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Identification of target genes of synovial sarcoma-associated fusion oncoprotein using human pluripotent stem cells

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ABSTRACT

Synovial sarcoma (SS) is a malignant soft tissue tumor harboring chromosomal translocation t(X; 18)(p11.2; q11.2), which produces SS-specific fusion gene, *SYT–SSX*. Although precise function of SYT–SSX remains to be investigated, accumulating evidences suggest its role in gene regulation via epigenetic mechanisms, and the product of SYT–SSX target genes may serve as biomarkers of SS. Lack of knowledge about the cell-of-origin of SS, however, has placed obstacle in the way of target identification. Here we report a novel approach to identify SYT–SSX2 target genes using human pluripotent stem cells (hPSCs) containing a doxycycline-inducible *SYT–SSX2* gene. SYT–SSX2 was efficiently induced both at mRNA and protein levels within three hours after doxycycline administration, while no morphological change of hPSCs was observed until 24 h. Serial microarray analyses identified genes of which the expression level changed more than twofold within 24 h. Surprisingly, the majority (297/312, 95.2%) were up-regulated genes and a result inconsistent with the current concept of SYT–SSX as a transcriptional repressor. Comparing these genes with SS-related genes which were selected by a series of *in silico* analyses, 49 and 2 genes were finally identified as candidates of up- and down-regulated target of SYT–SSX, respectively. Association of these genes with SYT–SSX in SS cells was confirmed by knockdown experiments. Expression profiles of SS-related genes in hPSCs and human mesenchymal stem cells (hMSCs) were strikingly different in response to the induction of SYT–SSX, and more than half of SYT–SSX target genes in hPSCs were not induced in hMSCs. These results suggest the importance of cellular context for correct understanding of SYT–SSX function, and demonstrated how our new system will help to overcome this issue.

1. Introduction

Genes expressed in tumor cells are categorized into several groups based on their molecular backgrounds. One group consists of genes related to cell-of-origin of each tumor. Identification of this set of genes would help to understand the origin-unknown tumors. Genes in the second group are malignant phenotype-related genes, which are shared by most of malignant cells. Genes related to cell cycle, apoptosis, or invasiveness are categorized in this group. A third group of genes is those related to oncogenic alterations specific to each type of tumor. Typical examples are genes regulated by fusion oncoproteins identified in soft tissue sarcomas, which will be promising candidates of molecular target therapy. It is not a simple procedure, however, to classify genes into these groups, because each factor is not completely independent and may affect each other. For example, origin-related genes expressed in target cells may affect a set of genes induced by fusion oncogenes.

Synovial sarcoma (SS) is a soft tissue sarcomas caused by fusion oncogene expression. Although the name of SS was based on morphological similarities with synovial lining cells described in the original publication, subsequent studies have denied synovial origin of this tumor and therefore SS is categorized in sarcomas with unknown cellular origin. In most of cases, SS cells carry a reciprocal translocation t(X; 18), which occurs between the *SYT* gene on chromosome 18 and one of the three *SSX* genes (*SSX1*, *SSX2*, and *SSX4*) on chromosome X, leading to expression of a SYT–SSX fusion protein [1,2]. Since SYT and SSX harbor transcriptional activator and repressor domains respectively, SYT–SSX is thought to be involved in dysregulation of target gene expression [3].

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To identify target genes of SYT–SSX, there are at least two approaches, *e.g.*, loss-of-function approach using SS cells and gain-of-function approach using cells without fusion genes. For the former, knockdown experiments were performed with siRNA designed for fusion point of *SYT–SSX* or *SSX* [4–6], and showed important roles of SYT–SSX in cell growth and focal adhesion. As the latter approach, forced expression of SYT–SSX has been performed in several cell lines, *i.e.*, 3Y1 rat fibroblasts [7], HEK [8], and mesenchymal stem cells (MSCs) [9,10], and succeeded to identify several targets of SYT–SSX such as the *IGF2* gene. The results of these *in vitro* experiments, however, failed to match completely with expression profile of SS tumors identified by several microarray analyses [11–13]. For example, we previously identified *FZD10* as a representative gene specifically expressed in SS tumors and SS cell lines through genome-wide microarray analysis [14], but no report of SYT–SSX overexpression identified *FZD10* as a target gene. This raises a possibility that cellular contexts used in the previous experiments were not fully appropriate to identify target genes of SYT–SSX.

