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

Kazuo Hayakawa [a,b,e], Makoto Ikeya [b], Makoto Fukuta [a,b,e], Knut Woltjen [c], Sakura Tamaki [a], Naoko Takahara [a], Tomohisa Kato Jr. [a], Shingo Sato [a,1], Takanobu Otsuka [e], Junya Toguchida [a,b,d,*]

[a] Department of Tissue Regeneration, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan
[b] Department of Cell Growth and Differentiation, Center for iPS Cell Research and Application, Kyoto University, Kyoto, Japan
[c] Department of Reprogramming Sciences, Center for iPS Cell Research and Application, Kyoto University, Kyoto, Japan
[d] Department of Orthopaedic Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan
[e] Department of Orthopaedic Surgery, Graduate School of Medical Sciences, Nagoya City University, Nagoya, Japan

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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].

* Corresponding author. Address: Department of Tissue Regeneration, Institute for Frontier Medical Sciences, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. Fax: +81 75 751 4646.
E-mail address: togjun@frontier.kyoto-u.ac.jp (J. Toguchida).
[1] Current address: Department of Developmental and Stem Cell Biology, The Hospital for Sick Children, Toronto, Canada.

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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