［研究論文］

SKL Tagging

A Method for Generating Dominant-negative Inhibitors of the Homeobox Transcription Factor Superfamily and Smads

SKL タグトラップ法

二量体型転写因子および Smad における機能阻害法の構築

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Abstract: Peroxisome targeting signal 1 (PTS1) is a C-terminal tripeptide with a consensus sequence such as serine-lysine-leucine (SKL). Using this molecular mechanism, we developed a novel dominant-negative assay, designated the SKL-tag trap assay, to negatively regulate dimeric transcription factor, as typified by the homeobox transcription factor superfamily. We tested this method on the homeobox protein Siamois, a well-studied dorsalizing factor in vertebrates, with Vent1 and Vent2 as ventralizing factors. In both cases, protein functions were effectively blocked by the SKL-tag trap assay. Moreover, we showed that the technical feasibility of the SKL-tag trap assay was confirmed for Smad transcription factors. Taken together, we conclude that the SKL-tag trap assay can be used for the effective way to inhibit function of dimeric, trimeric, and oligomeric transcriptional factors in amphibian embryos.

ペルオキシソーム移行シグナル 1 (PTS1) とはタンパク質の C 末端に存在するセリン―リシン―ロイシンの 3 アミノ酸 (SKL) のことである。我々は、二量体型転写因子の働きを阻害する初めての手法として SKL タグトラップ法を構築することにした。脊椎動物の背側化を導くもしくは腹側化を導くホメオボックス分子、それぞれ Saimois もしくは Vent1 と Vent2 についてこの手法を試みた結果、いずれの場合においてもその有効性が確認された。さらに、この手法は転写因子 Smad に対しても効力を示すことが判明した。以上より、SKL タグトラップ法は両生類胚において有効な二量体、三量体、さらには多量体形成型転写因子の機能を阻害する手法であると言える。

Keywords: SKL motif, homeobox, dorsalization, ventralization, *Xenopus laevis*
SKL モチーフ、ホメオボックス、背側化、腹側化、アフリカツメガエル

1 Introduction

In eukaryotic cells proteins destined for single-membrane subcellular organelles, peroxisomes, which contain digestive enzymes for breaking down toxic materials in cells, have a carboxy (C)-terminal peroxisome targeting signal consisting of the tripeptide serine (S)-lysine (K)-leucine (L), which is well known as the most effective signal peptide in PTS1. This C-terminal signal sequence allows the protein to bind to the transporter protein PEX5 and to be translocated into the peroxisome through PEX13-14 protein channel (Fig. 1) (Brocard *et al*., 2006).

Xenopus laevis is a genus of African frogs that are commonly known as the African clawed frogs and broadly used as an invaluable tool to study vertebrate embryology and development because of the following features. Embryos tolerate extensive manipulation such as single cell or tissue transplantations. It is very easy to inject of a range of materials such as DNAs, mRNAs, proteins, and whole nuclei into whole embryo or specific cells. Cell fate of each early embryonic cell in *Xenopus* is known, allowing targeted gene knock-out, knockdown and overexpression studies eggs and embryos provide abundant source for high-throughput biochemical studies.

