF3-PTC* mRNA. The amplified products were cloned into the pcDNA5/FRT vector (pcDNA5/FRT-3xFLAG-SRSF3-TR). All constructs were confirmed to have the expected sequence by DNA sequencing. The plasmid was transfected using FuGENE HD (Promega, Madison, WI) according to the manufacturer's instructions.

### *Establishment of a cell line stably expressing SRSF3-TR*

Flp-In-293 cells were co-transfected with the pcDNA5/FRT-3xFLAG-SRSF3-TR vector and a Flp recombinase expression plasmid pOG44 according to the manufacturer's protocol of the Flp-In system (Invitrogen). A single colony resistant to hygromycin B (Invitrogen) was selected and subcultured. Selection of positive colonies (293/SRFS3-TR) was performed by Western blotting using an anti-SRSF3 antibody. Flp-In-293 cells were transfected with pcDNA5/FRT-3xFLAG-mock and pOG44 vectors (293/mock cells) and used as a control.

### *Quantitative real-time reverse transcription-PCR (qPCR)*

Total RNA was extracted from 293/SRSF3-TR or 293/mock cells using an RNAiso plus (Takara, Otsu, Japan). One microgram of isolated RNA was reverse-transcribed using ReverTra Ace reverse transcriptase (TOYOBO, Osaka, Japan). Primer sequences are shown in Table 1. Target mRNA levels were measured using SYBR green master mix and the 7500 real-time system (Applied Biosystems, Foster City, CA). *Glyceraldehyde 3-phosphate*

Table 1. Primer sets used for quantitative real-time RT-PCR (qPCR)

| Targets | | Primer Sequences (5' - 3') |
|---|---|---|
| *SRSF3-PTC* | forward | TCCACCTCGTCGCAGAGTCACCATC |
| | reverse | TCATGTGAAACGACACCAGCCAAGC |
| *CCND1* | forward | GAAGATCGTCGCCACCTG |
| | reverse | GACCTCCTCCTCGCACTTCT |
| *CCND3* | forward | GCTTACTGGATGCTGGAGGTA |
| | reverse | AAGACAGGTAGCGATCCAGGT |
| *CCNE1* | forward | GGCCAAAATCGACAGGAC |
| | reverse | GGGTCTGCACAGACTGCAT |
| *CDC25A* | forward | ATCTCTTCACACAGAGGCAGAA |
| | reverse | CCCTGGTTCACTGCTATCTCTT |
| *E2F1* | forward | TCCAAGAACCACATCCAGTG |
| | reverse | CTGGGTCAACCCCTCAAG |
| *JUN* | forward | CAGGTGGCACAGCTTAAACA |
| | reverse | GTTTGCAACTGCTGCGTTAG |
| *GAPDH* | forward | AGCCACATCGCTCAGACAC |
| | reverse | GCCCAATACGACCAAATCC |
| *18S* | forward | CCCTATCAACTTTCGATGGTAGTCG |
| | reverse | CCAATGGATCCTCGTTAAAGGATTT |

*dehydrogenase* (*GAPDH*) mRNA and *18S* were measured as internal controls for normalization. Using the ΔΔCt method, data are presented as fold changes in mRNA levels relative to those in 293/mock cells.

