ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

# Construction of a Yeast-Based Signaling Biosensor for Human Angiotensin II Type 1 Receptor via Functional Coupling Between Asn295-Mutated Receptor and Gpa1/$G_{i3}$ Chimeric Gα

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**ABSTRACT:** Angiotensin II (Ang II) type 1 receptor (AGTR1) is a G-protein-coupled receptor (GPCR). Its natural ligand, Ang II, is an important effector molecule controlling blood pressure and volume in the cardiovascular system, and is consequently involved in various diseases such as hypertension and heart failure. Thus, the signaling mediator, AGTR1, is a significant molecular target in medicinal and therapeutic fields. Yeast is a useful organism for sensing GPCR signaling because it provides a simplified version of the complicated machinery used by mammalian cells for signal transduction. Although yeast cells can successfully transmit a signal through a variety of human GPCRs expressed in the cell membrane, there have been no reports of the functional activation of AGTR1-mediated signaling in yeast cells. In the present study, we introduced a single mutation into human AGTR1 and used yeast-human chimeric Gα to exert the functional activation of AGTR1 in yeast cells. The engineered yeast cells expressing AGTR1 mutated at Asn295 and the chimeric Gα successfully transmitted the signal inside the yeast cells in response to Ang II peptide and its analogs (Ang III and Ang IV peptides) added to the assay medium. Further, we demonstrated that the autocrine Ang II peptide and its analog, produced and secreted by the engineered yeast cells, could by themselves promote AGTR1-mediated signaling. This means that screening for agonistic peptides with various sequences from a self-produced genetic library would be a viable strategy. Thus, the constructed yeast biosensor, integrating an Asn295-mutated AGTR1 receptor, will be valuable in the design of drugs to treat AGTR1-related diseases.

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**KEYWORDS:** G-protein-coupled receptor; angiotensin II type 1 receptor; G-protein signaling; *Saccharomyces cerevisiae*; peptide screening; green fluorescent protein

## Introduction

Angiotensin II (Ang II) is a naturally occurring, linear octapeptide that exhibits several extremely potent biological activities, including reversible blood pressure elevation and smooth muscle contraction (Printz et al., 1972). While Ang II binds to two major subtypes of Ang II receptors, Ang II type 1 receptor (AGTR1) and Ang II type 2 receptor (AGTR2), the most noted physiological and pathophysiological actions are mainly envoked through AGTR1. Both AGTR1 and AGTR2 are heterotrimeric guanine nucleotide binding protein (G-protein) coupled receptors (GPCRs), which is a large and diverse family of integral membrane proteins. GPCRs participate in the regulation of many cellular processes and, therefore, represent key targets for pharmacological treatment. Ang II regulates the cardiovascular response through AGTR1 (Griendling et al., 1996; Mehta and Griendling, 2007) and is involved in a wide range of diseases such as hypertension, atherosclerosis, heart failure, and diabetes (Chien, 1999; Dzau and Lopez-Ilasaca, 2005; MacLellan and Schneider, 1998; McKinsey and Olson, 1999; Sadoshima and Izumo, 1997). Consequently, AGTR1 is a significant molecular target in various research fields, including medicine, therapeutics, and pharmaceutics.

All eukaryotes conserve heterotrimeric G-proteins that comprise Gα-, Gβ-, and Gγ-subunits on the inner leaflets of plasma membranes. Mammalian cells possess several types of G-proteins that enable the control of diverse physiological responses mediated by interactions with a variety of transmembrane GPCRs (Hur and Kim, 2002). However, this diversity makes it difficult to identify which

G-protein-GPCR pair is responsible for specific signals (Ishii et al., 2010). Because haploid *Saccharomyces cerevisiae* expresses one type of GPCR (either the **a**-cell specific pheromone receptor, Ste2p, or the α-cell specific pheromone receptor, Ste3p) and one type of heterotrimeric complex of G-proteins (Gpa1p, Ste4p, and Ste18p, which encode yeast Gα, Gβ, and Gγ, respectively), it can offer a simple way to transduce the signal promoted by the agonistic ligand (Fukuda et al., 2011). Moreover, *S. cerevisiae* provides several advantages, including the possession of an eukaryotic secretory machinery, post-translational modifications, rapid cell growth, and well-established and versatile genetic techniques (Ishii et al., 2010). The introduction of several kinds of reporter genes such as green fluorescent protein (*GFP*) (Iguchi et al., 2010; Togawa et al., 2010), β-galactosidase (*lacZ*) (Brown et al., 2000), luciferase (*luc*) (Fukutani et al., 2012), and growth selection marker (*HIS3*) (Manfredi et al., 1996) downstream of the pheromone signaling (G-protein signaling) pathway further facilitates the detection of the agonist-promoted signal. Thus, the eukaryotic unicellular yeast *S. cerevisiae* is a useful microbial host organism for studying GPCRs as monomolecular models (Ishii et al., 2010; Minic et al., 2005).

If expression of human GPCR on the plasma membrane of *ste2*Δ yeast **a**-cells permits coupling with yeast monopolistic G-proteins, the promoted signal in response to the cognate ligand or analog agonist can be easily monitored with reporter gene assays (Fukuda et al., 2011; Ishii et al., 2010). The use of an established fluorescence-based reporter gene assay provides the most convenient measurement procedure: the cell culture is simply diluted into buffers and the fluorescence is read using fluorometric instruments (Ishii et al., 2012a,b). A flow cytometer is an especially powerful tool for comparative quantification and quantitative screening (cell sorting) (Ishii et al., 2012a,b). Signaling assays using fluorescent reporter genes have to date been applied to several GPCRs, including yeast endogenous Ste2p (Ishii et al., 2006), murine olfactory receptor (OR226) (Fukutani et al., 2012), human somatostatin receptors (SSTR5 and SSTR2) (Iguchi et al., 2010; Nakamura et al., 2013b; Togawa et al., 2010), and human neurotensin receptor (NTSR1) (Ishii et al., 2014; Nakamura et al., 2013b). However, there have been no reports regarding the functional activation of AGTR1-mediated signaling in yeast cells. Since AGTR1 is a peptidic ligand-responsive GPCR, functional expression of AGTR1 in yeast cells could be applied to screening Ang II analogs or entirely new peptidic backbones that work as agonists or antagonists.

In this study, we succeeded in functional signal activation of human AGTR1 in engineered yeast cells. AGTR1-mediated signaling in response to its agonists was easily monitored using a fluorescence reporter assay. To allow functional activation of AGTR1 in yeast cells, we introduced a single Ala or Ser mutation at Asn295, situated in the seventh transmembrane helix of AGTR1, and used yeast-human chimeric Gα in place of the intrinsic yeast protein, Gpa1p. In addition, we demonstrated that the autocrine Ang II peptide and its analog (Ang III peptide) produced and secreted by the engineered yeast cells could by themselves promote AGTR1-mediated signaling.

