Molecular cloning and pharmacological characterization of porcine melanocortin-3 receptor

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Abstract

Mouse gene targeting studies revealed that the melanocortin-3 receptor (MC3R) affected feeding efficiency and fat storage in mice. The functions of the MC3R in other mammalian species remain to be investigated. We are interested in exploring the functions of the porcine MC3R (pMC3R) in regulating fat storage because of the economical importance of swine industry. Although nucleotide sequences of MC3Rs from several species have been reported, pMC3R had not been cloned and sequenced. We reported herein the molecular cloning and pharmacological analysis of the pMC3R. Sequence analysis revealed that pMC3R was highly homologous (>80%) at nucleotide and amino acid sequences to human, rat, and mouse MC3Rs. With human MC3R (hMC3R) as a control, the binding and signaling properties of pMC3R were investigated using several agonists including α- and γ-melanocyte-stimulating hormone (α- and γ-MSH), D-Trp^8-γ-MSH, and [Nle^4-D-Phe^7]-MSH (NDP-MSH) and the natural antagonist agouti-related protein (AgRP). The results showed that pMC3R bound NDP-MSH with the highest affinity followed by D-Trp^8-γMSH, γ-, and α-MSH. The same ranking was also found for hMC3R, although pMC3R had two- to ninefold higher affinities for these ligands. Both pMC3R and hMC3R bound AgRP with high affinity. D-Trp^8-γ-MSH was the most potent agonist to stimulate cAMP generation followed by NDP-, α-, and γ-MSH. This ranking was the same as that of hMC3R. The availability of pMC3R and its pharmacological characteristics will facilitate the investigation of pMC3R in regulating food intake and fat storage.

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Introduction

Melanocortins including α-, β-, and γ-melanocyte-stimulating hormones (α-, β-, and γ-MSH) and adrenocorticotropic hormone (ACTH) are short peptide hormones derived from pro-opiomelanocortin (POMC) through tissue-specific post-translational processing (Smith & Funder 1988). The melanocortin receptors (MCRs) are members of rhodopsin-like (family A) G-protein-coupled receptors (GPCRs). They are expressed on the cell surface, predicted to have seven transmembrane domains (TMDs) connected by alternating extracellular and intracellular loops, with the N-terminus lying on the outside of the cell and the C-terminus locating on the inside of the cell. Once bound to MCRs, melanocortins induce conformational changes in MCRs facilitating their coupling to and activation of the stimulatory G-protein, Gs. Activated Gs enhances adenylyl cyclase activity resulting in increased production of the intracellular second messenger cAMP triggering downstream signal transduction pathways (Gantz & Fong 2003). Agouti and agouti-related protein (AgRP) are the endogenous antagonists of the MCRs. So far, five MCRs have been cloned, named MC1R to MC5R according to the sequence of their cloning (Gantz & Fong 2003). Of the MCRs, MC3R and MC4R are the main subtypes expressed in the brain. They are expressed in the hypothalamic paraventricular nucleus and arcuate nucleus as well as in other brain regions including the cortex, thalamus, and hippocampus (Gantz *et al.* 1993*a*,*b*, Roselli-Rehfuss *et al.* 1993, Mountjoy *et al.* 1994). The MC3R is also expressed in the placenta and gut (Gantz *et al.* 1993*a*) and immune cells such as macrophages (Getting *et al.* 1999).

Although both the MC3R and the MC4R are involved in the central control of energy homeostasis, it is clear that the MC4R primarily regulates food intake and energy expenditure (reviewed in Cone 2005). On the other hand, the MC3R does not regulate food intake. MC3R knockout mice did not have hyperphagia and obesity; they had similar or even decreased levels of food intake and normal energy expenditure as compared with their wild-type littermates (Butler *et al.* 2000, Chen *et al.* 2000). Of particular interest was the observation that these mice have increased fat mass and decreased lean mass. Therefore, the MC3R is involved in regulation of feeding efficiency and fat storage. When compared with MC3R or MC4R single gene knockout mice, mice lacking both MC3R and MC4R developed exacerbated obesity, suggesting that the two central MCRs regulate different aspects of energy homeostasis (Chen *et al.* 2000). However, a recent gene knockout study performed with an obesity-resistant mice strain (Black Swiss 129) indicated that MC3R single gene knockout mice developed

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a comparable degree of increased adiposity as the MC4R knockout mice (Zhang *et al.* 2005) suggesting that the MC3R might also be involved in regulating food intake and energy expenditure. A potential pathway that the MC3R might regulate food intake is by releasing the inhibitory effect of the MC3R on POMC neurons (Marks *et al.* 2006).

To begin to understand what role(s) the MC3R might play in regulating fat storage and energy homeostasis in the pig, we report herein the molecular cloning and pharmacological characterization of the pMC3R. For the pharmacological characterizations, we tested several ligands including the endogenous agonists α- and γ-MSH, a superpotent agonist specific for the MC3R, D-Trp^8-γ-MSH (Grieco *et al.* 2000), and a superpotent analog of α-MSH specific for all MCRs except MC2R (the ACTH receptor), [Nle^4-D-Phe^7]-MSH (NDP-MSH; Sawyer *et al.* 1980). We also tested the binding of the cloned receptor to the natural antagonist of the MC3/4R, AgRP.

