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Identification of a chemical inhibitor for nuclear speckle formation: Implications for the function of nuclear speckles in regulation of alternative pre-mRNA splicing

Yutaro Kurogi [a], Yota Matsuo [a], Yuki Mihara [a], Hiroaki Yagi [a], Kaya Shigaki-Miyamoto [a], Syukichi Toyota [a], Yuko Azuma [a], Masayuki Igarashi [b], Tokio Tani [a,*]

[a] Department of Biological Sciences, Graduate School of Science and Technology, Kumamoto University, Chuo-ku, Kumamoto 860-8555, Japan
[b] Laboratory of Disease Biology, Institute of Microbial Chemistry, Shinagawa-ku, Tokyo 141-0021, Japan

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ABSTRACT

Nuclear speckles are subnuclear structures enriched with RNA processing factors and poly (A)$^+$ RNAs comprising mRNAs and poly (A)$^+$ non-coding RNAs (ncRNAs). Nuclear speckles are thought to be involved in post-transcriptional regulation of gene expression, such as pre-mRNA splicing. By screening 3585 culture extracts of actinomycetes with *in situ* hybridization using an oligo dT probe, we identified tubercidin, an analogue of adenosine, as an inhibitor of speckle formation, which induces the delocalization of poly (A)$^+$ RNA and dispersion of splicing factor SRSF1/SF2 from nuclear speckles in HeLa cells. Treatment with tubercidin also decreased steady-state MALAT1 long ncRNA, thought to be involved in the retention of SRSF1/SF2 in nuclear speckles. In addition, we found that tubercidin treatment promoted exon skipping in the alternative splicing of *Clk1* pre-mRNA. These results suggest that nuclear speckles play a role in modulating the concentration of splicing factors in the nucleoplasm to regulate alternative pre-mRNA splicing.

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1. Introduction

In eukaryotic cells, the nucleus is compartmentalized into more than a dozen nuclear structures, such as nuclear speckles, paraspeckles, promyelocytic leukemia bodies, polycomb group bodies and Cajal bodies, in addition to the well-known nuclear structure, the nucleoli (for reviews, see [1,2]). Of those, nuclear speckles are visible as 20–50 punctate structures per nucleus, with various sizes and irregular shapes, when stained with an antibody against a splicing factor, such as SRSF1 (serine/arginine rich splicing factor 1, also referred to previously as SF2) or SRSF2 (also referred to previously as SC35) [3,4]. At an electron microscopic level, they correspond to interchromatin granule clusters (IGCs) consisting of 20–25 nm granules [5]. Proteomic analysis of IGCs purified from mouse liver revealed that nuclear speckles contain at least 146 proteins, of which 81% of IGC proteins are associated with RNA metabolism, such as transcription, pre-mRNA splicing, 3′ end mRNA processing and nucleocytoplasmic transport of mRNAs, and among them, 54% are related to pre-mRNA splicing [6]. Live cell imaging showed that SRSF1 tagged with GFP associates and dissociates rapidly with nuclear speckles, suggesting that components of nuclear speckles are in dynamic and continuous flux [7–9]. In addition, a population of poly (A)$^+$ RNAs is enriched in nuclear speckles [4]. These poly (A)$^+$ RNAs represent not only protein-coding mRNAs, but also poly (A)$^+$ long non-coding RNAs (lncRNAs). MALAT1 (metastasis-associated lung adenocarcinoma transcript 1) is a speckle-localized lncRNA [10] and was suggested to be involved in the localization of SRSF1 to nuclear speckles [11].

Several lines of experiments have suggested that nuclear speckles are sites for the storage, assembly, and modification of splicing factors to be supplied to active transcription sites [4,12–14], whereas other groups have argued that nuclear speckles play more direct roles in the pre-mRNA splicing reaction (reviewed in [15]). Precise functions of nuclear speckles in gene expression and the mechanisms for their formation in the nucleus, however, remain to be solved.