## Materials and Methods

### Plasmid Construction

All plasmids used in this study are listed in Table I. All primers used for plasmid construction are listed in Supplementary Table SI. Plasmid maps are presented in Supplementary Fig. S1.

A DNA fragment encoding the human *AGTR1* gene was PCR-amplified from pPR3-AGTR1 (Nakamura et al., 2013a)

**Table I.** Yeast strains and plasmids used in this study.

| Strain or plasmid | Specific features | Source |
|---|---|---|
| Strain | | |
| BY4741 | *MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0* | Brachmann et al. (1998) |
| IMFD-70ZsD | BY4741 *sst2*Δ::*AUR1-C ste2*Δ::*LEU2 fig1*Δ::*ZsGreen his3*Δ::$P_{FIG1}$-*ZsGreen far1*Δ | Nakamura et al. (2013b) |
| IMFD-72ZsD | BY4741 *sst2*Δ::*AUR1-C ste2*Δ::*LEU2 fig1*Δ::*ZsGreen his3*Δ::$P_{FIG1}$-*ZsGreen far1*Δ *gpa1*Δ::$G_{i3}$*tp* | Nakamura et al. (2013b) |
| Plasmid | | |
| pGK421 | Yeast expression vector containing *PGK1* promoter, *PGK1* terminator, 2$\mu$ origin and *MET15* marker | Togawa et al. (2010) |
| pGK421-AGTR1 | Human AGTR1 receptor expression in pGK421 | This study |
| pGK421-AGTR1-N111G | Human AGTR1 receptor (N111G) mutant expression in pGK421 | This study |
| pGK421-AGTR1-N111A | Human AGTR1 receptor (N111A) mutant expression in pGK421 | This study |
| pGK421-AGTR1-N111S | Human AGTR1 receptor (N111S) mutant expression in pGK421 | This study |
| pGK421-AGTR1-N295A | Human AGTR1 receptor (N295A) mutant expression in pGK421 | This study |
| pGK421-AGTR1-N295S | Human AGTR1 receptor (N295S) mutant expression in pGK421 | This study |
| pGK421-AGTR1-L305Q | Human AGTR1 receptor (L305Q) mutant expression in pGK421 | This study |
| pGK426 | Yeast expression vector containing *PGK1* promoter, *PGK1* terminator, 2$\mu$ origin and *URA3* marker | Ishii et al. (2009) |
| pGK426-ssAGII | Ang II secretory expression in pGK426 | This study |
| pGK426-ssAGIII | Ang III secretory expression in pGK426 | This study |
| pGK426-ssAGIV | Ang IV secretory expression in pGK426 | This study |

using primers #1 and #2, digested with *Nhe*I + *Bgl*II, and inserted into the same sites between the *PGK1* promoter ($P_{PGK1}$) and the *PGK1* terminator ($T_{PGK1}$) on pGK421 (Togawa et al., 2010) to yield plasmid pGK421-AGTR1.

The plasmids for expressing mutated AGTR1 proteins were constructed as follows. To introduce point mutations, gene fragments encoding the upstream and downstream parts of mutated AGTR1 were respectively PCR-amplified from pGK421-AGTR1 using primers #1 + #3 and #4 + #2 (for N111G); #1 + #5 and #6 + #2 (for N111A); #1 + #7 and #8 + #2 (for N111S); #1 + #9 and #10 + #2 (for N295A); #1 + #11 and #12 + #2 (for N295S); #1 + #13 and #14 + #2 (for L305Q). These amplified fragments then were used as the templates for overlap PCR with primers #1 and #2. The resulting linear mutated *AGTR1* fragments were digested with *Nhe*I + *Bgl*II and ligated into similarly digested pGK421, resulting in plasmids designated pGK421-AGTR1-N111G, -N111A, -N111S, -N295A, -N295S, or -L305Q, respectively.

To secrete the peptidic ligands, the plasmids expressing the fusions of secretion signal sequence (s.s.; containing the pre, pro α-factor leader region) and mature regions of several peptidic ligands were constructed as follows. A DNA fragment encoding the fusion between the s.s. of α-factor and Ang II (AGII) was PCR-amplified from pGK426-S1442 (Ishii et al., 2012a) using primers #15 and #16. The *s.s.-AGII* fragment was digested with *Nhe*I + *Sal*I and ligated into similarly digested pGK426 (Ishii et al., 2009), resulting in the plasmid pGK426-ssAGII. A DNA fragment encoding the fusion between the s.s. of α-factor and Ang III (AGIII) was PCR-amplified from pGK426-ssAGII using primers #15 and #17. The *s.s.-AGIII* fragment was digested with *Nhe*I + *Sal*I and ligated into similarly digested pGK426, resulting in the plasmid pGK426-ssAGIII. A DNA fragment encoding the fusion between the s.s. of α-factor and Ang IV (AGIV) was PCR-amplified from pGK426-ssAGII using primers #15 and #18. The *s.s.-AGIV* fragment was digested with *Nhe*I + *Sal*I and ligated into similarly digested pGK426, resulting in the plasmid pGK426-ssAGIV.

### Yeast Transformation and Media

All yeast strains used in this study are listed in Table I. Transformation with plasmids was performed using the lithium acetate method (Gietz et al., 1992).

Synthetic dextrose (SD) medium contained 6.7 g/L yeast nitrogen base without amino acids (YNB) (BD-Diagnostic Systems, Sparks, MD) and 20 g/L glucose. For SDM71 medium, SD medium was adjusted to pH 7.1 with 200 mM 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid (MOPSO) (Nacalai Tesque, Kyoto, Japan). YPD medium contained 10 g/L yeast extract (Nacalai Tesque), 20 g/L peptone (BD-Diagnostic Systems) and 20 g/L glucose. Amino acids and nucleotides (20 mg/L histidine, 60 mg/L leucine, and/or 20 mg/L uracil) were supplemented into SD medium to provide the relevant auxotrophic components. For solid medium, agar was added at 20 g/L.