Materials and Methods

Plasmid and peptides

Human MC3R cDNA inserted in a mammalian expression vector, pcDNA3.1, was kindly provided by Dr Ira Gantz (University of Michigan, Ann Arbor, MI, USA). α-, γ-, and NDP-MSH were purchased from Phoenix Pharmaceuticals (Belmont, CA, USA). D-Trp^8-γ-MSH and AgRP (86–132) were obtained from Peptides International Inc. (Louisville, KY, USA). ^{125}I-iodinated NDP-MSH was purchased from Peptide Radioiodination Service Center, The University of Mississippi (University, MS, USA).

Molecular cloning of porcine MC3R

Sequence analysis performed with DNAman program (Lynnon Corp., Quebec, Canada) suggested that the putative coding region of the *pMC3R* gene consists of a single exon of 960 bp. Thus, the pMC3R coding region was amplified directly from porcine genomic DNA (Novagen, San Diego, CA, USA) using a primer pair (sense primer, 5′-AAGAATTCATGAATGCTTC-GTGCTGC-3′ and antisense primer, 5′-CCTCTAGAGCCT-CCTACCCCAGGTTC-3′) designed based on the published pig genomic DNA sequence from clone CH242-163M14 on chromosome 17 (GenBank accession no. CR956393). To facilitate the cloning, EcoRI and XbaI sites (underlined) were incorporated in sense and antisense primers respectively. The PCR was performed in a 50 μl mixture containing 100 ng porcine genomic DNA, 0·25 mM dNTPs, 0·4 μM of each of the primers, 1× pfu Turbo DNA polymerase buffer, 1·5 mM $MgCl_2$, and 2·5 U pfu Turbo DNA polymerase (Stratagene, La Jolla, CA, USA) with the following parameters: 2 min at 95 °C for one cycle and 1 min at 95 °C, 45 s at 56 °C, and 90 s at 72 °C for 45 cycles followed by a final cycle at 72 °C for 10 min. Subsequently, the 960 bp PCR fragment was visualized after electrophoresis with ethidium bromide using a 1% agarose gel, purified with Qiagen PCR purification kit (Qiagen), double digested with EcoRI and XbaI (New England Biolabs, Beverly, MA, USA). The EcoRI–XbaI DNA fragment was purified again with Qiagen PCR purification kit and then cloned into the expression vector pcDNA3.1(+) using T4 DNA ligase (Roche) according to the manufacturer's protocol. The nucleotide sequence of the cloned *pMC3R* gene was determined by sequencing three independent plasmids and deposited in NCBI GenBank with an accession number EU091085. Plasmids were prepared with Qiagen plasmid maxi kit for transfection of human embryonic kidney (HEK) 293T cells as described below.

Homology and phylogenetic analysis of pMC3R

Homology and phylogenetic analyses at nucleotide and amino acid levels were performed between different species including porcine, human, mouse, and rat using DNAman program and MacVector 8.0 (Accelrys Software Inc., San Diego, CA, USA) respectively, according to the manufacturer's protocols.

Transient expression of plasmids

Transient expression of plasmids was performed in HEK293T cells (American Type Culture Collection, Manassas, VA, USA). HEK293T cells were cultured at 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum, 10 mM HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen). Cells were plated on gelatin-coated 35 mm six-well plates (Corning, Corning, NY, USA) or on gelatin-coated 100 mm plates. Transient transfection was performed using the calcium precipitation method (Chen & Okayama 1987). For each 35 mm well of the six-well plates or 100 mm plates, 2 ml media containing 4 μg plasmid or 10 ml media containing 20 μg plasmid were added respectively. Cells were used for measuring ligand binding and agonist-stimulated cAMP generation 48-h post-transfection as described below.

Radioligand-binding assay with agonists

The method for binding assay has been described in detail before (Tao & Segaloff 2003). Briefly, 48 h after transfection, cells were washed twice with warm Waymouth's MB752/1 media (Sigma–Aldrich) modified to contain 1 mg/ml BSA (referred herein as Waymouth/BSA). One milliliter of fresh Waymouth/BSA with or without different concentrations of unlabeled agonists including α-, γ-, NDP-, and D-Trp^8-γ-MSH and 100 000 c.p.m. of ^{125}I-NDP-MSH (50 μl) was added to each well. The final concentration of unlabeled ligands ranged from 10^{-10} to 10^{-5} M (for α-, γ-, and D-Trp^8-γ-MSH) or from 10^{-11} to 10^{-6} M (for NDP-MSH). After incubation at 37 °C for 1 h, cells were washed twice with cold Hanks' balanced salt solution (Sigma–Aldrich) modified to contain 1 mg/ml BSA (referred herein as HBSS/BSA). Then, 100 μl of 0·5 M NaOH were added to each well. Lysed cells were collected from each well using cotton swabs to plastic tubes, and ligand binding was counted in a γ-counter. All determinations were performed in duplicates.

Radioligand-binding assay with AgRP

For AgRP-binding assay, transient transfection of plasmids was performed in 100 mm dishes as described above. Twenty-four hrs after transfection, cells were trypsinized, split into 24-well plates, and incubated for an additional 24 h. Cells were washed twice with warm Waymouth/BSA. Fresh Waymouth/BSA containing 20 μl of ^{125}I-NDP-MSH (40 000 c.p.m.) and 20 μl different concentrations (including zero) of unlabeled antagonist AgRP were added to each well to a total volume of 200 μl per well. The final concentration of the unlabeled AgRP ranged from 10^{-11} to 10^{-6} M. After incubation at 37 °C for 1 h, cells were washed twice with cold HBSS/BSA. Then, 50 μl of 0·5 M NaOH were added to each well. Cell lysate collection and ligand binding were performed as described above. All determinations were performed in triplicates.