*Western blotting*

Whole-cell lysates were prepared in RIPA buffer (10 mM Tris-HCl, pH 7.4 ; 150 mM NaCl ; 1 mM EDTA ; 1 mM DTT ; 0.1% sodium dodecyl sulfate ; 1% Nonidet P-40) containing a protease and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The extracted proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (BioRad, Hercules, CA). After blocking with 5% non-fat dry milk, the membranes were incubated overnight at 4°C with a mouse monoclonal anti-SRSF3 (1 : 1000 dilution, Sigma-Aldrich), anti-FLAG (1 : 1000, Sigma-Aldrich), anti-cyclin D1 (1 : 1000, Cell Signaling Tech., Danvers, MA), anti-cyclin D3 (1 : 1000, Cell Signaling Tech.), anti-cyclin E (1 : 1000, Santa Cruz Biotech., Santa Cruz, CA), anti-CDC25A (1 : 1000, Cell Signaling Tech.), anti-E2F1 (1 : 1000, Cell Signaling Tech.), anti-c-Jun (1 : 1000 ; Cell Signaling Tech.), anti-Sp1 (1 : 1000, Sigma-Aldrich), or anti-GAPDH (1 : 5000 ; Santa Cruz Biotech.) antibody. Following incubation with an appropriate secondary antibody for 1 h at room temperature, bound antibodies were detected with an ECL or ECL Prime Western Blotting Detection System (GE Healthcare, Piscataway, NJ). The membrane was re-blotted using an anti-GAPDH antibody as a loading control.

*Promoter activity assay*

The 5' flank of the human JUN gene was cloned into the pGL3-basic luciferase reporter vector (Promega). In brief, the first PCR was performed using the human genomic DNA as a template. The *JUN* proximal promoter region (from -1,266 to +170 bp) was amplified using the following primer set : 5'-AAAAAGGTACCTTACATGTGTCTAGCGCTTC-3' (forward) and 5'-AAAAACTCGAGGGGAGCCA-CAGGCGCTAGCTCT-3' (reverse). Subsequently, the amplified product was used as a template to generate deletion constructs consisting of the -798 to +170 bp, -331 to +170 bp, and -32 to +170 bp regions using the same reverse primer and one of the following forward primers : 5'-AAAAAGGTACCTC-TCGGCTTCTACGAGCAGC-3' for -798 to +170 bp, 5'-AAAAAGGTACCCCAAGACGTCAGCCCACAA-TG-3' for -331 to +170 bp and 5'-AAAAAGGTACC-AGCCAATGGGAAGGCCTTG-3' for -32 to +170 bp. The amplified products were subcloned into the pGL3-basic vector using KpnI and SacI restriction sites. Using FuGENE HD (Promega), pGL-3 luciferase constructs (100 ng) and pRL-CMV vector (50 ng) were co-transfected into 293/mock or 293/SRFS3-TR cells ($7.5 \times 10^4$ cells) growing on 24-well plates. Twenty-four hours after the transfection, cells were harvested, and the firefly and renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega).

## RESULTS

*Effect of SRSF3-TR on cell growth*

To disclose novel function(s) resided in a truncated SRSF3 (SRSF3-TR), we established a cell line stably expressing FLAG-tagged SRSF3-TR using the Flp-In system (Invitrogen). After transfection of Flp-In-293 cells with the vectors encoding 3xFLAG-SRSF3-TR and Flp recombinase, a positive clone (293/SRSF3-TR) was selected. The 293/SRSF3-TR cells stably expressed similar amounts of both endogenous SRSF3-FL and FLAG-tagged SRSF3-TR (Figure 1A). The stable expression of

SRSF3-TR did not change endogenous SRSF3-FL levels (Figure 1A). Unexpectedly, SRSF3-TR-expressing cells showed significantly accelerated growth, compared with control 293/mock cells (Figure 1B).

*Effects of SRSF3-TR on expression of genes crucial for G1 to S phase transition*

