Truncated SSX protein suppresses synovial sarcoma cell proliferation by inhibiting the localization of SS18-SSX fusion protein

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Abstract

Synovial sarcoma is a relatively rare high-grade soft tissue sarcoma that often develops in the limbs of young people and induces the lung and the lymph node metastasis resulting in poor prognosis. In patients with synovial sarcoma, specific chromosomal translocation of t(X;18) (p11.2;q11.2) is observed, and SS18-SSX fusion protein expressed by this translocation is reported to be associated with pathogenesis. However, role of the fusion protein in the pathogenesis of synovial sarcoma has not yet been completely clarified. In this study, we focused on the localization patterns of SS18-SSX fusion protein. We constructed expression plasmids coding for the full length SS18-SSX, the truncated SS18 moiety (tSS18) and the truncated SSX moiety (tSSX) of SS18-SSX, tagged with fluorescent proteins. These plasmids were transfected in synovial sarcoma SYO-1 cells and we observed the expression of these proteins using a fluorescence microscope. The SS18-SSX fusion protein showed a characteristic speckle pattern in the nucleus. However, when SS18-SSX was co-expressed with tSSX, localization of SS18-SSX changed from speckle patterns to the diffused pattern similar to the localization pattern of tSSX and SSX. Furthermore, cell proliferation and colony formation of synovial sarcoma SYO-1 and YaFuSS cells were suppressed by exogenous tSSX expression. Our results suggest that the characteristic speckle localization pattern of SS18-SSX is strongly involved in the tumorigenesis through the SSX moiety of the SS18-SSX fusion protein. These findings could be applied to further understand the pathogenic mechanisms, and towards the development of molecular targeting approach for synovial sarcoma.

Introduction

Synovial sarcoma is a relatively rare high-grade soft tissue sarcoma that often develops in the limbs of young people. Recent advancements in surgery, chemotherapy, radiotherapy, and multidisciplinary therapy have improved the prognosis. The overall 5-year survival rate in synovial sarcoma patients without metastasis is reported to be 61–80% [1-4]. However, in long-term, synovial sarcoma cells sometimes metastasize to the lung and the lymph node, turning into a life-threatening condition resulting in a poor prognosis. The pathogenic mechanisms have not yet been completely elucidated. However, specific chromosomal translocation of t(X;18) (p11.2;q11.2) has been identified in patients with synovial sarcoma [5]. The fusion of the *SS18* gene on chromosome 18 to the *SSX* gene on chromosome X results in the expression of fusion protein SS18-SSX composed of the NH_3-terminal half of the amino acids from SS18 and COOH-terminal amino acids of the SSX. Expression of the fusion protein has been observed in more than 97% of synovial sarcoma cells [6-8]. This suggests that the SS18-SSX fusion protein is specifically expressed in the synovial sarcoma cells, and is important in the pathogenesis of the disease. SS18 and SSX proteins are localized in the nucleus and are associated with transcriptional regulation, although neither have a distinct DNA binding-domain [6-8]. Therefore, they are thought to regulate transcription

by interacting with other proteins that can bind directly to the DNA in the nucleus.

The wild-type SS18 protein comprises 387 amino acid residues, being ubiquitously expressed in normal cells [6]. It has been reported that SS18 interacts with SNF/SWI complexes (a chromatin remodeling factor) [9-13], Sin3A (a factor of histone deacetylase complex) [14, 15], p300 [16], and AF10 [17]. SS18 is regarded as a transcriptional co-activator because it promotes transcription.

Wild-type SSX protein consists of 188 amino acid residues [7], and it is expressed in the testis and the thyroid, along with melanoma and lung cancer tumor cells, and is one of the cancer/testis antigens [18-21]. Major fusion partners of *SS18* in synovial sarcoma are *SSX1* and *SSX2*, and *SSX4* has been reported in rare cases [22, 23]. The *SSX1* to *SSX9* genes have been identified [24]. SSX is reported to interact with transcriptional repressors such as the polycomb-group (PcG) [25-29], core histone [11], RAB3IP, and SSX2IP [27, 28, 30], and is regarded as a transcriptional co-repressor since it suppresses transcription.