Intracellular cAMP generation

HEK293T cells were plated and transfected as described above. Forty-eight hours after transfection, cells were washed twice with warm Waymouth/BSA. Then, 1 ml fresh Waymouth/BSA containing 0·5 mM isobutylmethylxanthine (Sigma–Aldrich) was added to each well. After incubation at 37 °C for 15 min, either buffer alone or different concentrations of ligands (final concentrations of 10^{-11}–10^{-5} M for α- and γ-MSH, and 10^{-12}–10^{-6} M for NDP-MSH and D-Trp^{8}-γ-MSH) were added, and the incubation was continued for an additional 1 h. Cells were then placed on ice, media were aspirated, and intracellular cAMPs were extracted by the addition of 1·5 ml fresh 0·5 M perchloric acid containing 180 μg/ml theophylline, and measured using RIA. All determinations were performed in triplicate.

Statistical analysis

Maximal binding (B_{max}), IC_{50}, maximal responses (R_{max}), and EC_{50} were calculated using Prism 4.0 (GraphPad, San Diego, CA, USA). Data were presented as the mean and S.E.M. All statistical analyses were done using Prism 4.0.

Results

Nucleotide and deduced amino acid sequences of the putative pMC3R

Civanova *et al.* (2004) reported the partial sequence of the pMC3R and mapped the *pMC3R* gene to porcine chromosome 17. Recently, the complete nucleotide sequence of porcine chromosome 17 was deposited in GenBank. We performed nucleotide sequence alignment among the partial nucleotide sequence of *pMC3R* gene (GenBank accession no. AJ744762), nucleotide sequence of the clone CH242-163M14 on porcine chromosome 17 (GenBank accession no. CR956393), and nucleotide sequences of human, rat, and mouse MC3Rs (GenBank accession nos. NM_019888, NM_001025270, and NM_008561 respectively). We found that the putative *pMC3R* gene was intronless and contained an open reading frame of 960 bp encoding a putative protein of 319 amino acids (Fig. 1A). The TATA-box, characteristic of the mammalian promoter region, was identified 382 bp upstream of the translation initiation site of the putative pMC3R; a potential poly(A)$^{+}$ signal was recognized 1381 bp downstream from the translation stop site of the putative pMC3R (highlighted in bold in Fig. 1A). Comparison of the putative pMC3R sequence with that of the MC3Rs from other species showed that pMC3R is 88·2, 85·3, and 86·0% homologous at nucleotide level (Fig. 1B) and 87·5, 83·1, and 83·7% identical at amino acid level (Fig. 2A and B) to human, mouse, and rat MC3Rs respectively. Further comparison indicated that pMC3R is significantly conserved in amino acid sequence to other species in the TMDs, extracellular, and intracellular loops. But lower conservation is observed at the amino and carboxyl termini. In addition, the putative pMC3R is four amino acids shorter at the carboxyl terminus than human, rat, and mouse MC3Rs, and 37 amino acids shorter at the N-terminus than human MC3R (but the same as that of rat and mouse MC3Rs). Phylogenetic analysis indicates that pMC3R clusters together with MC3Rs from other species at nucleotide level (Fig. 1C) but is significantly divergent from human MC3R and to a less degree from mouse and rat MC3Rs at amino acid level (Fig. 2C).

Expression and functional analysis of the cloned pMC3R in HEK293T cells

Based on the sequence analysis described above, primers were designed and the 960 bp of putative pMC3R was directly amplified from porcine genomic DNA by PCR and cloned into mammalian expression vector pcDNA3.1 for functional analysis. To demonstrate whether the cloned pMC3R is capable of producing a biologically active receptor protein, we transfected the pMC3R construct obtained into HEK293T cells and analyzed its ligand binding and signaling properties using the natural agonist for MC3R, γ-MSH, and ligand-binding property with the natural antagonist for MC3R, AgRP. Human MC3R was used for comparison. As shown in Fig. 3 and Table 1, γ-MSH bound the cloned pMC3R with an IC_{50} of 129·07 nM and hMC3R with an IC_{50} of 293·00 nM. Both the cloned pMC3R and hMC3R bound AgRP with similar high affinity: the cloned pMC3R with an IC_{50} of 0·67 nM and hMC3R with an IC_{50} of 2·35 nM. Furthermore, γ-MSH also induced dose-dependent accumulations of intracellular cAMP with EC_{50} of 79·16 and 111·13 nM for the cloned pMC3R and hMC3R respectively. These results suggested that the cloned pMC3R encoded a functional MC3R protein.

Binding and signaling of pMC3R to three other MC3R agonists

For *in vivo* investigation of the role that pMC3R plays in pigs, the most potent and selective agonist for pMC3R must be selected from the pool of known MC3R agonists. Herein, we tested the

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-430                                  GGCTGCACTGAAGATTAAGTAGGAGTTGTCTGCCTCCAGCGATGTATAAAACCAG
-375   TGTTCACCCCTCACTCCCAACGACTCCTACCACAGCCCTCTCTTGCTATGCAAAGAGCCGTAACTGTAGCAACCG
-300   GGGGCTGGTGTGTTCCATTCCCAGGTGGGGAGGTGAGGAGAGGAAGGTAAGACAGGAGAGAGCTAAGAGCATCTA
-225   GAAAACTGCTTGCCCTGGAGAAGCAAAGTTCTCTGCATGTCTCGGAGCTGACTCTCTCTCTCTCCCTCTTCTC
-150   TCCATCTCGGGCAGAAGAGAATCAAAGTCCAGACTGGACTAGCATCCAAAACAAGCACCTGCAGGGAGATTTTTC
-75    TCTTTCCTGGAAGCAGCAGCCACAGCAGCCTCTGCACCCTGCTGGAGCCCGGGCTCCGATCCCCGGCCGTGAGCA
+1     ATGAATGCTTCGTGCTGCCTGCTCTCTGCTCCACCGGCGCTGCCTAACAGCTCAGAGCACCTCCCCGCCCCTTCC
+1      M  N  A  S  C  C  L  L  S  A  P  P  A  L  P  N  S  S  E  H  L  P  A  P  S

