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GENERATION OF MAMMALIAN CELL LINES WITH GENE KNOCK-DOWN FOR HUMAN MCHR2

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Abstract – Appetite regulating neuropeptide melanin-concentrating hormone (MCH) has been implicated in obesity. It functions through its two receptors MCHR1 and MCHR2. While MCH and MCHR1 have been studied more extensively, the function of MCHR2 remains largely unknown, due to the lack of suitable *in vitro* and *in vivo* models. To create an *in vitro* system of genetic knock-down of MCHR2 in mammalian cells, we constructed four small hairpin RNAs (shRNAs) against human MCHR2 in eukaryotic expression vector, and transfected the plasmids into CHO cells that stably express human MCHR2. Using the empty vector or a negative shRNA control plasmid, we show that MCHR2-shRNAs suppressed 45.8% - 66.4% of MCHR2 expression at both mRNA and protein levels. As the result, in cells carrying the MCHR2-shRNAs, binding of MCHR2 to MCH was decreased by 39.4% - 78.7% accompanied by a similar decrease in affinity of the receptor to ligand by 40.9% - 81.9%. These cells still respond to MCH treatment, but intracellular Ca^{2+} release as the downstream signaling event was also decreased by 114.8% - 822.4%. Together, this study generated a set of shRNAs and cell lines as valuable reagents for further study on MCHR2 functions. These results will ultimately help to advance our knowledge about appetite regulating neuropeptide receptors.

Key words: Melanin-concentrating hormone, melanin-concentrating hormone receptor 2, obesity, shRNA, gene expression, receptor binding, intracellular calcium.

INTRODUCTION

Obesity has become a common problem and a trophopathy disease in the modern society, causing cardiac and neuronal diseases, diabetes mellitus, hypertension and hyperlipoidemia. It has also been linked to the neuroendocrine regulation of ingestion (17). For this reason, a new appetite regulating neuropeptide melanin-concentrating hormone (MCH), as well as its two receptor subtypes, namely melanin-concentrating hormone receptor 1 and 2 (MCHR1 and MCHR2), have attracted much of scientific attention. At present, while the relations between MCH, MCHR1 and obesity have been better understood through the studies of transgenic and gene knock-out models (12), the function of MCHR2 remains largely unknown due to the lack of suitable *in vitro* and *in vivo* models.

RNA interference (RNAi) is a powerful technology to knock-down gene expression. It has been successfully applied in numerous studies on the functions of specific genes (10,20). In this study, we established mammalian cell lines with genetic knock-down of MCHR2 and confirmed the impaired functions of MCHR2 in the new *in vitro* model.

MATERIALS AND METHODS

Materials

Reagents

ReverTra Ace-α-cDNA synthesis kit was purchased from Takara Co. Ltd., Japan. Lipofectamine[TM]2000 and Trizol reagents were purchased from Invitrogen Co., USA. G418 was from Amresco Co., USA. Goat polyclonal IgG anti-human MCHR2 antibody was from Santa Cruz Co, USA. Horseradish peroxidase-conjugated rabbit anti-goat IgG secondary antibody was purchased from Beijing Dingguo Biotech. Co., China, Supersignal Chemiluminescence substrate solution and iodo-beads were purchased from Pierce Co. Ltd, USA. Japan. Calcium assay kit containing Pluronic-F12 and Fura-2 was purchased from Molecular Devices Co, USA. Human MCH was from Bachem company, USA. Membrane protein extraction kit was purchased from Nanjing Keygen Biotech Co., China. $Na^{125}I$ was purchased from Chengdu Zhonghe Gaotong Isotope Corporation, China.

PCR primers

Upstream and downstream primers for MCHR2 gene were 5′-ACCATGAATCCATTTCATGCATCTTG-3′ and 5′-CTAAAAGTGTGATTTCAGAGTGT-3′, respectively, and the amplified fragment was 1023 bp. The upstream and downstream primers for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were 5′-ACCACAGTCCATGC CATCAC-3′ and 5′-TCCACCACCCTGTTGCTGT-3′, respectively, and the amplified fragment was 467 bp. These primers were synthesized from Takara Co., Japan.

Construction of eukaryotic expression vector to deliver shRNA against human MCHR2

Four short hairpin RNA (shRNA) sequences were designed by siRNA Target Finder online tool provided by Ambion. They corresponded to the MCHR2 cDNA sequences from Genbank (gi:93277082) at 727-745nt, 849-867nt, 1273-1291nt, 762-780nt respectively. They were cloned into a eukaryotic expression plasmid pGenesil-1 and given the names pGenesil-1-MCHR2-shRNA1-4. The shRNAs in the vector were confirmed by sequencing (Table 1). At the same time, a non-specific shRNA fragment of similar length and GC content was also designed by siRNA Target Finder online tool based on a previous report (16). It was synthesized and cloned into the same vector to be used as a negative control (pGenesil-1-MCHR2-shRNAhk) along with the empty vector pGenesil-1.