SS18-SSX fusion protein is also known to be localized in the nucleus [25, 26, 31, 32], and is reported to interact with a variety of proteins [10−12, 25, 26, 29, 33]. Recently, gene expression profiles using DNA microarray has revealed various downstream genes that are targeted by the SS18-SSX fusion protein [34−42]. The control of gene

expression by SS18-SSX is believed to involve chromatin remodeling, due to its colocalization with both Trithorax (TrxG) and Polycomb group (PcG) complexes, maintaining chromatin in a poised bivalent state [26, 39, 43]. Lubieniecka reported that *EGR1* is repressed by the SS18-SSX protein through trimethylation of histone H3, and HDAC inhibitor reverts the histone modifications and reactivates *EGR1* expression in synovial sarcoma cells [43]. Su et al identify ATF2 as the DNA-binding partner of SS18-SSX and showed that HDAC inhibitors reverse the epigenetic repressor activity of the SS18-SSX oncoprotein complex by preventing TLE1 recruitment. [44]. Several studies have demonstrated that synovial sarcoma cells express mRNA transcripts of pluripotency factors Sox2, Oct3/4, and Nanog [45] and harbor stem-cell-like gene expression profiles [46], and that tumor cells lacking the BAF47 tumor suppressor subunit express stem-cell-like signatures [47]. Kadoch and Crabtree demonstrated that SS18-SSX fusion protein binds to SWI/SNF-like BAF (chromatin-remodeling) complexes and evicts both the wild-type SS18 and the tumor suppressor BAF47. This altered complex binds to and activates the Sox2 locus by disrupting H3K27me3-mediated repression, and drives proliferation of these cells [48]. In transgenic mice, conditional overexpression of SS18-SSX2 in the myogenic progenitor

compartment, but not other compartments, leads to the appearance of both monophasic and biphasic synovial sarcoma tumors with full penetrance [49].

Generally, proteins that function as transcriptional factors are believed to form complicated complexes, localize at specific region, and carry out their own functions. Synovial sarcoma cell line SYO-1 bearing the *SS18-SSX2* translocation was established previously [50]. We investigated the localization pattern of each component of synovial sarcoma-related fusion protein, and examined the inhibiting effect of the localization of SS18-SSX protein in order to understand the mechanisms by which SS18-SSX contributes towards the synovial sarcoma pathogenesis.

Results

Localization patterns of synovial sarcoma-related proteins in the SYO-1 cells

Localization of full-length SS18 and SSX proteins tagged to GFP was observed under fluorescence microscope, after the constructs pEGFP-*SS18*, pEGFP-*SSX1*, and pEGFP-*SSX2* were transfected into SYO-1 cells. SS18 localized to the nucleus and showed a speckled distribution pattern (Fig. 1A). Both SSX1 and SSX2 localized in the nucleus and displayed a diffuse localization pattern. SSX1 also displayed a speckled pattern and the number of these speckles were relatively more in cells transfected with SSX1 than (Fig. 1B) compared to cells with SSX2 (Fig. 1C). Next, SS18-SSX fusion

proteins were observed after transfection of pEGFP-*SS18-SSX1* and pEGFP-*SS18-SSX2*

into SYO-1 cells. Both SS18-SSX1 and SS18-SSX2 were localized in the nucleus

showing clear speckles similar to that observed for SS18. However, when we examined

closely, compared to SS18, the fusion proteins displayed a pattern in which densely

packed oval dots were more evenly distributed (Figs. 1G and 1H). There was no

remarkable difference in the localization between SS18-SSX1 and SS18-SSX2. To

examine the affect of the fluorescent protein on the localization, SS18, SSX2, and

SS18-SSX2 proteins without GFP in transfected HEK293 cells were analyzed by

fluorescence immunocytochemistry with anti-SS18 and anti-SSX antibodies. SS18 and

SS18-SSX2 showed a speckled pattern and SSX2 displayed a diffuse pattern, which are

similar to their localization of the proteins fused with GFP (Supplementary Fig. 1),

suggesting that addition of the fusion protein doesn't affect localization. Then, the

GFP-fusion plasmids harboring *SS18* moiety and *SSX* moiety were constructed.

