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Research paper

Functional characterization of cell lines for high-throughput screening of human neuromedin U receptor subtype 2 specific agonists using a luciferase reporter gene assay

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Abstract

We developed a functional cell-based high-throughput screening (HTS) assay to identify modulators of the human neuromedin subtype 2 receptor. This assay utilized the signal transduction pathway of hNMU2R, which is positively coupled to adenylyl cyclase and downstream calcium signal pathways. We describe in detail a robust, sensitive, and functional assay for the hNMU2R G-protein-coupled receptor expressed in human embryonic kidney (HEK)-293 cells, whose activity was reflected by a luciferase reporter gene transcriptionally regulated by a 3-repeat serum response element (SRE)-3 repeat multiple response element (MRE)-3 repeat cyclic AMP (cAMP) response element (CRE)-VIP mini promoter. The HEK 293 clonal cell line, stably co-transfected with the $3\times$SRE/$3\times$MRE/$3\times$CRE/VIP mini promoter-driven luciferase and pCDNA3.1-NMU2R plasmid, was selected by active geneticin sulfate and their ability to express luciferase with a forskolin challenge following hNMU plus forskolin, known to activate intracellular signal transduction. Then the cell density, incubation time, dimethyl sulfoxide (DMSO) concentration used to screen the hNMU receptor subtype 2 specific agonist were optimized, and whether intrinsic luminescent substance of extracts isolated from traditional Chinese herbs disturbs luminescence of luciferase expressed in HEK293 cells was considered. The optimal incubation time was found to be between 8 and 9 h, the cell density and DMSO concentrations were optimized from 3×10^4 to 6×10^4, and less than 2%, respectively. Our data show that hNMU2R luci-HEK293 cells and their assay exhibit a low background and ideal model for high-throughput screening. These results demonstrate that this reporter gene assay is useful for pharmacological analysis, and is amenable to HTS for human NMU2R agonists.

Keywords: NMU2 receptor; Reporter gene; Luciferase; G-protein-coupled receptor; Cell-based assay; Traditional Chinese herbs

1. Introduction

Neuromedin U (NMU) is a brain-gut peptide involved in various physiological functions. In the periphery, NMU regulates muscle contractile activity (gastrointestinal), intestinal ion transport, blood pressure, local blood flow (cardiovascular) [1], and adrenocortical function [2]. In the CNS, NMU contributes to various functions including feeding behavior, energy balance [3], and spinal nociceptive transmission and processing [4]. Two human G-protein-coupled receptors, named NMUR1 and NMUR2, have been identified as NMU-specific receptors. NMUR1 is abundantly expressed in peripheral tissues, whereas NMUR2, which shares 55% amino acid identity with NMUR1, is specifically expressed in various brain regions, including hypothalamic areas consistent with a role for NMU in regulating food intake [1]. The identification of the two receptor subtypes for NMU and the characterization of its central effects on feeding helped stimulate research on the biochemical and physiological functions of this neuropeptide. However, to date, no NMUR2 selective agonists are available, which hinders our ability to

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thoroughly profile this receptor subtype. To better describe its pharmacological and physiological function, it is therefore necessary to clone and characterize the human NMU-specific receptor, NMUR2.

In the present study, we have cloned the NMUR2 receptor and established a stable cell line in order to perform high-throughput screening for NMUR2 selective agonists. Specifically, we co-transfected the NMUR2 gene with a luciferase reporter gene, driven by a VIP mini promoter flanked by $3 \times$SRE/$3 \times$MRE/$3 \times$CRE response elements into HEK-293 cells. The entire assay was carefully optimized, including.cell density, incubation time, dimethyl sulfoxide (DMSO) concentration, and the effects of the intrinsic luminescence of extracts from traditional Chinese herbs on background of hNMU2R-luci-HEK293 cell assay system. Our findings suggest that the hNMU2R-luci-HEK293 cells exhibit low luminescence background suitable for high-throughput screening.

2. Materials and methods

2.1. Reagents

The luciferase substrate Bright-Glo™ and the pGL3 vector were obtained from Promega (USA). The human hypothalamus cDNA was obtained from Clon Tech (USA). The pCR2.1-TOPO and pCDNA3.1 vectors were purchased from Invitrogen (Carlsbad, CA, USA). G418 and lipofectamine were obtained from Gibco/BRL. NMU, forskolin, and dimethyl sulfoxide (DMSO) were purchased from Sigma except culture media (from Hyclone™). The HEK293 and CHO cell line used in this study were obtained from the Shanghai Shenergy Biocolor BioScience and Technology Company.

2.2. Plasmids

The pGL3-Basic-Luci plasmid, contains the luciferase coding sequence driven by a VIP mini promoter flanked by synthesized $3 \times$SRE/$3 \times$*MRE*/$3 \times$*CRE* response elements. The pcDNA3.1-NMU2R recombinant plasmid was constructed by ligation of the human NMU2R receptor cDNA amplified from human hypothalamic cDNA and cloned into the pCR2.1-TOPO vector. Both strands of all constructs were sequenced by automated sequencing.

2.3. Development of NMU2R-luci stable cell lines

The HEK293 cells or CHO cells were grown in a 5% CO_2 humidified atmosphere at 37 °C using DMEM (whereas CHO cells using RPI1640) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 0.1 mmol/L nonessential amino acid (not used CHO cells), and 1.0 mM sodium pyrurate (not used CHO cells). Cells were plated in 96-well plates prior to transfection at a density of 1×10^5 cells/well (24 h of incubation; HEK293 cells were grown to 50–80% confluence and transfected in batch).