### AGTR1 Signaling Assay

AGTR1 signaling assays basically followed previously described procedures (Nakamura et al., 2013b, 2014) except for changes in the ligands. In brief, to assay signal activation from human AGTR1, the yeast strains transformed with the wild-type or mutated AGTR1 expression plasmids were grown in SD medium (supplemented as needed) at 30°C overnight, then the cells were inoculated into 5 mL of the respective fresh SD medium to give an initial optical density of 0.03 at 600 nm ($OD_{600} = 0.03$). The cells were incubated at 30°C on a rotary shaker at 150 rpm for up to 18 h, then harvested, washed, and resuspended in water to yield an $OD_{600} = 10$. The resulting yeast cell suspensions were added (at 10 μL/well; $OD_{600} = 1$) to the wells of 96-well cluster dishes containing fresh SDM71 medium (80 μL/well) supplemented with 10-times concentrated Ang II (Calbiochem, Darmstadt, Germany), Ang III (AnaSpec, Fremont, CA) or Ang IV (Ang II (3-8)) (GenScript, Piscataway, NJ) (10 μL/well) at various concentrations or with distilled water (without ligand; control). The plates were incubated at 30°C with shaking (150 rpm) for 4 h. After incubation, the samples containing the yeast cells were observed using a fluorescence microscope or diluted with 1 mL of sheath fluid, then fluorescence was analyzed by flow cytometry. Half maximal effective concentration ($EC_{50}$) values were determined using KaleidaGraph4.0 fits to a dose response logistic model.

### Flow Cytometry Analysis

Flow cytometry measurements of green fluorescence followed a previously described procedure (Iguchi et al., 2010; Togawa et al., 2010). In brief, fluorescent cells were detected using a BD FACSCanto II flow cytometer equipped with a 488-nm blue laser (Becton, Dickinson and Company, Franklin Lakes, NJ); the data were analyzed using BD FACSDiva software (v5.0; Becton, Dickinson and Company). The ZsGreen fluorescence signal was collected through a 530/30 nm band-pass filter; a GFP-A mean of 10,000 cells was defined as "green fluorescence intensity."

### Fluorescence Microscopy Imaging

The cultured cells were washed and suspended in distilled water, and observed using a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan). Green fluorescence images were acquired with a 470/40 band-pass filter for excitation and a 535/50 band-pass filter for emission.

### AGTR1 Signaling Assay Using a Secretory Expression System for Peptide Ligands

AGTR1 signaling assays using a secretory expression system for the peptide ligands basically followed a previously reported procedure (Nakamura et al., 2013b) with some modifications. In brief, to assay signal activation from human AGTR1, yeast strains harboring pGK421-AGTR1-N295A or

-N295S receptor expression plasmids and pGK426-based peptide expression plasmids were grown in SD medium (supplemented as needed) at 30°C overnight, then the cells were inoculated into 5 mL of the respective fresh SDM71 medium to give an initial $OD_{600} = 0.1$. The cells were incubated at 30°C on a rotary shaker at 150 rpm for 9 h. The yeast cells were observed using a fluorescence microscope, or diluted with 1 mL of sheath fluid and fluorescence was analyzed by flow cytometry.

## Results

### Mutated AGTR1 Is Capable of Coupling to the Yeast Signal Transduction Pathway

To monitor the signal activation of human AGTR1 receptor, previously engineered yeast cells in which the *GFP* reporter genes have been integrated into the genome were used as the host strains (Nakamura et al., 2013b). In the engineered strains, the tetrameric *Zoanthus* sp. green fluorescent protein (ZsGreen), the fluorescence of which is brighter than enhanced green fluorescent protein (EGFP), was used as the GFP reporter. The expression of ZsGreen is controlled by the signal responsive *FIG1* promoter. Engineered yeast strains in which two copies of $P_{FIG1}$-*ZsGreen* reporter cassettes were integrated into the genome were used to obtain even brighter fluorescence (Table I). Therefore, signaling activation by agonist stimulus results in the generation of a green fluorescence signal (Nakamura et al., 2013b).

In addition, all engineered yeast strains have the *ste2*Δ, *sst2*Δ, and *far1*Δ alleles and are thus deficient in the yeast single GPCR, the yeast principal negative regulator of G-protein signaling (RGS), and the yeast G1-cyclin-dependent kinase inhibitor, an inhibitor that induces G1 cell cycle arrest in response to signaling (Fukuda et al., 2011; Iguchi et al., 2010, Ishii et al., 2012b; Togawa et al., 2010). These deletions respectively allow expression of human GPCRs without competitive receptor expression (Iguchi et al., 2010; Ishii et al., 2010; Togawa et al., 2010), hypersensitive agonist stimulation even at low ligand concentrations (Ishii et al., 2006), and growth in association with plasmid retention under signal-activated states (Ishii et al., 2008).

Since there have been no reports of the functional activation of AGTR1-mediated signaling in yeast cells, we constructed multicopy episomal plasmids for expressing wild-type and six mutants of AGTR1 receptor under the control of the constitutive *PGK1* promoter (Supplementary Fig. S2, Table I). The selected six AGTR1 mutants have increased affinities and potencies for Ang II, although they present the constitutive activation of signaling in mammalian cells (Balmforth et al., 1997; Feng et al., 1998, 2005; Groblewski et al., 1997; Noda et al., 1996; Parnot et al., 2000). Yeast IMFD-70ZsD strain, which specifically possesses intrinsic Gα (Gpa1p) (Table I), was used to transform AGTR1 and its mutant expression plasmids, then AGTR1 signaling assays were carried out. As shown in Figure 1A, none of the IMFD-70ZsD yeast cells expressing wild-type AGTR1 or its mutants showed green fluorescence, even in the presence of 1 mM Ang II. This result indicated that wild-type and mutant AGTR1 cannot transduce signals in yeast cells through the endogenous yeast Gα-subunit (Gpa1p).