Methods

Cell culture and transfection

CHO cells stably expressing human MCHR2 gene were established from our earlier work (19). They were routinely grown in DMEM/F12 medium supplemented with 10% fetal bovine serum as well as penicillin and streptomycin and maintained under 5% CO_2 at 37°C. At 70-80% confluency, the cells were transfected with eukaryotic expression vectors carrying either the shRNA sequences (pGenesil-1-MCHR2-shRNA1-4) or the negative control sequence (pGenesil-1-MCHR2-shRNAhk), or the pGenesil-1 empty vector using Lipofectamine^{TM} 2000 as described by the manufacturer's protocol. 48 h after transfection, the cells were treated with G418 (1300 μg/ml) for 3 weeks and the clones derived from single cells resistant to the drug were isolated and expanded for the following experiments, or preserved in liquid nitrogen.

RT-PCR analysis for MCHR2 mRNA expression

Total RNA was prepared from CHO cells transfected with plasmids using the Trizol reagent and quantified by spectrophotometry using the Gene Quant 1300 Bioanalyzer (Biochrom Ltd, UK). Then, 1.0 μg RNA sample was subjected to reverse-transcription with a First Strand cDNA Synthesis Kit, and the resulted cDNA was used as template for PCR. A 15 μl PCR reaction mixture contained 2 μl cDNA template, 1.5 μl 10×PCR buffer, 1.0 μl $MgCl_2$, 1.0μl dNTPs (2.5 m*M* each), 0.25 μl primers (20 μ*M*), and 0.2 μl Taq DNA polymerase. The PCR was carried out under the following conditions: an initial denaturation at 95°C for 2 min, and 29 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 20 s, followed by a final extension at 72°C for 7 min. As an internal control, GAPDH mRNA expression in CHO cells from each sample was also analyzed by RT-PCR under similar conditions. The PCR products were then visualized on a 1% agarose gel and the image was photographed. The optical densities of the DNA bands for MCHR2 and GAPDH on the gel were qualified by Quantity One software, and the relative expression level of MCHR2 was expressed as the OD of MCHR2 divided by that of GAPDH. The experiment was repeated for three times.

Table 1. The sequences of shRNA oligonucleotides

shRNA	The sequences of shRNA oligonucleotide
MCHR2-shRNA1	5'-GATCCGCCATTTCGACTGACACGTTTTCAAGACGAACGTGTCAGTCGAAATGGTTTTTTGTCGACA—3'
	3'---GCGGTAAAGCTGACTGTGCAAAAGTTCTGCTTGCACAGTCAGCTTTACC AAAAAACAGCTGTTCGA-5'
MCHR2-shRNA2	5'-GATCCGAGACGGTGTTGAGAGTTGTTTCAAGACGACAACTCTCAACACCGTCT TTTTTTGTCGACA—3'
	3'---GCTCTGCCACAACTCTCAACAAAGTTCTGCTGTTGAGAGTTGTGGCAGA AAAAAACAGCTGTTCGA-5'
MCHR2-shRNA3	5'-GATCCGATCCAAAGAAGAGCGACTGTTCAAGACGCAGTCGCTCTTCTTTGGATTTTTTTGTCGACA—3'
	3'---GCTAGGTTTCTTCTCGCTGACAAGTTCTGCGTCAGCGAGAAGAAACCTA AAAAAACAGCTGTTCGA-5'
MCHR2-shRNA4	5'-GATCCGACCATCCGGATCAATTTGTTCAAGACG CAAATTGATCCGGATGGTCTTTTTTGTCGACA—3'
	3'---GCTGGTAGGCCTAGTTAAACAAGTTCTGCGTTTAACTAGGCCTACCAG AAAAAACAGCTGTTCGA-5'
MCHR2-shRNAhk	5'-GATCCGACTTCATAAGGCGCATGCTTCAAGACGGCATGCGCCTTATGAAGTCTTTTTT GTCGACA -3'
	3'-GCTGAAGTATTCCGCGTACGAAGTTCTGCCGTACGCGGAATACTTCAGAAAAAAACAGCTGTTCGA-5'

Western blot analysis for MCHR2 protein expression

Membrane protein was prepared from CHO cells transfected with the plasmids using membrane protein extraction kit as recommended by the manufacturer. Protein sample (20 μg) was then subjected to 10% SDS-PAGE followed by immunoblotting with anti-MCHR2 antibody (1:200). After chemiluminescence using ECL Plus reagent, the blot was photographed by Cool bioluminescence imager (Made in China) that captures chemiluminescent signals directly in the blot without exposure to x-way film, and protein band signals were quantified as integral optical density (IOD) by Quantity One software (Bio-Rad, US). The experiment was repeated for three times.