The HEK293 cells and CHO cells were co-transfected with the human NMU2R receptor (pcDNA3.1-NMU2R, 0.2 μg/μL) and the pGL3-Basic-Luci vector containing the $3 \times$SRE/$3 \times$*MRE*/$3 \times$*CRE*/VIP mini promoter-luciferase gene (p3S3M3C/VIP/Luci, 1 μg/μL) by using lipofectamine according to the manufacturer's instructions. Selective medium containing 0.8 mg/mL active geneticin sulfate (G418) was applied to the cells after 48 h. Cells showing resistance to G418 were screened for inducible luciferase expression in response to forskolin, or by combining with the endogenous ligand, NMU. Positive clones exhibiting the greatest fold induction of luciferase in response to forskolin or NMU in the presence of forskolin were chosen as the reporter cell line and used in all subsequent experiments. The negative HEK293 cell line was generated by co-transfecting pcDNA3.1 in the absence of hNMU2R and the pGL3-Basic-Luci vector containing the $3 \times$SRE/$3 \times$*MRE*/$3 \times$*CRE*/VIP mini promoter-luciferase gene (p3S3M3C/VIP/Luci) using the same lipofectamine approach described above.

2.4. Luciferase reporter assay

Cells were detached by agitation, centrifuged, and resuspended in DMEM supplemented with 2% fetal calf serum. Cell viability was determined by trypan blue exclusion. Cells were then seeded in 100 μL aliquots into clear-bottomed white 96-well plates with lids (Corning Incorporated Costar, USA) at a density of 3×10^4 or 4×10^4 cells per well. After overnight incubation, cells were treated with 10 μL forskolin or a mixture of forskolin with an extract from traditional Chinese herbs diluted in 1% DMSO. After a 6–8 h incubation period, the luciferase activity was conducted by adding 100 μL luciferase substrate Bright-Glo™ into each well. Standardization of this assay consisted of varying the cell density and incubation time to optimize signal to background ratio. Wells containing negative control and positive control (hNMU2R HEK293-luci cell line stimulated with forskolin in the presence of NMU) were also included at the start and at the end of the series of experiments to ensure the stability of the assay cells throughout the course of the experiment.

2.5. Compounds

A total of 324 reference non-peptidic compounds were obtained by ASE200 (Accelerated Solvent Extractor, Dionex, USA) at 60 °C and 1500 psi. These compounds were dissolved in water and used as candidates for screening.

2.6. Data analysis

The effects of cell concentration, incubation time, and DMSO on the luciferase activity were monitored using the luminescence value (relative light units) and the

percentage of the maximal luciferase expression induced by forskolin in the absence and presence of NMU at various concentrations. Data points represent mean values of at least three independent experiments carried out in triplicate. Evaluation and validation of the high-throughput screening assays were made according to the methods described in a previous report [5].

3. Results

3.1. Functional characterization of the recombinant reporter hNMU2R-luci-HEK293 cell line

Stable transfections were carried out using a fivefold excess of reporter DNA over hNMU2R DNA with lipofectamine because the reporter vector does not possess an antibiotic resistance gene for selection in eukaryotic cells. Thus, the selection was driven by the neomycin resistance gene in the vector containing hNMU2R. After cell growth under the selection with the antibiotic G418, the selected clones were stored under liquid nitrogen. When the hNMU2R-luci-HEK293 stable cell clones were stimulated with 10 μM forskolin or 1 μM hNMU in the presence of 5 μM forskolin, a significant luciferase response was generated (Fig. 1). Relative to forskolin, hNMU plus forskolin stimulated greater levels of luciferase activity, but when forskolin was absent, hNMU did not activate significant luciferase activity. Therefore, there is some synergism between hNMU and forskolin.

Cell clones with the forskolin-induced luciferase expression lower than twofold of basal levels were discarded. Only those clones with significant expression (greater than twofold induction) were harvested. The observed difference in the expression levels may reflect different integration positions of the reporter gene into the cell genome and its accessibility to transcription factors such as CREB (cAMP response element binding protein). To analyze the concentration-dependent activation of adenylyl cyclase by forskolin, forskolin was added at concentrations ranging from 0.1 to 300 μM. The luciferase expression stimulated by forskolin was dose-dependent, with a calculated EC_{50} of 5 μM (Fig. 2a). Consequently, all cell clones were used for functional analysis with 5 μM forskolin. In order to select HEK293 cell clones that expressed both hNMU2R and luciferase, a functional selection strategy was utilized (see Fig. 2). Initially, 1 μM hNMU was used, but later the agonist concentration was increased to 5 μM in order to increase putative functional responses. hNMU-mediated increases in forskolin-stimulated luciferase expression were also dose-dependent with a calculated EC_{50} value of 1.2 μM. The clones with the best signal-to-background ratio that showed the strongest increase of stimulated luciferase expression were chosen for further characterization. These cell clones were designated as hNMU2R-luci-HEK293 cell line (Fig. 2b).