In a previous study, we showed that SRSF3 knockdown caused G1 cell cycle arrest through inhibition of cyclin D/CDK4/6 activation and reduction of E2F1 expression (7). Based on the data, we compared mRNA and protein levels of cyclins (D1, D3, and E1), E2F1, and CDC25A between 293/SRSF3-TR and 293/mock cells. As shown in Figure 2, SRSF3-TR-expressing cells significantly up-regulated expression of *CCND1*, *CCND3*, *CCNE1*, *E2F1*, and *CDC25A* mRNAs (Figure 2A) and increased amounts of cyclin D1, cyclin D3, CDC25A, and E2F1 (Figure 2B). The first kinase complexes to become active in G1 phase are cyclin D/CDK4/6 that initiate phosphorylation of retinoblastoma (RB) proteins and release E2F1 from the RB-repressor complex, permitting transcription essential for the G1/S transition. Induction of cyclin D1 is important for the activation of cyclin D/CDK4/6. We, therefore, focused on the mechanism how SRSF3-TR increased expression of cyclin D1. Several lines of evidence suggest that c-Jun is one of the crucial factor for cyclin D1 expression (9, 10). In fact, 293/SRSF3-TR cells increased *JUN* mRNA (Figure 3A) and c-Jun protein (Figure 3B) levels, compared with 293/mock cells. We also confirmed that the stable expression of SRSF3-TR did not change the stability of *JUN* mRNA, suggesting that SRSF3-TR might activate transcription of the *JUN* gene.

*Effects of SRSF3-TR on JUN promoter activity*

To test whether SRSF3-TR regulated c-Jun expression at the transcriptional level, we cloned the 5'-flank of *JUN* (from -1,266 to +170 bp), and 293/SRSF-TR or 293/mock cells were transfected with a luciferase reporter construct containing -1,266/+170, -798/+170, -331/+170, or -32/+170 bp region of the human *JUN* promoter (Figure 5A). As shown in Figure 4A, 293/SRSF3-TR cells significantly increased the luciferase reporter activity of the -1,266/+170 and -798/+170 bp regions of the *JUN* promoter, compared with 293/mock cells. The -331/+170 bp construct partially reduced the promoter activity in 293/SRSF3-TR cells, and the -32/+170 bp region almost completely lost the promoter activity (Figure 4A). The -331/-32 bp region contains two AP-1-binding sites (-190 to -183 and -71 to -64) (Figure 4C), and the proximal AP-1-binding

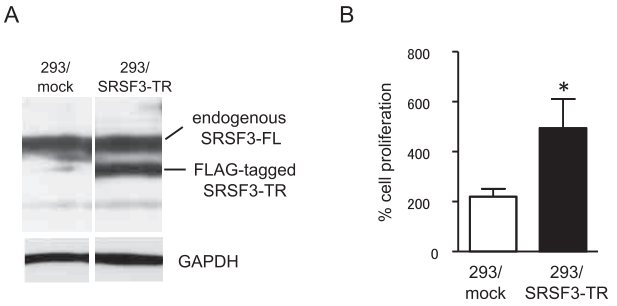


Figure 1. Cell proliferation of 293/SRSF3-TR cells.
(A) Flp-In-293 cells stably expressing FLAG tagged-SRSF3-TR (293/SRSF3-TR) or mock (293/mock) were established using the Flp-In system as described in Materials and Methods. Whole-cell lysates were prepared from them and subjected to Western blotting using the SRSF3 antibody. GAPDH was served as a loading control. (B) Numbers of growing cells in 35 mm cloture dishes were counted on day 4 after plating. Values represent means± SD, n=3. *$P<0.05$ by ANOVA and Bonferroni test.