The homeobox gene *Siamois* is expressed in a localized fashion in

the blastula chordin- and noggin-expressing (BCNE) center of amphibian blastula and works as a central player of dorsalizing activity of *Xenopus laevis* embryos (Lemaire *et al*., 1995; Carnac *et al*., 1996; Laurent *et al*., 1997; Ishibashi *et al*., 2008). Siamois homologue has never been reported in mammalians. However, human organizer cells express DUXO (DUX of the Organizer), a novel member of the double-homeobox (DUX) family of transcription factors, and both of DUXO's homeodomains share very high similarity with the amino acid sequence of the *Xenopus laevis* proteins Siamois (Sharon *et al*., 2012). Overexpression of DUXO upregulates the prominent organizer markers such as *goosecoid* and *cerberus1*, suggesting that DUXO is working as dorsalizing factor in mammalian and can be substitute for Siaomis (Sharon *et al*., 2012). Other homeobox genes *Vent1* and *Vent2* are widely expressed at the ventral side of gastrula embryos under the control of the BMP (bone morphogenetic protein) signaling and have a role of ventralizing factor (Sander *et al*., 2007). Interestingly, the putative homolog of the *Xenopus Vent2* gene, *VENTX* is isolated in human and has a normal and malignant myelopoiesis (Rawat *et al*., 2010). Smad transcription factors are working as the main cytoplasmic regulator of the TGFβ signaling. Five of the mammalian Smads—Smad1, Smad2, Smad3, Smad5, and Smad8—act as receptor-regulated Smads (R-Smad). Smads 1, 5, and 8 are substrates for the receptors targeted by ventralizing factors, BMPs, and Smads 2 and 3 for receptors targeted by mesoderm inducers such as activin and Nodal. Smad4, also referred to as Co-Smad, serves as a common partner for all R-Smads. Smad6 and Smad7 are inhibitory Smads (I-Smad) that interfere with Smad-receptor or Smad-Smad interactions.

In this study, we introduce a novel and simple approach for inhibiting homeobox transcription factor superfamily proteins. Homeobox proteins are dimerized by hydrogen bonds to attain biological activity in nucleus (Kuroiwa, 1987). The phenomena described above prompted us to investigate the following idea named as the SKL-tag trap assay. Artificially, SKL-

conjugated homeobox proteins may dimerize with endogenous homeobox protein. If so, target homeobox superfamily molecules would be trapped by PEX5 (Lanyon-Hogg *et al*., 2010), and as a result they should shuttle into peroxisome and never function in nucleus (Fig. 1). We find that this is indeed the case and also adaptable for other transcription factors such as Smads. PTS are observed in all eukaryotes, so the SKL-tag trap method could be widely applicable to inhibit the function of oligomeric transcriptional factors. In order to block the function of target gene, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR/Cas methods have been developed as powerful tool to make gene knockout creatures (Gaj *et al*., 2013), and RNA interference technique (RNAi) and antisense morpholino oligomer (MO) as gene knockdown (Flynt *et al*., 2011; Heasman, 2002). However, these methods are not so effective when functionally interchangeable proteins are existed. On the other hand, the SKL-tag trap assay is also able to inhibit such interchangeable proteins by dimerization.

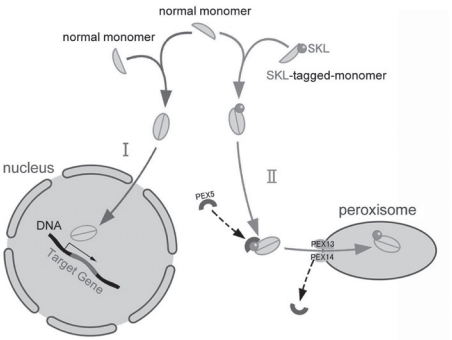

Fig. 1 Schematic representation of the SKL-tag trap assay

I: Normal pathway of homodimer-typed transcriptional factors such as the homeobox transcriptional factor superfamily proteins. II: Scheme of the SKL-tag trap assay. An experimentally generated carboxy-terminal SKL-conjugated subunit may bind subunits in the complementary endogenous heterodimeric protein via the formation of a hydrogen bond. The resultant protein complex should then be trapped by PEX-5, resulting in its continuous shuttling to the peroxisome.

2 Experimental procedures

To conjugate the SKL motif to the carboxy-terminal end of a target protein, Siamois, five nucleotides were added to the 5' end of the forward primer, followed by a stop codon sequence, while four nucleotides were added to the reverse primer (Fig. 2). In the cases for conjugating the SKL motif to Vent1, Smad1, Smad2, and Smad4, nine nucleotides were added to the 5' end of the forward primer. Plasmids pRN3-Siamois, pCS2-Vent1, pCS2-Smad1, pCS2-Smad2, and pCS2-Smad4 were used for creating SKL-tag mutants. The primers 5'-AATTATGACTTCTTCGGCCGCG-3' and 5'-TACTGTTTGGGTAGGGCTCTCTAT-3' were used for constructing Siamois-SKL. The forward primer 5'-AGTAAACTATAAGCCTCTAGAACTATAGTGAGTCG-3' was used for constructing Vent1-SKL, Smad1-SKL, Smad2-SKL, and Smad4-SKL. The reverse primers 5'-CATATACTGAGCCCCAAAGAGTG-3', 5'-ATCCATTAACGAGACCGAGGAG-3', 5'-GGACATGCTTGAGCAGCGG-3', and 5'-GTCCAGAGGCTGAGGATCAGC-3' were used for constructing Vent1-SKL, Smad1-SKL, Smad2-SKL, and Smad4-SKL, respectively. The underlined sequences in the primers were used for adding SKL. pUC57-Vent2-SKL was