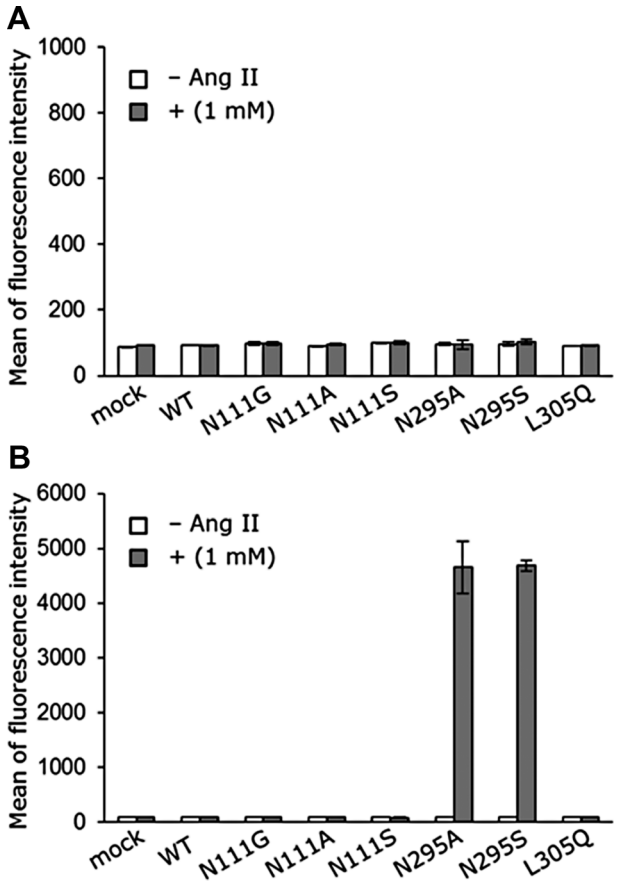


**Figure 1.** Activation of human AGTR1 produced in yeast following the exogenous addition of Ang II. Yeast strains IMFD-70ZsD (Gpa1p) (**A**) and IMFD-72ZsD ($G_{i3}$tp) (**B**) were transformed with pGK421 (mock) or pGK421-AGTR1-based plasmids (WT AGTR1 or its mutants). All transformants were grown in SD selective medium for 18 h. The cells then were incubated for another 4 h in pH-adjusted SD selective medium with or without 1 mM Ang II. The ZsGreen fluorescence of 10,000 cells was measured by flow cytometry. Mean values of the green fluorescence signal of 10,000 cells. Error bars represent the standard deviation from three independent transformants ($n = 3$).

A yeast-human chimeric Gα-subunit, in which the carboxyl-terminal 5 amino acid residues of Gpa1p (KIGII) are replaced with the equivalent residues from human $G\alpha_{i3}$ (ECGLY) (Gpa1/$G\alpha_{i3}$ transplant, $G_{i3}$tp), has been shown to improve signal transmission via several GPCRs expressed in yeast cells (Brown et al., 2000; Ishii et al., 2014; Nakamura et al., 2013b). Therefore, yeast IMFD-72ZsD strains, which specifically possess the chimeric Gα ($G_{i3}$tp) (Table I), were used to transform AGTR1 and its mutant expression plasmids, then AGTR1 signaling assays were performed. As shown in Figure 1B, the yeast strains expressing wild-type, and Asn111 and Leu305 mutants, of AGTR1 receptor showed no fluorescence. In contrast, the yeast strains expressing Asn295 mutants (N295A and N295S) displayed a 53-fold

increase in fluorescence intensity in response to agonistic Ang II stimulation (Fig. 1B). Microscope images of green fluorescence visually displayed clear differences in brightness in response to the Ang II-induced signal (Fig. 2). These results indicated that the mutation of AGTR1 at Asn295 to either an alanine or serine residue permits functional coupling with yeast-human chimeric G-protein ($G_{i3}$tp) and enables signal transmission in the engineered yeast cells.

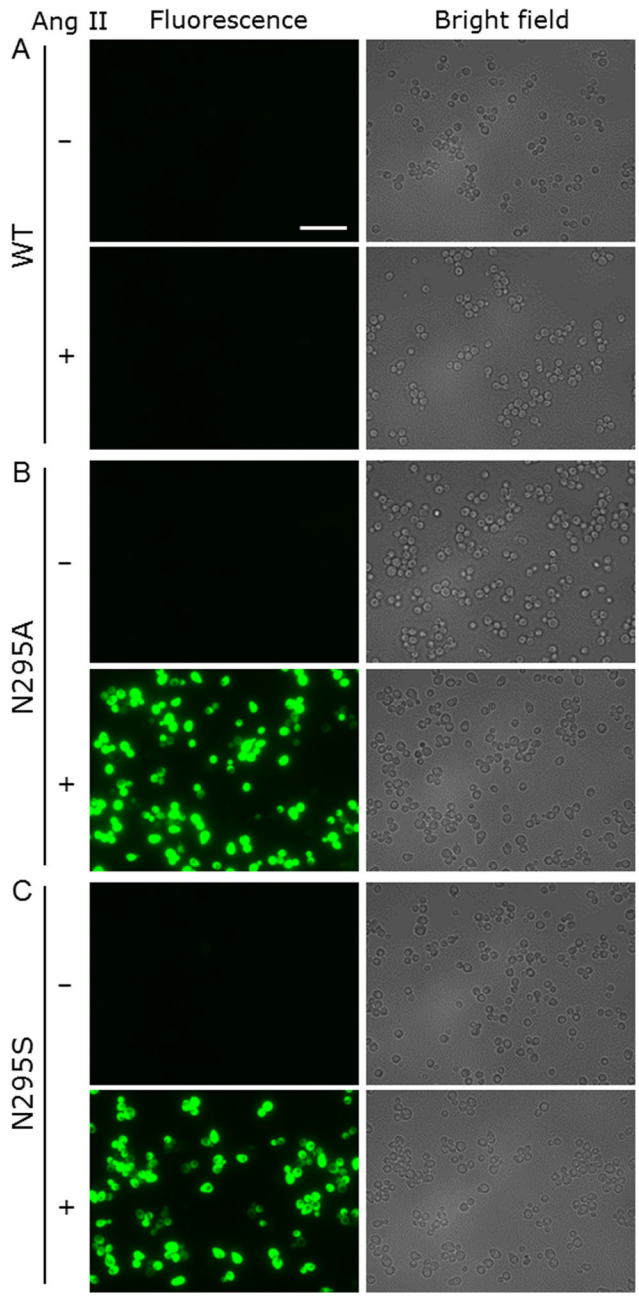


**Figure 2.** Visualization of activated human AGTR1 (WT, N295A, and N295S) produced in yeast following the exogenous addition of Ang II. Yeast strain IMFD-72ZsD was transformed with pGK421-AGTR1 (**A**), pGK421-AGTR1-N295A (**B**), or pGK421-AGTR1-N295S (**C**). All transformants were grown in SD selective medium for 18 h. The cells then were incubated for another 4 h in pH-adjusted SD selective medium with or without 1 mM Ang II. Cells were examined using the 100× objective lens of a fluorescence microscope. Exposure time was 0.5 s. The left photographs are fluorescence micrographs, and the right photographs are bright-field micrographs. Scale bar: 25 μm.

### Dose-Dependency of Asn295-Mutated AGTR1 Signaling for Ang II in Gα-Engineered Yeast Cells

To investigate the dose-dependency of AGTR1 receptors with an Asn295 mutation, yeast IMFD-72ZsD strains harboring pGK421-AGTR1-N295A or -N295S were used in signaling assays. Figure 3 shows the dose-response curve for the pharmacological efficacy of ligand-specific signaling of Asn295-mutated AGTR1 in yeast cells that express yeast-human chimeric $G_{i3}$tp (IMFD-72ZsD). Changing the Ang II concentration allowed confirmation of dose-dependent increases in fluorescence intensity in both cell types (Fig. 3). The half-maximal effective concentration ($EC_{50}$) values were 368 μM (N295A) and 402 μM (N295S). These results suggested that the affinity or sensitivity of the N295A mutant for Ang II was higher than that of the N295S mutant.