Alternative splicing occurs in pre-mRNAs with multiple introns and generates diversity in gene expression in the eukaryotic cells (for a review, see [16]). Alternative pre-mRNA splicing is found in as many as 95% of human genes [17,18] and is observed also in fungi [19]. Alternative splicing is regulated principally by specific RNA

* Corresponding author. Address: Department of Biological Sciences, Graduate School of Science and Technology, Kumamoto University, Kurokami, Chuo-ku, Kumamoto 860-8555, Japan. Fax: +81 96 342 3461.
E-mail address: ttani@sci.kumamoto-u.ac.jp (T. Tani).

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binding proteins, splicing factors, and their interactions with *cis*-acting elements in pre-mRNAs [20]. SR proteins with an Arg/Ser-rich (RS) domain, such as SRSF1 and SRSF2, are major splicing regulators essential for constitutive splicing and regulation of alternative pre-mRNA splicing by affecting the splice site choice [20,21]. In addition, several lines of evidence have suggested that splicing reactions are performed co-transcriptionally [22]. It has emerged that the chromatin state affecting the elongation rate of RNA polymerase II serves as another important regulator for the determination of splice sites and hence regulation of alternative pre-mRNA splicing [23,24].

We previously screened for compounds that affect the formation of nuclear speckles using immunofluorescence of SRSF1 and identified staurosporine as a compound inducing the accumulation of SRSF1 and SRSF2 in enlarged nuclear speckles [25]. Small chemical molecules are useful tools to analyze complicated biological processes and phenomena. In this study, we screened a library of actinomycete culture samples with visualization of poly $(A)^+$ RNA to obtain chemical inhibitors for the analysis of nuclear speckles and identified a compound that induces the delocalization of poly $(A)^+$ RNA and dispersion of SRSF1 from nuclear speckles. We revealed that aberrant formation of nuclear speckles affects the patterns of alternative pre-mRNA splicing.

2. Materials and methods

2.1. Cell culture

HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Carlsbad, USA) supplemented with 10% fetal bovine serum (Life Technologies), 1.2 units/ml penicillin, and 1.2 μg/ml streptomycin at 37 °C in 5% CO_2. Cells were grown to 70–80% confluence for each experiment.

2.2. Preparation of extract samples from actinomycete cultures

To prepare extract samples, actinomycete strains stocked in Institute of Microbial Chemistry (Tokyo, Japan) were cultured on barley media by solid-state fermentation for 14 days at 30 °C. The cultured barley media were then extracted with ethanol to make extract samples.

2.3. Screening with in situ hybridization

HeLa cells were incubated in DMEM medium supplemented with 2.5% (v/v) extract samples of actinomycete cultures for one hour in glass-bottomed 96-well plates, followed by fixation with 3% (w/v) formaldehyde for 15 min. Cells were then permeabilized with 0.5% Triton X-100 for 5 min on ice, rinsed with phosphate buffered saline (PBS) and 2X SSC. Hybridization was performed at 42 °C in a solution containing 2X SSC, 20% formamide, 1 mg/ml of tRNA, 10% dextran sulfate and an oligo dT probe labeled with digoxigenin. After hybridization for 16– to 20 h, cells were washed three times 10 min each in 2X SSC, and incubated with a mouse anti-digoxin antibody (Sigma–Aldrich, St. Louis, USA) followed by treatment with an anti-mouse IgG antibody conjugated with fluorescein isothiocyanate (FITC) (MP Biomedicals, Santa Ana, USA). First screening was done using mixed pools of four extract samples. Positive pools were then divided into a single sample to be tested as a second screening.

2.4. Immunofluorescence

HeLa cells were fixed with 4% paraformaldehyde/PBS for 10 min and permeabilized with 0.5% Triton-X100/PBS for 5 min at room temperature. Cells were then treated with the antibody against SRSF1/SF2 (Life Technologies) or SRSF2/SC35 (Becton Dickinson, Franklin Lakes, USA) for 3 h, followed by treatment with a FITC or Alexa Fluor 488-conjugated secondary antibody for one hour. After counterstaining with 4′,6-diamidino-2-phenylindole (DAPI), cells were observed with an Olympus AX70 fluorescence microscope (Tokyo, Japan) equipped with a Photometrics Quantix cooled CCD camera (Munich, Germany).