A large number of studies have emphasized the role of epigenetic modification in gene expression, and pluripotent stem cells have a transcriptionally permissive chromatin structure [15]. Information concerning a set of genes directly induced by SYT–SSX in pluripotent stem cells may help to identify target genes in cell-of-origin of SS. Here, we report successful generation of SYT–SSX2 inducible-PSC lines using *piggyBac* (PB) transposon-delivered Tet-on system, and identified a set of up-regulated genes after induction. Comparing these genes with SS-related genes selected by *in silico* analyses, we finally identified 36 genes up-regulated by SYT–SSX2. All of them were down-regulated by knockdown experiments using SS cell lines, indicating the authenticity of our approach.

2. Materials and methods

2.1. Plasmid DNA construction

The entire coding region of the *SYT–SSX2* gene with FLAG tag was cloned into pCR8/GW/TOPO/TA vector (Life Technologies, Carlsbad, CA) (ST, TK and JT, manuscript in preparation) and transferred into KW111/GW, a derivative of PB-TET [16] containing the rtTA transactivator, via LR clonase reaction, resulting in KW111-FLAG-SYT–SSX2. KW111-stuffer vector, harboring only IRES-mCherry, was also constructed in the same manner.

2.2. Cell culture

For PSCs, we used human ESCs (KhES1 and KhES3) [17] and human iPSCs (414C2) [18]. They were maintained in Primate ES cell medium (ReproCELL, Tokyo, Japan) supplemented with 4 ng/ml recombinant human basic fibroblast growth factor (bFGF; WAKO, Osaka, Japan) on SNL feeder cells as described previously [19]. Human SS cell lines used in this study were described previously [20], and cultured with Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich, St. Louis, MO) supplemented with 10% FBS (Nichirei bioscience, Inc., Tokyo, Japan). All cells were maintained at 37 °C and 5% CO_2.

2.3. Establishment of drug inducible hPSC lines by PB transposon system

hPSCs were seeded onto SNL feeder cells two days before transfection. One μg of each KW111 and PBaseII plasmid DNA were co-transfected into hPSCs with FuGENE® HD (Promega, Tokyo, Japan). After 48 h, we started selection with 100 μg/ml Geneticin (Life Technologies) continuously. After expansion, we validated the expression of SYT–SSX2 both at mRNA and protein levels at each time point after doxycycline administration (LKT laboratory, Inc., St. Paul, USA) with indicated concentrations. Morphology and mCherry expression were observed using BIOREVO with BZ analyzer (KEYENCE, Osaka, Japan). Experiments were performed at least two times independently using all PSC lines.

2.4. RT-PCR and qPCR

Total RNA preparation (RNeasy kit, Qiagen, Valencia, CA), reverse transcription using oligodT and Superscript III reverse transcriptase (Invitrogen, California, USA), PCR with ExTaq (Takara, Shiga, Japan), and quantitative PCR with Thunderbird SYBR qPCR Mix (TOYOBO, Osaka, Japan) were performed as described previously [21]. qPCR was performed with StepOne real-time PCR system (Applied Biosystems, Forester City, CA) with duplicate. Primer sequences are listed in Table S1.

2.5. Western blotting

SDS–PAGE and blotting with whole-cell lysates were performed by standard procedures. Protein bands were detected with Amercham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare, Tokyo, Japan) and visualized using BIO-RAD Molecular Imager® Chemi-Doc™ XRS+ with Image Lab™ software. The antibodies used in this study are described in Supplemental Experimental procedures.

2.6. DNA microarray

Microarray analysis using total RNA was performed according to standard procedures (see Supplemental Experimental procedures). Reported data from our microarray analysis was deposited in the public database Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) under accession no. GSE43080.

For SS-related gene search, we retrieved data sets from GEO. All studies used the Human Genome U133 Plus 2.0 Arrays (Affymetrix). The accession numbers of public database used in this study are described in Table S2.

2.7. RNA interference

Procedures of siRNA designed for *SYT–SSX2* will be described elsewhere (ST and JT, manuscript in preparation).

