J. Biochem. 2010;147(6):875–884 doi:10.1093/jb/mvq023

JB THE JOURNAL OF BIOCHEMISTRY

Control of signalling properties of human somatostatin receptor subtype-5 by additional signal sequences on its amino-terminus in yeast

Received January 7, 2010; accepted February 22, 2010; published online March 5, 2010

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The yeast *Saccharomyces cerevisiae* is known as an available host for human G-protein-coupled receptor (GPCR) ligand screening. Although several types of yeast signal sequences (SS) attached with the GPCRs could improve their productivities and facilitate transportation of the GPCRs to the yeast plasma membrane, the effects of additional SS on ligand-specific signalling functions of GPCRs are not reported. Here, we demonstrated the controlling signalling properties by addition of SS using engineered yeast as a host. Prepro and pre regions of α-factor and amino-terminal sequence of Ste2 (Ste2N) were used as SS, and somatostatin (SST) receptor subtype-5 (SSTR5) was used as a model GPCR. We also constructed a yeast-based fluorescent assay system for monitoring the activation levels of SSTR5 signalling by a green fluorescent protein (*GFP*) reporter gene. The production levels and localisation patterns of the SS-attached SSTR5 were more significantly improved than those of wild-type SSTR5. In addition, we successfully controlled the pharmacological efficacy and potency by introducing SS. Among four types of SSTR5 receptors, Ste2N–SSTR5 responded at the lowest ligand concentration. This finding will be informative for constructing optimal yeast-based ligand screening systems to discriminate the cells on the basis of signalling levels.

Keywords: fluorescent protein/G-protein-coupled receptor/signal sequence/somatostatin/yeast.

Abbreviations: G-protein, guanine nucleotide binding proteins, GPCR, G-protein-coupled receptor; SS, signal sequence, Ste2N, amino-terminal sequence of Ste2; SST, somatostatin, SSTR5, somatostatin receptor; SSTR5, somatostatin receptor subtype-5, GFP, green fluorescent protein; CFP, cyan fluorescent protein, RGS, regulator of G-protein signalling; *PGK5′*, *PGK1* promoter, *PGK3′*, *PGK1* terminator; *5′FIG1*, *FIG1* promoter, *5′GPA1*, *GPA1* promoter; *MCS*, multiple cloning site, *CEN/ARS*, centromere-autonomously replicating sequence; *EGFP*, enhanced green fluorescent protein, *ECFP*, enhanced cyan fluorescent protein; OD, optical density, MOPSO, 3-(N-morpholino)-2-hydroxypropanesulfonic acid; green-RFU, relative GFP fluorescence unit, cyan-RFU, relative CFP fluorescence unit; EC_{50}, half maximal effective concentration, Gi3tp, Gpa1-$G\alpha_{i3}$ transplant; prepro, prepro-α-factor, pre, pre-α-factor.

Somatostatin (SST) is a natural ligand known as a growth hormone release-inhibiting factor that regulates the human endocrine system by binding with somatostatin receptors (SSTRs) belonging to the G-protein-coupled receptor (GPCR) superfamily. Following natural ligand-binding, GPCRs can transduce external stimuli into internal signals via the intracellular heterotrimeric guanine nucleotide binding proteins (G-proteins) that comprise Gα-, Gβ- and Gγ-subunits (*1*). SSTRs are basically classified into five subtypes (SSTRx; x = 1–5), and they are widely expressed in several human organs with tissue-specific distribution patterns and significance for the therapeutic targets of several diseases (*2*). For example, SSTRs appear to predominantly regulate growth hormone secretion in acromegaly patients (*3*), and SSTR2 and SSTR5 in particular are expressed in almost every growth-hormone secreting tumor (*4–6*). Therefore, to support beneficial treatment of acromegaly patients, an easy-to-use system to screen selective drug candidates for each SSTR subtype is desired.

The eukaryotic unicellular yeast *Saccharomyces cerevisiae* is considered a powerful tool for human GPCR studies because it can simply examine signalling activation because of its uncompetitive and monopolistic G-protein signalling pathway (pheromone signalling pathway mediated by single yeast GPCR, Ste2 pheromone receptor) as compared to other higher mammalian cell lines (*7*, *8*). The yeast cells have successfully expressed many types of human GPCRs and have been adeptly used for various applications such as ligand screening and receptor mutagenesis using enzymatic and growth reporter genes (*9–11*). However, GPCRs seemingly include limitations for the arrangements of seven transmembrane structures and the signalling machineries in yeasts, and actually, some types of human GPCRs are still unreported to

transduce the signals responding to the agonists in yeast cells.

It has been reported that several types of signal sequences (SS) were attached to the amino-terminus to facilitate productivity and transportation of the receptors to the yeast plasma membrane. One of the most frequently-used leader sequences for the secretion of foreign proteins in yeast is the prepro region that comprises the amino-terminal 85 aa of the α-factor pheromone, which have been previously used to express heterologous GPCRs in yeast cells (*12*). The pre regions of several secretory proteins that are cleaved off by the signal peptidase are also often used for the protein secretions (*13*, *14*). In addition, the amino-terminal sequence of the yeast endogenous Ste2 receptor has been introduced to the amino-terminus of the heterologous receptors, such as human β_2-adrenergic receptor and human D_{2s} dopamine receptor (*15*, *16*). Although SS addition can improve GPCR productivity and transportation, the signalling functions of GPCR in yeast compared to natural GPCR without SS have not been reported.

In this study, we demonstrated controlling of signalling potency and efficacy in yeast by introduction of SS to the SSTR5 amino-terminus. For the applications to the practical primary screening of drug discovery, high sensitivity is needed even if lower ligand concentration (*i.e.* higher pharmacological potency). To monitor SSTR5 signalling activation levels, we used a yeast-based fluorescent signalling assay system that expresses green fluorescent protein (*GFP*) reporter gene in response to SST-specific SSTR5 activation. We first engineered the intracellular G-protein α-subunit to optimise the agonist-specific signalling function of human SSTR5 in yeast. We then evaluated the signalling activities of the natural form of SSTR5 receptor without SS and the engineered form of SSTR5 receptors with SS. In addition, we examined the SSTR5 receptor expression levels and localisations using a cyan fluorescent protein (CFP) tag protein fused to the SSTR5 carboxyl-terminus.