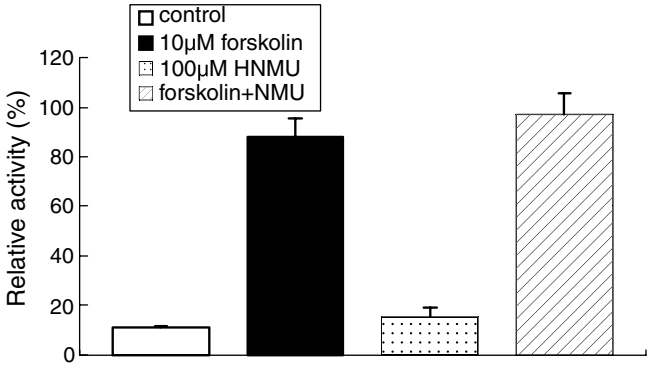

Fig. 1. Effect of hNMU on luciferase activity in the presence or absence of forskolin in the recombinant hNMU2R-luci-HEK293 cell line and negative control cell line. The recombinant hNMU2R-luci-HEK293 cell lines were plated in 96-well plates at a density of 4×10^4 cells/mL per well for 8 h stimulation with forskolin or hNMU in the presence or absence of forskolin. The negative control cell line was similarly plated in 96-well plates for 8 h stimulation with hNMU in the absence of forskolin. Data points were obtained in triplicate in each of three independent experiments. Values represent means ± SEM. Results are presented as percent of maximal luciferase expression (% response). The white bar indicates the negative control, the black bar represents forskolin, and the gray bars with dashed or diagonal lines represent hNMU and hNMU in the presence of forskolin, respectively.

To further examine the specificity of the response of recombinant hNMU2R-luci-HEK293 cells to hNMU, we tested the effects of various concentrations of forskolin, hNMU, acetylcholine (Ach) and neuropeptide Y (NPY) on luciferase activity in the chosen hNMU2R-luci-HEK293 clones. Neither Ach nor NPY induced luciferase activity in the hNMU2R-luci-HEK293 host cells, furthermore, NMU and α-MSH, at concentrations ranging from 0.1 to 5 μM, failed to induce a dose–response curve in negative control HEK 293 cells whereas forskolin did stimulate dose-dependent luciferase activity in these cells (Figs. 2c–d), suggesting that another three G-coupled receptors do not modulate the reporter system (Fig. 2a), and the negative control cells are functional, because the responses of negative control cells to forskolin or to hNMU in the presence of forskolin were similar. These data indicate that the stably transfected hNMU2R-luci-HEK293 cells can be specifically activated through the hNMU2R-sensitive Gq proteins and via correct coupling of the hNMU2R in this cell line. Based on these results, the combined 5 μM forskolin and 1 μM hNMU were used as a specific activator in the subsequent experiments. At these concentrations, the combination of the two activators induced luciferase activity in the linear portion of the curve.

In subsequent experiments, the effects of various conditions on the luciferase reporter assay sensitivity were examined. Spontaneous luminescent values of the Chinese herb extracts at background levels, which were very low and not considered, cannot interfere with the precision of the data. Fig. 2e shows that biological and experimental variation may not impair data in experiments. Our assay system appear to be specific to receptor.