2.5. RT-PCR and Western blot analyses

Total RNAs were purified from HeLa cells using TRIzol reagents (Invitrogen) according to the manufacturer's protocol. RT-PCR analysis was carried out using 1 μg total RNA and primers for *Clk* pre-mRNA as described [26]. Nucleotide sequences of primers used for RT-PCR analysis was as follows: Clk forward; 5′-GCATAGTAGCA AGTCCTCTG-3′, Clk reverse; 5′-TACTGCTACACGTCTACCTC-3′, GAPDH forward; 5′-CTGGGCTACACTGAGCAC-3′, GAPDH reverse; 5′-GG TCCACCACCCTGTTGC-3′. MALAT1 forward; 5′- GAAGGAGCGCTA ACGATTTG-3′, MALAT1 reverse; 5′- TCTCCTGGACTTGGCAGTCT-3′. Western blot analysis using the anti-SRSF1/SF2 antibody (Life Technologies) was performed as described [25].

3. Results

3.1. Screening for compounds that affect the formation of nuclear speckles with in situ hybridization

To examine the roles of nuclear speckles in gene expression, we screened the actinomycete cultures for natural compounds that affect the formation of nuclear speckles. As nuclear speckles are enriched with splicing factors and also poly $(A)^+$ RNAs [3,4], we decided to screen the culture library with *in situ* hybridization using an oligo dT probe to detect the nuclear distribution of poly $(A)^+$ RNAs. Fig. 1A shows the screening outline. After culturing HeLa cells with culture extracts of actinomycetes (2.5% in DMEM medium) for 1 h, we performed *in situ* hybridization with the oligo dT probe and visually screened the hybridized cells that cause aberrant formation of nuclear speckles with a fluorescence microscope. Of 3585 extract samples screened, we identified 44 samples that induce aberrant formation of nuclear speckles. The positive extracts were categorized into 3 groups based on their phenotypes, that is, extracts accumulating poly $(A)^+$ RNAs in nuclear speckles, causing the speckles to enlarge (enlargement; 23 samples), extracts inducing delocalization or dispersion of poly $(A)^+$ RNAs from nuclear speckles (diffusion; 17 samples) and extracts resulting in the formation of cytoplasmic poly $(A)^+$ granules similar to the stress granules [27] after treatment (SG formation; 4 samples).

Fig. 1B shows representative phenotypes of HeLa cells treated with positive samples. 1870-14a caused the enlargement of speckles with poly $(A)^+$ RNAs, 1891-1a induced the dispersion of poly $(A)^+$ RNA speckles and 1876-72a resulted in the formation of cytoplasmic granules with poly $(A)^+$ RNAs. Among 17 extract samples that caused diffusion of poly $(A)^+$ RNAs from speckles, 1891-1a induced complete delocalization of poly $(A)^+$ RNAs from nuclear speckles within one hour treatment. We chose 1891-1a for further analysis in this study.

3.2. Purification of the active compound from the 1891-1a extract

We then purified the compound inducing the dispersion of poly $(A)^+$ RNA speckles from the 1891-1a extract, which was prepared from the actinomycete strain MK565-86F15. We extracted the 1891-1a extract with ethyl acetate and found that the activity of speckle dispersion was in the water layer, not in the ethyl acetate