3. Results

3.1. Generation of drug-inducible SYT–SSX2 PSC lines

For efficient generation of PSCs harboring inducible expression of SYT–SSX2, we combined PB and Tet-on systems (Fig. 1A). We successfully generated two ESC lines (KhES1-SYT–SSX2 and KhES3-SYT–SSX2) and one iPSC line (414C2-SYT–SSX2) (Fig. 1B). All lines retained their original pluripotency as demonstrated by RT-qPCR, surface marker analysis, and embryoid body formation assay (Fig. S1). The expression of SYT–SSX2 was induced both at mRNA and protein level in dose- and time-dependent manner in KhES1-SYT–SSX2 (Fig. 1C–F). Expression of *FZD10* and *IGF2* genes, which are putative SYT–SSX targets based on previous studies, was induced within 6 h (Fig. 1G), and cells migrated out from the colonies 24 h after the induction (Fig. 1H). The expression of pluripotent markers, however, retained similar levels in these cells (Fig. S2), suggesting that the mRNA and morphological changes associated with SYT–SSX2 expression were not merely caused by

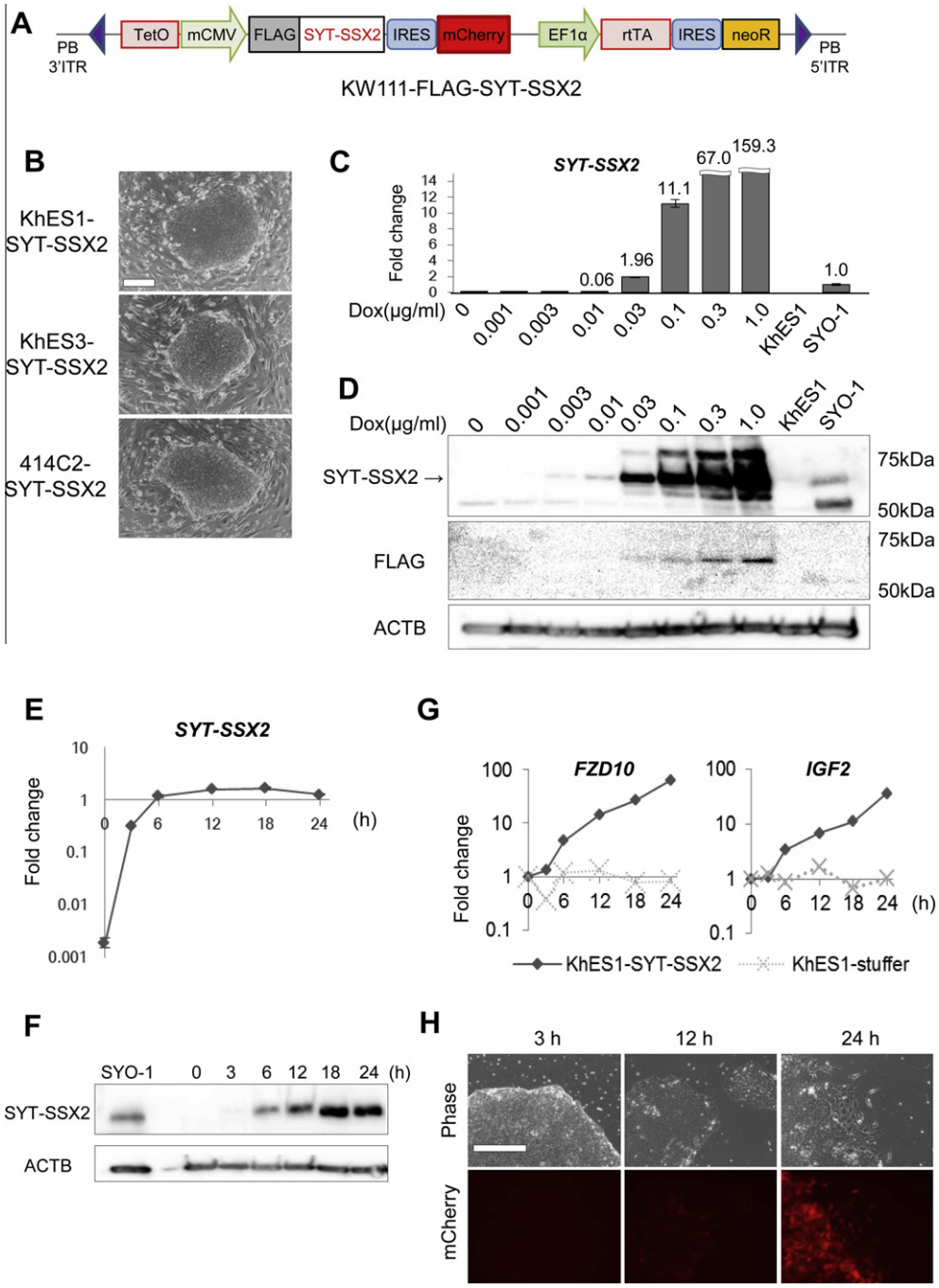

Fig. 1. Generation and characterization of PSC lines with inducible SYT–SSX2. (A) Schematic of the inducible system containing *SYT–SSX2* gene in a PB vector. (B) Morphology of doxycycline-inducible PSC lines. Doxycycline-dose-dependent induction of SYT–SSX at mRNA (C) and protein (D) levels in KhES1-SYT–SSX2. (C) RT-qPCR data, expression relative to the level of SYO-1. (D) FLAG-SYT–SSX2 protein was detected by anti-SYT (top panel) and anti-FLAG (middle panel) antibody. Time-dependent induction of