Localization of tSS18 (truncated SS18 composing of 1-379 amino acids), tSSX1, and

tSSX2 (truncated SSX composing of 111-188 amino acids) was observed after

transfection of pEGFP-*tSS18*, pEGFP-*tSSX1* and pEGFP-*tSSX2* into SYO-1 cells. The

tSS18 showed a similar localization pattern in the nucleus as that of SS18, although the

intensity of the fluorescence was weak (Fig. 1D). Localization pattern of tSSX1 and

tSSX2 did not differ remarkably from those of SSX1 and SSX2, respectively (Fig. 1E

and F). We also looked at the localization patterns of these synovial sarcoma-related

proteins in HEK293 cells transfected with these plasmids, the localization patterns were

similar to that observed in SYO-1 cells (Supplementary Fig. 2). We confirmed that the

proteins from these plasmid constructs were successfully expressed in HEK293 cells, by

Western blotting with anti-SS18 and anti-SSX antibodies (Supplementary Fig. 3).

Change in the localization pattern of SS18-SSX upon co-expression with truncated

SS18 and SSX proteins

Effect of tSS18 and tSSX truncated proteins on localization of SS18-SSX was examined

in synovial sarcoma cells. When GFP-tagged *SS18-SSX2* was co-transfected along with

DsRedmonomer-*tSS18* into SYO-1 cells, the localization pattern of SS18-SSX2 was not

remarkably different from that observed in cells that were transfected with SS18-SSX2

alone, but the localization of tSS18 was similar with that of SS18-SSX2 (Fig. 2, A1, and

A2). We then looked at the changes in the localization pattern if any of the GFP-tagged

SS18-SSX2 when co-expressed with DsRedmonomer-*tSSX2* fusion in SYO-1 cells. The

localization of SS18-SSX2 significantly changed from a speckled pattern to a diffuse

localization pattern, and was similar to that of tSSX2 (Fig. 2, B1, and B2). When

GFP-tagged *SS18-SSX2* and DsRedmonomer-*tSSX1* fusion were co-expressed,

localization of SS18-SSX2 also changed from speckled to a diffuse pattern, and was similar to that of tSSX1 (data not shown). When *SS18-SSX1* was co-expressed with *tSSX1*, the localization of SS18-SSX1 also showed to a diffuse pattern (Supplementary Fig. 4). However, when pEGFP-*SS18-SSX2* and pDsRedmonomer empty vector were co-expressed, localization of SS18-SSX2 did not change (Fig. 2, C1, and C2). When we transfected SYO-1 cells with DsRedmonomer-fused *SS18*, *SSX1* and *SSX2* full-length genes instead of using the truncated genes, the localization of SS18-SSX was similar to the localization pattern observed earlier (data not shown). We obtained similar results for the localization of these fusion proteins in HEK293 cells transfected with the above plasmids (Supplementary Figs. 5 and 6). All the cells showing a diffuse pattern we examined were observed to have red-fluorescence of tSSX2 in their nuclei. Therefore, we analyzed quantitatively the effect of increasing expression of tSSX on localization of SS18-SSX. Transfection of increased amount of DsRedmonomer-*tSSX2* plasmid (0, 2, 4, and 6 μg) showed significant loss of cells with speckled pattern, while transfection of increased amount of DsRedmonomer-*tSS18* plasmid never showed loss (Fig. 3). We examined effect of DsRedmonomer-tSSX2 and -tSS18 on GFP-SS18-SSX2 expression in transfected HEK293 cells by Western blotting analysis. However, the result showed that protein expression of SS18-SSX2 was not affected by that of tSSX2 and tSS18

(Supplementary Fig. 7).

Suppression of cell proliferation of SYO-1 cells by exogenous expression of tSSX2

As described above, the localization of SS18-SSX2 was affected by the co-expression of tSSX2 in SYO-1 cells. Therefore, we examined the effect of expression of *tSSX2* on proliferation of SYO-1 cells harboring the *SS18-SSX2* fusion gene. SYO-1 cells were transfected with GFP-fused *tSSX2* or GFP vector alone as control, split 48 h after transfection, and the cells expressing the fluorescence proteins were observed and counted on day 4, 6, and 8 after transfection. The cell proliferation ratio was normalized by dividing the numbers of GFP expressing cells on days 6 and 8 by the number on day 4 in SYO-1 cells transfected with *tSSX2* and control group. The cell proliferation was significantly suppressed in the tSSX2 group on days 6 and 8 (Fig. 4). A time-course experiment showing the change in the number of GFP expressing cells is shown in Fig. 5.