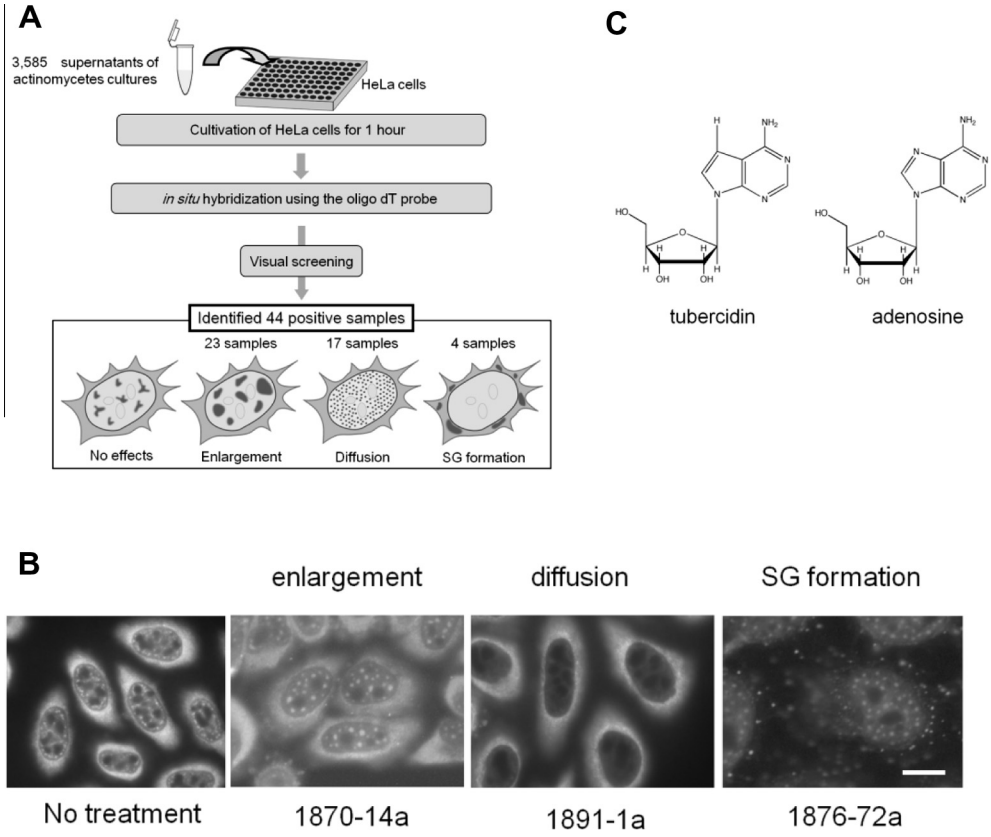

Fig. 1. (A) Outline of the screening procedure. After addition of cultured extract samples of actinomycetes to the media (2.5%, v/v), HeLa cells were incubated for one hour in a glass-bottomed 96-well plate and then subjected to *in situ* hybridization with a digoxigenin-labeled oligo dT probe. Hybridized cells were visually screened with a fluorescence microscope to identify the samples affecting the nuclear localization of poly $(A)^+$ RNAs. Of 3585 samples screened, we identified 44 samples that induced the aberrant localization of poly $(A)^+$ RNAs to nuclear speckles. Positive samples were categorized into three groups (enlargement, diffusion and SG formation) according to their phenotype of poly $(A)^+$ RNA localization. (B) Representative phenotypes of HeLa cells treated with positive extracts. Treatment with 1870-14a resulted in the accumulation of poly $(A)^+$ RNAs in enlarged speckles, 1891-1a caused delocalization of poly $(A)^+$ RNAs from nuclear speckles and 1876-72a caused speckles to become round and generated poly $(A)^+$ RNA granules in the cytoplasm. (C) Structure of tubercidin. The N-7 of adenosine is replaced with C–H in tubercidin.

layer (data not shown). The water layer was then fractionated with HPLC. *In situ* hybridization of HeLa cells treated with each HPLC fraction showed that the compound inducing poly $(A)^+$ RNA dispersion resided in fractions 6 and 7 (Fig. S1). Analysis of the positive fractions revealed that they contain tubercidin, an adenosine analogue in which the N-7 of adenosine is replaced with C–H (7-deaza-adenosine) (Fig. 1C).

Treatment of HeLa cells with tubercidin resulted in delocalization of poly $(A)^+$ RNA from the speckles (Fig. 2A), the same phenotype as that of the 1891-1a extract, indicating that tubercidin is a causative compound for the phenotype of poly $(A)^+$ RNA dispersion.