Radioligand binding assay (RBA)
Labeling of ^{125}I-MCH

The labeling was carried out with the iodo-beads kit according to the manufacturer's protocol and also based on some previous studies (12). The sequence of (^{125}I-Tyr13)-labeled MCH was as follows: Asp-Phe-Asp-Met-Leu-Arg-Cys-Met-Leu-Gly-Arg-Val-Tyr**^{13}**-Arg-Pro-Cys-Trp-Gln-Val.

The membrane fractions were prepared from cells as described in previous studies (2,4,9). For the binding assay, 15 μg membrane proteins were added into 4 sets of 4 detection tubes, and then ^{125}I-MCH was added into each set of 4 tubes at 50, 100, 200, 400, and 1000pmol/L, respectively. In two of the 4 tubes containing same amount of ^{125}I-MCH, unlabeled MCH at 1000-fold in excess over the amount of ^{125}I-MCH was added but no cold MCH was added to the other tubes. Finally, all the tubes were made up to a total volume of 300 μl with addition of the binding buffer (25 mmol/L HEPES, 10 mmol/L $MgCl_2$, 10 mmol/L NaCl, 5 mmol/L $MnCl_2$, 0.1% BSA). The reactions were incubated at room temperature for 3 h, and then terminated by 3 ml of ice-cold, 5-fold diluted binding buffer. After suction through a glass fiber filters, ^{125}I-MCH bound to membrane proteins was captured in the filter, and the radioactivity was measured in a γ-ray Counter (GC-911). Radioactivities obtained from tubes without cold MCH represented the total binding of radio-ligand both nonspecificly to any membrane proteins and the specific binding to MCH receptor. Radioactivities captured from tubes with excess cold MCH represented the non-specific binding of radioligand to any membrane proteins. Then, the specific binding (SB) activities were calculated by subtracting the non-specific binding from the total binding obtained from the tubes with the same ^{125}I-MCH concentration. Experiments were performed for three times. Data were further processed with PRISM4.0 software (GraphPad, San Diego, CA) and maximum binding (B_{max}) and dissociation constant (K_d) were calculated.

Calcium influx assay

To observe the intracellular Ca^{2+} release upon stimulation by MCH, cells were grown in six-well plates at a density of 0.5×10^5/ml. After 24 h, cells were washed with and changed to 1 mL HPSS buffer (20 mmol/L HEPES, 115 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 0.8 mmol/L $MgCl_2$, 13.8 mmol/L Glucose), and then Fura 2-AM was added together with 10 μl 0.1% Pluronic-F127 in the dark to a final concentration of 1 μmol/L. The cells were incubated at 37°C under 5% CO2 for 60 min, washed for three times with HEPES buffer, and placed under an inverted fluorescence microscope (Nikon, Japan, TE2000U). The cells were treated with 1 μmol/L MCH and Ca^{2+} release from single cells was measured with MetaMorph Imaging Software (3,6,7).

In a separate experiment, cells were grown in a 96-well plate at 37°C under 5% CO2. After 24 h, the media were replaced with 100 μl FLIPR buffer, and Fluo 3-AM dye was then added in the dark to a final concentration of 4 μmol/L. The plate was incubated at 37°C under 5% CO2 for 60 min. The wells were then washed twice and refilled with 150 μl FLIPR buffer. Upon addition of 50 μl MCH at 0.01-1000 nmol/L into triplicate wells, real time Ca^{2+} release was measured immediately in a fluorescence imaging reader (American Molecular Devices Co. Ltd.) for 3 min at 488 nm (15). The fluorescence intensity under 488nm was also obtained as Ratio A. MCH-induced calcium release in real time in single cells was plotted as change of fluorescent intensities over time (70 seconds), and a concentration curve of MCH effect on the level of intracellular free calcium was also plotted over the range of MCH concentrations at a fixed time point. The EC50 values for MCH were obtained from sigmoidal fits of a nonlinear curve-fitting program (Prism version 4.0; GraphPad software, San Diego , CA) based on the fluorescence intensity obtained from each well. The experiment was repeated for three times.

Statistical analysis

All data were presented as mean ± SD of measurements obtained from triplicate experiments. t-test was conducted to compare differences in values between groups and P values < 0.05 were considered statistically significant.