Materials and Methods

Yeast strains and media

Yeast strains used in this study are listed in Table I. *Saccharomyces cerevisiae* MI-170 is *gpa1*Δ *ste2*Δ *sst2*Δ *far1*Δ quadruple mutant strain, which was originated from the BY4741 starin. The *gpa1*Δ allele is deficient in the yeast Gα-subunit to introduce different types of Gα-subunits. The *ste2*Δ allele is deficient in the yeast single GPCR to express human receptor without competitive expression. The *sst2*Δ allele is deficient in the yeast principal negative regulator of G-protein signalling (RGS) to produce signalling hypersensitivity for the lower agonist concentration. The *far1*Δ allele is deficient in the yeast G1-cyclin-dependent kinase inhibitor to prevent the induction of G1 arrest in response to signalling.

As the initial host strain, the BY4741 *gpa1*Δ single mutant strain was used to generate MI-170 with homologous recombination and marker recycle method (*17*) using three types of DNA fragments: *5′STE2-URA3-hrSTE2-3′STE2*, *5′SST2-URA3-hrSST2-3′SST2* and *5′FAR1-URA3-hrFAR1-3′FAR1*. These fragments were amplified by PCR using suitable oligonucleotide primer pairs and DNA templates as listed in Tables II and III. Sequential disruption of the *STE2*, *SST2*, and *FAR1* genes resulted in generation of the MI-100 (*gpa1*Δ *ste2*Δ), MI-150 (*gpa1*Δ *ste2*Δ *sst2*Δ), and MI-170 (*gpa1*Δ *ste2*Δ *sst2*Δ *far1*Δ) strains, respectively.

Yeast strains were cultivated in SD media containing 6.7 g/l yeast nitrogen base without amino acids (Becton, Dickinson and Company, Flanklin Lakes, NJ, USA), 20 g/l glucose and supplemented with appropriate amino acids and a nucleotide depending on the desired selectable marker (20 mg/l histidine, 60 mg/l leucine, 20 mg/l methionine and 20 mg/l uracil).

Plasmid constructions and yeast transformations

Plasmids constructed in this study are listed in Table IV. The DNA fragments were generated by PCR amplification from the respective template DNA using oligonucleotide primer pairs as listed in Tables II and III, then inserted into cloning vectors at the corresponding restriction sites.

Eventually, the following 12 plasmids were used for the yeast transformations with the lithium acetate method (*18*). To investigate agonist-induced SSTR5 signalling activity, seven plasmids—pGK-SSTR5-HA, pGKalpha-SSTR5-HA, pGKal-SSTR5-HA, pGKst-SSTR5-HA, pMHG-FIG1, pSL-GPA1 and pSL-Gi3tp—were used to transform MI-170 (MI-170-*x*; $x = 1$–5) (Table I). For estimation of receptor expression levels and observation of receptor localisations in yeast cells, five plasmids—pSSTR5-CF2, pSSTR5-CF2alpha, pSSTR5-CF2al, pSSTR5-CF2st and pGK421—were used to transform MI-170 (MI-170-*x*; $x = 6$–10) (Table I).

Flow cytometric analysis

CFP and GFP fluorescence were, respectively, analysed on the BD FACSCanto II flow cytometer equipped with both a 405-nm violet laser and a 488-nm blue laser (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and the data were analysed using the BD FACSDiva software (v5.0; Becton, Dickinson and Company). The CFP fluorescence signal was collected through a 480/30 nm band-pass filter and the CFP-A mean of 10,000 cells was defined as 'cyan fluorescent intensity'; similarly, the GFP fluorescence signal was collected through a 530/30 nm band-pass filter and the GFP-A mean of 10,000 cells was defined as 'green fluorescent intensity'.

GPCR Signalling Assay

Yeast transformants (MI-170-*x*; $x = 1$–5) were grown in plain SD media (without buffering agent and pH control) at 30°C over night and were inoculated into 20 ml of the same SD media to give an initial optical density of 0.03 at 600 nm ($OD_{600} = 0.03$). The cells were then grown at 30°C on a rotary shaker at 150 rpm for up to 18 h and harvested. After washing, the cells were adjusted to give an $OD_{600} = 10$ with pH 7.1-adjusted SD media containing 200 mM 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid (MOPSO). The yeast cell suspension (10 µl; to give an $OD_{600} = 1$) and the SST (14 aa peptide; Calbiochem, Darmstadt, Germany) (10 µl; to give an indicated final concentration, respectively) were added to the wells of 96-well cluster dishes containing fresh SD media (80 µl; adjusted to pH 7.1). The plates were incubated at 30°C and shaken at 150 rpm for 4 h. After incubation, the samples containing the yeast cells were diluted with 1 ml of sheath fluid, and GFP fluorescence was analysed by the flow cytometer. The relative GFP fluorescence units (green-RFU) were normalized by dividing the green fluorescent intensities of the yeast strains in the presence of SST by those of respective strains in the absence of SST. Assays were performed using three independent transformants. Half maximal effective concentrations (EC_{50}) values were determined using KaleidaGraph4.0 Fits to a dosersplgst model.

Determination of expression levels of SSTR5-CFP fusion proteins

Yeast transformants (MI-170-*x*; $x = 6$–10) were grown in plain SD media at 30°C overnight and the cells were inoculated into 20 ml of the same media to give an initial $OD_{600} = 0.03$. The cells were further cultivated at 30°C on a rotary shaker at 150 rpm for up to 18 h and then harvested. After washing, the cells were diluted with 1 ml of sheath fluid to give an $OD_{600} = 0.1$, and the CFP fluorescence was analysed with a flow cytometer. The relative CFP fluorescence units (cyan-RFU) were normalized by dividing the cyan fluorescent intensities of the yeast strains (MI-170-*x*; $x = 6$–9) by that of MI-170-10 control strain. Assays were performed using three independent transformants.

Table I. Yeast strains.