+76    TTCAGCAACCAGAGCAGCAGCGGCTTCTGCGAGCAGGTCGTCATCCAGCCCGAGGTCTTCCTGGCTCTGGGCATC
+26     F  S  N  Q  S  S  S  G  F  C  E  Q  V  V  I  Q  P  E  V  F  L  A  L  G  I

+151   CTCAGCCTGCTGGAGAACGTGCTGGTCATCCTGGCCGTGGCCAGGAACGGCAACCTGCACTCGCCCATGTACCTC
+51     L  S  L  L  E  N  V  L  V  I  L  A  V  A  R  N  G  N  L  H  S  P  M  Y  L

+226   TTCCTCTGCAGCCTGGCCGTGGCCGACCTGCTGGTGAGCGTGTCCAACGCCCTGGAGACCATCATGATCGCCGTG
+76     F  L  C  S  L  A  V  A  D  L  L  V  S  V  S  N  A  L  E  T  I  M  I  A  V

+301   GTCAACAGCGACGCCCTGACCTTCGAGGACCAGTTCGTCCAGCACATGGACAACGTCTTCGACTCCATGATCTGC
+101    V  N  S  D  A  L  T  F  E  D  Q  F  V  Q  H  M  D  N  V  F  D  S  M  I  C

+376   ATCTCGCTGGTGGCCTCCATCTGCAACCTCTTGGCCATCGCCGTGGACAGGTACGTCACCATCTTCTACGCGCTG
+126    I  S  L  V  A  S  I  C  N  L  L  A  I  A  V  D  R  Y  V  T  I  F  Y  A  L

+451   CGCTACCACAGCATCATGACCGTGCGGAAGGCGGGGGCCCTGATCGCGGCCATCTGGGTGTGCTGCGGCGTCTGC
+151    R  Y  H  S  I  M  T  V  R  K  A  G  A  L  I  A  A  I  W  V  C  C  G  V  C

+526   GGCGTGGTCTTCATCGTCTACTCCGAGAGCAAGATGGTCATCGTGTGCCTTGTCGTCATCTTCTTCGCCATGCTG
+176    G  V  V  F  I  V  Y  S  E  S  K  M  V  I  V  C  L  V  V  I  F  F  A  M  L

+601   CTCCTCATGGGCACCCTCTACGTGCACATGTTTCTCTTCGCCCGGCTGCACGTCCAGCGCATCGCCGCGCTGCCG
+201    L  L  M  G  T  L  Y  V  H  M  F  L  F  A  R  L  H  V  Q  R  I  A  A  L  P

+676   CCCGCCGACGGGGGGCCCCCCCCCGCAGCGCTCGTGCCTGAAGGGGGCCGTGACCATCTCCCTCCTGCTGGGGGTC
+226    P  A  D  G  G  P  P  P  Q  R  S  C  L  K  G  A  V  T  I  S  L  L  L  G  V

+751   TTCATCTTCTGCTGGGCCCCCTTCTTTCTCCACCTGGTTCTCATCATCACCTGCCCCACCCACCCCTACTGCATC
+251    F  I  F  C  W  A  P  F  F  L  H  L  V  L  I  I  T  C  P  T  H  P  Y  C  I

+826   TGCTACACCGCCCACTTCAACACCTACCTGGTCCTCATCATGTGCAACTCGGTCATCGACCCCCTGATCTACGCC
+276    C  Y  T  A  H  F  N  T  Y  L  V  L  I  M  C  N  S  V  I  D  P  L  I  Y  A