RESULTS

The effects of shRNAs on mRNA expression of MCHR2

CHO cells were transfected with one of the pGenesil-1-MCHR2-shRNAs or the control vectors, and total RNA was extracted and MCHR2 mRNA levels were analyzed by RT-PCR (Fig.1). While the mRNA levels of the house keeping gene GAPDH were similar in all the cell groups, the normalized MCHR2 mRNA levels (the ratios of MCHR2/GAPDH band intensities) decreased in cells transfected with pGenesil-1-MCHR2-shRNA1-4 ($p<0.05$) by 54.9%, 66.4%, 56.9%, and 45.8%, respectively, over those in the negative control group, whereas no significant difference was found between the negative control and the empty vector groups ($p>0.05$). These results suggest that shRNAs efficiently suppressed MCHR2 mRNA expression and the effect of the interference sequences is specific.

The effects of shRNAs on protein expression of MCHR2

The same groups of cells in the experiment above were also subjected to Western blot analysis for MCHR2 expression at the protein level. As shown in Fig. 2, transfected with pGenesil-1-MCHR2-shRNA1-4 also signify-cantly decreased MCHR2 protein expression

($p<0.05$) in CHO cells. Compared with cells transfected with the negative control vector, each of the shRNA sequences (pGenesil-1-MCHR2-shRNA1-4) brought about a reduction by 62.0%, 81.0%, 73.3%, and 44.2%, respectively, whereas there was no significant difference between the negative control and the empty vector groups ($P>0.05$). There results further confirm the functionality and specificity of these interference sequences, and the successful silencing of MCHR2 gene in these cells.

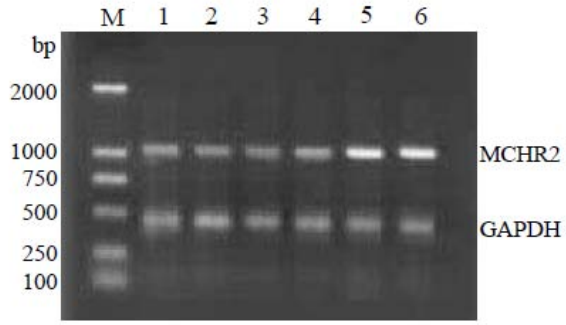

Figure 1. The effects of the shRNAs against MCHR2 on MCHR2 mRNA expression. RT-PCR analysis for total RNA samples extracted from CHO cells transfected with pGenesil-1-MCHR2-shRNA1-4 (Lane 1 - 4), the pGenesil-1-MCHR2-shRNAhk negative control (Lane 5), and the empty pGenesil-1 vector (Lane 6). Lane M, DL2000 DNA ladder.

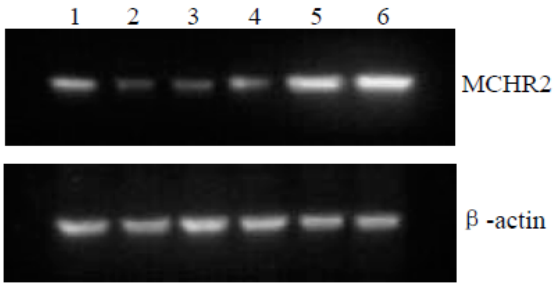

Figure 2. The effects of the shRNAs against MCHR2 on MCHR2 protein expression. Western-blot analysis on protein samples extracted from CHO cells transfected with pGenesil-1-MCHR2-shRNA1-4 (Lane 1 - 4), the pGenesil-1-MCHR2-shRNAhk negative control (Lane 5), and the empty pGenesil-1 vector (Lane 6).

The effects of shRNAs on the binding of MCHR2 to its ligand

Intact membrane fractions were prepared from all the CHO cell groups above, and the binding of MCHR2 to radio-labeled MCH was characterized *in vitro*. Compared with those in the pGenesil-1 group, in CHO cells with MCHR2 gene knock-down, B_{max} value decreased by 59.3%, 78.7%, 68.3%, and 39.4%, and K_d value increased by 57.1%, 81.%, 63.6%, and 40.9%, respectively. There were no significant differences in both the B_{max} and K_d values between the two control groups ($p>0.05$). The decrease in B_{max} indicates the decrease in the number of the receptors, and the increase of K_d values indicates diminished affinity of MCH with MCHR2 receptors. Therefore, the decreased expression of MCHR2 resulted in decreasing the number of the receptors and their binding affinity.