Strain	Relevant genotype	Reference or source
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	(*24*)
BY4741 *gpa1Δ*	BY4741 *gpa1Δ::kanMX4*	(*25*) purchased from Invitrogen, Carlsbad, CA, USA
MI-100	BY4741 *gpa1Δ::kanMX4 sst2Δ*	This study
MI-150	BY4741 *gpa1Δ::kanMX4 sst2Δ ste2Δ*	This study
MI-170	BY4741 *gpa1Δ::kanMX4 sst2Δ ste2Δ far1Δ*	This study
MI-170-1	MI-170 harbouring pMHG-FIG1, pSL-GPA1and pGK-SSTR5-HA	This study
MI-170-2	MI-170 harbouring pMHG-IG1, pSL-Gi3tp and pGK-SSTR5-HA	This study
MI-170-3	MI-170 harbouring pMHG-FIG1, pSL-Gi3tp and pGKakpha-SSTR5-HA	This study
MI-170-4	MI-170 harbouring pMHG-FIG1, pSL-Gi3tp and pGKal-SSTR5-HA	This study
MI-170-5	MI-170 harbouring pMHG-FIG1, pSL-Gi3tp and pGKst-SSTR5-HA	This study
MI-170-6	MI-170 harbouring pSSTR5-CF2	This study
MI-170-7	MI-170 harbouring pSSTR5-CF2alpha	This study
MI-170-8	MI-170 harbouring pSSTR5-CF2al	This study
MI-170-9	MI-170 harbouring pSSTR5-CF2st	This study
MI-170-10	MI-170 harbouring pGK421	This study

Fluorescence microscope observation of localisation patterns of SSTR5-CFP fusion proteins

Yeast transformants (MI-170-*x*; $x = 6$–9) were grown in plain SD media at 30°C overnight, and the cells were inoculated into 20 ml of the same media to give an initial $OD_{600} = 0.03$. The cells were further cultivated at 30°C on a rotary shaker at 150 rpm for up to 18 h and then harvested. After washing, the cells were diluted with 1 ml of distilled water to give an $OD_{600} = 1$, and the CFP fluorescence images were analysed by a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). Exposure time was 2 s for MI-170-6; and 1 s for MI-170-7, MI-170-8 and MI-170-9.

Results

Optimisation of G-protein α-subunit for evaluating SST-specific SSTR5 signalling activity

To evaluate signalling activity of the SSTR5 responding to SST binding in yeast, we used a yeast-based fluorescent signalling assay system that induces expression of the *GFP* reporter gene by SST-dependent activation of GPCR signalling. To improve the functional coupling of the human SSTR5 to the yeast pheromone signalling pathway, the yeast–human chimeric Gα subunit, in which the carboxyl-terminal 5 aa residues of yeast endogenous Gα subunit (Gpa1) was substituted by that of human $G\alpha_{i3}$ (Gpa1-$G\alpha_{i3}$ transplant; Gi3tp), was produced as an alternative form of Gpa1 because of its effective signal transduction of the $G\alpha_{i3}$ familiar receptors (*19*). Then, the transduction efficiency coupled with the human SSTR5 was compared between Gpa1- and Gi3tp-expressing yeast cells (Fig. 1).

Figure 1A shows the dose–response curve for the pharmacological efficacy of ligand-specific SSTR5 signalling in yeast cells that express the endogenous Gα-subunit Gpa1 or the yeast–human chimeric Gα-subunit Gi3tp. In both cells, depending on the SST concentration, the appearance of green fluorescence was observed, clearly indicating that SSTR5 signalling is quantitatively measurable by the fluorescent signalling assay system using the *GFP* reporter gene. The maximum value for Green-RFU, which represented the maximum effect of the dose-response, was higher in the Gi3tp-expressing cells than in the Gpa1-expressing cells.

Figure 1B shows the pharmacological potency of ligand-specific SSTR5 signalling in Gpa1- or Gi3tp-expressing yeast cells. The higher EC_{50} value of the Gi3tp-expressing yeast cells than the Gpa1-expressing yeast cells suggests that the SST-induced interaction between the human SSTR5 and the yeast–human chimeric Gα subunit is more efficient for the signalling activation machinery on the intracellular peripheral yeast membrane (*19*). Therefore, for further experiments, Gi3tp-expressing yeast cells were used to investigate the effect of SS introduction to the SSTR5 amino-terminus on SST-dependent signalling activity in yeast.

Effect of yeast SS attachment at amino-terminus of human SSTR5 on SST-specific SSTR5 signalling activity in yeast

To investigate SST-specific signalling activity of human SSTR5 fused with SS at the amino-terminus in yeast, three types of yeast SS—prepro-α-factor (prepro), pre-α-factor (pre) and Ste2 amino-terminal 20 aa (Ste2N)—were selected because these particular SS could enhance the productivity and signalling abilities of human GPCRs in yeast cells (*12*–*16*). The three SS were genetically connected to the SSTR5 amino-terminus, and the SST-specific signalling activation levels of the SS-attached SSTR5 receptors were evaluated in the Gi3tp-expressing yeast cells using the *GFP* reporter gene.

Figure 2A shows the efficacies for SST-specific signalling activation mediated by SSTR5 and the three types of SS-SSTR5 receptors. SST-specific signalling activities were observed in all three SS-inserted SSTR5 receptors, although the maximal effects of the dose-responses (green-RFU) in the cells expressing the SS-attached SSTR5 receptors were lower than those of the cells expressing the natural SSTR5 receptor without SS addition. In the case of the Ste2N-SSTR5-expressing cells, the dose–response levels at lower SST concentrations were highest among all strains.

Figure 2B shows the pharmacological potencies of SST for the SSTR5 receptors expressed in yeast. The EC_{50} values of the cells expressing the pre-SSTR5 and Ste2N-SSTR5 were lower than those of the cells expressing the native form of the SSTR5 receptor,

Table II. Oligonucleotide primer pairs.