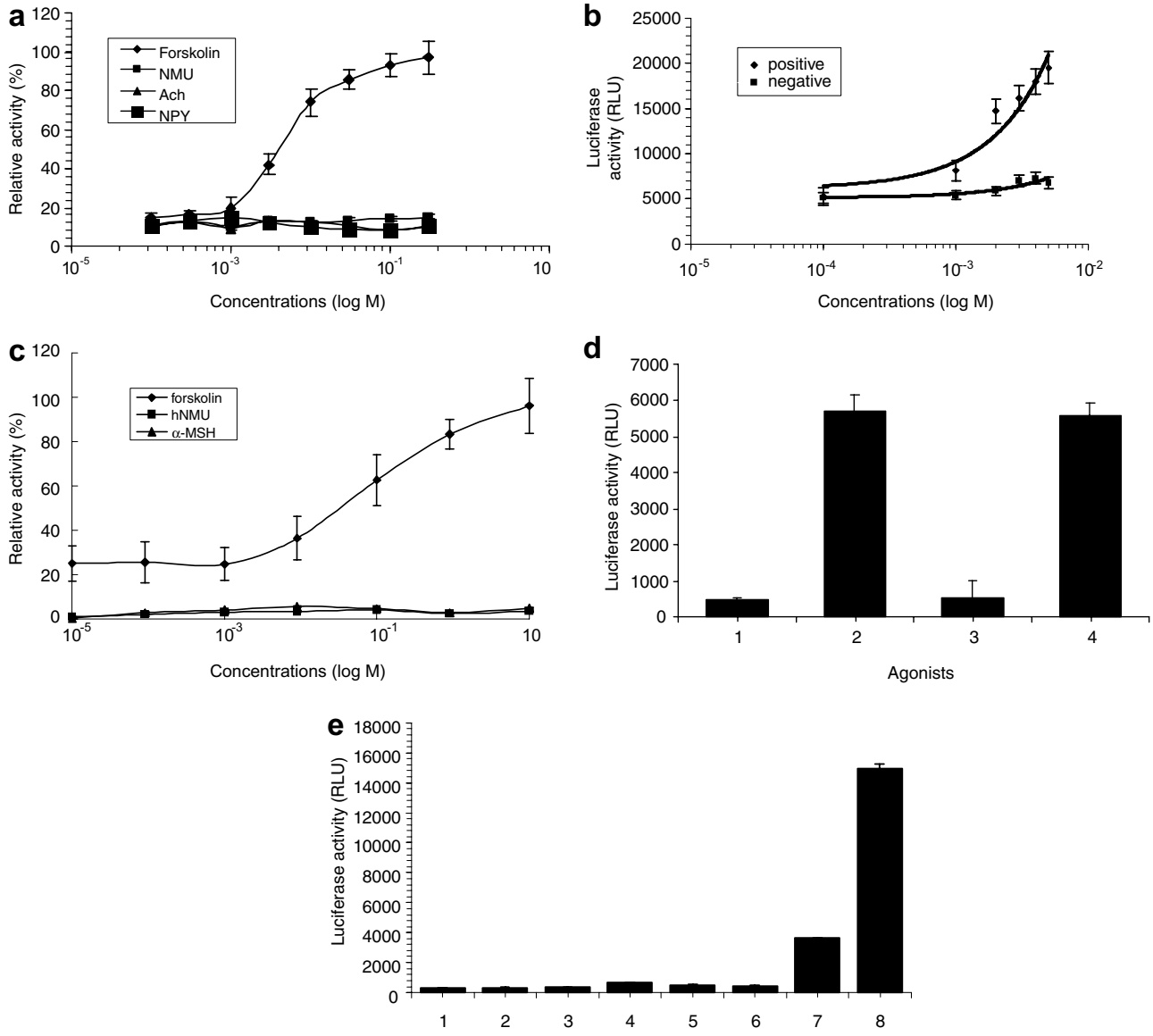

Fig. 2. (a) Functional characterization of the recombinant hNMU2R-luci-HEK293 cell line in the reporter gene assay. Cell lines were plated in 96-well plates at a density of 4×10^4 cells/mL per well for 8 h stimulation with agonist. The effects of various concentrations of forskolin, NMU, Ach and NPY on luciferase expression were evaluated using the recombinant hNMU2R-luci-HEK293 cell line. Data points were obtained in triplicate in each of three independent experiments. Values represent means ± SEM. Results are presented as percent of maximal luciferase expression (% response). (b) Functional characterization of the recombinant hNMU2R-luci-HEK293 cell line in the reporter gene assay. The positive cell line and negative cell line were plated in 96-well plates at a density of 4×10^4 cells/mL per well for 8 h stimulation with agonist. Concentration–response curves of the agonist NMU in the presence of 5 μM forskolin were obtained using the hNMU2R-luci-HEK293 cell line and the negative control cell line. Data points were obtained in triplicate in each of three independent experiments. Values represent means ± SEM. Results are presented as relative light units (RLU). (c) Functional characterization of the recombinant negative control cell line with forskolin. Cells were plated in 96-well plates at a density of 4×10^4 cells/mL per well for 8 h stimulation with agonist. Data points were obtained in triplicate in each of three independent experiments. Values represent means ± SEM. The effects of various concentrations of forskolin, NMU, and α-MSH on luciferase expression using the negative control cell line are presented. Results are presented as percent of maximal luciferase expression (% response). (d) Functional characterization of the recombinant negative control cell line. Cell lines were plated in 96-well plates for 8 h stimulation at a density of 4×10^4 cells/mL per well for 8 h stimulation with agonist. Data points were obtained in triplicate in each of three independent experiments. Values represent means ± SEM. The responses of the recombinant negative control cell line to forskolin and NMU are presented. 1:Blank control; 2:5 μM forskolin; 3:1 μMNMU; 4:1 μMNMU in the presence of 5 μM forskolin. Results are presented as relative light units (RLU). (e) Effects of various factors on the recombinant reporter hNMU2R-luci-HEK293 cell line in the reporter gene assay. Cell lines were plated in 96-well plates at a density of 4×10^4 cells/mL per well for 8 h stimulation with agonist. Data points were obtained in triplicate in each of three independent experiments. Values represent means ± SEM. The intrinsic luminescence values of the cells, Bright-Glo™, and extracts from traditional medicines are presented. 1, hNMU2R-luci-HEK293 cell free; 2, hNMU2R-luci- HEK293 cells in the absence of Bright-Glo; 3, hNMU2R-luci-HEK293 cells in the absence of Forskolin; 4, hNMU2R- luci HEK293 cells in the presence of Cirsium japonicum extract; 5, blank; 6, hNMU2R-luci-HEK293 cells in the presence of NMU; 7, hNMU2R-luci-HEK293 cells in the presence of forskolin; 8, hNMU2R-luci-HEK293 cells stimulated with NMU in the presence of forskolin.

3.2. Optimization of functional reporter assay for HTS

During the optimization, we performed a series of experiments to optimize assay conditions for the various cell lines. We investigated the abilities of two different tissue culture cell lines to support the human NMU2R. The lines chosen were the standard HEK293 line and CHO line. Both cell lines were co-transfected with the hNMU2R cDNA together with the $3\times$SRE/$3\times$MRE/$3\times$CRE/VIP mini promoter-luciferase construct. Luciferase activity was generated by stimulation with forskolin or a mixture of forskolin with hNMU (Fig. 3). Both cell lines supported a functional assay for human hNMU2R.

Subsequently, we examined the effects of the number of hNMU2R-luci-HEK293 cells per well on the assay. Luciferase activity was measured following 10 μM forskolin stimulation with cell counts ranging from 10×10^3 to 90×10^3 per well. Luciferase activity changed modestly relative to cell number, but no cell concentration produced a significantly more robust response to forskolin than any other (Fig. 4). Because the sensitivity of the assay was similar for all cell counts, we conducted subsequent high-throughput screening (HTS) experiments using a cell density of 30×10^3 to 60×10^3 cells/mL per well.

Luciferase activity values were obtained after 4–11 h of stimulation to determine the optimal incubation time (Fig. 5). Although there was a linear increase in luciferase activity across incubation times, optimal time occurred at the 8- and 9-h time points. Longer incubations did not lead to a greater increase in the luminescence signal. Therefore, an 8 or 9 h exposure to forskolin or its mixture with hNMU was chosen for subsequent experiments.