The effects of shRNAs on the intracellular Ca^{2+} release

Using cell-permeable fluorescent dye as intracellular calcium indicator, changes in the levels of intracellular free calcium were monitored in real time in all the CHO cell groups in response to MCH (1 μmol/L). Compared with the pGenesil-1 control group, all the experimental groups had decreased calcium influx in selected single cells (Fig. 3), indicating the ability of pGenesil-1-MCHR2-shRNAs to inhibit the MCH-induced Ca^{2+} release. When the changes in the intracellular calcium levels were plotted against MCH concentration, the concentration curves of all MCHR2 knock-down groups shifted to the right of those for both control groups (Fig. 4). EC_{50} values for all the cell groups were calculated based on these curves and are shown in Table 2. Compared with that in the pGenesil-1 group, EC_{50} increased significantly ($p<0.05$) in the experimental groups pGenesil-1-MCHR2-shRNA1-4 by 276.3%, 822.4%, 476.7% and 114.8%, respectively, whereas no significant change in the EC50 values was found in the shRNA negative control group ($p>0.05$).

DISCUSSION

A cyclic peptide of 19 amino acids, melanin-concentrating hormone (MCH) participates in many physiological functions including sensation, stress response, and learning. It also plays a critical role in food intake as well as energy balance (13), and functions as a neuropeptide enhancing appetite. Previous studies showed that acute intraventricular or nucleus paraventricularis injection of MCH (1) can stimulate food intake, and chronic intraventricular injection can result in weight gain in mice and rats due to increased food intake, ultimately leading to obesity (5). MCH gene knockout in mice has resulted in emaciation syndrome manifested as decrease