3.3. Treatment with tubercidin induces dispersion of splicing factors from the speckles

Nuclear speckles are known to contain abundant splicing factors and poly $(A)^+$ RNAs [3,4]. To examine whether localization of splicing factors is also affected by treatment with tubercidin, we next immunostained splicing factors, SRSF1 and SRSF2, after treatment with tubercidin in HeLa cells. SRSF1 and SRSF2 are major speckle-localized splicing factors containing an RS domain rich in arginine and serine dipeptides, which are categorized in the SR proteins [4,28]. It has been shown that they are involved in the regulation of alternative splicing, as well as constitutive splicing, as components of the spliceosome [29].

In HeLa cells, SRSF2 is localized discretely in speckles, whereas SRSF1 is enriched in speckles with diffuse nuclear distribution (Fig. 2B). Interestingly, treatment of HeLa cells with tubercidin decreased the intensities of SRSF1 fluorescence signals in speckles and made the SRSF1 distribution more uniform in the nucleus (Fig. 2B, upper panels). In the case of SRSF2, SRSF2 speckles stained with the antibody became smaller and their number increased with the treatment time (Fig. 2B, lower panels).

To examine if the decrease of fluorescent signals of SRSF1 in nuclear speckles resulted from the repression of SRSF1 expression or dispersion of SRSF1 from the nuclear speckles, we performed Western blot analysis of SRSF1 using extracts from HeLa cells treated with tubercidin. As shown in Fig. 2C, Western blot analysis showed no reduction of the amount of SRSF1 protein after tubercidin treatment, suggesting that the reduction of SRSF1 signals in nuclear speckles is due to the dispersion of SRSF1 from nuclear speckles to the nucleoplasm, not to the decreased expression of SRSF1. As for the effects on poly $(A)^+$ RNA, further analysis is necessary to determine whether speckle-localized poly $(A)^+$ RNAs were dispersed from nuclear speckles or they were degraded by tubercidin treatment.

3.4. Steady-state amount of speckle-localized MLAT1 lncRNA is decreased by tubercidin treatment

It was recently shown that the speckle-localized long non-coding RNA MALAT1 interacts with SRSF1 and other SR proteins and plays a role in their retention in nuclear speckles [11]. To examine the action mechanisms of tubercidin on the nuclear speckles, we analyzed MALAT1 lncRNA after tubercidin treatment. We isolated total RNA from HeLa cells treated with tubercidin and subjected them to RT-PCR analysis. As a result, we found that the