+901   TTCCGGAGCCTGGAACTGCGCAACACCTTCAAGGAGATCCTGTGCAGCTGCAACGCCTGAACCTGGGGTAGGAGG
+301    F  R  S  L  E  L  R  N  T  F  K  E  I  L  C  S  C  N  A  *
+976   CAGGGCCCCGCCGGAGCGGTCCTCAGCCTGGTCGCGTTTCACCTGCCGGCCAGCCAAGGTGTTTAGGAAGCGGGG
+1051  AGAAACGCACTCAAAAGATGGAAAGATGTGTTCACGGTCATGATCATACAGGTCCTTTTGTTTTTAAGCTTACAA
+1126  AACGTTGAGAAAGAAGGGGGGGGGCTTTCTGAAAGGACGCCAACTTGGGTAAGTCCCCAACTCTGCTTTCCCAA
+1201  AGAGTCGCGGGGGCAAATCCGTGGAGACGGCTCTGGGTGCTGTCTGCGTTCGTTTCCATGCACCCGGGATCCGTG
+1276  ATGCCCCGCCTGCTCCCCGGCTTCCCACCCACGCCTCCGTGTTTCAAGTTCAAACCTCTCCCAAGCAAACCTGTT
+1351  CCAGCTATTGACCTTCAGCTACCAGCTGCTCGGGTGGCTTTGCCCTCATTTTGTGAGACCCTGAGTCTCTATTAC
+1426  TCTCTTTACAAGAAAACGGTTTCCTAAAAACAATGACCTCTTTGTGGAAGAAGTGATGCTATTTGCCCCTCCTCT
+1501  TGACCCCAGAGCTCCCCTGCAAAAATAGGGTGGGAATGTAGCCCACTTCTAGTCTGTTGTTATTGTTATTGTTGC
+1576  TGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTTGTTAGGCTTTTATGCAGGCAACATAGGGCAGCTT
+1651  TCAAAACTCTTCTGAAAGAGAAAGTGCTTTGGAAAATCAGAACATACCCATCTACAAAGTATTTGGAGACCTTGC
+1726  TACTTTGAGGTGTACATTTTCCAATTTTGCAAAAGTCATGTTTTCCTAATATGCCTTTTTTCGTCTTTAAAGCAA
+1801  GGAAGCAACTCCTGGGAGGGAGGGCATCTGTCTCAAGAAATTGCTGGACTGTAGTTTCCGAGAGTTTTAGTGCCA
+1876  GGGAGCTGCCTGCGGCTTTCGTGGAAGCTGTTTTTGCTCAGAGAAACTGGGTCTCTACCACGTGTGGTCTATGGG
+1951  ATGTGGGCGATGAAGGAGAATGGGGTTTTGGTTCACTGTTCCTTTTGCTTGACATGATGCTAAAGCTGGGTGACC
+2026  CTCCTGTCGAGGTCTACACTGCATCAAGTAAAGCGATCCATTCCTGCGAGCGAGCGCAGGGTGAGATTCGCTGAG
+2101  CAGTGTTGTACACCAGTGCGCTGCAAGCATCCATCAGACCCCTTTCTGCCTCAGATGACTTCCGGCTGTGCTCTC
+2176  CAGCTGTCCTCACCCCCCCCCCCCCCATGAATCCTTATATTACGCTGTGTCTTTGGTATACGACTGTGTCTTTTTC
+2251  TTGTGCTGGAATATATTGGGGGTTTTTTGTACTTGATATGTGCTCGTGTGCATACTATTGTTTCTTGCTTTGATT
+2326  GTGATGTTTGAAAATAATAAACATCTTCTGTGGCTGATAAACATGTGTCACTTTAAACTACCCCACCTGGACGCA
+2401  CCCTAGTGACGTCTGCACGTGGCCATCATATGTGACACTG
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B

	Pig	Human	Mouse	Rat
Pig	100·0	88·2	85·3	86·0
Human		100·0	87·6	87·6
Mouse			100·0	93·0
Rat				100·0

C

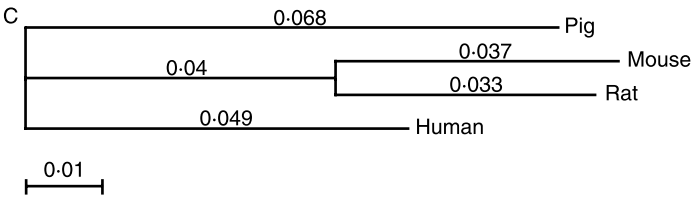

Figure 1 Deduced nucleotide and amino acid sequences of pMC3R and comparison with MC3Rs from other mammal. (A) Sequence of the full-length pMC3R cDNA with *in silico* translation is shown. Arrows indicate the position and direction of the primers used for amplification of the cDNA by PCR. (B) Homology and (C) phylogenetic analysis of MC3R nucleotide sequences from several species.

A

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                                        Extracellular Amino Terminus
pMC3R:  ------------------------------------MNASCCLLSAPPALPNSSEHLPA   23
hMC3R:  MSIQKTYLEGDFVFPVSSSSFLRTLLEPQLGSALLTAMNASCCLPSVQPTLPNLSQHPAA   60
mMC3R:  ------------------------------------MNSSCCLSSVSPMLPNLSEHPAA   23
rMC3R:  ------------------------------------MNSSCCPSSSYPTLPNGSEHLQA   23

                                    TMD1                IL1       TMD2
pMC3R:  PSFSNQSSSGFCEQVVIQPEVFLALGILSLLENVLVILAVARNGNLHSPMYLFLCSLAVA   83
hMC3R:  PFFSNQSSSAFCEQVFIKPEVFLSLGIVSLLENILVILAVVRNGNLHSPMYFFLCSLAVA  120
mMC3R:  PPASNRSGSGFCEQVFIKPEVFLALGIVSLMENILVILAVVRNGNLHSPMYFFLCSLAAA   83
rMC3R:  PSASNRSGSGFCEQVFIKPEVFLALGIVSLMENILVILAVVRNGNLHSPMYFFLLSLLQA   83

                                EL1                     TMD3
pMC3R:  DLLVSVSNALETIMIAVVNSDALTFEDQFVQHMDNVFDSMICISLVASICNLLAIAVDRY  143
hMC3R:  DMLVSVSNALETIMIAIVHSDYLTFEDQFIQHMDNIFDSMICISLVASICNLLAIAVDRY  180
mMC3R:  DMLVSLSNSLETIMIAVINSDSLTLEDQFIQHMDNIFDSMICISLVASICNLLAIAIDRY  143
rMC3R:  DMLVSLSNSLETIMIVVINSDSLTLEDQFIQHMDNIFDSMICISLVASICNLLAIAVDRY  143

                    IL2                 TMD4            EL2         TMD5
pMC3R:  VTIFYALRYHSIMTVRKAGALIAAIWVCCGVCGVVFIVYSESKMVIVCLVVIFFAMLLLM  203
hMC3R:  VTIFYALRYHSIMTVRKALTLIVAIWVCCGVCGVVFIVYSESKMVIVCLITMFFAMMLLM  240
mMC3R:  VTIFYALRYHSIMTVRKALTLIGVIWVCCGICGVMFIIYSESKMVIVCLITMFFAMVLLM  203
rMC3R:  VTIFYALRYHSIMTVRKALSLIVAIWVCCGICGVMFIVYSESKMVIVCLITMFFAMVLLM  203