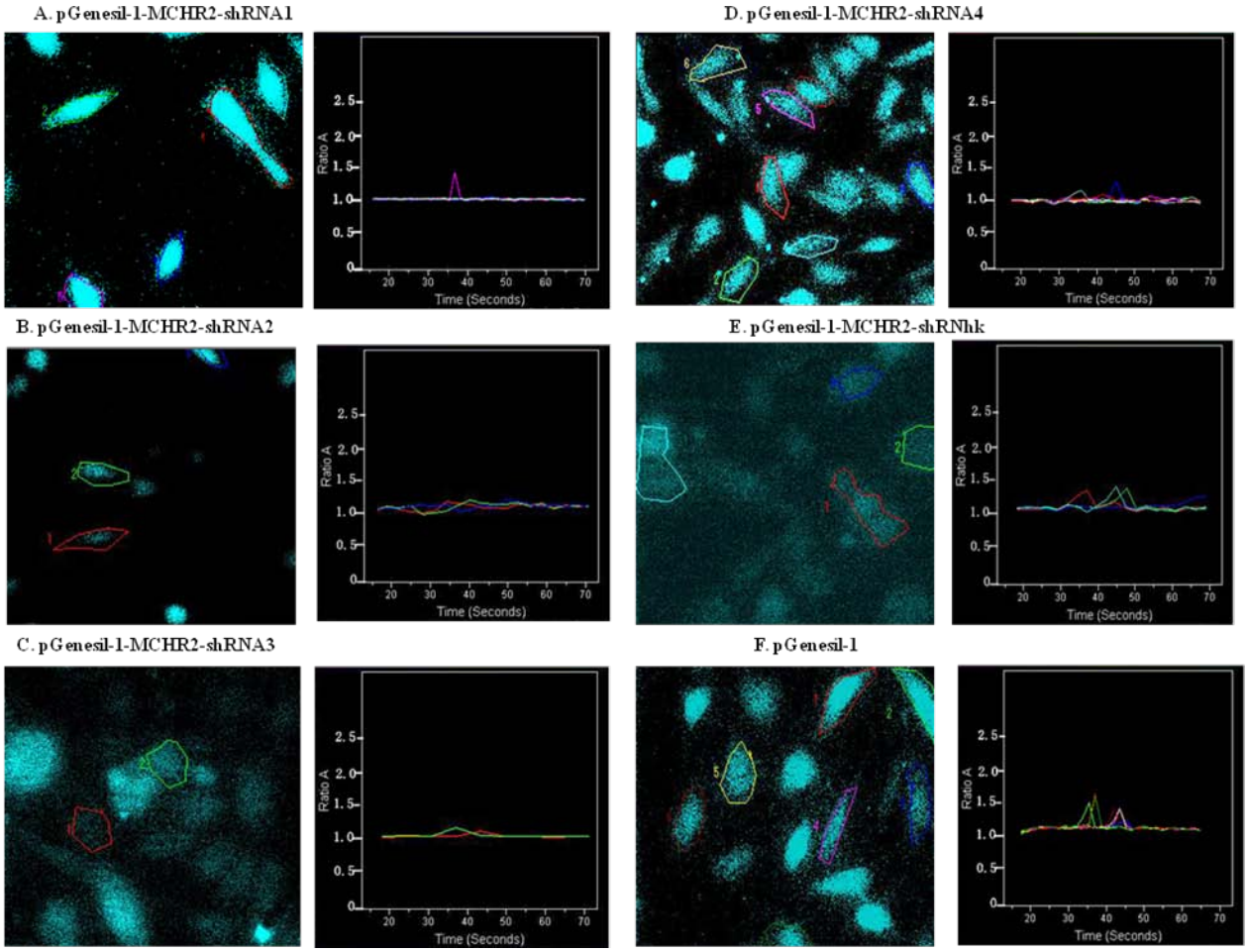

Figure 3. Effects of shRNAs against MCHR2 on Ca^{2+} influx in single cells CHO cells transfected with pGenesil-1-MCHR2-shRNA1-4, or the two control plasmids as indicated (A-F) were treated with 1 μmol/L Fura 2-AM and 0.1% Pluronic-F127, and then stimulated with 1 μmol/L MCH. Ca^{2+} release from single cells was measured with MetaMorph Imaging Software. Left panels: images of single cells; right panels: Ratio A plot in real time over 70 seconds.

Table 2. The effects of MCHR2-shRNAs on the MCHR2 expression and physiological functions ($\bar{x}$ ±s, n=3)

physiological functions ($\bar{x}$ ±s，n=3)

Cell group	mRNA	protein	B_{max} (of ligand binding)	K_d (of ligand binging)	EC_{50} (Ca^{++} release)
	MCHR2/GAPDH	(IOD)	$fmolL^{-1} \cdot mg^{-1}$ pro	fmol/L	nmol/L
pGenesil-1-MCHR2-shRNA1	1.16±0.02[ab]	17.17±4.95[ab]	125.33±3.04[ab]	269.88±0.61[ab]	8.74±0.64[ab]
pGenesil-1-MCHR2-shRNA2	0.86±0.01[ab]	8.65±3.72[ab]	64.01±2.19[ab]	310.77±0.89[ab]	21.43±0.99[ab]
pGenesil-1-MCHR2-shRNA3	1.11±0.02[ab]	12.08±3.97[ab]	97.65±3.85[ab]	280.10±0.58[ab]	13.39±0.61[ab]
pGenesil-1-MCHR2-shRNA4	1.39±0.03[ab]	24.80±1.91[ab]	186.83±6.09[ab]	240.81±1.10[ab]	4.99±0.24[ab]
pGenesil-1-MCHR2-shRNAhk	2.46±0.06[c]	43.77±3.84[c]	302.88±8.59[c]	171.72±1.12[c]	2.36±0.03[c]
pGenesil-1 vector	2.58±0.01	44.73±5.04	308.26±6.90	170.89±0.91	2.32±0.02

a: $p < 0.05$, *vs.* pGenesil-1 group, b: $p < 0.05$, *vs.* pGenesil-1-MCHR2-shRNAhk group, c: $p > 0.05$, *vs.* pGenesil-1 group

food intake, increased metabolism, and weight loss. All these previous data support a crucial role of MCH in regulating weight.

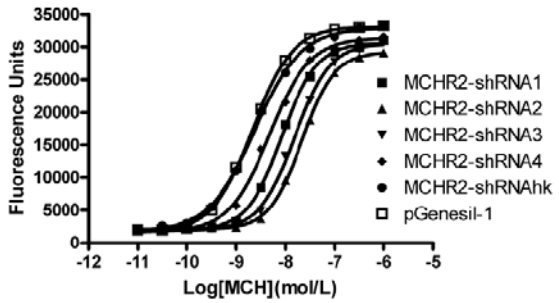

Figure 4. Concentration curves of calcium influx in response to MCH treatment in different CHO cell groups. CHO cells in a 96-well plate were treated with 4 μmol/L Fluo 3-AM dye and MCH at 0.01-1000 nmol/L as indicated. Real time Ca^{2+} release was measured in a fluorescence imaging reader at 488 nm and fluorescence intensities were plotted with the log change of MCH concentration.Data were presented as mean values from three experiments.

The two receptor subtypes of MCH (MCHR1 and MCHR2) belong to the subfamily of G-protein-coupled receptors. MCHR1 is known to induce Ca^{2+} release mainly through activating G_i, G_o and G_q proteins (18). Mice with MCHR1 gene knockout are thin and less susceptible to obesity when taking high-fat diet (11). Since the inactivation of MCHR1 renders resistant to obesity even with high-fat diet, MCHR1 antagonist has been developed to treat obesity (8). However, up to date, the function of MCHR2 and whether MCHR2 is also related to obesity has not been reported.

MCHR2 gene is located on chromosome 6q16.2-6q21. Its full length cDNA is 1023 bp, and the protein consists of 340 amino acids. MCHR2 has been shown to be mainly expressed in the brain, with particularly high expression in the anterior hypothalamic area, lateral hypothalamic area and hypothalamus ventromedial nucleus (14). Since the nerve center regulating food intake is also located in the hypothalamus, MCHR2 may play an important role in regulating feeding behavior. In addition, in patients with obesity, cytogenetic changes are identified in the long arm of chromosome 6 where MCHR2 is located, further supporting a correlation between MCHR2 and obesity. Therefore further study on the function of MCHR2 is needed to confirm its relationship with obesity, which may offer the new target for the prevention and treatment of obesity.