### Activation of Asn295-Mutated AGTR1 by Secretory Expressed Angiotensin Peptides

Finally, several Ang II peptidic analogs that differ in affinity or activity toward AGTR1 were employed to test whether the secretory expression system of various peptides could also

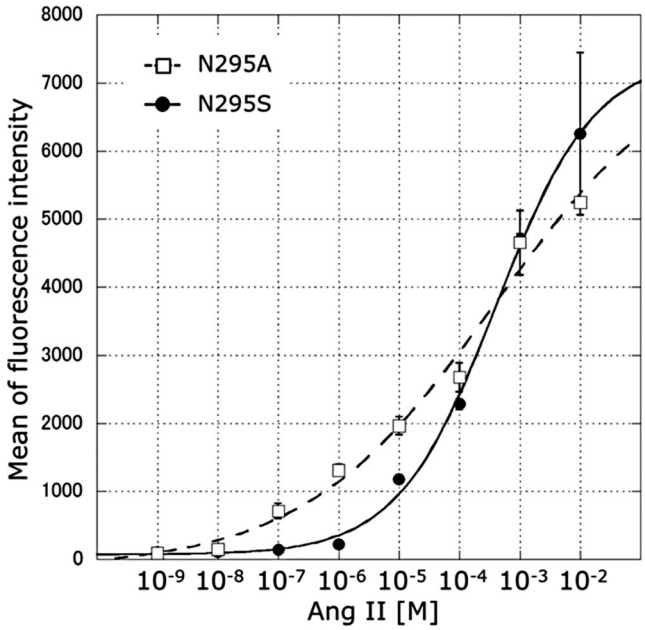


**Figure 3.** Dose-response curves for Ang II-specific AGTR1 signaling activities of recombinant yeast cells. Yeast strain IMFD-72ZsD was transformed with pGK421-AGTR1-N295A or -N295S. All transformants were grown in SD selective medium for 18 h. The cells then were incubated for another 4 h in pH-adjusted SD selective medium with Ang II at various concentrations. The ZsGreen fluorescence of 10,000 cells was measured by flow cytometry. Each data point is presented as the mean ± standard deviation of three independent transformants ($n = 3$ each).

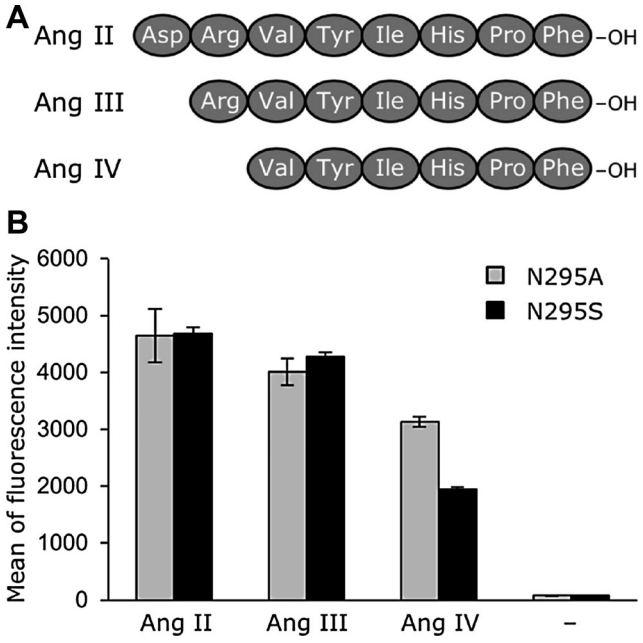


**Figure 4.** Activation of human AGTR1 by the exogenous addition of Ang II and its analog. **A**: Amino acid sequences of Ang II peptide and its analogs. **B**: Yeast strain IMFD-72ZsD was transformed with pGK421-AGTR1-N295A or -N295S. All transformants were grown in SD selective medium for 18 h. The cells then were incubated for another 4 h in pH-adjusted SD selective medium with 1 mM Ang II or its analogs. The ZsGreen fluorescence of 10,000 cells was measured by flow cytometry. Mean values of the green fluorescence signal of 10,000 cells. Error bars represent the standard deviation from three independent transformants ($n=3$).

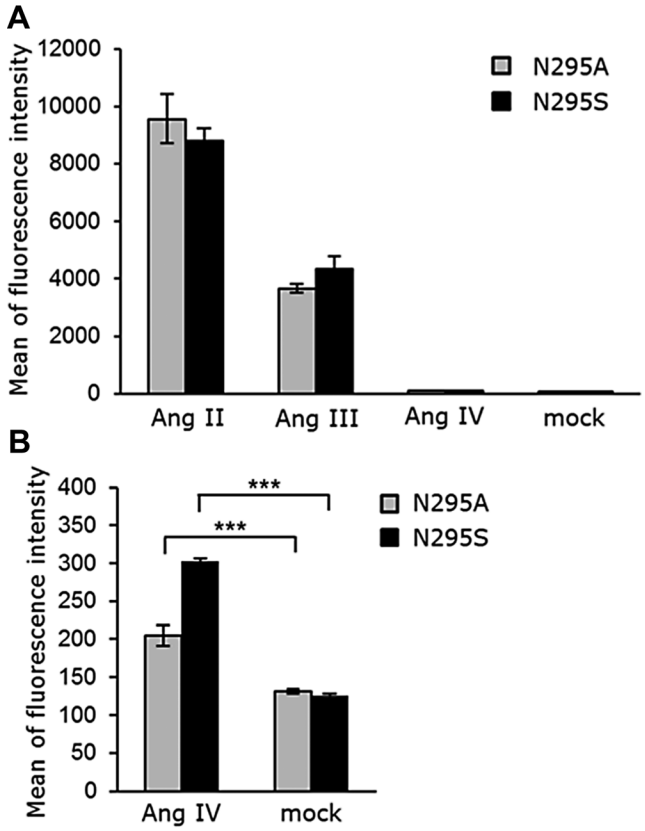


**Figure 5.** Activation of human AGTR1 by secretory expressed Ang II. Yeast strain IMFD-72ZsD, which coexpresses either pGK421-AGTR1-N295A or -N295S, and either pGK426-ssAGII (Ang II), pGK426-ssAGIII (Ang III), pGK426-ssAGIV (Ang IV), or pGK426 (mock), were incubated in pH-adjusted SD selective medium for 9 h (**A**) and 18 h (**B**). The ZsGreen fluorescence of 10,000 cells was measured by flow cytometry. Mean values of the green fluorescence signal of 10,000 cells. Error bars represent the standard deviation from three independent transformants ($n=3$). Statistical significance was assessed by the *t*-test (***$P<0.001$).

permit the functional signaling activation of Asn295-mutated AGTR1 in yeast cells (Fig. 4A). Ang III is a heptapeptide that has 40% of the pressor activity of Ang II (Salhan et al., 2012). Ang IV is a hexapeptide that, like Ang III, has decreased activity compared to Ang II (Wright et al., 2008). Yeast cells were engineered to synthesize and secret these peptidic Ang II analogs by fusing with a secretion signal sequence.