                                IL3                         TMD6
pMC3R:  GTLYVHMFLFARLHVQRIAALPPADGGPPPQRSCLKGAVTISLLLGVFIFCWAPFFLHLV  263
hMC3R:  GTLYVHMFLFARLHVKRIAALPPADGVAPQQHSCMKGAVTITILLGVFIFCWAPFFLHLV  300
mMC3R:  GTLYIHMFLFARLHVQRIAVLPPAGVVAPQQHSCMKGAVTITILLGVFIFCWAPFFLHLV  263
rMC3R:  GTLYIHMFLFARLHVQRIAALPPADGVAPQQHSCMKGAVTITILLGVFIFCWAPFFLHLV  263

                    EL3                 TMD7            Cytoplasmic Tail
pMC3R:  LIITCPTHPYCICYTAHFNTYLVLIMCNSVIDPLIYAFRSLELRNTFKEILCSCNA----  319
hMC3R:  LIITCPTNPYCICYTAHFNTYLVLIMCNSVIDPLIYAFRSLELRNTFREILCGCNGMNLG  360
mMC3R:  LIITCPTNPYCICYTAHFNTYLVLIMCNSVIDPLIYAFRSLELRNTFKEILCGCNSMNLG  323
rMC3R:  LIITCPTNPYCICYTAHFNTYLVLIMCNSVIDPLIYAFRSLELRNTFKEILCGCNGMNVG  323
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B

	Pig	Human	Mouse	Rat
Pig	100·0	87·5	83·1	83·7
Human		100·0	88·7	89·3
Mouse			100·0	94·0
Rat				100·0

C

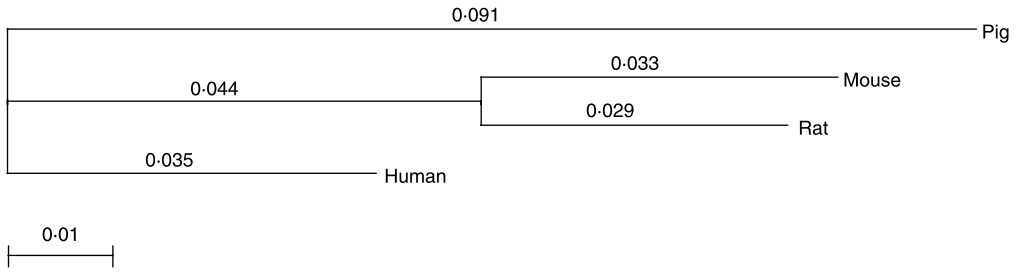

Figure 2 Comparison of amino acid sequences between pMC3R and MC3Rs from other species. Alignment of the amino acid sequences between pMC3R and human, mouse and rat MC3Rs. Positions of different regions of the receptor are indicated above the sequences and labeled as follows: transmembrane domains as TMD 1–7, extracellular loops as EL 1–3, intracellular loops as IL 1–3, amino and carboxyl termini as extracellular amino terminus and cytoplasmic tail respectively. The conserved residues are shaded in gray. The transmembrane residues are in bold. (B) Homology and (C) phylogenetic analysis of MC3R amino acid sequences from several species.