Name	Oligonucleotide primer pair sequences
o1	5′-TTTCTTTTCACCTGCTCTGGCTATAATTATAATTGGTTACTTAAAAATGCACCGTTAAGAACCATATCCAAGAATCAAAATTTTTTGTTCTTTTTTTTGA-3′ and 5′-TCTTAACGGTGCATTTTTAAGGGTAATAACTGATATAATT-3′
o2	5′-TCGTACGTTCCTTCTAGGTTTTGCACGCACTATCTGAGGCGTTATAGGTTCAATTTGGTAATTAAAGATAGAGTTGTAAGTTTTTTGTTCTTTTTTTTGA-3′ and 5′-TACCAAATTGAACCTATAACGGGTAATAACTGATATAATT-3′
o3	5′-TTTATATTTCTTGACCATCCTTTACACAAAGTCTATAGATCCACTGGAAAGCTTCGTGGGCGTAAGAAGGCAATCTATTATTTTTTGTTCTTTTTTTTGA-3′ and 5′-GGGCCTCGATTCCCGAACTAGGGTAATAACTGATATAATT-3′
o4	5′-AATTATATCAGTTATTACCCTTAAAAATGCACCGTTAAGAACCATATCCAAGAATCAAAATCAAAATTTACGGCTTTGAAAAAGTAATTTCGTGACCTTC-3′ and 5′-AAGATTAACTGTATATATTGCCTGAGAGTTCTAGATCATG-3′
o5	5′-AATTATATCAGTTATTACCCGTTATAGGTTCAATTTGGTAATTAAAGATAGAGTTGTAAGTTTCATTGAGAGTCTTACTCATCTTCAGGTACAATTGCAC-3′ and 5′-AAAAAAAAGTTTTCAGACGGACTTAAACCGCCAACCAGCC-3′
o6	5′-AATTATATCAGTTATTACCCCCACTGGAAAGCTTCGTGGGCGTAAGAAGGCAATCTATTATAGTTCGGGAATCGAGGCCCGTATTTCGAGGCTTTTGCTT-3′ and 5′-GCATATATGACGAGATTTAATTATCGCCAATAGGTTCTTT-3′
o7	5′-TTTCTTTTCACCTGCTCTGGCTATAATTATAATTGGTTACTTAAAAATGCACCGTTAAGAACCATATCCAAGAATCAAAATTTTTTGTTCTTTTTTTTGA-3′ and 5′-AAGATTAACTGTATATATTGCCTGAGAGTTCTAGATCATG-3′
o8	5′-TCGTACGTTCCTTCTAGGTTTTGCACGCACTATCTGAGGCGTTATAGGTTCAATTTGGTAATTAAAGATAGAGTTGTAAGTTTTTTGTTCTTTTTTTTGA-3′ and 5′-AAAAAAAAGTTTTCAGACGGACTTAAACCGCCAACCAGCC-3′
o9	5′-TTTATATTTCTTGACCATCCTTTACACAAAGTCTATAGATCCACTGGAAAGCTTCGTGGGCGTAAGAAGGCAATCTATTATTTTTTGTTCTTTTTTTTGA-3′ and 5′-GCATATATGACGAGATTTAATTATCGCCAATAGGTTCTTT-3′
o10	5′-TTTTGCTAGCATGGAGCCCCTGTTCCCAGC-3′ and 5′-TTGGAGATCTTCACAGCTTGCTGGTCTGCA-3′
o11	5′-CCCGACGTCCACGGACTATAGACTATACTA-3′ and 5′-CCTGACGTCTGAACCAGTCCTAAAACGAGT-3′
o12	5′-TTTTGGATCCGTGAGCAAGGGCGAGGAGCT-3′ and 5′-GGGGAGATCTTTACTTGTACAGCTCGTCCA-3′.
o13	5′-TTTTGCTAGCATGGAGCCCCTGTTCCCAGC-3′ and 5′-GGGAAGATCTCAGCTTGCTGGTCTGCATAA-3′
o14	5′-GGGGGCTAGCATGAGATTTCCTTCAATTTT-3′ and 5′-TGGGAACAGGGGCTCTCTTTTATCCAAAGA-3′
o15	5′-TCTTTGGATAAAAGAGAGCCCCTGTTCCCA-3′ and 5′-TTGGAGATCTTCACAGCTTGCTGGTCTGCA-3′
o16	5′-GGGGGCTAGCATGAGATTTCCTTCAATTTT-3′ and 5′TTGGAGATCTTCACAGCTTGCTGGTCTGCA-3′.
o17	5′-TTTTGCTAGCATGAGATTTCCTTCAATTTTTACTGCAGTTTTATTCGCAGCATCCTCCGCATTAGCTGAGCCCCTGTTCCCAGCCTCCACGCCCAGC-3′ and 5′-GGGAAGATCTCAGCTTGCTGGTCTGCATAA-3′
o18	5′-AAAAGCTAGCATGTCTGATGCGGCTCCTTCATTGAGCAATCTATTTTATGATCCAACGTATAATCCTGGTGAGCCCCTGTTCCCAGCCTCCACGCCCAGC-3′ and 5′-GGGAAGATCTCAGCTTGCTGGTCTGCATAA-3′
o19	5′-TTTTGCTAGCATGGAGCCCCTGTTCCCAGC-3′ and 5′-TTGGAGATCTTCAAGCGTAATCTGGAACATCGTATGGGTACAGCTTGCTGGTCTGCA-3′
o20	5′-GGGGGCTAGCATGAGATTTCCTTCAATTTT-3′ and 5′-TTGGAGATCTTCAAGCGTAATCTGGAACATCGTATGGGTACAGCTTGCTGGTCTGCA-3′
o21	5′-TTTTGCTAGCATGAGATTTCCTTCAATTTTTACTGCAGTTTTATTCGCAGCATCCTCCGCATTAGCTGAGCCCCTGTTCCCAGCCTCCACGCCCAGC-3′ and 5′-TTGGAGATCTTCAAGCGTAATCTGGAACATCGTATGGGTACAGCTTGCTGGTCTGCA-3′
o22	5′-AAAAGCTAGCATGTCTGATGCGGCTCCTTCATTGAGCAATCTATTTTATGATCCAACGTATAATCCTGGTGAGCCCCTGTTCCCAGCCTCCACGCCCAGC-3′ and 5′-TTGGAGATCTTCAAGCGTAATCTGGAACATCGTATGGGTACAGCTTGCTGGTCTGCA-3′
o23	5′-TTTTGGATCCATGGTGAGCAAGGGCGAGGA-3′ and 5′-AAAACCGCGGTTACTTGTACAGCTCGTCCA-3′
o24	5′-CCCCCCGCGGGAAATAAATTGAATTGAATT-3′ and 5′-TTTTGAGCTCAGCTTTAACGAACGCAGAAT-3′
o25	5′-GAAACTCGAGATCACCCTGCATTGCCTCTT-3′ and 5′-CCCCGGATCCTTTTTTTTTTTTTTTTTTGT-3′
o26	5′-GGGGCTCGAGTCATACTTAGAAAAACATGT-3′ and 5′GGGGAGATCTTCATATAATACCAATTTTTT-3′
o27	5′-GGGGCTCGAGTCATACTTAGAAAAACATGT-3′ and 5′-CCCCAGATCTTCAGTACAAACCACATTCTTTAAGGTTTTGCTGGATGATTAGATCGGTGACTGCACTCAA-3′

Table III. PCR-amplified DNA fragments used for yeast gene disruptions and plasmid constructions.