To test whether Ang II analogs could also activate Asn295-mutated AGTR1, the yeast IMFD-72ZsD strains harboring pGK421-AGTR1-N295A or -N295S were used in signaling assays. As shown in Figure 4B, the yeast strains expressing Asn295 mutants (N295A and N295S) displayed a 45- and 49-fold increase in fluorescence intensity, respectively, in response to agonistic Ang III stimulation. Addition of Ang IV to yeast cells resulted in 35- and 22-fold higher fluorescence than in the absence of agonist (N295A and N295S, respectively) (Fig. 4B).

Subsequently, the human Asn295-mutated AGTR1 expression plasmids (pGK421-AGTR1-N295A or -N295S) and peptide expression plasmids (pGK426-ssAGII, -ssAGIII, -ssAGIV, or pGK426 (mock)) were co-transformed into the IMFD-72ZsD strain (Table I). As shown in Figure 5A, the yeast strains concomitantly expressing AGTR1 (N295A and N295S) and secretory Ang II exhibited a greater than 94- and 91-fold increase in fluorescence intensity compared with the mock strain after 9 h cultivation. The secretory expressed Ang III also induced ZsGreen fluorescence, exhibiting a greater than 36- and 45-fold increase in fluorescence intensity compared with mock after 9 h cultivation (N295A and N295S, respectively) (Fig. 5A). Although the strain secreting Ang IV showed no green fluorescence after 9 h cultivation (Fig. 5A), it exhibited a greater than 1.5- and 2.4-fold increase in fluorescence intensity compared with mock after 18 h cultivation (N295A and N295S, respectively) (Fig. 5B). Microscope images of green fluorescence visually displayed clear differences in brightness arising from the distinct sequences of the secretory expressed angiotensin peptides (Fig. 6 and Supplementary Fig. S3). These results demonstrated that the secretory expression system could screen the peptidic angiotensin analogs, which can activate AGTR1-mediated signaling in yeast cells. Furthermore, the fluorescence intensity was clearly correlated with the agonistic activity of the ligands. Thus, it is expected that this yeast biosensor, integrating an Asn295-mutated AGTR1 receptor, will be useful for screening angiotensin agonists from peptidic libraries and for identifying their pharmacophores.

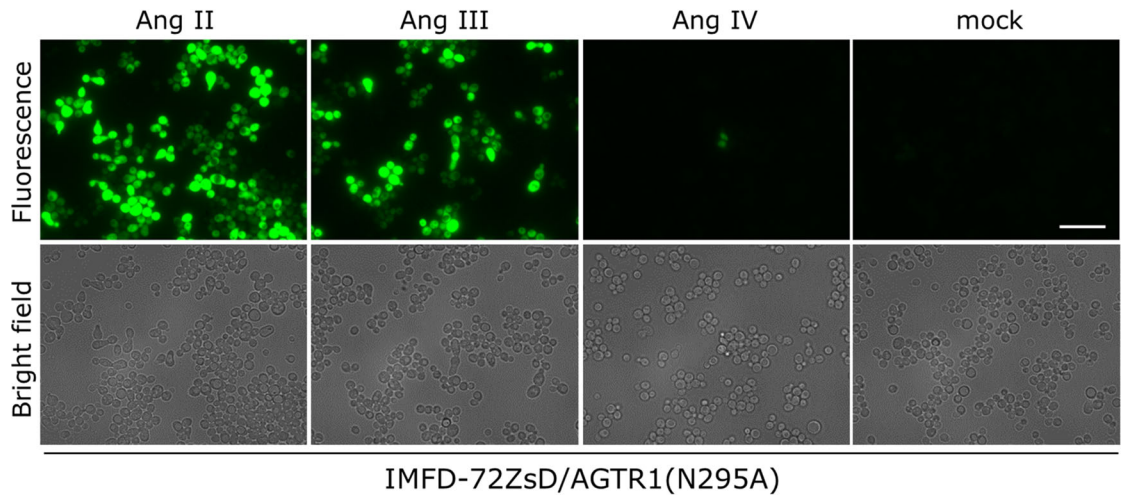


**Figure 6.** Visualization of activated human AGTR1 (N295A) by secretory expressed Ang II. Yeast strain IMFD-72ZsD, which coexpresses pGK421-AGTR1-N295A and either pGK426-ssAGII (Ang II), pGK426-ssAGIII (Ang III), pGK426-ssAGIV (Ang IV), or pGK426 (mock), were incubated in pH-adjusted SD selective medium for 9 h. Cells were examined using the 100× objective lens of a fluorescence microscope. Exposure time was 0.33 s. The upper photographs are fluorescence micrographs, and the lower photographs are bright-field micrographs. Scale bar: 25 μm.

The methodology presented in this study could also be useful for other human GPCRs.

## Discussion

We constructed a yeast-based signaling biosensor using human AGTR1 mutants. The results showed that Asn295-mutated AGTR1 functionally coupled with the yeast-human chimeric Gα ($G_{i3}$tp) in yeast cells. Furthermore, the autocrine Ang II peptide and its analogs could promote AGTR1-mediated signaling in the engineered yeast cells.

Noda et al. (1996) and Feng et al. (1998) proposed that activation of AGTR1 requires two distinct Ang II-dependent steps: an initial conformational change in the ligand binding pocket produced by Ang II side chains, then the Ang II-dependent stabilization of the active state (R*) in AGTR1. In previous reports, an interaction between Asn111 and Tyr292 (Groblewski et al., 1997) and/or Asn295 (Balmforth et al., 1997) was proposed as a major determinant of the inactive conformation of AGTR1. Li et al. (2002) reported that interaction of Asn111 (TM III) with Tyr292 and/or Asn295 (TM VII) in the basal state stabilizes the inactive conformation (R) of AGTR1. In addition, the second amino acid residue (Arg2) of Ang II interacts with Asp281 (TM VII). A conformational rearrangement or movement of TM VII disrupts the interaction between Asn111 and Tyr292 and/or Asn295. The receptor "relaxes" into a preactivated state (R′) and the transmembrane binding site opens to accommodate the C-terminal part of the octapeptide Ang II (Li et al., 2002). Feng et al. (1998) reported that Tyr4 of Ang II must be capable of displacing the Asn111 side chain in the wild-type AGTR1 to provoke the conformational transition from the subsequent R′ state to the fully activated R* state via the nonaromatic Ang II interaction. Taken together, these reports provide insights into the mechanism of AGTR1 activation by Ang II peptide.