SYT-SSX2 at mRNA (E) and protein (F) levels in KhES1-SYT–SSX2 by doxycycline treatment (0.03 μg/ml). (E) Expression relative to the level of SYO-1. (F) FLAG-SYT–SSX2 protein was detected using an anti-SYT antibody. (G) Induction of *FZD10* and *IGF2* genes. Expression relative to levels in uninduced cells. (H) Morphology (upper panel) and expression of mCherry (lower panel) 24 h after induction. Scale bar, 200 μm.

the loss of pluripotency. Two additional cell lines showed similar profiles (Fig. S3). These results indicate that our strategy is appropriate to identify SYT–SSX2 target genes within 24 h.

3.2. Identification of candidates of SYT–SSX2 target genes in PSCs

Using these three lines, we next performed microarray analysis at 0, 6, 12, 18, and 24 h after SYT–SSX2 induction. All three lines demonstrated quite comparable responses to SYT–SSX2 induction in terms of global gene expression change and number of up- or down-regulated genes at each time point (Fig. S4). Intriguingly, among the genes showing more than twofold change, the majority were up-regulated. The number of up-regulated genes increased over time, and 297 genes were identified as up-regulated at 24 h after induction (Fig. 2A). Most displayed continuous up-regulation and only few (23/297, 7.7%) showed a transient change. On the contrary, down-regulated genes were not found within 12 h, and only 15 genes were identified as down-regulated at 24 h after induction (Fig. 2B). Based on these results, genes up- or down-regulated within 24 h in microarray analysis were selected as