In this study, we showed that several shRNA sequences specifically targeting the human MCHR2 effectively suppressed MCHR2 expression at both the mRNA and protein levels. The specificity of these shRNA sequences was confirmed by using a randomly assembled shRNA sequence as a negative control. At the same time, MCHR2 knock-down also significantly decreased B_{max} and increased Kd values of MCHR2 binding to its ligand. While decrease in B_{max} indicates the decrease in the number of the receptors, which is consistent with decreased protein expression, the increase of K_d values indicates diminished affinity of MCHR2 to MCH, which cannot be simply explained by the change in receptor amount. Therefore, it is highly possible that the gene know-down also caused other changes to the protein such as conformation of the receptor at the cell surface. This possibility requires further investigation. The measurement of intracellular Ca^{2+} release can reflect the downstream signaling event of MCH-MCHR2 interaction. We showed that shRNAs against MCHR2 also inhibited intracellular calcium release in response to MCH stimuli, most likely due to the reduced number of MCHR2 presented at the cell surface.

From this study, we created four eukaryotic expression vectors carrying shRNA sequences against human MCHR2 and established CHO cells with MCHR2 gene knock-down. We showed the specificity and efficiency of these shRNA sequences, as well as their functionality in suppressing the expression and biological activity of MCHR2. In these cells, the reduced expression in MCHR2 mRNA and protein was accompanied by the decrease in B_{max} and increase in K_d of the receptor binding to MCH, and an increased EC_{50} value for MCH-induced calcium influx.

Together, we successfully applied RNAi technology to silence MCHR2 gene expression in mammalian cells. These shRNAs and cell lines can serve as valuable reagents for further studies on the function of MCHR2, which will ultimately help to advance our knowledge about appetite regulating neuropeptide receptors.

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REFERENCES

1. Abbott, C.R., Kennedy, A.R., Wren, A.M., Rossi, M., Murphy, K.G., Seal, L.J., Todd, J.F., Ghatei, M.A., Small, C.J. and Bloom, S.R., Identification of hypothalamic nuclei involved in the orexigenic effect of melanin concentrating hormone. *Endocrinology*. 2003, **144**: 3943– 3949.

2. Chambers, J., Ames, R.S., Bergsma, D., Muir, A., Fitzgerald, L.R., Hervieu, G., Dytko, G.M., Foley, J.J., Martin, J., Liu, W.S., Park, J., Ellis, C., Ganguly, S., Konchar, S., Cluderay, J., Leslie, R., Wilson, S. and Sarau, H.M., Melanin-concentrating hormone is the cognate ligand for the orphan G-protein-coupled receptor SLC-1. *Nature*. 1999, **400**: 261-265.

3. Francke, F., Ward, R.J., Jenkins, L., Kellett, E., Richter, D., Milligan, G. and Bächner, D., Interaction of neurochondrin with the melanin concentrating hormone-receptor 1 interferes with G protein-coupled signal transduction but not agonist-mediated internalization. *J Biol Chem*. 2006, **281**: 32496-32507.

4. Hawes, B.E., Kil, E., Green, B., O'Neill, K., Fried, S. and Graziano, M.P., The melanin-concentration hormone receptor couples to multiple G proteins to activate diverse intracellular signaling pathways. *Endocrinology*. 2000, **141**: 4524-4532.

5. Ito, M., Gomori, A., Ishihara, A., Oda, Z., Mashiko, S., Matsushita, H., Yumoto, M., Sano, H., Tokita, S., Moriya, M., Iwaasa, H. and Kanatani, A., Characterization of MCH-mediated obesity in mice. *Am J Physiol Endocrinol Metabol*. 2003, **284**: E940–E945.

6. Liu, S., Carrillo, J.J., Pediani, J. and Milligan, G., Effective information transfer from the α_{1b}-adrenoceptor to $G\alpha_{11}$ requires bothβ/γ interactions and an aromatic group four amino acids from the C terminus of the G protein. *J. Biol. Chem*. 2002, **277**: 25707-25714.

7. Murdoch, H., Feng, G.J., Bachner, D., Ormiston, L., White, J.H., Richter, D. and Milligan, G., Periplakin Interferes with G Protein Activation by the Melanin-concentrating Hormone Receptor-1 by Binding to the Proximal Segment of the Receptor C-terminal Tail. *J Biol Chem*. 2005, **280**: 8208-8220.