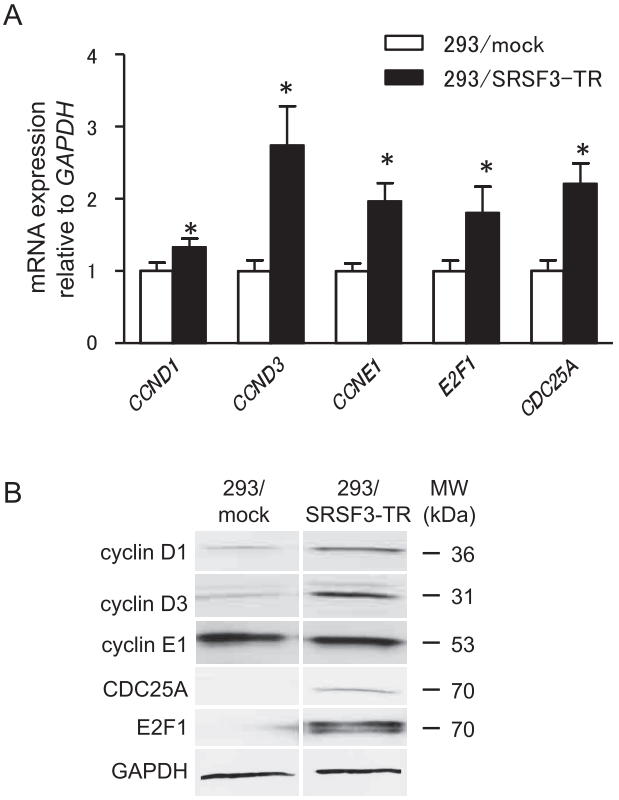


Figure 2. Expression of G1 to S phase transition regulators in 293/SRSF3-TR cells.
(A) Levels of *CCND1*, *CCND3*, *CCNE1*, *CDC25A*, and *E2F1* mRNAs in 293/SRSF3-TR cells were measured by qPCR using *GAPDH* mRNA as an endogenous quantity control. Values (means± SD, n=3) are expressed as fold changes, compared with those of 293/mock cells. *$P<0.05$ by ANOVA and Bonferroni test. (B) Amounts of cyclins (D1, D3, and E1), CDC25A, and E2F1 proteins in these cells were measured by Western blotting using GAPDH as a loading control.

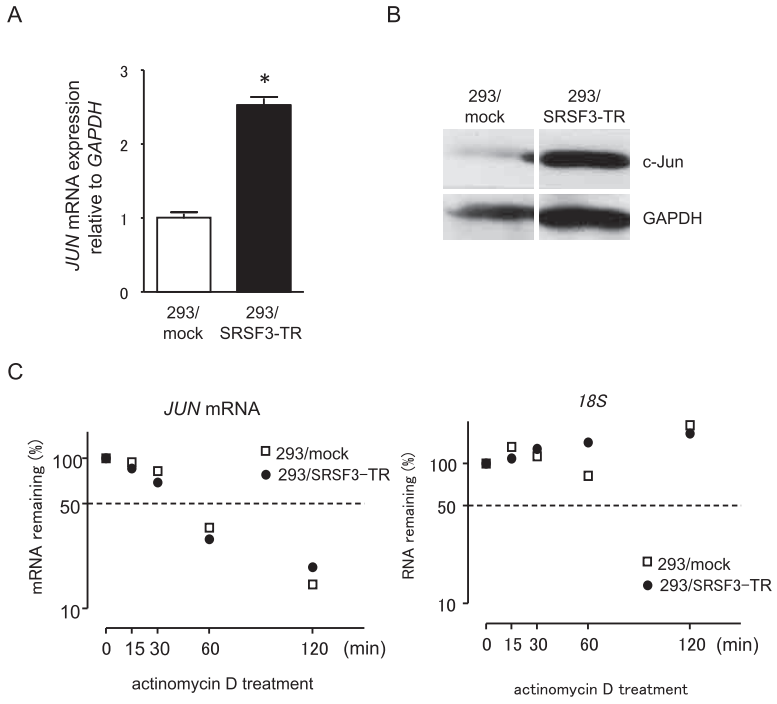


Figure 3. Up-regulation of c-Jun expression in 293/SRSF3-TR cells.
(A) Levels of *JUN* mRNA in 293/SRSF3-TR cells were measured by qPCR using *GAPDH* mRNA as an endogenous quantity control. Values (means± SD, n=4) are expressed as fold changes, compared with those of 293/mock cells. **P*< 0.05 by ANOVA and Bonferroni test. (B) Amounts of c-Jun proteins were measured by Western blotting using GAPDH as a loading control. (C) To compare the stability of *JUN* mRNA between 293/mock (□) and 293/SRSF3-TR (●) cells, both cells were incubated with 2.5 μg/ml actinomycin D for the indicated times. Amounts of *JUN* mRNA remained were measured by qPCR. Amounts of *18S* were also measured as a control.