Suppression of colony formation of synovial sarcoma cells by exogenous expression of tSSX

We examined the effect of *tSSX2* expression on colony formation of SYO-1 cell line.

SYO-1 cells were transfected with GFP-tagged *tSSX2* or GFP vector alone, split 48 h

after transfection, and selected with G418 for three weeks. The colonies were observed

after cell fixation and staining. The number of colonies formed as well as the size of the

colony was decreased in the *tSSX2* group as compared with that of the control group

(Fig. 6A). We also studied the colony forming ability of another synovial sarcoma cell

line YaFuSS harboring the *SS18-SSX1* fusion gene. Reduced number of colonies was

observed in YaFuSS cell line transfected with GFP-tagged *tSSX1* (Fig. 6B).

Discussion

In the present study, the wild-type SS18 clearly showed a speckled localization pattern,

while SSX showed primarily a diffuse pattern. The localization pattern of SS18-SSX

fusion proteins was clearly nuclear with a speckled pattern and clear elliptical dots were

densely distributed, which differed from those of the wild-type SS18 and SSX. In

addition, localization of tSS18 and tSSX, which are components of SS18-SSX fusion

protein did not remarkably differ from that of the wild-type SS18 and SSX, respectively.

Hence, the localization pattern of SS18-SSX is thought to be the distinct feature

obtained only when both SS18 and SSX form a fusion protein. The subtle difference of

tSSX1 and tSSX2 localization is considered to depend on 11 different amino acids

between them.

Several investigators have reported regarding the localization of synovial sarcoma-associated proteins. dos Santos et al. have reported that the SS18 displays a nuclear punctated localization pattern and SSX a diffuse pattern in the nucleus of transfected COS-1 cells [31]. They suggested that SS18 might influence the manifestation of the tumor since the SS18-SSX fusion protein is also localized in the nucleus and displays a similar punctated pattern [31]. Brett et al. also reported that SS18 and SSX1 displayed a speckled and uniform distribution pattern in the nucleus, respectively, and that the localization pattern of SS18-SSX2 fusion protein is similar to that of SS18 in transfected NIH3T3, Cos-7, HT1080, and MRC-5 cells [32]. On the other hand, Soulez et al. reported that the co-localization of SS18-SSX fusion protein and SSX with RING1 and BMI1, which belong to polycomb group (PcG), but not SS18 [26]. dos Santos et al. subsequently reported that HeLa and COS-1 cells harboring the *SSX* expression vector displayed speckles in the diffuse distribution, and the localization of speckles of SS18-SSX coincided with that of SSX [25]. Furthermore, when the C-terminus of the SSX region called the SSX repression domain was removed, the localization of SS18-SSX coincided with that of SS18 [26, 51]. Therefore, they concluded that SSX region played a dominant role over SS18 region in localization of

SS18-SSX and that the C-terminus of SSX was especially important [25].

In our study, we demonstrate that the localization pattern of SS18-SSX changes significantly when co-expressed with tSSX, suggesting that the localization of SS18-SSX can be antagonized at least by tSSX. These results indicate that SS18-SSX might bind to other proteins via its SSX region; this agrees well with the results of Soulez et al. and dos Santos et al.[25, 26]. As the localization of SS18-SSX changed to a diffuse pattern upon co-expression of tSSX and this seems to coincide with the localization pattern of SSX and tSSX, the localization of SS18-SSX might be guided through the SSX region of SS18-SSX. Interestingly, since co-expression of tSSX2 suppressed cell proliferation and colony formation of the synovial sarcoma SYO-1 and YaFuSS cell lines, the speckle distribution pattern characterized by SS18-SSX might be strongly involved in tumorigenesis of synovial sarcoma cells. Recently, Kadoch and Crabtree demonstrated that SS18-SSX protein binds to SWI/SNF-like BAF (chromatin-remodeling) complexes, and SS18-SSX-driven altered BAF complex formation depends on two amino acids of SSX [48]. Our results showing disappear of SS18-SSX speckles by exogenous tSSX transfection do not contradict their results, and the phenomenon we found might show the disruption of SS18-SSX-driven altered BAF complex antagonized by tSSX. The effect of tSSX on SS18-SSX speckle disruption