Feng et al. (2005) reported that N295S, L305Q, and N111G mutant receptors show significantly increased binding affinities for Ang II. Similarly, N111A, N111S, and N295S mutant receptors show significantly increased binding affinities for Ang III (Balmforth et al., 1997). In addition, Ang IV peptide could activate N111G, N295S, and L305Q receptors, but did not activate wild-type AGTR1 (Feng et al., 2005). In our study, none of the AGTR1 mutants (N111G, N111A, N111S, N295A, N295S, and L305Q) expressed in yeast cells showed functional coupling with yeast endogenous G-protein (Gpa1p). However, our results indicated that Asn295-mutated AGTR1 permits functional coupling with yeast-human chimeric G-protein ($G_{i3}$tp) and enables signal transmission in the engineered yeast cells, although the Asn111- and Leu305-mutated AGTR1 did not transduce signal inside $G_{i3}$tp-expressing yeast cells. Since the Asn295 mutation in AGTR1 likely disrupts the interaction between Asn111 (TM III) and Asn295 (TM VII), the receptor might be in a "relaxed" conformation and thus easily accessible to the binding sites. Therefore, the Ang II-promoted steric conformation of Asn295-mutated AGTR1 probably permitted functional coupling to $G_{i3}$tp mutants with different amino acids in the last five C-terminal positions (Brown et al., 2000; Minic et al., 2005) in yeast cells. Another explanation is that lipid membrane composition and organization might change the activity of GPCRs (Lagane et al., 2000) like other transmembrane proteins (Baenziger et al., 2000; Dumas et al., 1999).

It should be noted that the autocrine Ang II peptide could promote AGTR1-mediated signaling. Surprisingly, the

fluorescence intensity in the yeast expressing autocrine Ang II peptide was higher than when Ang II peptide was exogenously added. The regional concentration of exogenous Ang II peptide on the cell membrane was estimated to be over 100 μM (Supplementary Fig. S4). The higher accessibility of ligands to GPCRs might have facilitated efficient activation. Another possibility is that the autocrine system could avoid the negative impact of degradation of Ang II peptide. In contrast, the fluorescence intensity in yeast expressing autocrine Ang IV peptide was lower than that when Ang IV peptide was exogenously added. Because Ang IV has much lower affinity for AGTR1 than Ang II, autocrine Ang IV peptide might diffuse out of the cell and into the medium without binding to the receptor. As in the case of Ang II peptide, the autocrine system has evolved to detect high-affinity or highly-active agonists. Thus, the secretory expression system holds promise for screening agonistic peptides by using plasmid libraries to express autocrine peptides with random or site-directed mutations.

## Conclusion

In this study, we established a fluorescence-based yeast biosensor based on an Asn295-mutated AGTR1 receptor. To exert the functional activation of AGTR1 in yeast cells, we introduced a single Ala or Ser mutation at Asn295 in AGTR1 and used yeast-human chimeric Gα. In addition, we demonstrated that the autocrine Ang II peptide and its analog (Ang III peptide) produced and secreted by the engineered yeast cells also could promote AGTR1-mediated signaling. Since our system is applicable to AGTR1, this system can likely be extended to other human GPCRs, which comprise one of the most important families of drug targets being pursued today. Consequently, the proposed system has the potential to be an effective tool for screening angiotensin agonists from peptidic libraries and to identify their pharmacophores. This work thus opens up a way for generating new devices in various pharmaceutical research areas.

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### References

Baenziger JE, Morris ML, Darsaut TE, Ryan SE. 2000. Effect of membrane lipid composition on the conformational equilibria of the nicotinic acetylcholine receptor. J Biol Chem 275(2):777–784.

Balmforth AJ, Lee AJ, Warburton P, Donnelly D, Ball SG. 1997. The conformational change responsible for AT1 receptor activation is dependent upon two juxtaposed asparagine residues on transmembrane helices III and VII. J Biol Chem 272(7):4245–4251.

Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P, Boeke JD. 1998. Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: A useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast 14(2):115–132.

Brown AJ, Dyos SL, Whiteway MS, White JH, Watson MA, Marzioch M, Clare JJ, Cousens DJ, Paddon C, Plumpton C, Romanos MA, Dowell SJ. 2000. Functional coupling of mammalian receptors to the yeast mating pathway using novel yeast/mammalian G protein α-subunit chimeras. Yeast 16(1):11–22.

Chien KR. 1999. Stress pathways and heart failure. Cell 98(5):555–558.

Dumas F, Lebrun MC, Tocanne JF. 1999. Is the protein/lipid hydrophobic matching principle relevant to membrane organization and functions? FEBS Lett 458(3):271–277.

Dzau VJ, Lopez-Ilasaca M. 2005. Searching for transcriptional regulators of Ang II-induced vascular pathology. J Clin Invest 115(9):2319–2322.

Feng YH, Miura S, Husain A, Karnik SS. 1998. Mechanism of constitutive activation of the AT1 receptor: Influence of the size of the agonist switch binding residue Asn(111). Biochemistry 37(45):15791–15798.

Feng YH, Zhou L, Qiu R, Zeng R. 2005. Single mutations at Asn295 and Leu305 in the cytoplasmic half of transmembrane α-helix domain 7 of the AT1 receptor induce promiscuous agonist specificity for angiotensin II fragments: A pseudo-constitutive activity. Mol Pharmacol 68(2): 347–355.

Fukuda N, Ishii J, Kaishima M, Kondo A. 2011. Amplification of agonist stimulation of human G-protein-coupled receptor signaling in yeast. Anal Biochem 417(2):182–187.

Fukutani Y, Ishii J, Noguchi K, Kondo A, Yohda M. 2012. An improved bioluminescence-based signaling assay for odor sensing with a yeast expressing a chimeric olfactory receptor. Biotechnol Bioeng 109(12): 3143–3151.

Gietz D, St Jean A, Woods RA, Schiestl RH. 1992. Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res 20(6): 1425.

Griendling KK, Lassègue B, Alexander RW. 1996. Angiotensin receptors and their therapeutic implications. Annu Rev Pharmacol Toxicol 36: 281–306.