We also evaluated the effects of various concentrations of DMSO, the diluent for extracts, on the response to forskolin (Fig. 6). Luciferase activity was enhanced by 1–2% DMSO, but concentrations greater than 2% caused a decrease in luciferase activity, and a complete loss of signal occurred with concentrations $\geqslant$8% DMSO. It is likely that these findings are due to a cytotoxic effect of DMSO at these concentrations. Therefore, for subsequent HTS studies, a concentration of 1% DMSO was used.

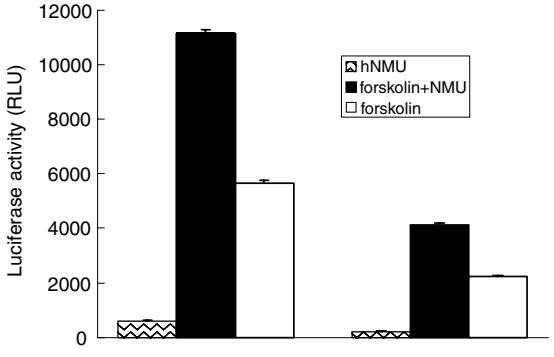

Fig. 3. Response of hNMU2R-luci-HEK293 and hNMU2R-luci-CHO host cell lines to human NMU peptide and forskolin. hNMU2R-luci-HEK293 and hNMU2R-luci-CHO host cell lines were incubated with forskolin and human hNMU peptide in the presence of forskolin for 8 h at a density of 4×10^4 cells/mL per well. The luciferase activity was monitored by TURNER DESIGN equipment measuring relative light units. Data points were obtained in triplicate in each of three independent experiments. The gray bar represents NMU, the black bar represents forskolin plus NMU, the white bar represents forskolin.

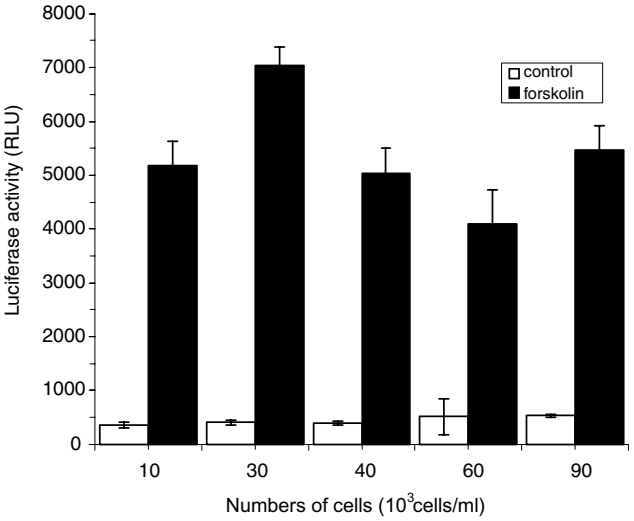

Fig. 4. The effect cell density on luciferase activity of the hNMU2R-luci-HEK293 and negative control cell. Cells were plated at 96-well plates overnight at densities ranging from 10×10^3 to 90×10^3 cells per well. Cells were then incubated with 5 μM forskolin for 8 h. Data points were obtained in triplicate in each of three independent experiments. Data are expressed as relative light units. The white bar represents the negative control, the black bar represented forskolin.

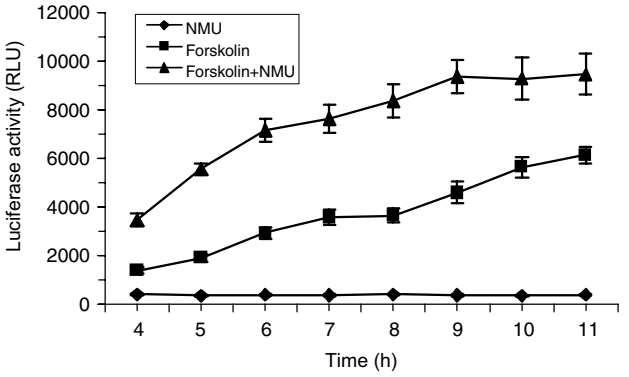

Fig. 5. Effect of incubation time on forskolin- and hNMU +forskolin-induced luciferase activity. Plates were seeded with 4×10^4 hNMU2R-luci-HEK293 cells and challenged for 4, 5, 6, 7, 8, 9, 10, 11 h with 5 μM forskolin or 1 μM hNMU in the presence of 5 μM forskolin. Data points were obtained in triplicate in each of three independent experiments. Data are expressed as relative light units. ♦, represent hNMU, ■, represent forskolin, and ▲ represent NMU in the presence of forskolin.

3.3. An assessment of the screening data variability in the design and validation of HTS assays

Assays for HTS not only require small sample volume, high throughput, and robust signals, but also require adequate sensitivity, reproducibility, and accuracy in order to

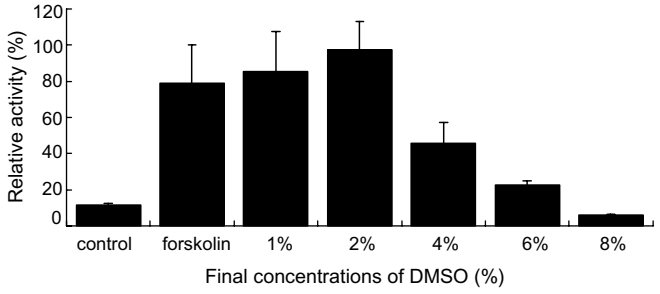

Fig. 6. Effects of DMSO concentrations on the response of the hNMU2R-luci-HEK293 cells to forskolin. Plates were seeded with 3×10^4 hNMU2R-luci-HEK293 cells and challenged for 8 h with DMSO at various concentrations in the presence of forskolin. Data are expressed as a percentage of the maximum response to forskolin with 0% DMSO present. Data points were obtained in triplicate in each of three independent experiments.