Amplified DNA fragments	Relevant genetic descriptions	Template DNAs	Primer pairs
5′STE2-URA3	80-bp *STE2*-5′ flanking region and *URA3*	pRS406	1
5′SST2- URA3	80-bp *SST2*-5′ flanking region and *URA3*	pRS406	2
5′FAR1- URA3	80-bp *FAR1*-5′ flanking region and *URA3*	pRS406	3
hrSTE2-3′STE2	40-bp *STE2*-5′ flanking region and 1-kbp *STE2*-3′ flanking region	BY4741 genomic DNA	4
hrSST2-3′SST2	40-bp *SST2*-5′ flanking region and 1-kbp *SST2*-3′ flanking region	BY4741 genomic DNA	5
hrFAR1-3′FAR1	40-bp *FAR1*-5′ flanking region and 1-kbp *FAR1*-3′ flanking region	BY4741 genomic DNA	6
5′STE2-URA3-hrSTE2-3′STE2	*5′STE2- URA3* and *hrSTE2-3′STE2*	*5′STE2-URA3* and *hrSTE2-3′STE2*	7
5′SST2-URA3-hrSST2-3′SST2	*5′SST2-URA3* and *hrSST2-3′SST2*	*5′SST2-URA3* and *hrSST2-3′SST2*	8
5′FAR1-URA3-hrFAR1-3′FAR1	*5′FAR1-URA3* and *hrFAR1-3′FAR1*	*5′FAR1-URA3* and *hrFAR1-3′FAR1*	9
SSTR5-HA	The *SSTR5-HA* fragment in pBlue-SSTR5-HA	Human brain cDNA (Invitrogen, Carlsbad, CA, USA)	10
2μ origin	The *Aat*II 2μ origin fragment in pRS401+2 µm	pWI3	11
ECFP	The *Bam*HI-*Bgl*II *ECFP* fragment in pGK421-ECFP	pECFP-C1	12
SSTR5	The *Nhe*I-*Bgl*II *SSTR5* fragment in pSSTR5-CF2	pBlue-SSTR5-HA	13
Prepro-α-factor	Template DNA of overlap PCR for the *prepro-SSTR5* amplification	pWI3α	14
hr-SSTR5	Template DNA of overlap PCR for the *prepro-SSTR5* amplification	pBlue-SSTR5-HA	15
Prepro-SSTR5	The *Nhe*I-*Bam*HI *prepro-SSTR5* fragment in pSSTR5-CF2alpha	*prepro-α-factor and hr-SSTR5*	16
Pre-SSTR5	The *Nhe*I-*Bgl*II *pre-SSTR5* fragment in pSSTR5-CF2al	pBlue-SSTR5-HA	17
Ste2N-SSTR5	The *Nhe*I-*Bgl*II *Ste2N-SSTR5* fragment in pSSTR5-CF2st	pBlue-SSTR5-HA	18
SSTR5-HA	The *Nhe*I-*Bgl*II *SSTR5-HA* fragment in pGK-SSTR5-HA	pBlue-SSTR5-HA	19
Prepro-SSTR5-HA	The *Nhe*I-*Bgl*II *prepro-SSTR5-HA* fragment in pGKalpha-SSTR5-HA	pBlue-SSTR5-HA	20
Pre-SSTR5-HA	The *Nhe*I-*Bgl*II *pre-SSTR5-HA* fragment in pGKal-SSTR5-HA	pBlue-SSTR5-HA	21
STE2N-SSTR5-HA	The *Nhe*I-*Bgl*II *STE2N-SSTR5-HA* fragment in pGKst-SSTR5-HA	pBlue-SSTR5-HA	22
EGFP	The *Bam*HI-*Sac*II *EGFP* fragment in pMHG-FIG1	pEGFP-C1	23
PGK3′	The *Sac*II-*Sac*I *PGK3′* fragment in pMHG-FIG1	pTA2-PGK	24
5′FIG1	The *Xho*I-*Bam*HI 450-bp *FIG1*-5′ flanking region in pMHG-FIG1	BY4741 genomic DNA	25
5′GPA1 -GPA1	The *Xho*I-*Bgl*II 1-kp *GPA1*-5′ flanking region–*GPA1* in pSL-GPA1	BY4741 genomic DNA	26
5′GPA1-Gi3tp	The *Xho*I-*Bgl*II 1-kb *GPA1*-5′ flanking region–*Gi3tp* in pSL-Gi3tp	BY4741 genomic DNA	27

suggesting that introduction of SS improves ligand sensitivity (Fig. 2B and Table V).

Estimation of expression levels and observation of localisation of CFP fluorescent-tagged SSTR5 receptors with or without introduction of SS in yeast

To estimate expression levels and observe the subcellular localisations of the SSTR5 receptors with or without addition of SS in yeast, the CFP fluorescent tag protein was introduced at the carboxyl-terminus of the natural SSTR5 and the engineered SS-introduced SSTR5 receptors (SSTR5-CFP and three types of SS-SSTR5-CFPs) were expressed in the yeasts. Figure 3 shows the average intensities of CFP fluorescence of 10,000 cells normalized with the control strain harbouring mock vector. Compared with the SSTR5-CFP-expressing cells without addition of SS, the yeast cells expressing the all three types of SS-SSTR5-CFPs displayed higher fluorescence intensities in the following order: SSTR5-CFP < prepro-SSTR5-CFP < pre-SSTR5-CFP <Ste2N -SSTR5-CFP. The result suggests that introduction of SS accelerates SSTR5 receptor expression levels in yeast cells and also corresponds to findings of previous reports (*12*, *15*).

To confirm the localisations of SSTR5 receptors in yeast, SSTR5-CFP-expressing cells were subsequently observed by fluorescence microscopy (Fig. 4). Among the four SSTR5-CFP receptors, the Ste2N-SSTR5-CFP receptor was localised to the plasma membranes. In the case of yeast cells expressing SSTR5-CFP without addition of SS, CFP fluorescence appeared to be mainly accumulated in vacuoles and was partially observed on the plasma membranes and inside the cells. On the other hand, the prepro-SSTR5-CFP receptor was predominantly localised to the endoplasmic reticulum and Golgi apparatus accompanied with strong CFP fluorescence in yeast cells. The pre-SSTR5-CFP receptor displayed an intermediate localisation pattern between prepro-SSTR5-CFP and Ste2N-SSTR5-CFP.