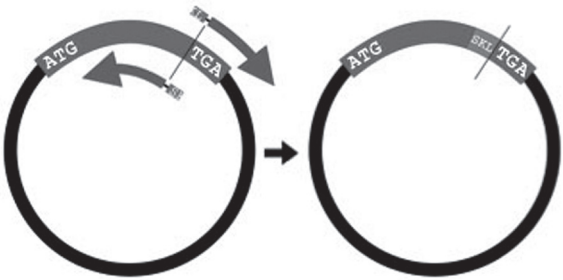

Fig. 2 Schematic diagram of SKL conjugation

An SKL conjugate method to the carboxy-terminal end of target molecules. Only a single long-range polymerase chain reaction is required. Primer regions are indicated by arrow and box. The sequence of the arrow region is exactly same as the sequence in plasmid. The box region has the sequence of Serine-Lysine-Leucine (SKL). This is merely an example of gene construction way to conjugate SKL, many other ways can be applicable for it as we noted about gene construction for Vent1-SKL, Smad1-SKL, Smad2-SKL, and Smad4-SKL.

used for constructing Vent2-SKL. We performed long-length polymerase chain reaction involving these primers and high-fidelity DNA polymerase (KOD-plus Neo, Toyobo, Japan). The 5' ends of the DNA fragments were phosphorylated using T4 polynucleotide kinase, followed by self-ligation with T4 DNA ligase. The mRNAs used for microinjection experiments were transcribed from linearized plasmids by using mMessage mMachine kit (Ambion). Note that the lengths of plasmid that we used for inverse PCR in this study were 3 to 5 kbps.

3 Results and Discussions

3.1 The SKL-tag trap assay blocks activity of Siamois

Only one polymerase chain reaction is required to conjugate the three SKL amino acids at the carboxy-terminal end of a target protein. To evaluate whether the mutated molecules constructed by this simple method actually functions, we used our experimental model *Xenopus* Siamois, a homeobox transcription factor superfamily that functions as a strong dorsalizing factor during *Xenopus* embryogenesis.

Siamois proteins, which belong to the homeobox transcription factor superfamily, are known to be essential for dorsal formation during vertebrate embryonic development (Ishibashi *et al*., 2008). In the *Xenopus* species, *Siamois* is expressed on the dorsal-animal side of blastula embryos prior to any other BCNE players (Kuroda *et al*., 2004). Therefore, ectopic activation of Siamois (Fig. 3A) can easily induce complete secondary axis containing head structure (Fig. 3B-E, I). On the other hand, most embryos injected with mRNA encoding SKL-tagged Siamois (*Siamois-SKL*) did not have secondary axis formation (Fig. 3F, G, I), and only one embryo had an incomplete secondary axis (Fig. 3H, I). Next, we tried to examine whether Siamois-SKL works as an inhibitor of the endogenous Siamois. If the nuclear localization step in Siamois were to be inhibited using the SKL-tag trap assay, the anterior structure would fail to develop. MOs are used as loss-of-functional methods

to inhibit translation of target gene (Heasman, 2002). As reported (Ishibashi *et al*., 2008), microinjection of MOs targeting *Siamois* mRNA (Siamois-MO) into dorsal side (Fig. 4A) caused anterior defects and ventralization (Fig. 4B, C, E, G). Interestingly, almost same phenotypical effects were observed in *Siamois-SKL* mRNA-injected embryos (Fig. 4D, F, G). These results also suggest that Siamois can dimerize with Twin, which is thought as multiple protein for Siamois (Ishibashi *et al*., 2008), in endogenous condition.