might depend on two amino acids of SSX at position 43 and 44. They still demonstrated that assembly of wild-type complexes and proliferative quiescence can be achieved by increasing the concentration of wild-type SS18. However, we haven't performed a cell growth assay using tSS18 transfection, because we could not find any change of SS18-SSX localization by tSS18 transfection due to similarity of localization of SS18-SSX and tSS18. Our finding that tSS18 and SS18 co-localize with SS18-SSX spatially in nuclear might explain their results that increased expression of SS18 displaces SS18-SSX from SWI/SNF-like BAF complexes and lead to reduced growth. Perani et al. reported that SS18 forms an oligomer with SS18 itself or with SS18-SSX [9]. If SS18-SSX forms an oligomer with tSS18, it could account for the same localization pattern observed for SS18-SSX and tSS18.

SSX1 and SSX2 interact with BMI1 and RING1A, which belong to PcG and with LHX4, RAB3IP, and SSX2IP which are transcription factors [27, 28]. RAB3IP and SSX2IP interact with the N-terminal domain of SSX [27, 30]. Since SS18-SSX fusion proteins do not consist of the interaction domains, RAB3IP and SSX2IP are quite unlikely to be the candidate proteins interacting with SS18-SSX. Our results using SSX were similar between the two subtypes of SSX, and it is known that PcGs such as BMI1 and RING1A interact with SSX1 and SSX2 commonly. Therefore, BMI1 and RING1A

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could be the candidate proteins interacting with the SSX1 or SSX2 region of SS18-SSX fusion protein.

Our results revealed the possibility that SS18-SSX is involved in tumor proliferation because of its interaction with some specific proteins interacting with the wild-type SSX via the SSX region of SS18-SSX. Further study is needed to identify these interacting proteins, which will provide a better understanding on the pathways involved in the pathogenesis of synovial sarcoma. This could provide new target molecules that could help in the development of newer treatment options for synovial sarcoma using molecular targeting approach.

Materials and methods

Cell lines

Human synovial sarcoma cell line SYO-1 expressing the *SS18-SSX2* fusion gene was established in our laboratory [50]. Human synovial sarcoma cell line YaFuSS expressing the *SS18-SSX1* fusion gene was kindly provided by Dr. J. Toguchida (Institute for Frontier Medical Sciences, Kyoto University, Japan) [52]. Human normal embryonic kidney cell line HEK293 was purchased from American Type Culture Collection. These cell lines were grown in Dulbecco's modified Eagle's medium or RPMI-1640

(Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum

(Invitrogen), 100 units/ml of penicillin G and 100 µg/ml of streptomycin (Meiji Seika,

Tokyo, Japan). All cells were incubated at 37°C in a humidified atmosphere containing

5% CO_2.

Plasmid construction

The coding regions of the human *SS18-SSX1*, *SS18-SSX2*, *SS18*, *SSX1*, *SSX2*, *tSS18*

(truncated *SS18* coding #1-379 amino acids), *tSSX1* and *tSSX2* (truncated *SSX* coding

#111-188 amino acids) were amplified by PCR with cDNA derived from synovial

sarcoma cells as described earlier [14]. The amplified cDNAs were inserted downstream

of green fluorescent protein (GFP) of pEGFP-C vector (Clontech), and the expression

plasmids were constructed to produce GFP tagged-*SS18-SSX1*, -*SS18-SSX2*, -*SS18*,

-*SSX1*, -*SSX2*, -*tSS18*, -*tSSX1* and -*tSSX2* proteins. Furthermore, pDsRedmonomer

plasmids bearing the *SS18*, *SSX1*, *SSX2*, *tSS18*, *tSSX1* and *tSSX2* cDNAs were inserted

downstream of DsRedmonomer, to produce DsRedmonomer fusion proteins. To detect

the localization of SS18, SSX2, and SS18-SSX2 by fluorescence immunocytochemistry,

pCMV-Tag2B expression plasmids with FLAG tag containing these cDNAs were used

for transfection as described [53]