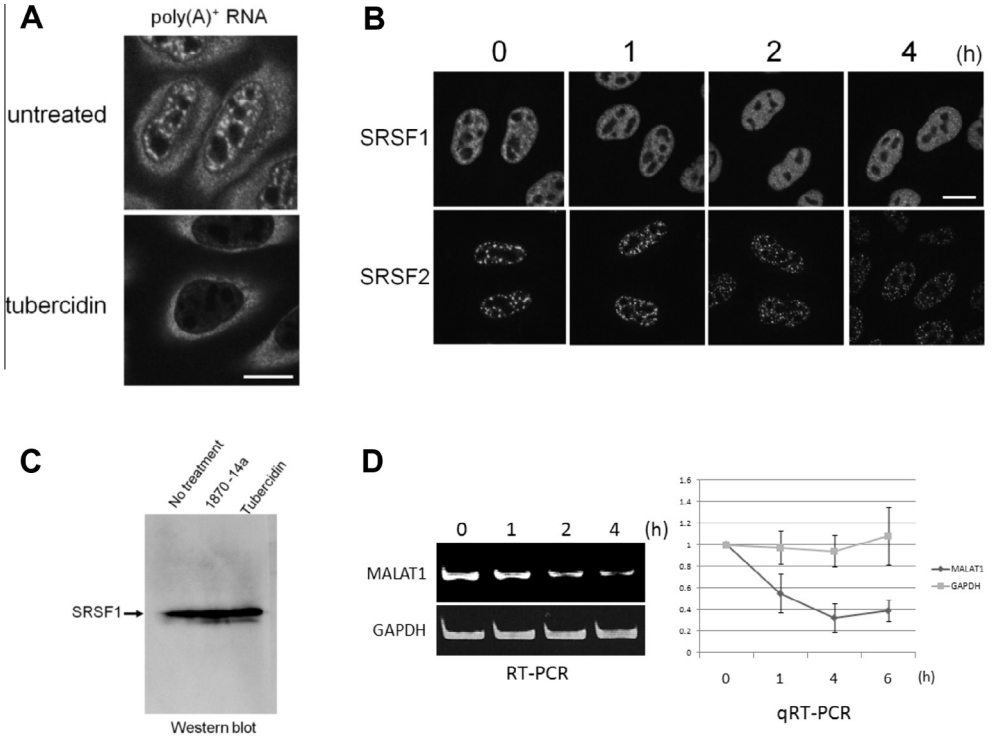

Fig. 2. (A) Treatment of HeLa cells with tubercidin induced the delocalization of poly $(A)^+$ RNAs from the speckles, the same phenotype as in the 1891-1a extract. HeLa cells were incubated with tubercidin (5 μg/ml) for 1 h and then subjected to *in situ* hybridization with the oligo dT probe. Scale bar: 10 μm. (B) Time course of localization changes of splicing factors SRSF1 and SRSF2 after tubercidin treatment. HeLa cells were incubated with tubercidin (5 μg/ml) for the indicated times and then stained with the antibody against SRSF1 (upper panels) or SRSF2 (lower panels). Scale bar: 10 μm. (C) Western blot analysis of SRSF1. Protein extracts prepared from HeLa cells treated with tubercidin (5 μg/ml) and 1870-14a compound (5 μg/ml) for 1 h were subjected to Western blot analysis with the SRSF1 antibody. The amount of SRSF1 was not changed after tubercidin treatment. 1870-14a isolated from the 1870-14a extract was used as a control for the compound inducing enlargement of nuclear speckles. (D) Tubercidin decreased the steady-state amount of MALAT1 lncRNA in HeLa cells. In contrast, the amount of GAPDH mRNA was not changed after tubercidin treatment. Total RNAs isolated from HeLa cells treated with tubercidin for the indicated times were subjected to RT-PCR (left) and qRT-PCR (right) analyses. qRT-PCR experiments were repeated four times ($n = 4$).

steady-state amount of MALAT1 lncRNA decreased after tubercidin treatment, whereas the amount of GAPDH mRNA did not (Fig. 2D), suggesting that tubercidin reduces the stability of MALAT1 lncRNA or inhibits transcription of the MALAT1 gene.

3.5. Tubercidin promotes exon skipping in alternative pre-mRNA splicing of Clk1 pre-mRNA

As tubercidin affected the nuclear distribution of the splicing factor SRSF1 and SRSF2 (Fig. 2B), we next analyzed the effects of tubercidin on constitutive and alternative pre-mRNA splicing. RT-PCR analyses of pre-mRNAs with short introns, such as the 7th intron in the GAPDH gene, showed that tubercidin does not inhibit constitutive pre-mRNA splicing (Fig. 3B). However, we found that treatment with tubercidin changes the alternative pre-mRNA splicing of *Clk1* pre-mRNA (Fig. 3). In HeLa cells, the *Clk1* gene generates two alternative mRNAs, Clk1 mRNA with the exon 4 and $Clk1^T$ mRNA excluding the exon 4 (exon skipping) [30] (Fig. 3A). Interestingly, tubercidin treatment promoted the exon 4 skipping in alternative splicing of *Clk* pre-mRNA (Fig. 3B). In contrast, 1870-14a, which induced the speckle-accumulation of poly $(A)^+$ RNAs (Fig. 1B) and SRSF1 (Yota Matsuo and Tokio Tani, unpublished results), promoted the inclusion of exon 4 in *Clk1* alternative pre-mRNA splicing after treatment.