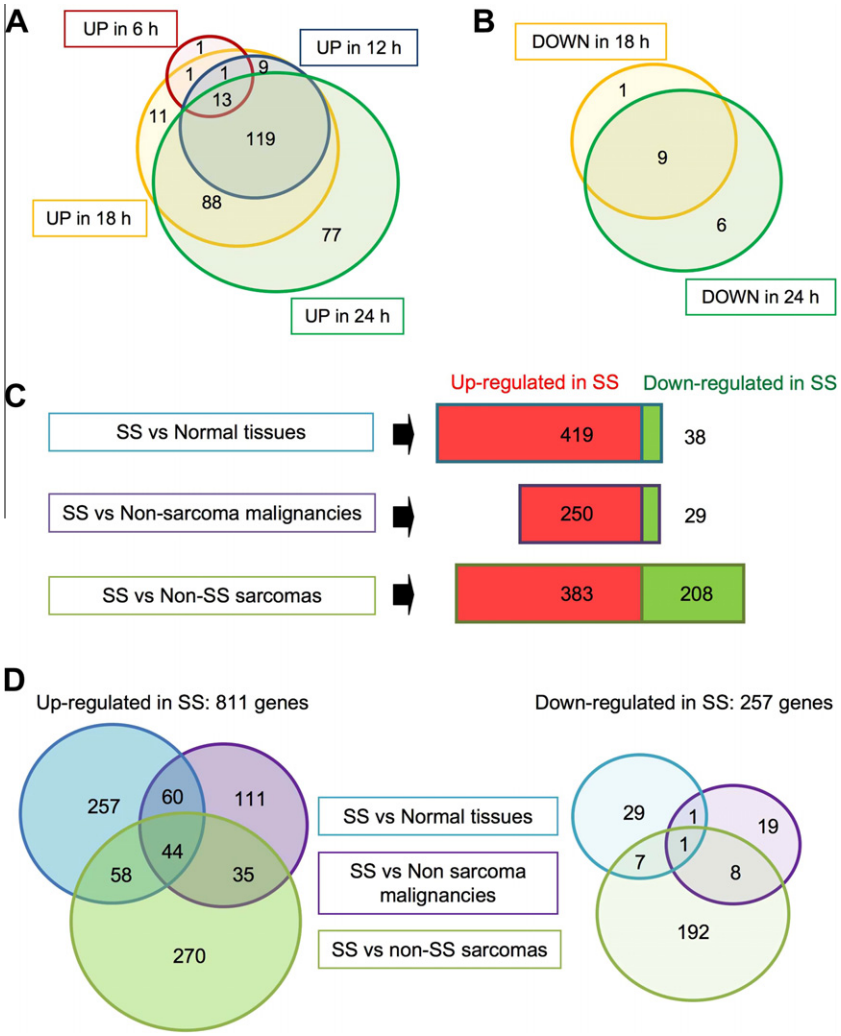

Fig. 2. Identification of candidates of SYT–SSX2 target genes in PSCs and SS-related genes. Venn-diagram showing up- (A) and down-regulated (B) genes in PSCs at 6, 12, 18, and 24 h after induction. Each gene showed a fold change more than two comparing expression before and after induction. *p*-value <0.05 (ANOVA with Benjamini Hochberg FDR). (C) *In silico* search for SS-related genes. Numbers of up- and down-regulated genes in each comparison are shown in each column. (D) Venn-diagram showing SS-related genes selected by each comparison. Numbers of up- (left panel) and down- (right panel) regulated genes are shown in the diagrams.

candidate SYT–SSX2 targets in PSCs and used for further analyses (Table S3).

3.3. Identification of SS-related genes by in silico analyses

To investigate significance of these candidate genes, we next sought to select SS-related genes by a series of *in silico* analyses. We compared the gene expression profile of SS tumors with SYT–SSX2 to those of normal tissues, non-sarcoma malignancies, and non-SS sarcomas (Fig. 2C). These analyses identified a total of 811 up-regulated and 257 down-regulated genes (Fig. 2D and Table S4). GO term analysis revealed that up-regulated genes compared to normal tissues were mainly related with mitosis and cell cycle, while those to non-sarcoma malignancies and non-SS sarcomas were related with developmental processes (Table S5).

3.4. Identification of SYT–SSX2 target genes in PSCs

Comparing SYT–SSX2 target genes in PSCs to SS-related genes selected by *in silico* analyses, we identified 49 and 2 genes as commonly up-regulated and down-regulated genes, respectively (Fig. 3A). Among 49 up-regulated genes, 27 (55.1%) showed up-regulation more than twofold within 12 h (Fig. 3A), when no morphological changes were observed (Figs. 1G and S3). To validate these microarray data, induction of these 49 genes by SYT–SSX2 in PSCs was further analyzed by RT-qPCR (Fig. S5). Induction was confirmed for all genes, although RT-qPCR detected the induction of these genes earlier than by microarray, such that 36 (73.4%) genes showed up-regulation within 12 h (Fig. S5).

3.5. Validation of the effect of SYT–SSX2 on the expression of target genes

To confirm that up-regulated genes identified in PSCs are truly regulated by SYT–SSX2 in SS cells, knockdown experiments using siRNA against *SYT–SSX2* were performed. The 36 genes identified in the former experiments as SYT–SSX target genes were analyzed in this experiment. Two SYT–SSX2 positive SS cell lines, SYO-1 and 1273/99, were used, and the efficacy of knockdown by siRNAs was confirmed by mRNA analyses of *SYT–SSX2* and also known target genes (Fig. S6). SYO-1 cells expressed all 36 of the SYT–SSX2 target genes, and after treatment with siRNA against *SYT–SSX2*, significant reduction of expression was observed for 29 genes (Fig. 3B, left column). In the case of 1273/99, the expression of three genes were below the detectable level, and among the remaining 33 genes, the expression of 29 genes showed a reduction by siRNA (Fig. 3B, right column). When the data from two lines were combined, all