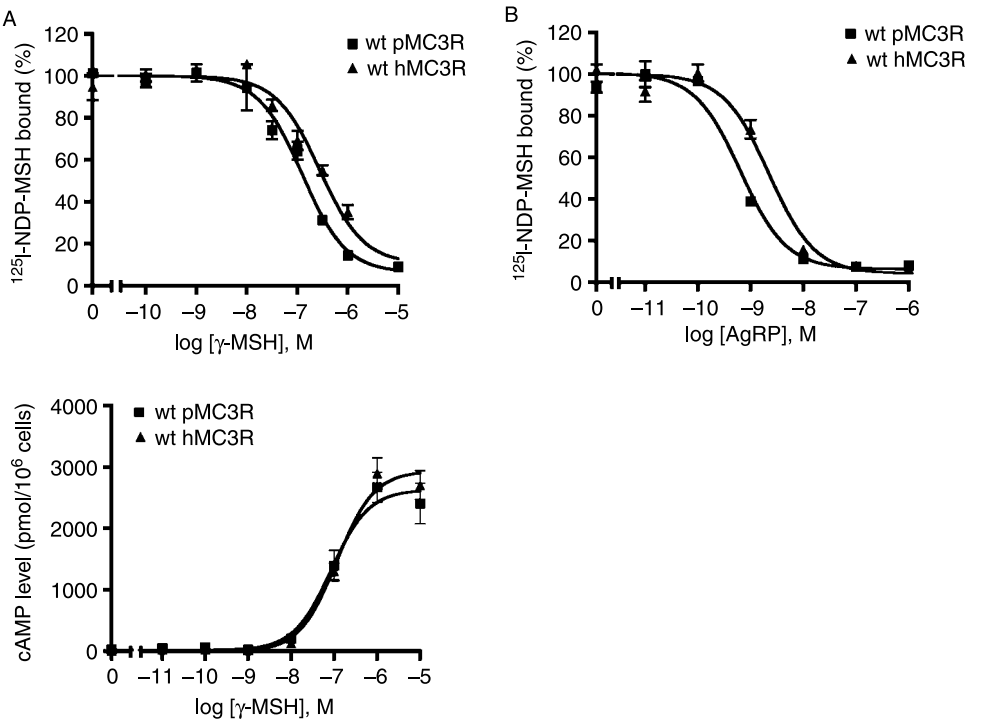

Figure 3 Expression and functional analysis of the cloned pMC3R in HEK293T cells. HEK293T cells were transiently transfected with pMC3R and hMC3R, and binding and signaling assays were performed as described in Materials and Methods. For ligand binding, different concentrations of unlabeled (A) γ-MSH or (B) AgRP were used to displace the binding of ^{125}I-NDP-MSH to pMC3R or hMC3R on intact cells. Results shown are expressed as the percentage ± S.E.M. of the maximal binding from duplicate determinations within one experiment. All experiments were performed at least thrice. For measurement of cAMP accumulation, the transfected cells were stimulated with various concentrations of γ-MSH, and intracellular cAMP levels were measured as described in Materials and Methods. Results are expressed as the mean ± S.E.M. of triplicate determinations within one experiment, and all experiments were performed at least thrice.

pharmacological properties of three MC3R agonists on the cloned pMC3R. These agonists include the natural agonist of MC3R, α-MSH; a superpotent analog of α-MSH for MC3R and MC4R, NDP-MSH; and a superpotent analog specific for MC3R, D-Trp^8-γ-MSH. As shown in Fig. 4, ligand-binding assays revealed that pMC3R bound NDP-MSH with the highest affinity (IC_{50} of 4·36 nM) followed by D-Trp^8-γ-MSH (IC_{50} of 10·65 nM) and α-MSH (IC_{50} of 674·90 nM). The same

Table 1 Ligand binding and agonist-stimulated cAMP response of porcine melanocortin-3 receptor (pMC3R) and human MC3R (hMC3R). Data shown are the mean ± S.E.M. of the indicated number of experiments

			Ligand binding		Ligand-stimulated cAMP response	
	MC3R	***n***	IC_{50} (nM)	***n***	EC_{50} (nM)	Rmax (pmol/10^6 cells)
Ligands						
α-MSH	Porcine	3	674·90 ± 83·76	4	59·03 ± 7·51	2854 ± 96
	Human	3	1151·33 ± 85·54	4	70·27 ± 11·97	2765 ± 238
NDP-MSH	Porcine	5	4·36 ± 0·43	4	2·24 ± 0·24	1286 ± 160
	Human	5	21·71 ± 2·64	4	5·01 ± 0·87	2438 ± 333
γ-MSH	Porcine	3	129·07 ± 3·28	4	79·16 ± 6·05	2354 ± 147
	Human	3	293·00 ± 21·64	4	111·13 ± 11·78	2623 ± 185
D-Trp^8-γ-MSH	Porcine	3	10·65 ± 3·31	4	1·24 ± 0·58	1894 ± 256
	Human	3	90·74 ± 12·46	4	1·95 ± 0·64	1972 ± 258
AgRP	Porcine	3	0·67 ± 0·06	NA	NA	NA
	Human	3	2·35 ± 0·42	NA	NA	NA

IC_{50} is the concentration of ligand that is needed to cause 50% inhibition in the binding assay. EC_{50} is the concentration of agonist that results in 50% stimulation of the maximal response. NA, not applicable.