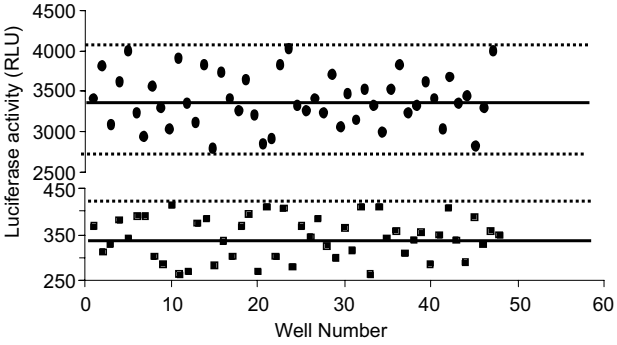

Fig. 7. Analysis of the variability of the screening system. In order to assess the well-to-well and plate-to-plate signal variation at the defined screening conditions, assay hNMU2R-luci-HEK293 cells were harvested and adjusted to a cell count of 3×10^4 cells/mL plated in 96-well plates. Into 48 of the wells 10 μL of 5 μM forskolin was added, and the remaining 48 wells contained only culture medium. Cells were exposed to treatment or control for 8 h. The solid horizontal lines indicate the means of the positive and negative control data in the well-to-well comparison. Dashed lines represent the standard deviation (SD) from the mean of each data set. ● represent the positive control, and ■ the negative control. The data are expressed as relative light units.

discriminate hits among a very large number of compounds that span a wide range of activity. Therefore, a simple and dimensionless parameter is desirable when comparing and evaluating the quality and suitability of an HTS assay. The Z'-factor can be used to evaluate the quality or performance of any given HTS assay. If the Z'-factor is an arbitrary value less than 1, and greater than or equal to 0.5, the assay is suitable for HTS. In order to evaluate the suitability of the reporter assay for HTS, the intra-plate well-to-well variability and inter-plate variability were determined. In our experiments, after the assay method was determined, several plates containing both positive and negative controls were assayed in order to assess the well-to-well and plate-to-plate signal variation at the defined screening conditions. hNMU2R-luci-HEK293 cells were harvested and adjusted to a cell count of 3×10^4 cells/mL in 90 μL culture medium, and maintained overnight in a 5% CO_2 humidified atmosphere at 37 °C using DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 0.1 mmol/L nonessential amino acid, and G418 selection medium. Cells were then placed in 96-well plates; 10 μL of 5 μM forskolin was added to 48 wells, and the remaining 48 wells served as negative controls exposed for 8 h to the culture medium only. When comparing the signal variation between plates, the cells were plated in 10 wells each in 8 different plates. The activity data for each individual plate were evaluated, including standard deviations, and coefficients of variation (CV) from the average values of the positive and negative controls were determined (Fig. 7). CVs were used to calculate the Z-factor. CVs in the well-to-well and plate-to-plate analysis are shown in Table 1. Because the CV of the average value varied very little, and the Z-factor was 0.70, the assay is suitable for HTS to identify hits with confidence.

To identify hNMU2R agonists, compounds extracted from traditional Chinese herbs described previously to be anti-obesity dissolved in water were tested. Extracts of 324 traditional Chinese herbs were used to stimulate hNMU2R-luci-HEK293 cells in the presence of forskolin (Fig. 8). Of these, 49 extracts increased luciferase activity greater than or equal to twofold higher than the established cut-off value of luciferase stimulated by forskolin in the absence of hNMU. These extracts were identified as potential candidates of agonists for hNMU2R, whose EC_{50} will be determined in future studies of extract activity at the hNMU2R.

4. Discussion

Impairment of G-protein-coupled receptor (GPCR) function has been implicated in a wide variety of various pathological conditions, including nervous system, immunological, and endocrine conditions, suggesting that the discovery of specific agonists may lead to the development of successful therapeutic agents [6]. Our ability to screen active compounds from large chemical libraries against specific GPCRs requires the development of efficient methods to evaluate functional responses.

In order to develop a double-recombinant HEK293 cell line that expressed the human NMU subtype two receptor (hNMU2R) with a luciferase reporter gene, a co-transfection strategy was employed. This strategy is faster than sequential transfection strategies in which only one gene is transfected at a time and requires one antibiotic to select for recombinant cells. Here, the human NMU2R cDNA

Table 1
The stability of screening system

	Well	Plate		Well	Plate
μ_{c+}	3400.96	3538.19	μ_{c-}	343.86	380.08
σ_{c+}	277.32	266.12	σ_{c-}	30.58	35.03
	CV = 8.1%	CV = 7.5%		CV = 8.9%	CV = 9.2%
	$Z' = 0.70$	$Z' = 0.71$			