Groblewski T, Maigret B, Larguier R, Lombard C, Bonnafous JC, Marie J. 1997. Mutation of Asn111 in the third transmembrane domain of the AT1A angiotensin II receptor induces its constitutive activation. J Biol Chem 272(3):1822–1826.

Hur EM, Kim KT. 2002. G protein-coupled receptor signalling and cross-talk: Achieving rapidity and specificity. Cell Signal 14(5):397–405.

Iguchi Y, Ishii J, Nakayama H, Ishikura A, Izawa K, Tanaka T, Ogino C, Kondo A. 2010. Control of signalling properties of human somatostatin receptor subtype-5 by additional signal sequences on its amino-terminus in yeast. J Biochem 147(6):875–884.

Ishii J, Fukuda N, Tanaka T, Ogino C, Kondo A. 2010. Protein–protein interactions and selection: Yeast-based approaches that exploit guanine nucleotide-binding protein signaling. FEBS J 277(9):1982–1995.

Ishii J, Izawa K, Matsumura S, Wakamura K, Tanino T, Tanaka T, Ogino C, Fukuda H, Kondo A. 2009. A simple and immediate method for simultaneously evaluating expression level and plasmid maintenance in yeast. J Biochem 145(6):701–708.

Ishii J, Matsumura S, Kimura S, Tatematsu K, Kuroda S, Fukuda H, Kondo A. 2006. Quantitative and dynamic analyses of G protein-coupled receptor signaling in yeast using Fus1, enhanced green fluorescence protein (EGFP), and His3 fusion protein. Biotechnol Prog 22(4):954–960.

Ishii J, Moriguchi M, Hara KY, Shibasaki S, Fukuda H, Kondo A. 2012b. Improved identification of agonist-mediated Gα(i)-specific human G-protein-coupled receptor signaling in yeast cells by flow cytometry. Anal Biochem 426(2):129–133.

Ishii J, Oda A, Togawa S, Fukao A, Fujiwara T, Ogino C, Kondo A. 2014. Microbial fluorescence sensing for human neurotensin receptor type 1 using Gα-engineered yeast cells. Anal Biochem 446:37–43.

Ishii J, Tanaka T, Matsumura S, Tatematsu K, Kuroda S, Ogino C, Fukuda H, Kondo A. 2008. Yeast-based fluorescence reporter assay of G protein-coupled receptor signalling for flow cytometric screening: FAR1-disruption recovers loss of episomal plasmid caused by signalling in yeast. J Biochem 143(5):667–674.

Ishii J, Yoshimoto N, Tatematsu K, Kuroda S, Ogino C, Fukuda H, Kondo A. 2012a. Cell wall trapping of autocrine peptides for human G-protein-coupled receptors on the yeast cell surface. PLoS ONE 7(5):e37136.

Lagane B, Gaibelet G, Meilhoc E, Masson JM, Cézanne L, Lopez A. 2000. Role of sterols in modulating the human μ-opioid receptor function in *Saccharomyces cerevisiae*. J Biol Chem 275(43):33197–33200.

Li MT, Vanderheyden PM, Szaszák M, Hunyady L, Vauquelin G. 2002. Angiotensin IV is a potent agonist for constitutive active human AT1 receptors. Distinct roles of the N- and C-terminal residues of angiotensin II during AT1 receptor activation. J Biol Chem 277(26):23107–23110.

MacLellan WR, Schneider MD. 1998. Success in failure: Modeling cardiac decompensation in transgenic mice. Circulation 97(15):1433–1435.

Manfredi JP, Klein C, Herrero JJ, Byrd DR, Trueheart J, Wiesler WT, Fowlkes DM, Broach JR. 1996. Yeast α mating factor structure-activity relationship derived from genetically selected peptide agonists and antagonists of Ste2p. Mol Cell Biol 16(9):4700–4709.

McKinsey TA, Olson EN. 1999. Cardiac hypertrophy: Sorting out the circuitry. Curr Opin Genet Dev 9(3):267–274.

Mehta PK, Griendling KK. 2007. Angiotensin II cell signaling: Physiological and pathological effects in the cardiovascular system. Am J Physiol Cell Physiol 292(1):C82–C97.

Minic J, Sautel M, Salesse R, Pajot-Augy E. 2005. Yeast system as a screening tool for pharmacological assessment of G protein coupled receptors. Curr Med Chem 12(8):961–969.

Nakamura Y, Ishii J, Kondo A. 2013a. Rapid, facile detection of heterodimer partners for target human G-protein-coupled receptors using a modified split-ubiquitin membrane yeast two-hybrid system. PLoS ONE 8(6): e66793.

Nakamura Y, Ishii J, Kondo A. 2013b. Bright fluorescence monitoring system utilizing *Zoanthus* sp. green fluorescent protein (*ZsGreen*) for human G-protein-coupled receptor signaling in microbial yeast cells. PLoS ONE 8(12):e82237.

Nakamura Y, Takemoto N, Ishii J, Kondo A. 2014. Simultaneous method for analyzing dimerization and signaling of G-protein-coupled receptor in yeast by dual-color reporter system. Biotechnol Bioeng 111(3): 586–596.

Noda K, Feng YH, Liu XP, Saad Y, Husain A, Karnik SS. 1996. The active state of the AT1 angiotensin receptor is generated by angiotensin II induction. Biochemistry 35(51):16435–16442.

Parnot C, Bardin S, Miserey-Lenkei S, Guedin D, Corvol P, Clauser E. 2000. Systematic identification of mutations that constitutively activate the angiotensin II type 1A receptor by screening a randomly mutated cDNA library with an original pharmacological bioassay. Proc Natl Acad Sci USA 97(13):7615–7620.

Printz MP, Némethy G, Bleich H. 1972. Proposed models for angiotensin II in aqueous solution and conclusions about receptor topography. Nat New Biol 237(74):135–140.

Sadoshima J, Izumo S. 1997. The cellular and molecular response of cardiac myocytes to mechanical stress. Annu Rev Physiol 59:551–571.

Salhan D, Sagar A, Kumar D, Rattanavich R, Rai P, Maheshwari S, Adabala M, Husain M, Ding G, Malhotra A, Chander PN, Singhal PC. 2012. HIV-associated nephropathy: Role of AT2R. Cell Signal 24(3):734–741.

Togawa S, Ishii J, Ishikura A, Tanaka T, Ogino C, Kondo A. 2010. Importance of asparagine residues at positions 13 and 26 on the amino-terminal domain of human somatostatin receptor subtype-5 in signaling. J Biochem 147(6):867–873.

Wright JW, Yamamoto BJ, Harding JW. 2008. Angiotensin receptor subtype mediated physiologies and behaviors: New discoveries and clinical targets. Prog Neurobiol 84(2):157–181.

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