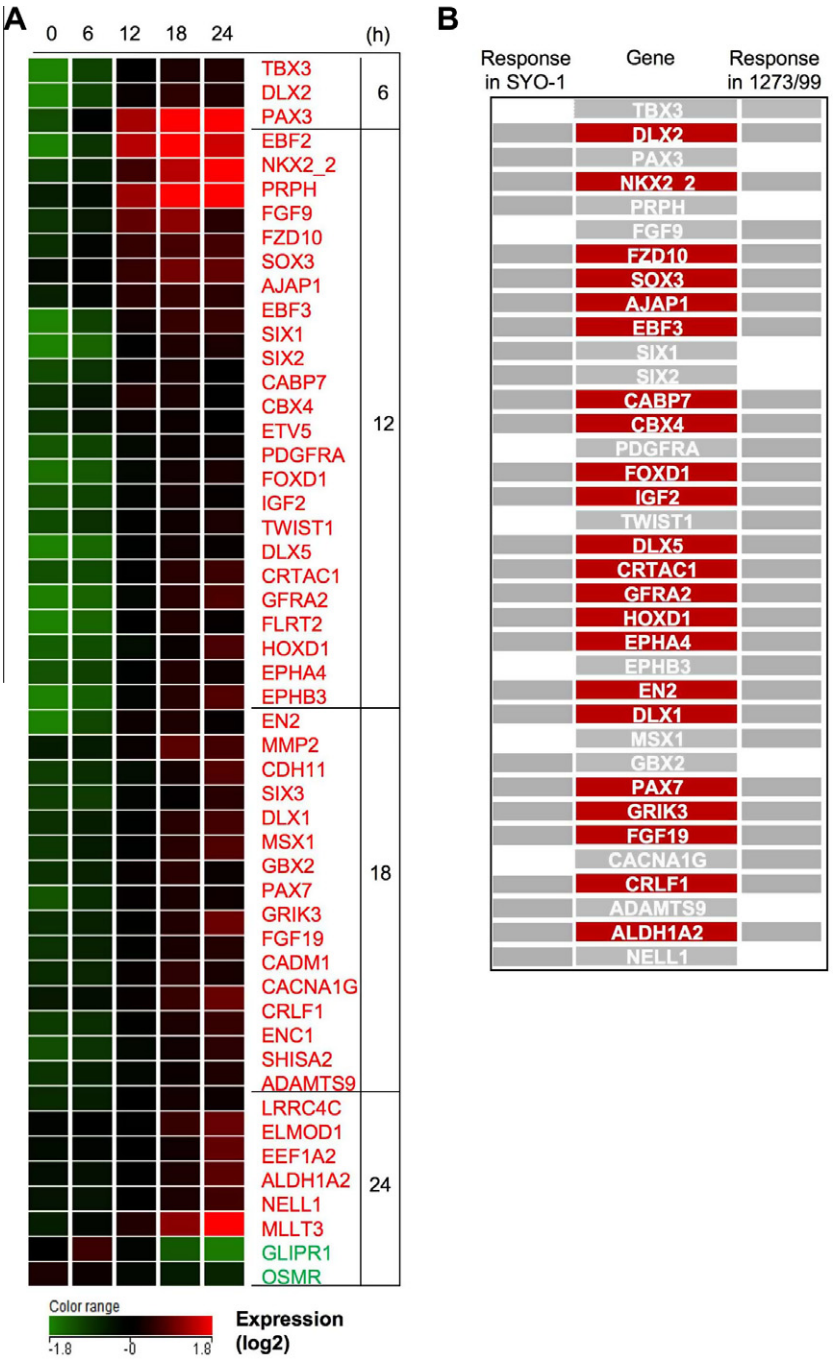

Fig. 3. Identification of SYT–SSX2 target genes. (A) Hierarchical clustering made of 49 up-regulated genes and 2 down-regulated genes. Genes shown in the right column are organized according their first time point of twofold up-regulation. (B) Summary of knockdown experiments using siRNA against the *SYT–SSX2* gene. Genes down-regulated by siRNA in one cell line are highlighted in gray in the corresponding left or right column and also the center column. Genes highlighted in red in the center column indicate those down-regulated in both cell lines.