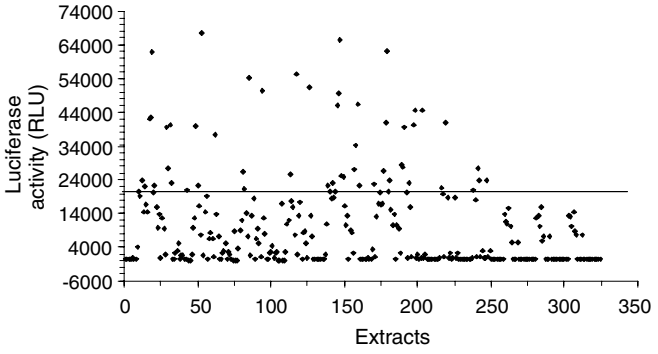

Fig. 8. Response of the hNMU2R-luci-HEK293 cell line to various compounds. Extracts of 324 traditional Chinese herbs identified as anti-obesity candidates were incubated with hNMU2R-luci-HEK293 cells for 8 h in the presence of forskolin at a density of 4×10^4 cells/mL per well. Forty-nine extracts, when combined with forskolin, increased luciferase activity driven by the $3 \times$SRE/$3 \times$MRE/$3 \times$CRE/VIP mini promoter to levels twofold or greater relative to 5 μM forskolin stimulation alone. The solid horizontal lines represent the value greater than twofold above luciferase activity with forskolin alone. Each extract was tested in triplicate, and the average relatively light unit value for each extract is represented as ♦.

and the luciferase reporter gene, whose upstream sequence contained a $3 \times$SRE/$3 \times$MRE/$3 \times$CRE/VIP-mini promoter, were co-transfected. The $3 \times$SRE/$3 \times$MRE/$3 \times$CRE/VIP-mini promoter drives luciferase expression, which relies on an increase in intracellular cAMP and/or calcium levels for transcriptional activation of the reporter gene and its concomitant translation [7]. Thus an increase in intracellular cAMP and/or calcium levels can be inferred by increases in luciferase expression. Reporter assays using the MRE/CRE/VIP promoter and CRE-directed promoters have been successfully used to examine the pharmacology of Gq-, Gs-, or Gi-coupled receptors [8,9], Our assay measured the response of a GPCR by detecting changes in $3 \times$SRE/$3 \times$MRE/$3 \times$CRE/VIP promoter-controlled transcription levels of luciferase; SRE could extend the utility of the MRE/CRE/VIP reporter vector [10,11].

Numerous studies have demonstrated that an increase in intracellular calcium or cAMP can activate cAMP- or calcium-dependent protein kinases and result in the phosphorylation of CREB and SRE-, MRE-, and/or CRE-directed transcription [12,13]. Furthermore, some studies have confirmed a synergistic effect of forskolin with mediators of calcium mobilization on CREB phosphorylation [14]. In these cases, the activation of either Gq- or Gs- coupled receptors, and the increase of intracellular calcium enhanced luciferase expression only in the presence of forskolin. Likewise, in the present study, similar synergistic effects were seen both in the presence of hNMU and forskolin, indicating that it is unlikely that the extracts and NMU act directly through CREB activation, and likely synergize with calcium-dependent protein kinase pathway. The hNMU2R-luci-HEK293 cell line was highly responsive to stimulation with forskolin, as well as hNMU in the presence of forskolin (Fig. 1), whereas the negative control cell line did not show the same response. These results suggest that hNMU specifically stimulates hNMU2R. This result was also confirmed by the functional characterization of the recombinant reporter hNMU2R-luci-HEK293 cell line (Fig. 2). Ach binds M1 muscarinic G_q-coupled receptors to activate phospholipase C. NPY positively couples to Ca^{2+} pathways via specific NPY receptor subtypes exerting various biological functions in the central and peripheral nervous system, including the activation of feeding behavior, regulation of circadian rhythm, and modulation of memory. The hNMU2R-luci-HEK293 cell line exhibited a dose-dependent response to forskolin, but not to acetylcholine or NPY, suggesting that the cAMP signal pathway had been selectively activated via the hNMU2R. α-MSH is the endogenous ligand at the melanortin (MC)-3 and MC4 receptors, which belong to a family of G_s-protein-coupled receptors. These receptors are abundantly expressed in the CNS and regulate energy metabolism by activating adenylyl cyclase. MC receptors and the hNMU2R activate similar downstream transduction pathways, but MC activation did not result in luciferase activity in the hNMU2R-luci-HEK293 cell line. These results suggest that the hNMU2R-luci-HEK293 cell line does not express the endogenous muscarinic, melanortin, or NPY receptors, and additionally there appears to be no crosstalk between these receptors with hNMU2R. Moreover, the response of the hNMU2R-luci-HEK293 cell line to hNMU in the presence of forskolin increased in a dose-dependent manner. Therefore, the hNMU2R-luci-HEK293 cell line is useful and specific for pharmacological analysis of hNMU2R agonists.

Although the maximum response differed between HEK293 and CHO cell lines, the difference most likely reflected in the cells' responsiveness to the forskolin or the hNMU in the presence of forskolin, rather than transfection efficiencies (Fig. 3). Transfection efficiency can be influenced by many factors, such as transfection reagent to DNA ratio, insufficient amount of transfection reagent, incubation time, vector, cell density, and poor DNA quality. If a human GPCR fails to function in one cell line, it can be reevaluated rapidly in a range of alternative lines since many of the fundamental properties of functional assay systems are determined by the nature of the host cells themselves. These properties include (i) the ability to express the target GPCR with appropriate posttranslational modifications and in the required cellular location, and (ii) the presence (or absence) of endogenous GPCRs, G-proteins, effector proteins, scaffolding proteins, and accessory/chaperone molecules. Each cell platform presents various advantages and disadvantages. Because the HEK293 line exhibited a greater response to the forskolin and the hNMU in the presence of forskolin, these cells were chosen and the cell density was adjusted for HTS. If the cell density is too high during transfection reagent, addition, cells may not be at the optimal phase of growth. This can lead to insufficient uptake of the complexes into the cells or insufficient expression of the gene of interest.