Discussion

In this study, to guide the easy-to-use yeast-based system to comfortably screen primary drug candidates

Table IV. Plasmids.

Plasmid	Relevant genotype	Reference or source
pRS406	Containing *URA3* marker	American Type Culture Collection, Manassas, VA, USA
pBluescript II KS(+)	Cloning vector	Stratagene, La Jolla, CA, USA
pBlue-SSTR5-HA	*SSTR5-HA* in pBluescript II KS(+)	This study
pWI3	Containing 2μ origin	(*26*)
pRS401	Containing *MET15* marker	American Type Culture Collection, Manassas, VA, USA
pTA2-PGK	Containing multiple cloning sites (*MCS*) between the yeast *PGK1* promoter (*PGK5′*) and *PGK1* terminator (*PGK3′*)	(*27*)
pRS401+2 μm	2μ origin in pRS401	This study
pGK421	The *XhoI-NheI PGK5′-MCS-PGK3′* digested from pTA2-PGK in pRS401+2 μm	This study
pECFP-C1	Containing *ECFP*	Clontech Laboratories, Mountain View, CA, USA
pGK421-ECFP	*ECFP* in pGK421	This study
pSSTR5-CF2	*SSTR5* in pGK421-ECFP	This study
pWI3α	Containing *prepro-α-factor*	(*26*)
pSSTR5-CF2alpha	*Prepro-SSTR5-ECFP* in pGK421	This study
pSSTR5-CF2al	*Pre-SSTR5-ECFP* in pGK421	This study
pSSTR5-CF2st	*STE2N-SSTR5-ECFP* in pGK421	This study
pGK-SSTR5-HA	*SSTR5-HA* in pGK421	This study
pGKalpha-SSTR5-HA	*Prepro-SSTR5-HA* in pGK421	This study
pGKal-SSTR5-HA	*Pre-SSTR5-HA* in pGK421	This study
pGKstSSTR5-HA	*STE2N-SSTR5-HA* in pGK421	This study
pEGFP-C1	Containing *EGFP*	Clontech Laboratories, Mountain View, CA, USA
pRS403 + 2 μm	Containing *HIS3* marker and 2μ origin	(*28*)
pMHG-FIG1	*5′FIG1-EGFP-PGK3′* in pRS403+2 μm	This study
pGK415	Containing *PGK5′-MCS-PGK3′*, *LEU2* marker and *CEN/ARS* origin	(*28*)
pSL-GPA1	*5′GPA1-GPA1-PGK3′* in pGK415	This study
pSL-Gi3tp	*5′GPA1-Gi3tp-PGK3′* in pGK415	This study

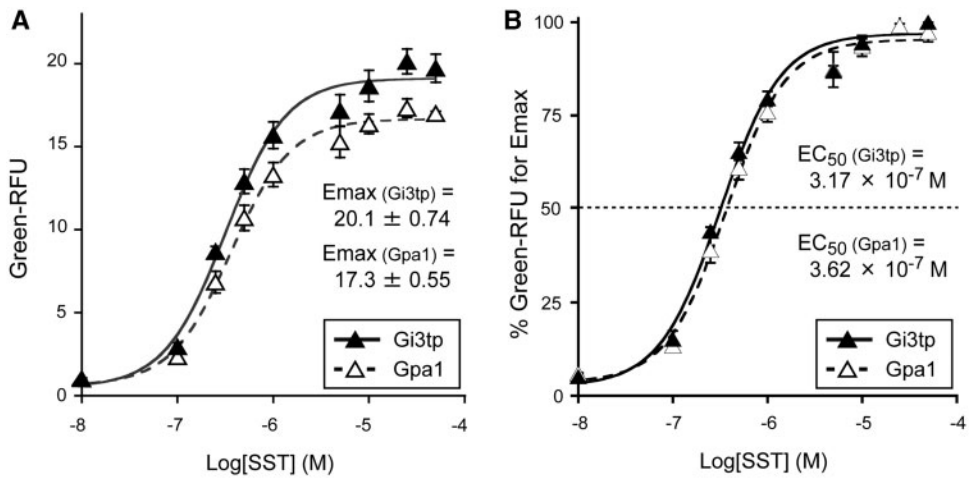

Fig. 1 Dose–response curves for SST-specific SSTR5 signalling activities of recombinant yeast cells expressing different types of intracellular peripheral G-protein α-subunits. As Gα-subunits for the functional SST-specific SSTR5 signalling, yeast endogenous Gpa1 and yeast–human chimeric Gi3tp were compared using the *GFP* reporter gene. The signalling activities of recombinant strains MI-170-1 (Gpa1, open symbol) and MI-170-2 (Gi3tp, closed symbol) were evaluated as described in 'MATERIALS AND METHODS' section. (**A**) Pharmacological efficacies of yeast transformants were represented by green-RFU normalized with the green fluorescent intensities of the SST-untreated yeast cells as the reference values, respectively. (**B**) Pharmacological potencies of yeast transformants were represented by relative green-RFU normalized with the values of maximal effects of SST-specific dose–responses as the reference values, respectively. Data points represent the mean of triplicate independent experiments and error bars represent the standard deviation. EC_{50} values were determined using KaleidaGraph4.0 fits to a dosersplgst model.

for human GPCRs, we aimed to control human SSTR5 signalling activity in yeast via addition of SS. We used yeast-based fluorescent assay systems for evaluation of SSTR5 signalling by the *GFP* reporter gene. SST-specific stimulation of human SSTR5 receptor can be converted into yeast pheromone signalling via the yeast endogenous Gα-subunit protein (Gpa1), and the resulting green fluorescent signals from the activated cells are quantitatively and immediately measurable by direct analysis of non-destructive cells using a flow cytometer.