Transfection

To increase the transfection efficiency, reverse transfection method was used. The vectors (total DNA amount of 0.25 µg in cases where one kind of vector was used, and 0.125 µg each in case of two kinds of vectors) were mixed with 1 µL of Effectene (QIAGEN), 3.25 µL EC-buffer, 1 µL enhancer and 0.6 µL of 1.5 M sucrose; 9 µL of gelatin was added 15 min later, and the mixture was dropped into a well (12 mm × 10 mm) of a 8 well tissue culture chamber slide (Lab-Tec, Nunc) and allowed to dry. The cells were plated in the wells, and the localization of fluorescent proteins was observed under fluorescence microscope after replacement of the medium with DMEM without phenol red.

Fluorescence immunocytochemistry

To determine the localization of SS18 and SSX in transfected HEK293 cells, cells were seeded on glass culture slides (BD Falcon 8-well CultureSlide; BD Biosciences), and grown to 80% confluence. Cells were fixed in 1% formaldehyde for 10 min at room temperature, permeabilized, blocked with 1% bovine serum albumin (BSA) in PBS for 30 min at room temperature, and then incubated with anti-SS18 antibody (SYT; C-19,

Santa Cruz Biotechnology, California, USA) or anti-SSX1 antibody (FL-188, Santa Cruz Biotechnology) overnight. Cells were washed and then incubated simultaneously with Alexa 594-conjugated secondary antibody (Invitrogen, Eugene, OR) for 1 h and Hoechst 33342 (1 mg/mL) (ICN Biomedicals, Aurora, OH) for nuclear staining. Images were acquired with SenSys0401E (Roper Scientific Germany, Ottobrunn, Germany), DMRA2 (Leica Microsystems, Wetzlar, Germany) and Leica Cytogenetic Workstation (CW4000; Leica Microsystems Imaging Ltd, Cambridge, UK).

Western blotting analysis

The protein samples (10 µg total proteins) were combined with gel loading buffer, heated to 95°C for 10 min, and then separated on 12% polyacrylamide gels. The proteins were subsequently transblotted onto PVDF membranes (Invitrogen) and blocked overnight at 4^oC in 3% BSA/PBS. The immunoblots were incubated for 4 h at room temperature with anti-SS18 antibody (SYT; H-80, Santa Cruz Biotechnology) or anti-SSX1 antibody (FL-188, Santa Cruz Biotechnology). β-actin (Sigma, Saint Louis, USA) was used as a loading control. After washing with PBS/0.05% Tween-20, the filters were incubated with alkaline phosphatase-conjugated antibodies. The protein signal was visualized using FLA-3000 (Fujifilm).

Cell proliferation assay

The plasmid pEGFP (control group) or pEGFP-*tSSX2* (tSSX2 group) was transfected into SYO-1 cells cultured in 60 mm culture dish, and split into 4 plates of 60 mm cell culture dishes 48 h after transfection. The transfectants expressing the fluorescent proteins were observed and counted under a fluorescence microscope 4, 6 and 8 days after transfection. Ratio of the number of cells on days 6 and 8 to that of day 4 was calculated, and compared between tSSX2 and the control group.

Colony formation assay

SYO-1 cells were grown in 90 mm culture dish and were transfected with the plasmid pEGFP (control group) or pEGFP-*tSSX2* (tSSX2 group), split into 10 plates of 60 mm culture dishes 48 h after transfection, selected with 400 µg/mL of G418 for two weeks, and stained with Giemsa stain solution after 4% formaldehyde fixation.

Statistical analysis

Comparison between the two groups in the cell proliferation assay was performed using *t*-test and $p < 0.05$ were considered statistically significant. StatView version 5.0 (SAS

Institute Inc., Cary, North Carolina) was used for statistical analysis.

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Figure Legends

Figure 1. Localization of synovial sarcoma-related proteins in synovial sarcoma SYO-1 cells.