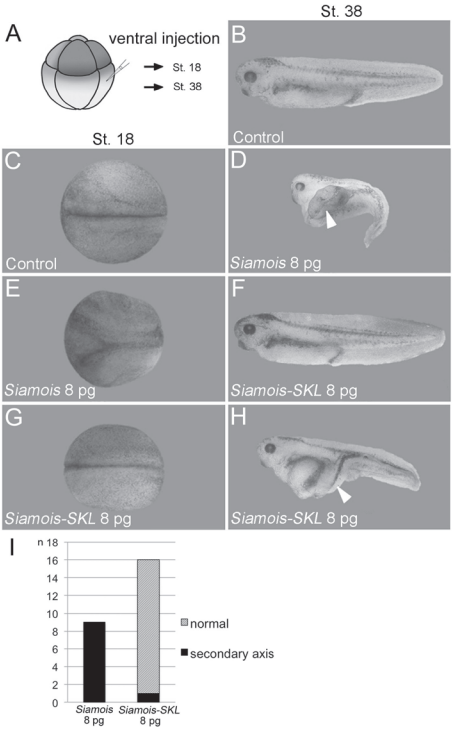

Fig. 3 Siamois-SKL does not have dorsalizing activity

(A) Experimental procedure of microinjection and embryo culture. mRNAs were injected into single site of ventral vegetal blastomere at 8-cell stage. (B) Control embryo at stage 38. (C) Control embryo at stage 18. (D) *Siamois* mRNA-injected embryo at stage 38. (E) *Siamois* mRNA-injected embryo at stage 18. (F) *Siamois-SKL* mRNA-injected embryo at stage 38. (G) *Siamois-SKL* mRNA-injected embryo at stage 18. (H) Weak secondary axis formation observed in *Siamois-SKL* mRNA-injected embryo at stage 38. (I) Graph to show the histogram of D-H.

3.2 The SKL-tag trap assay blocks activity of Vent1 and Vent2

Vents were originally isolated as the downstream molecules of ventralizing signal dependent on BMPs, and positive feedback loop of Vent/BMP is thought as the major ventralization mechanism of vertebrate early embryogenesis (Onichtchouk *et al*., 1996). We next asked whether the SKL-tag trap assay is adaptable for Vent1 and Vent2. As shown in Fig. 5, we designed SKL-tagged Vent1 and Vent2 (Vent1-SKL and Vent2-SKL) to block BMP signaling. Then we found that ventralization activity by Vent2 was completely diminished in the case of Vent2-SKL (Fig. 5A, B, C, D). Moreover, a small secondary axis formation, which is generally defined as a

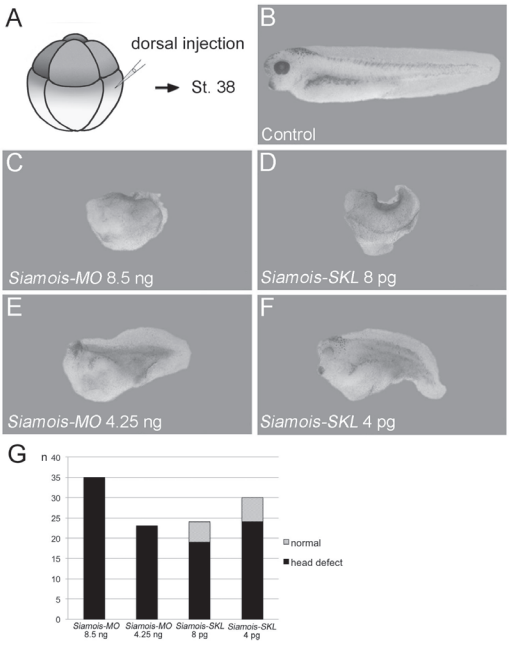

Fig. 4 Siamois-SKL mimics Siamois-MO

(A) Experimental procedure of microinjection and embryo culture. mRNAs were injected into two sites of dorsal blastomere at 8-cell stage. (B) Control embryo at stage 38. (C, E) Siamois-MO-injected embryo at stage 38. (D, F) *Siamois-SKL* mRNA-injected embryo at stage 38. (G) Graph to show the histogram of C-F.

dorsalized effect, was observed by single ventral injection of *Vent1-SKL* or *Vent2-SKL* mRNAs (Fig. 5E, F, G), and coinjection of *Vent1-SKL* and *Vent2-SKL* mRNAs improved the quality of ectopic secondary axis structure (Fig. 5H, I). These results suggest that the SKL-tagging should be an effective dominant negative way for the homeobox transcriptional factor superfamily.