The substitution of carboxyl-terminal 5 aa residues of the endogenous Gpa1 for the yeast Gpa1-human $G\alpha_{i3}$ chimeric Gα-subunit protein, Gi3tp, was effective

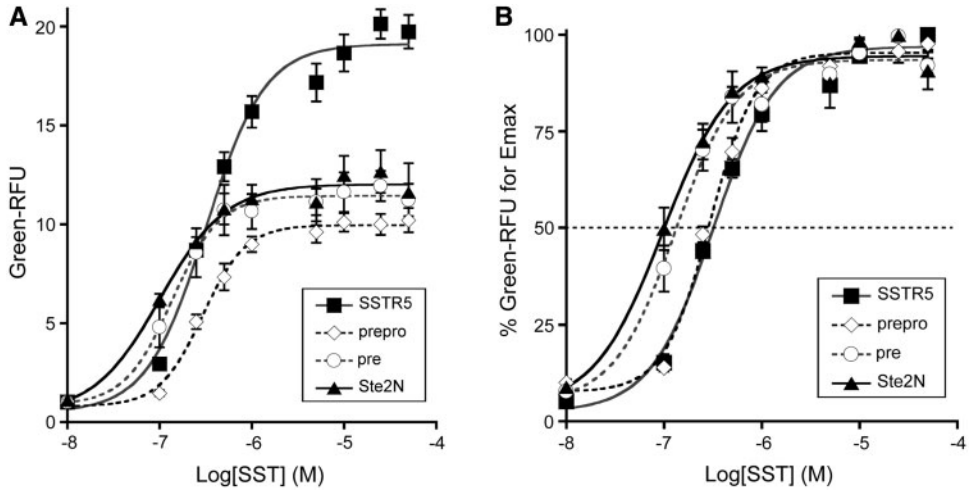

Fig. 2 Dose–response curves for SST-specific signalling activities of SSTR5 with or without additional SS in yeast cells. To examine the effects of SS for the SST-specific SSTR5 signalling, prepro-SSTR5, pre-SSTR5 and Ste2N-SSTR5 were prepared. The signalling activities of recombinant strains MI-170-3 (prepro), MI-170-4 (pre) and MI-170-5 (Ste2N) were evaluated using the *GFP* reporter gene as described in 'MATERIALS AND METHODS' section. (**A**) Pharmacological efficacies of yeast transformants were represented by green-RFU normalized with the green fluorescent intensities of the SST-untreated yeast cells as the reference values, respectively. (**B**) Pharmacological potencies of yeast transformants were represented by relative green-RFU normalized with the values of maximal effects of SST-specific dose responses as the reference values, respectively. MI-170-2 data shown in Fig. 1 are displayed for comparison. Data points represent the mean of triplicate independent experiments and error bars represent the standard deviation.

Table V. Characterization of SSTR5 receptors with and without SS expressed in MI-170 yeast cells.

Receptor	Expression level[a]	Maximal effect[b]	EC_{50} value[c]
SSTR5	2.58 ± 0.54	20.1 ± 0.74	3.17×10^{-7}
Prepro-SSTR5	4.81 ± 0.99	10.2 ± 0.62	2.92×10^{-7}
Pre-SSTR5	6.12 ± 0.49	11.9 ± 0.76	1.37×10^{-7}
Ste2N-SSTR5	8.43 ± 1.37	12.7 ± 1.1	1.01×10^{-7}

[a]Expression levels cited the values in Fig. 3.
[b]Maximal effects cited the values in Fig. 2A.
[c]EC_{50} values cited the values in Fig. 2B.

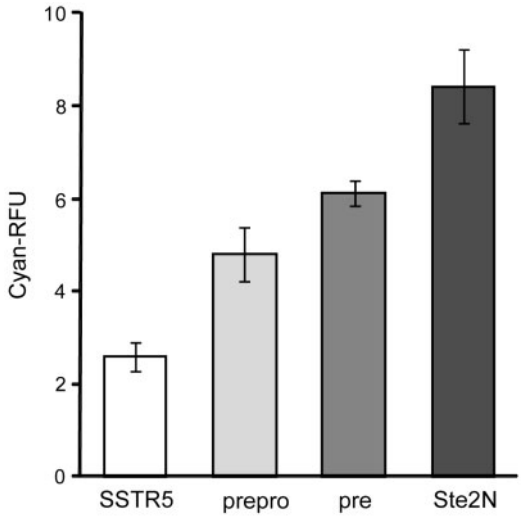

Fig. 3 Estimation of expression levels of CFP-fluorescent tagged SSTR5 receptors with or without additional SS sequences. Four CFP-fluorescent tagged SSTR5 constructs—SSTR5-CFP, prepro-SSTR5-CFP, pre-SSTR5-CFP and Ste2N-SSTR5-CFP—were prepared to generate recombinant strains MI-170-6 (SSTR5), MI-170-7 (prepro), MI-170-8 (pre) and MI-170-9 (Ste2N), respectively. Cellular cyan-RFU was calculated as described in 'MATERIALS AND METHODS' section. Data represent the mean of triplicate independent experiments and error bars represent the standard deviation.

to functionally couple the SSTR5 to the yeast pheromone signalling pathway as shown in a previous report (*19*). Obviously, the maximum effect responding to the highest concentration of SST was enhanced in the Gi3tp-expressing yeast cells and the dose–response curves exposed the suitable pharmacological potency and efficacy of Gi3tp for the SSTR5 (Fig. 1). Therefore, the SSTR5 Gα-subunit was successfully optimised by substitution of chimeric Gi3tp for endogenous Gpa1 in the yeast-based fluorescent signalling assay system. Although productivity and transportation of the heterogous GPCRs could be enhanced via introduction of the yeast SS in yeast cells (*12*, *15*, *16*), the signalling functions of SS-attached GPCRs have been rarely described. When constructing a superior ligand screening method using a yeast-based fluorescent signalling assay system, either higher sensitivity or a higher maximal effect should be achieved in the system, although it is difficult to simultaneously achieve both. Indeed, the signalling properties desired in yeast-based signalling assay systems differ depending on the aims for varied assay applications. For example, systems with high sensitivity are useful for the agonist detection assay at low concentrations or the competitive assay with antagonists; on the other hand, systems with high maximal effect are useful for the high-throughput ligand screening system by FACS. By controlling the signalling property, we could construct a suitable yeast-based assay system depending on the aims of the assays. Therefore, to control signalling ability of human SSTR5 in a yeast-based signalling assay system, we constructed four types of SSTR5 receptor variants and investigated their SST-specific signalling activities in yeast cells.