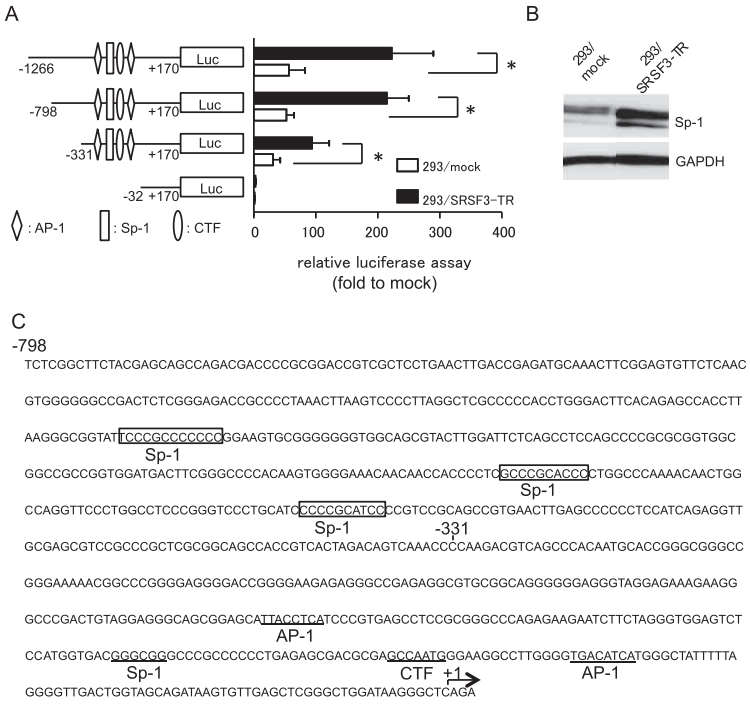


Figure 4. *JUN* promoter activity in 293/SRSF3-TR cells.
(A) Luciferase reporter plasmids (pGL3) driven by 1,266-, 798-, 331-, 32-bp *JUN* promoter fragments were transfected into 293/SRSF3-TR and 293/mock cells. After transfection for 24 h, these cells were harvested, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System. Values are expressed as fold changes (means± SD, n=6). **P*< 0.05 by ANOVA and Bonferroni test. (B) Amounts of Sp-1 proteins were measured by Western blotting using GAPDH as a loading control. (C) Nucleotide sequence of the 5'-flanking region of the human *JUN* gene. Putative binding sites for Sp-1, CTF and AP-1 are indicated with underlines. Sp-1 predicted binding sites between -798 and -332 bp based on the JASPER database (relative profile score threshold 85%) are shown by a square. Putative transcription initiation site is indicated by a bent arrow.

site is responsible for *trans*-activation of the JUN promoter by its gene product (11). Sp-1 is also known as a crucial transcription factor for *JUN* (12), and several Sp-1-binding sites are distributed in both -331/-32 bp and -798/-332 bp regions. Moreover, 293/SRSF3-TR significantly induced Sp-1 (Figure 4B), suggesting that multiple *cis* elements distributed in the -798/-32 bp region might be involved in the SRSF3-TR-mediated *JUN* promoter activity.