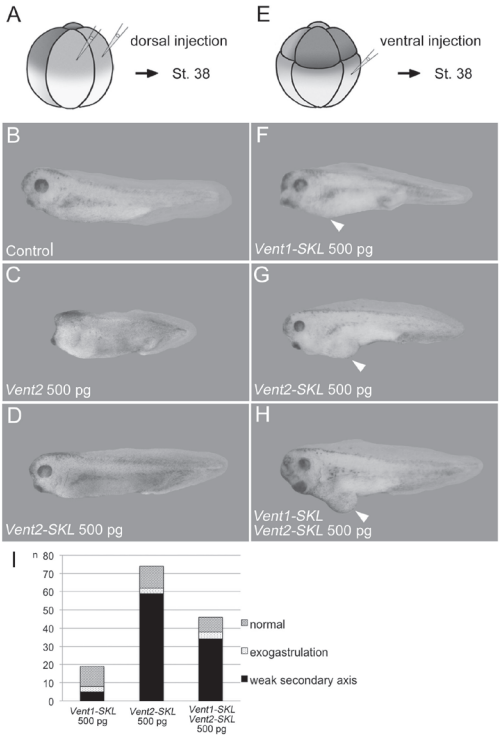

Fig. 5 Vent1-SKL and Vent2-SKL have dorsalizing activity

(A) Experimental procedure of microinjection and embryo culture. mRNAs were injected into two sites of dorsal blastomere at the 4-cell stage. (B) Control embryo at stage 38. (C) *Vent2* mRNA-injected embryo. (D) *Vent2-SKL* mRNA-injected embryo. No obvious effect was observed. (E) Experimental procedure of microinjection and embryo culture. mRNAs were injected into single site of ventral vegetal blastomere at 8-cell stage. (F) *Vent1-SKL* mRNA-injected embryo. White arrowhead indicates weak secondary axis formation. (G) *Vent2-SKL* mRNA-injected embryo. (H) *Vent1-SKL* and *Vent2-SKL* mRNAs-coinjected embryo. Siamois-MO-injected embryo at stage 38. (I) Graph to show the histogram of F-H.

3.3 The SKL-tag trap assay can target other transcription factors

Is the SKL-tag trap assay effective for the other dimer-typed or oligomer-typed transcription factors? In order to answer this question, Smad family is one of the best candidates for evaluating this assay because Smads can move into nucleus as heterotrimer or homotrimer when activated (Conidi *et al*., 2013). We added SKL at the carboxy-terminal regions of Smad1, Smad2, and Smad4 by PCR (Smad1-SKL, Smad2-SKL, and Smad4-SKL) and then injected each mRNA into the 2-cell stage embryos (Fig. 6A). Surprisingly, strong toxicity was observed in the case of *Smad1-SKL* mRNA (Fig. 6B, C, F), and severe posteriorization was induced in only a few survived embryos. Usually, the BMP/Smad1 signal is required for ventralization and epidermalization in ectoderm region, so loss-of-function of Smad1 should induce dorsalization or neuralization in epidermis. Interestingly, Smad1-MO in zebrafish embryos strongly upregulates transcription of ERK (Zhang *et al*., 2014). Strong posteriorization and toxicity are observed when ERK was activated under the control of FGF signal or Xnr3 (Hou *et al*., 2007; Yokota *et al*., 2003). Therefore, phenotypical effects observed in *Smad1-SKL* mRNA-injected embryos may be explained by overexpression of ERK. In the case of Smad2-SKL, most of embryos lost head structure and had shorten body axis (Fig. 6D, F). This level of shorten axis was not induced by microinjection of *Smad1-SKL* mRNA. Smad2 is required for mesoderm induction by Nodal signaling, so this result might be quite reasonable. Interestingly, in the case of Smad4-SKL, anterior defects were observed as the main phenotypical effects (Fig. 6E, F). Especially, optic structure was diminished in many embryos (Fig. 6E). Smad4 is usually working with Smad1 or Smad2 (Eivers *et al*., 2008), but Smad1 and Smad2 can be still working without Smad4 because R-Smad such as Smad1 and Smad2 can form a symmetric homotrimer (Conidi *et al*., 2013). Therefore, these obvious anterior defects were induced by combination of moderate inhibitions of the BMP/Smad1 and Nodal/Smad2 signaling.