36 genes were found to be down-regulated by siRNA against *SYT–SSX2* (Fig. 3B). We also performed the same experiments with another *SYT-SSX2* siRNA and confirmed similar tendency (data not shown). These results suggest that our approach could faithfully identify SYT–SSX2 target genes in SS cells, and these 36 genes were strong candidates of genes directly regulated by SYT–SSX2.

3.6. Comparison between PSCs and MSCs as host cells for overexpression

In this study, PSCs were used to identify SYT–SSX2 target genes, most of which were up-regulated, while only few were down-regulated after 24 h of induction (Fig. S4). Previously, gain-of-function experiments have been performed using MSCs since they are considered as a putative cell-of-origin for SS [9,10], and a set of genes were identified as targets by microarray analyses. We chose one such analysis (GSE26563) and compared the gene expression profile of SYT–SSX2 expressing PSCs with that of MSCs. When we focused on 811 genes identified as SS-related, up-regulated genes by *in silico* analyses (Fig. 2D and Table S2), we found significant differences between PSCs and MSCs with respect to the effect of SYT–SSX2. In PSCs, the change of these genes was unidirectional (up-regulation), which was compatible with the concept of SS-related genes (Fig. 4A). The change observed in MSCs was, however,

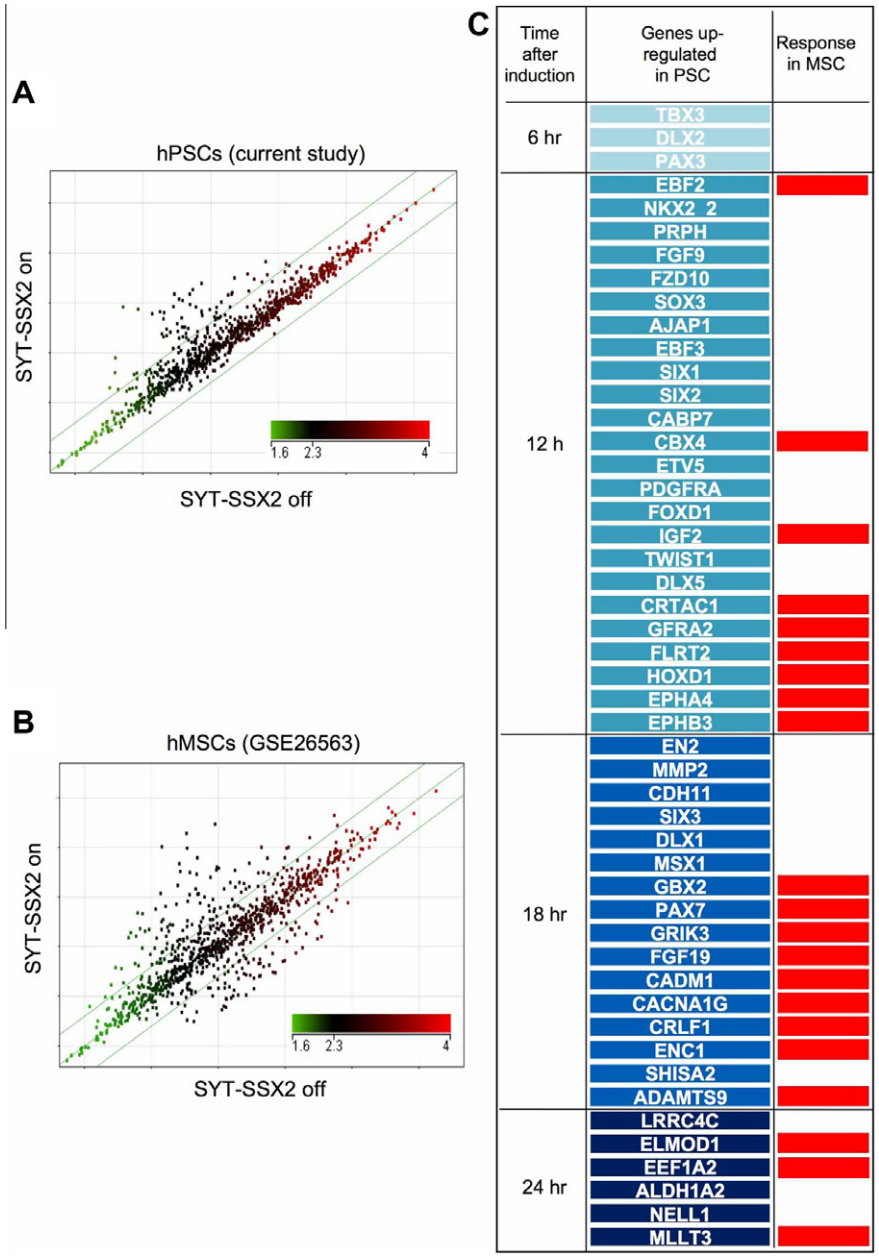

Fig. 4. Comparison between PSC and MSC with SYT–SSX2. Scatter plot dotted with SS-related up-regulated genes. (A) Comparison of expression profiles of SS-related up-regulated genes in hPSCs before and after SYT–SSX2 induction (24 h). (B) Comparison of expression profiles in hMSCs before and after SYT–SSX2 infection (48 h). (C) Comparison between hPSC and hMSC in terms of SYT–SSX target genes identified in this study. Genes up-regulated in hMSCs in response to SYT–SSX induction are highlighted in red.

bidirectional and some SS-related genes were down-regulated by SYT–SSX2 (Fig. 4B). Among the 49 genes identified as putative SYT–SSX2 targets by microarray analyses, 21 genes were up-regulated in MSCs by SYT–SSX2, but the remaining 28 genes were not (Fig. 4C). For example, *IGF2*, a known downstream gene of SYT–SSX, was shared in both gene lists, but *FZD10* was not (Fig. 4C). These results strongly indicated the importance of cell context to identify target genes for a given fusion oncoprotein.

4. Discussion