## DISCUSSION

Alternative splicing is important for generation of protein isoform diversity and for regulation of gene expression program. At the same time, however, up to one-third of alternative splicing of human genes generates PTC-containing mRNAs (13). All SRSF family member genes produce PTC-containing splice variants, which is coincided with the presence of UCEs (14). To avoid production of the C-terminally truncated proteins which may have potential dominant-negative or gain-of-function, PTC-containing mRNAs are expected to be degraded by NMD. However, several lines of evidence have shown that NMD activity is often inhibited by stresses, such as hypoxia, amino acid starvation, and generation of reactive oxygen species (15-17). Moreover, it has been shown that the impairment of NMD is involved in the oncogenic mechanism (18). In fact, several cancer cell lines (HCT116, RKO, and HeLa cells) constitutively expressed significant amounts of the *SRSF1* PTC variant. For example, HCT116 cells expressed the *SRSF1* PTC variant nearly equivalent to the major isoform (data not shown). These PTC variants are believed not to be translatable, whereas SRSF3-TR was documented to be translated from *SRSF3-PTC* mRNA (8). We also found that treatment of HCT116 cells with sodium arsenite induced SRSF3-TR possibly through inhibiting NMD (unpublished observations). The inclusion of exon 4 to mature SRSF3 mRNA was reported to occur under serum starvation (8), and the biological significance of exon 4-inclusion is considered to be an auto-regulatory feedback loop by SRSF3 itself (19).

To uncover unknown functions of SRSF3-TR, we established a cell line stably expressing SRSF3-TR using the Flp-In system, and found that SRSF3-TR-expressing cells (293/SRSF3-TR) significantly accelerated their growth, compared with control cells (293/mock). As to the mechanism for the accelerated cell growth, 293/SRSF3-TR significantly increased mRNA and protein levels of essential regulators for the G1 to S phase transition, which included cyclin D1, cyclin D3, CDC25A, and E2F1. c-Jun is required for progression through the G1 phase, the mechanism by which involves transcriptional control of the *cyclin D1* gene (*CCND1*). We also confirmed that SRSF3-TR-expressing cells significantly increased expression of *JUN* mRNA and induced c-Jun protein without any growth stimulus. c-Jun positively regulates cell proliferation through the induction of *CCND1* transcription and the repression of tumor suppressor gene expression and function (20). The regulatory elements crucial for the human *CCND1* transcription contain two AP-1 binding sites and several AP-1 proteins including c-Jun and c-FOS were shown to bind these sites and suggested to stimulate *CCND1* transcription (21-23). Along with this line, 293/SRSF3-TR cells exhibited a significantly augmented promoter activity of *JUN*. The proto-oncogene *JUN* is positively autoregulated by its product, c-Jun/AP-1 (11). It was reported that two AP-1-binding sites (-182 and -64) of the JUN promoter were important for basal activity and dexamethasone-mediated repression of the *JUN* promoter (24). The proximal -71 to -64 bp region is responsible for the autoregulation of the *JUN* promoter activity (11). Our luciferase reporter assay also indicated the importance of the -331 to -32 bp region for both basal and SRSF3-TR-dependent activities of the *JUN* promoter. At the same time, however, our results implicated that there might be SRSF3-TR responsive element(s) in the -798 to -332 bp region. In addition to c-Jun, Sp-1 plays an essential role for basal expression of *JUN* (12). The -331/-32 bp region of the *JUN* promoter contains one Sp-1-binding site, and several putative Sp-1-binding sites are scattered in the -798 to -332 bp region. Moreover, Sp-1 was induced in 293/SRSF3-TR cells. Thus, multiple *trans* factors and *cis* elements appear to be involved in the SRSF3-TR-mediated transcription of *JUN*. Further studies are needed to elucidate the mechanism how SRSF3-TR regulates c-Jun expression.

Although the mechanism how SRSF3-TR regulates c-Jun expression remains to be elucidated, our results suggest that a truncated SRSF3 derived from a PTC-containing splice variant of the *SRSF3* gene may have a mitogenic property. NMD was often inhibited under certain conditions such as hypoxia and oxidative stress. Cancer tissues are exposed

to similar microenviromental conditions, and it has been shown that the impairment of NMD is involved in the oncogenic mechanism (18). In such situations, SRSF3-TR could be induced and might participate in abnormal cell growth. Further studies are needed to fully elucidate the molecular mechanism for the SRSF3-TR-mediated acceleration of cell growth.

## CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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