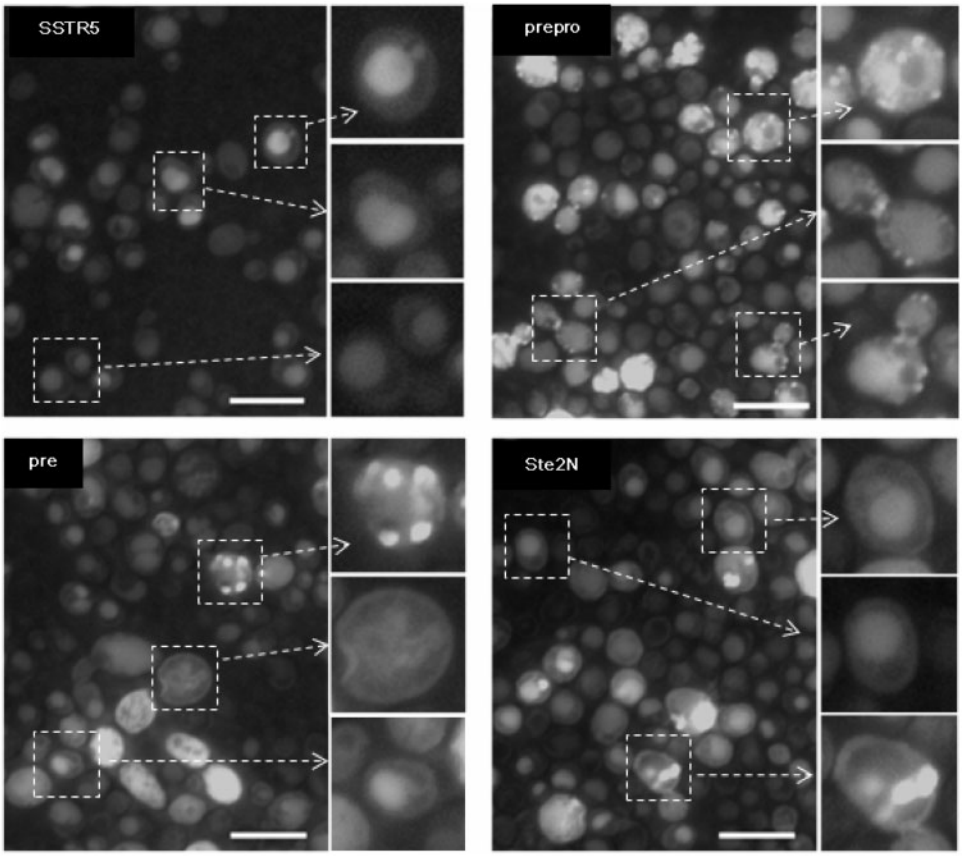

Fig. 4 Observation of localisation patterns of CFP-fluorescent tagged SSTR5 receptors with or without additional SS. The yeast strain MI-170 was used as the host strain to introduce CFP-fluorescent tagged SSTR5 receptors without or with additional SS. Four CFP-fluorescent tagged SSTR5 constructs—SSTR5-CFP, prepro-SSTR5-CFP, pre-SSTR5-CFP and Ste2N-SSTR5-CFP—were prepared to generate recombinant strains MI-170-6 (SSTR5), MI-170-7 (prepro), MI-170-8 (pre) and MI-170-9 (Ste2N), respectively. The cellular cyan fluorescence was observed with the fluorescence microscope as described in 'MATERIALS AND METHODS' section. White bars represent 10 μm.

Insertions of any three SS to the amino-terminus of SSTR5 drastically decreased the efficacies of the engineered receptors expressing cells compared to that of the native SSTR5 form (Fig. 2A and Table V). The pharmacological potencies of SST for the engineered SS-SSTR5 receptors were higher than that of the native form SSTR5 receptor; Ste2N-SSTR5 in particular displayed the highest potency for SST-dependent signalling activity (Fig. 2B and Table V). Therefore, the Ste2N-SSTR5 receptor is useful for constructing high-sensitivity yeast-based assay systems such as agonist detection assays at low concentrations and competitive assays with antagonists.

FACS analysis of the CFP fusion proteins indicated that addition of SS increased SSTR5 expression levels in yeast cells (Fig. 3). However, not all of the CFP-tagged SSTR5 molecules were localised to the plasma membrane because the CFP-tagged SSTR5 molecules were transported by different protein sorting pathways depending on the SS properties (Fig. 4). These results suggest that the SS of the typical secretory protein is not suitable for functional expression of seven transmembrane SSTR5 receptors at the yeast plasma membrane. The potency was decreased in the case of prepro-SSTR5, which may be caused by the lower amount of the receptors on yeast cell surface.

The large amounts of SSTR5 receptors at the yeast plasma membrane accelerate the ligand binding even low concentration of SST, as a result, the potency of Ste2N-SSTR5 was improved. Although ligand binding ability seemed to effect on the efficacy, Sander *et al.* (*16*) reported the introduction of Ste2-derived SS to human D_{2s} dopamine receptor did not affect the ligand binding ability when the receptor was expressed on the yeast cell surface. Therefore, we assumed the binding ability of SS-attached SSTR5 is almost same as that of SSTR5. One of the possible explanations for decreased efficacy is that introduction of SS at amino-terminus may inhibit the receptor dimerisation (*20*, *21*). Dimerisation of GPCRs should be required for desensitisation and internalisation, which are necessary events to refresh ligand-bound receptors and regenerate ligand-free receptors at the plasma membrane for responding to the redundant extracellular ligands (*22*, *23*).

In conclusion, we demonstrated SST-specific signalling functions of SSTR5 via introduction of SSs in yeast. Using a yeast–human Gi3tp chimera, we optimised the yeast-based fluorescent assay system. Introduction of Ste2N to the amino-terminus of human SSTR5 significantly improved pharmaceutical potency in the yeast-based fluorescent assay system. This finding will be an informative support to

construct optimal yeast-based flow cytometric signalling assay systems using other human GPCRs and will be helpful for execution of primary drug screening.

Acknowledgements

The authors thank Prof. Shun'ichi Kuroda and Dr Kenji Tatematsu for their help.

Funding

This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (Life surveyor) and in part by a Special Coordination Funds for Promoting Science and Technology, Creation of Innovation Centres for Advanced Interdisciplinary Research Areas (Innovative Bioproduction Kobe) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and was funded in part by AS ONE Corporation.

Conflict of interest

None declared.

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