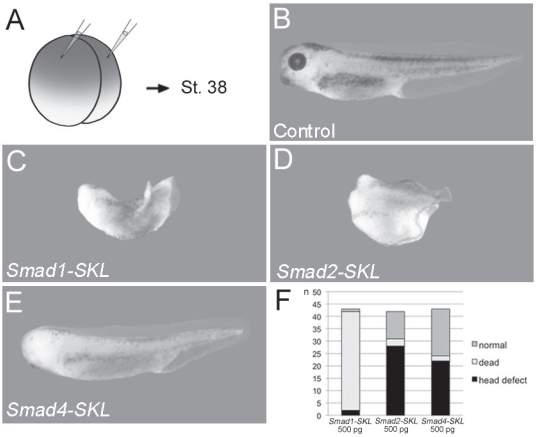

Fig. 6 SKL-conjugation is effective for Smad proteins

(A) Experimental procedure of microinjection and embryo culture. mRNAs were injected into two sites of animal side at 2-cell stage. (B) Control embryo at stage 38. (C) *Smad1-SKL* mRNA-injected embryo. (D) *Smad2-SKL* mRNA-injected embryo. (E) *Smad4-SKL* mRNA-injected embryo. (F) Graph to show the histogram of C-E.

3.4 Conclusions

In the present study, we have demonstrated a novel, yet simple and very effective technique for generating dominant-negative proteins by conjugating the SKL motif to the carboxy-terminal end of transcriptional factors. In this system, we used only one long-range polymerase chain reaction requiring two primers, each having several additional nucleomers, for adding the SKL motifs (Fig. 2). It may be quite reasonable way to add several nonsense amino acids between C-terminal region of target protein and SKL as linker, but at least in the cases of Siamois, Vents, and Smads it was not required. It must be next way when simple conjugation of SKL does not work for target protein. We did not perform typical control experiment such as conjugation of not-SKL tripeptide instead of SKL because we could not find proper tripeptide for it. One possible way is to use alanine (A), which is the simplest and uncharged asymmetric amino acid, instead of K in SKL, meaning to use

SAL instead of SKL, because a positively charged residue (K) is the most significant feature for keeping PTS activity (Brocard *et al*., 2006). However, this type of experiment may be not required because it is well known that C-terminal protein-tags with leucine at the C-terminal region such as Calmodulin-tag (KRRWKKNFIAVSAANRFKKISSSGAL) and Myc-tag (EQKLISEEDL) do not make any functional effect on target proteins.

The phenotypical observation using African clawed frog has long history and is thought as one of the most reliable methods to evaluate the effect of the BMP, the Wnt, and the Nodal signaling (De Robertis and Kuroda, 2004). In this study, obvious low-Wnt, high-BMP, or low-Nodal effects were respectively induced by overexpression of Siamois-SKL, Vent1-SKL and Vent2-SKL, or Smad2-SKL. Since many studies have been focused on Nodal, BMP, and Wnt proteins in general, the methods described in this study will be, at a minimum, useful to cell biologists who study these molecules. Furthermore, it may also be applicable in studies involving different molecules in various organisms. The uses of this SKL-tag trap assay should, therefore, allow researchers in molecular and cellular biology to readily generate new tools for inhibiting the activities of target dimeric, oligomeric, or polymeric transcriptional factors.

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