As our system is based on a cell assay, the number of cells can influence the response to stimulation with agonists for adenylyl cyclase and GPCRs. A high density of cells may result in high background, and be unsuitable for cell growth. Consequently, we determined an optimal cell number to be between 3 and 6×10^4 per well (Fig. 4). Ligand–receptor binding often induces a series of complex events, including signal transduction, which are associated with receptor density, drug concentration, and incubation time. In our study, we determined the optimal incubation time to be 8 h (Fig. 5) and the ideal concentration of the solvent to be 1% DMSO (Fig. 6), which did not induce the cytotoxicity seen at higher concentrations.

The assays can be performed in a simple homogeneous assay model, with no wash steps required. The inter-plate and intra-plate CVs are usually low enough that deconvolution of the data and identification of the active compounds are relatively easy to perform [15]. In this study, we employed a sample statistical calculation, denoted as a Z'factor [5] to evaluate the quality of the data. The advantage of this parameter is that it takes into consideration not only the difference between maximum and minimum signal, but also the standard deviation (SD) of both signals. The obtained Z' value of 0.7 suggests that this system is an excellent assay for HTS. In addition, the luminescence-based system is designed for HTS and is compatible not only with 96-well plates, but with higher density plates [16]. As a result, it is an ideal instrument not only assay for development, but also for the screening of bioactive pharmaceutical agents. The successful determination of EC_{50} values for forskolin and hNMU + forskolin demonstrates the quantitative nature of the assay data, and illustrates the potential use for HTS as well as downstream lead optimization. Moreover, the system can also be used to study Gαi and Gαs-coupled receptor-mediated inhibition and induction of cAMP [17], and to screen agonists or antagonists.

Our reporter assay containing a promoter with three SREs, three MREs and three CREs controlling the transcription of the luciferase gene is rapid and sensitive, and can measure the effect of agonists on Gq-, Gi-, and Gs-coupled receptors. The promoter can respond to increases in intracellular calcium, protein kinase C stimulation, and changes in cAMP levels (suggesting the stimulation of protein kinase A). This is in contrast to other reporter assays that require different sets of conditions for measuring Gq-, Gs-, or Gi-coupled receptors. An analogous assay has been described by Fitzgerald and colleagues [18], who used three MREs and a CRE as promoter. Furthermore, our assay can be applied in the pharmacological characterization of both agonist and antagonist ligands [19]. Although there are many different commercially available reporter gene systems that can be applied to HTS [20,21], firefly luciferase suited our particular screening needs for the following reasons [22]. First, since fewer cells per well can be plated and cell culture time is reduced, the activity represents a sensitive readout, which facilitates the optimization of the signal-to-noise ratio in a stable reporter system. Second, the enzyme is not secreted, and the medium containing test compounds need not be removed before cell lysis, thus avoiding the diminishment of assay signals via inhibition of luciferase activity or interference. Finally, since the assay is facile and sufficient signal can be obtained after only 8 h of incubation with agonist, the entire assay can be completed in a normal workday.

Although various technologies measuring intracellular calcium responses have been reported [23,24] in this study, a luciferase reporter assay technology was successfully used to demonstrate that the functional engagement of NMU2R upon ligand can be analyzed quantitatively by spectral concentration. This technology can be further extended to more GPCRs, where cAMP and calcium signaling pathways are ubiquitous second messengers in the activation pathway mediated by these proteins. Our reporter assay eliminates the need for setting up two or three separate assays to characterize different second-messenger pathways, and is therefore superior to conventional second-messenger assays that are technically cumbersome and require radioactivity, such as adenylyl cyclase activity assays, radioimmunoassays for measuring cAMP [25], and assays detecting the accumulation of tritiated-inositol phosphates [26]. Those methods measuring calcium mobilization have improved the throughput of signal transduction assays but require specialized equipment to detect a response [27].

It should be noted that some compounds may not be specific for the particular receptor pathway. For example, compounds having a partial agonist profile may bind at sites distal to the ligand binding site, inducing an allosyeric conformational change, and an ambiguous step in luciferase generation [28], but we cannot detect this looking. High-throughput screening involves testing a large number of compounds against a biological target to identify potential drug leads. If a drug is missed in the initial screening, a potentially valuable drug will not be developed and the chemical palate available to pharmacologists will be artificially limited. Therefore, two or three assays may need to be performed to ensure that the appropriate second-messenger response is evaluated. Thus, additional binding studies are also required to determine if the other compound interferes with the receptor–ligand interaction.

5. Conclusion

In the present study, we developed a stable double-recombinant HEK293 cell line (hNMU2R-luci-HEK293) which expressed the human NMU2R and a $3 \times$SRE/$3 \times$MRE/$3 \times$CRE-responsive luciferase reporter gene to monitor receptor activity. This cell line exhibited correct functional coupling of the NMU receptor to the intracellular signaling pathways that stimulated activity of adenylyl cyclase and calcium signaling pathways. The functional response of the cells supported the stability and specificity of this novel recombinant cell line. The luciferase reporter

system has many advantages compared to other reporter genes. It is highly sensitive, which makes it amenable to the 96 or 384-well plate format and can be used in any laboratory. Optimization of the functional assay resulted in application for automated HTS.

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