GFP fused proteins were observed using a fluorescence microscope. A, GFP-SS18; B, GFP-SSX1; C, GFP-SSX2; D, GFP-tSS18; E, GFP-tSSX1; F, GFP-tSSX2; G, GFP-SS18-SSX1; H, GFP-SS18-SSX2; I, GFP. Scale bars indicate 5 µm.

Figure 2. Representative images showing the co-expression of GFP-tagged SS18-SSX2 and of DsRed-monomer-tagged truncated proteins in transfected SYO-1 cells.

Fluorescent proteins were observed using a fluorescence microscope. A, co-expression of GFP-SS18-SSX2 (A1) and DsRed-monomer-tSS18 (A2); B, co-expression of GFP-SS18-SSX2 (B1) and DsRed-monomer-tSSX2 (B2); C, co-expression of GFP-SS18-SSX2 (C1) and DsRed-monomer empty vector (C2); D, co-transfection of GFP-SS18-SSX2 (D1) and DsRed-monomer-tSSX2 (D2) SYO-1 cell in which tSSX2 was not expressed after co-transfection. Scale bars indicate 5 µm.

Figure 3. Effect of increasing expression of tSSX on localization of SS18-SSX.

Plasmid pEGFP-SS18-SSX2 (2 µg) was co-transfected in HEK293 cells with pDsRedmonomer-*tSSX2* (0, 2, 4, and 6 µg) (*A*), or DsRedmonomer-*tSS18* (0, 2, 4, and 6 µg) (*B*). The total DNA amount of transfection was complimented by pCMV-Tag2B empty plasmid without fluorescent protein (6, 4, 2, and 0 µg). The cells showing a speckled pattern or a diffuse pattern of SS18-SSX2 were counted in 30 fields of fluorescence microscope, and relative ratio of cells with speckled pattern was calculated (*C*). Scale bars indicate 10 µm.

Figure 4. Exogenous expression of tSSX2 suppresses the proliferation of synovial sarcoma SYO-1 cells.

SYO-1 cells were transfected with pEGFP-*tSSX2* or pEGFP vector, split 48 h after transfection, and the cells expressing the GFP-tagged fusion proteins were counted on day 4, 6 and 8 after transfection. The cell proliferation ratio was normalized by dividing the number of cells expressing GFP-tagged proteins on days 6 and 8 by the number on day 4. Error bars indicate standard deviation, *$p < 0.001$, **$p < 0.0001$.

Figure 5. Representative images showing changes in proliferation of SYO-1 cells expressing GFP-*tSSX2* monitored for a period of 18 days.

SYO-1 cells were transfected with pEGFP-*tSSX2* (A) or pEGFP vector (B), split 48 h after transfection, and the cells expressing GFP-tagged proteins were observed under fluorescence microscope on day 4, 6, 8, 10, and 18 after transfection. 1, day 4; 2, day 6; 3, day 8; 4, day 10; 5, day18. Scale bars indicate 20 µm.

Figure 6. A. Exogenous expression of tSSX suppresses the colony formation in SYO-1 cells.

SYO-1 cells cultured in 90 mm dishes were transfected with pEGFP empty vector (A1) or pEGFP-*tSSX2* (A2), split into 10 plates of 60 mm dishes 48 h after transfection and selected with G418 for three weeks. The cells were fixed in 4% formaldehyde and stained with Giemsastain solution. B, YaFuSS cells were transfected with pEGFP empty vector (B1) or pEGFP-*tSSX2* (B2), and assessed for colony formation activity. The colonies were counted and the average number of colonies formed is shown as bar graphs for SYO-1 (A3) and YaFuSS (B3). Error bars indicate standard deviation, * *p* < 0.01.

Supplementary Figure 1. Localization of synovial sarcoma-related proteins by fluorescence immunocytochemistry.

HEK293 cells were transfected with pCMV-Tag2B-SS18, pCMV-Tag2B-SSX2, and pCMV-Tag2B-SS18-SSX2, and analyzed by fluorescence immunocytochemistry with anti-SS18 and anti-SSX antibodies. The transfected cells with SS18 and SS18-SSX2 were reacted with anti-SS18 antibody (*upper* and *middle*, respectively), and the SSX2 transfectant was reacted with anti-SSX antibody (*lower*). *Left*, antibody reaction using Alexa 594-conjugated secondary antibody; *middle*, Hoechst33342 staining; *right*, merged image. Scale bars indicate 5 µm.