8. Mcbriar, M.D., Guzik, H., Shapiro, S., Paruchova, J., Xu, R., Palani, A., Clader, J.W., Cox, K., Greenlee, W.J., Hawes, B.E., Kowalski, T.J., O'neill, K., Spar, B.D., Weig, B., Weston, D.J., Farley, C. and Cook, J., Discovery of orally efficacious melanin-concentrating hormone receptor-1 antagonists as antiobesity agents. Synthesis, SAR, and biological evaluation of bicyclo(3.1.0)hexyl ureas. *J Med Chem*. 2006, **49**: 2294-2310.

9. Macdonald, D., Murgolo, N., Zhang, R., Durkin, J.P., Yao, X., Strader, C.D. and Graziano, M.P., Molecular characterization of the melanin-concentration hormone/ receptor complex: Identification of critical residues involved in binding and activation. *Mol pharmacol*. 2000, **58**: 217-225.

10. Miyagishi, M., Sumimoto, H., Miyoshi, H., Kawakami, Y. and Taira, K., Optimization of an siRNA expression system with an improved hairpin and its significant suppressive effects in mammalian cells. *J Gene Med*. 2004, **6**: 715-723.

11. Marsh, D.J., Weingarth, DT., Novi, D.E., Chen, H.Y., Trumbauer, M.E., Chen, A.S., Guan, X.M., Jiang, M.M., Feng, Y., Camacho, R.E., Shen, Z., Frazier, E.G., Yu, H., Metzger, J.M., Kuca, S.J., Shearman, L.P., Gopal-Truter, S., MacNeil, D.J., Strack, A.M., MacIntyre, D.E., Van der Ploeg, L.H, and Qian, S., Melanin-concentrating hormone 1 receptor-deficient mice are lean, hyperactive, and hyperphagic and have altered metabolism. *Proc Natl Acad Sci USA*. 2002, **99**: 3240-3245.

12. Nothacker, H.P., Wang, Z., McNeill, A.M., Saito, Y., Merten, S., O'Dowd, B., Duckles, S.P. and Civelli, O., Identification of the natural ligand of an orphan G-protein-coupled receptor involved in the regulation of vasoconstriction. *Nature Cell Biol*. 1999, **1**: 383-385.

13. Pissios, P. and Maratos-Flier, E., Melanin-concentrating hormone:from fish skin to skinny mammals. *Trends Endocrinol Metab*. 2003, **14**: 243-248.

14. Sailer, A.W., Sano, H., Zeng, Z., McDonald, T.P., Pan, J., Pong, S.S., Feighner, S.D., Tan, C.P., Fukami, T., Iwaasa, H., Hreniuk, D.L., Morin, N.R., Sadowski, S.J., Ito, M., Ito, M., Bansal, A., KyB, Figueroa, D.J., Jiang, Q., Austin, C.P., MacNeil, D.J., Ishihara, A., Ihara, M., Kanatani, A., Van der Ploeg, L.H., Howard, A.D. and Liu, Q., Identification and characterization of a second melanin concentrating hormone receptor, MCH-2R, *Proc Natl Acad Sci USA*. 2001, **98**: 7564-7569.

15. Satio, Y., Tetsuka, M., Li, Y., Kurose, H. and Maruyama, K., Properties of rat melanin-concentrating hormone receptor 1 internalization. *Peptides*. 2004, **25**: 1597-1604.

16. Tuschl, T. and Borkhardt, A., Small interfering RNAs: a revolutionary tool for the analysis of gene function and gene therapy. *Mol Interv*. 2002, **2(3)**: 158-167.

17. Tritos, N.A. and Maratos-Flier, E.,Two important systems in energy homeostasis: melanocortins and melanin-concentrating hormone. *Neuropeptides*. 1999, **33**: 339 -349.

18. Tetsuka, M., Satio, Y. and Imai, K.,The basic residues in the membrane-proximal C-terminal tail of the rat melanin-concentrating hormone receptor 1 are required for receptor function. *Endocrinology*. 2004, **145**(**8**) : 3712-3723

19. Yuan, C.F., Bu, Y.Q., Sengupta, J., Yang, J.X., Huang, X.N., Cheng, li., Zhang, Q., Yi, F.P., Liu, G.L. and Song, F.Z., Establishment of CHO cell line expressing human MCHR2 gene and research of its molecular characteristics. *Indian J.Exp.Biol*. 2009, **8**: 862-870.

20. Yu, J.Y., Taylor, J., DeRuiter, S.L., Vojtek, A.B. and Turner, D.L., Simultaneous inhibition of GSK3alpha and GSK3beta using hairpin siRNA expression vectors. *Mol Ther*. 2003, **7**: 228-236.