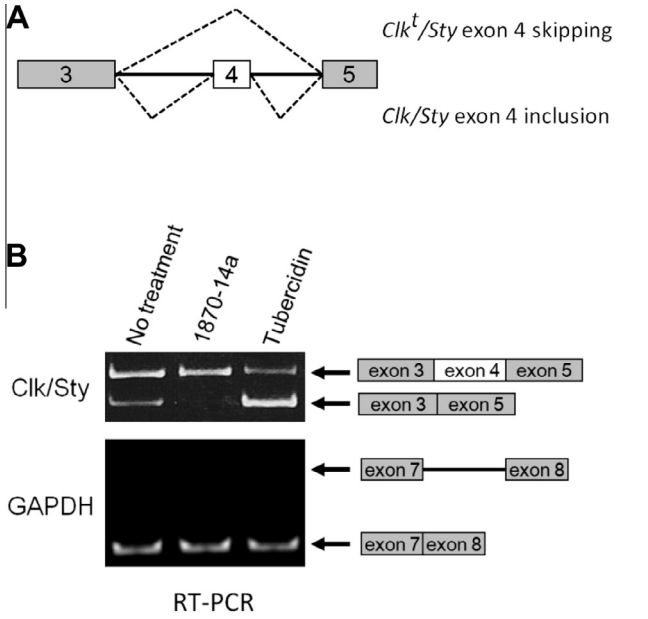

Fig. 3. (A) Schematic representation of alternative splicing of *Clk1/Sty* pre-mRNA. (B) Tubercidin promotes exon skipping in alternative splicing of *Clk1/Sty* pre-mRNA. Total RNAs prepared from HeLa cells treated with tubercidin or the 1870-14a compound for 4 h were analyzed by RT-PCR to examine the effects of compounds on alternative pre-mRNA splicing. Tubercidin treatment resulted in the promotion of exon 4 skipping in Clk1 alternative pre-mRNA splicing, whereas the 1870-14a compound enhanced exon 4 inclusion (upper panel). Tubercidin did not inhibit the constitutive splicing of GAPDH pre-mRNA (lower panel).

4. Discussion

In this study, we identified tubercidin as a compound that induces aberrant formation of nuclear speckles. Tubercidin abolished

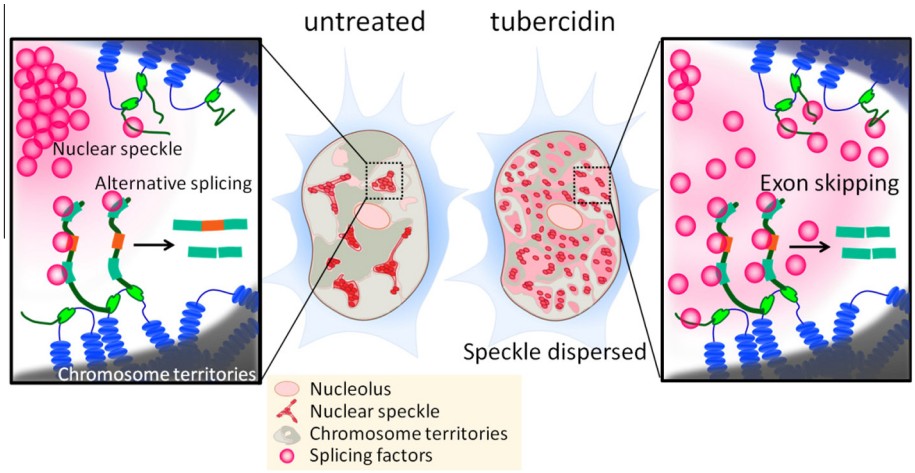

Fig. 4. A hypothetical model for the change of alternative pre-mRNA splicing in cells treated with tubercidin. Tubercidin caused the dispersion of splicing factors, such as SRSF1, from nuclear speckles, resulting in elevation of their concentrations at the sites where the splicing reactions occur and promotion of exon skipping in alternative splicing of several pre-mRNAs regulated by released splicing factors.

the localization of poly $(A)^+$ RNA in nuclear speckles (Figs. 1B and 2A) and also caused the dispersion of a splicing factor, SRSF1, from speckles (Fig. 2B). As far as we know, this is the first report on a compound inducing the delocalization of speckle-enriched poly $(A)^+$ RNAs.