Supplementary Figure 2. Localization of synovial sarcoma-related fusion proteins in HEK293 cells.

Cells expressing GFP-tagged proteins were observed under a fluorescence microscope. A, GFP-SS18; B, GFP-SSX1; C, GFP-SSX2; D, GFP-SS18-SSX1; E, GFP-SS18-SSX2; F, GFP. Scale bars indicate 5 µm.

Supplementary Figure 3. Confirmation of protein expression from plasmid constructs by Western blotting.

A, HEK293 cells were transfected with pEGFP (lane 1), pEGFP-SS18 (lane 2, about 79 kDa), pEGFP-SS18-SSX1 (lane 3, about 83 kDa), pEGFP-SS18-SSX2 (lane 4, about 83 kDa), pEGFP-tSS18 (lane 5, about 77 kDa), pDsRedmonomer-tSS18 (lane 6, about 81 kDa), and pDsRedmonomer (lane 7), and the cell extracts were detected by western blotting with anti-SS18 antibody. B, HEK293 cells were transfected with pEGFP (lane 1), pEGFP-SSX1 (lane 2, about 51 kDa), pEGFP-tSSX1 (lane 3, about 38 kDa), pDsRedmonomer-tSSX1 (lane 4, about 42 kDa), pDsRedmonomer (lane 5), pEGFP (lane 6), pEGFP-SSX2 (lane 7, about 51 kDa), pEGFP-tSSX2 (lane 8, about 38 kDa), pDsRedmonomer-tSSX2 (lane 9, about 42 kDa), and pDsRedmonomer (lane 10), and the cell extracts were detected by western blotting with anti-SSX antibody.

Supplementary Figure 4. Changes in the localization of SS18-SSX1 when co-expressed with DsRedmonomer tagged truncated SS18 or SSX1 proteins in SYO-1 cells.

A, co-expression of GFP-SS18-SSX1 (A1) and DsRedmonomer-tSS18 (A2); B, co-expression of GFP-SS18-SSX1 (B1) and DsRedmonomer-tSSX1 (B2). Scale bars indicate 5 µm

Supplementary Figure 5. Changes in the localization of SS18-SSX2 when co-expressed with DsRedmonomer-tSS18 or -tSSX2 proteins in HEK293 cells.

pEGFP-SS18-SSX2 (2 µg) was transfected in HEK293 cells with 6 µg of pDsRedmonomer (*left*), pDsRedmonomer-tSSX2 (*middle*), and pDsRedmonomer-tSS18 (*right*). *Upper*, GFP protein; *middle*, DsRedmonomer protein; *lower*, merged image. White arrow shows a cell with speckled pattern of SS18-SSX2 in which DsRedmonomer-tSSX2 was not expressed. Scale bars indicate 10 µm

Supplementary Figure 6. Changes in the localization of SS18-SSX when co-expressed with DsRedmonomer tagged truncated SS18, SSX1 or SSX2 proteins in HEK293 cells.

A, co-expression of GFP-SS18-SSX1 (A1) and DsRedmonomer-tSS18 (A2); B, co-expression of GFP-SS18-SSX2 (B1) and DsRedmonomer-tSS18 (B2): C, co-expression of GFP-SS18-SSX1 (C1) and DsRedmonomer-tSSX1 (C2); D, co-expression of GFP-SS18-SSX2 (D1) and DsRedmonomer-tSSX2 (D2). Scale bars indicate 5 µm.

Supplementary Figure 7. Effect of DsRedmonomer-tSSX2 on GFP-SS18-SSX2

expression in transfected HEK293 cells.

Plasmid pEGFP-SS18-SSX2 (2 µg) was transfected in HEK293 cells with 6 µg of pDsRedmonomer (*lane 1*), pDsRedmonomer-tSSX2 (*lane 2*), and pDsRedmonomer-tSS18 (*lane 3*), and the total extracts (10 µg) were analyzed by western blotting analysis with anti-SS18 antibody (*upper*), anti-FLAG antibody (middle), and anti-β actin antibody (*lower*). The pDsRedmonomer vector contains FLAG-tag.