Recent studies *in vitro* and *in vivo* have been important to disclose the molecular mechanism of SS development, particularly concerning the role of STY–SSX in epigenetic regulation. Garcia et al. performed ChIP-seq analysis and compared the data with their transcriptome data [22]. They showed that SYT–SSX2 occupied trimethylated histone H3 lysine 27- (H3K27me3) labeled regions, a marker for suppressive histone modification, and concluded that SYT–SSX2 mainly targets stable silenced differentiation programs [22]. More directly, serial mass-spectrometry and co-immunoprecipitation revealed that SYT–SSX2 bridges between ATF2 and TLE1, and generates suppressive complex upon ATF2 target genes [23]. Based on these results, our insights into the regulatory system of SYT–SSX have gradually increased, especially concerning inhibitory mechanisms of gene expression [24].

Besides well-characterized suppressive mechanisms, our knowledge regarding the activity of SYT–SSX in gene expression is only fragmental [25–30]. In this study, we observed a striking difference in expression profiles between PSCs and MSCs in response to SYT–SSX induction, indicating that cell context is an important factor to understand the function of SYT–SSX. In this sense, PSCs with inducible *SYT–SSX2* gene established in this study

will serve as a powerful tool. Although Myf5-positive immature myoblasts were proposed to be a cell-of-origin of SS [13], other types of cells may also serve as the origin [31]. In our previous study [11], we found that SS shared gene expression profiles with malignant peripheral nerve sheath tumors, of which the cell-of-origin are Schwann cells, suggesting that SSs develop from some type of neural crest derived cells. Stepwise differentiation of neural crest derived cells from PSCs and induction of SYT–SSX in cells at each stage of differentiation will help to verify our hypothesis.

Another importance of this report is that we obtained similar results using both ESCs and iPSCs, the latter of which were derived from adult tissue (Fig. S4). Gene expression is regulated by a number of individual-specific genetic factors including single nucleotide polymorphisms in regulatory regions, or aberrant genetic alterations that may be a cause or result of tumorigenesis. Establishment of SYT–SSX inducible iPSCs from SS patients will help resolve issues of genetic contribution, as we can now compare the expression profile of genes induced by SYT–SSX in transgenic iPSCs from normal tissue with those of the patients' own SS tumor cells.

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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.2013.01.003.

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