We also identified actinomycete cultured extracts inducing the accumulation of poly $(A)^+$ RNAs in the speckles, leading to speckle-enlargement, by the screening with *in situ* hybridization (Fig. 1B). It is possible that some of these extracts contain compounds inhibiting transport of mRNAs from the nucleus to the cytoplasm, as knockdown of proteins involved in nuclear mRNA export, such as UAP56 and Aly, was shown to cause accumulation of poly $(A)^+$ mRNA in nuclear speckles [32]. Purifications of active compounds from these extracts are also ongoing.

Tubercidin is an analogue for adenosine, firstly identified in culture filtrates of *Streptomyces tubercidicus*, and was reported to show antibiotic and anti-tumor effects [31]. How does tubercidin affect speckle formation, leading to aberrant localization of splicing factors and poly $(A)^+$ RNAs? Phosphorylation of SRSF1, which shuttles between the nucleus and cytoplasm, by SRPK1 and SRPK2 in the cytoplasm was shown to promote its import into the nucleus, resulting in the accumulation of SRSF1 in nuclear speckles [33,34]. SRSF1 is then hyperphosphorylated by a family of *cdc2*-like nuclear kinases, Clk1 to Clk4, at nuclear speckles, and causes their release from nuclear speckles to nearby sites where transcription and splicing reactions take place [14]. Tubercidin, an analogue of adenosine, may inhibit the kinases involved in the cellular localization of splicing factors as a competitor of ATP, which is essential for kinase activity. However, as the *in vitro* kinase assay demonstrated that tubercidin does not inhibit SRPK1 and Clk1 significantly (Yota Matsuo, Masatoshi Hagiwara and Tokio Tani, unpublished results), it is less likely that tubercidin functions as a competitor of ATP in the phosphorylation of splicing factors. Alternatively, as tubercidin was shown to be incorporated into RNAs during transcription *in vivo* [31], speckle-localized lncRNAs, such as MALAT1, incorporate tubercidin and might affect the localization of splicing factors. It is noteworthy that speckle-localized MALAT1 lncRNA was reported to modulate the cellular levels of SR proteins by retention in nuclear speckles and regulate the phosphorylation of SR proteins [11]. As shown in Fig. 2D, tubercidin decreased the steady-state level of MALAT1 lncRNA, although its mechanism is not known at present. It is possible that tubercidin affects nuclear speckles through synthesis or stability of the speckle-localized ncRNAs. Further analysis is now underway to elucidate the action mechanism of tubercidin on speckle formation and retention of poly $(A)^+$ RNAs in nuclear speckles.

We demonstrated that treatment with tubercidin promotes exon skipping in the alternative splicing of *Clk1* pre-mRNA (Fig. 3). Western blot analysis indicated that the amount of SRSF1 is not changed and immunofluorescence of SRSF1 displayed more homogenous nuclear distribution after tubercidin treatment (Fig. 2B and C). Thus, tubercidin is likely to induce the release of SRSF1 from speckles and increase its nucleoplasmic concentration at the sites where splicing reactions take place, resulting in changes in alternative pre-mRNA splicing (Fig. 4). We previously showed that staurosporine, a compound purified from the extract (1871-62a) inducing the enlargement of nuclear speckles, caused an opposite phenomenon, that is, it caused accumulation of SRSF1 in nuclear speckles and enhanced the exon 4 inclusion in *Clk1* alternative pre-mRNA splicing [25], like 1870-14a in this study. Large-scale analyses of changes in alternative pre-mRNA splicing after tubercidin treatment using exon arrays are now ongoing to characterize the features of pre-mRNAs affected. Our results in this study support the hypothesis that speckles are storage sites for splicing factors and regulate alternative pre-mRNA splicing by modulating the concentration of splicing factors in the nucleoplasm. Tubercidin is a promising chemical tool to analyze the mechanism of speckle formation and the functions of nuclear speckles in gene expression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.060.

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