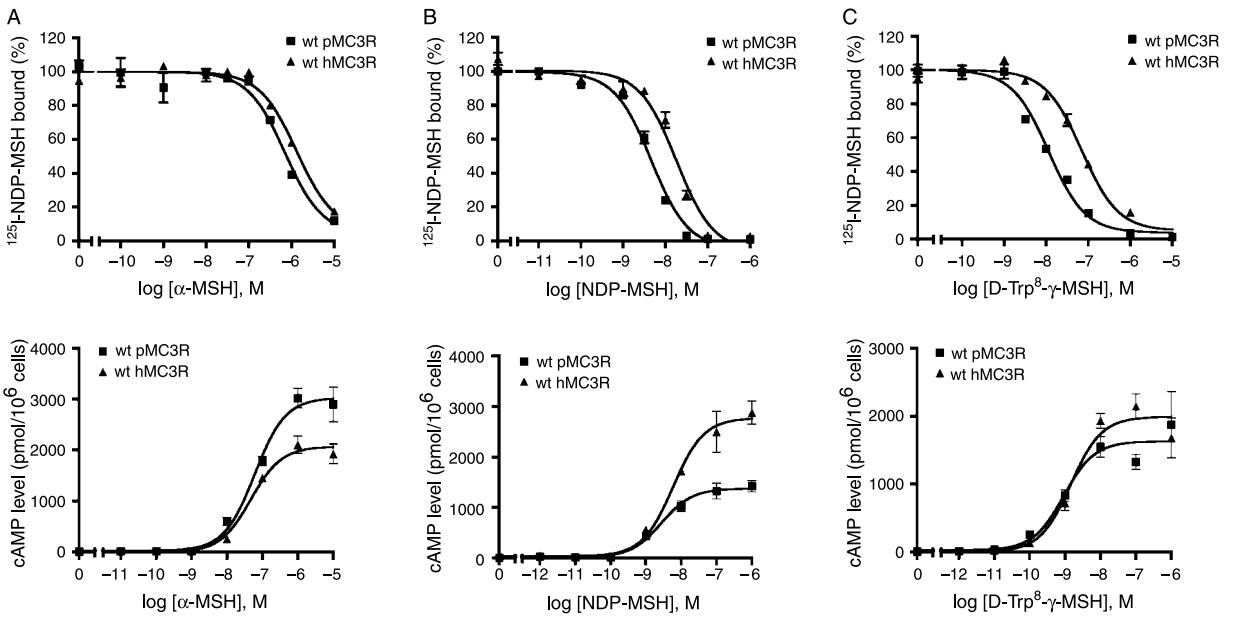

Figure 4 Binding and signaling of pMC3R to three other MC3R agonists. HEK293T cells were transiently transfected with pMC3R or hMC3R. Binding assays were performed as described in Materials and methods. Different concentrations of unlabeled (A) α-MSH, (B) NDP-MSH, or (C) D-Trp^8-γ-MSH were used to displace the binding of ^{125}I-NDP-MSH to pMC3R or hMC3R on intact cells. To measure accumulation of intracellular cAMP, the transfected cells were stimulated with various concentrations of (A) α-MSH, (B) NDP-MSH, or (C) D-Trp^8-γ-MSH, and intracellular cAMP levels were measured as described in Materials and methods. Results are expressed as the mean±S.E.M. of triplicate determinations within one experiment, and all experiments were performed at least thrice.

ranking was also observed for hMC3R with an IC_{50} of 21·71 nM for NDP-MSH, 90·74 nM for D-Trp^8-γ-MSH, and 1151·33 nM for α-MSH. It is interesting to note that pMC3R always had two- to ninefold higher affinities for these ligands than the hMC3R (Table 1). The data from the signaling assays demonstrated that D-Trp^8-γ-MSH was the most potent agonist to stimulate dose-dependent cAMP generation with an EC_{50} of 1·24 nM, followed by NDP-MSH (EC_{50} of 2·24 nM) and α-MSH (EC_{50} of 59·03 nM; Table 1). Human MC3R has the same ranking with an EC_{50} of 1·95 nM for D-Trp^8-γ-MSH, 5·01 nM for NDP-MSH, and 70·27 nM for α-MSH (Table 1). Taken together, these data demonstrated that D-Trp^8-γ-MSH was the most potent agonist in eliciting pMC3R signaling.

As can be seen from Table 1, the maximal responses to α-, γ-, and D-Trp^8-γ-MSH are similar between the pMC3R and hMC3R. However, the hMC3R has significantly larger maximal response to NDP-MSH than pMC3R. One reason might be that hMC3R is expressed at slightly higher level (148±8% of pMC3R, $n=12$). However, the maximal responses to the other three agonists are similar between the two MC3Rs. We suggest that the NDP-MSH binding to hMC3R induces a conformation with higher coupling efficiency than the pMC3R (Ballesteros *et al.* 1998).

Discussion

Previous pharmacological and genetic studies in rodents suggested that the MC3R was not involved in food intake regulation. Administration of MC3R-specific agonist such as γ-MSH did not inhibit food intake (Kask *et al.* 2000). In fact, a recent study showed that peripheral administration of D-Trp^8-γ-MSH stimulated food intake in rats by releasing the inhibitory effect of the MC3R on POMC neurons (Marks *et al.* 2006). Changes in energy balance such as food restriction or diet-induced obesity did not change MC3R expression level in the brain, whereas the MC4R density was modulated (Harrold *et al.* 1999). Finally, MC3R knockout mice did not have increased food intake as did the MC4R knockout mice (Huszar *et al.* 1997, Butler *et al.* 2000, Chen *et al.* 2000). Mice lacking the MC3R have increased fat contents but are normal in terms offood intake and body weight (Butler *et al.* 2000, Chen *et al.* 2000), suggesting that the MC3R pathway modulates feeding efficiency and fat storage. The recent gene targeting study by Zhang *et al.* (2005) showed that Black Swiss 129 mice lacking *MC3R* gene had a comparable level of adiposity as mice lacking the *MC4R* gene. In humans, only a few naturally occurring mutations in the *MC3R* gene have been identified (Lee *et al.* 2002, 2007, Tao 2007). These mutations were found to cause defects in signaling (Rached *et al.* 2004, Tao & Segaloff 2004, Lee *et al.* 2007, Tao 2007). In contrast, in the MC4R, numerous mutations have been identified from various patient populations (reviewed in Tao 2005, 2006). Therefore, the roles of the MC3R in energy homeostasis are much less understood.

Pork is an important protein source in our diet. About 60% of the saturated fat comes from animal products in typical American diet. Leaner meat will decrease intake of saturated

fat and decrease blood cholesterol levels. To increase the economic return for hog farmers and reduce the obesity epidemic, it is of great interest to decrease the fat content in pork. Therefore, a better understanding of fat storage in pigs is of great economic interest.

In the present study, as the first step towards elucidating the role(s) of the MC3R in pig energy homeostasis, we describe the molecular cloning and pharmacological characterization of the porcine MC3R. Through bioinformatics analysis and progress in pig genome sequencing, we were able to identify the putative full-length coding region of pMC3R. Primers designed based on this sequence were used to amplify the coding region from pig genomic DNA by taking advantage of the fact that the mammalian MCRs, including the putative pMC3R, are intronless. This amplified fragment was inserted into pcDNA3.1 and the sequence verified.

This construct was used for detailed pharmacological analysis using a series of ligands. From ligand-binding studies, we showed that of the four agonists tested, the rank order in terms of affinity is: NDP-MSH > D-Trp^8-γ-MSH > γ-MSH > α-MSH. Human MC3R has the same rank order for these ligands. From signaling experiments, we showed that the rank order in terms of potency for these four ligands is: D-Trp^8-γ-MSH > NDP-MSH > α-MSH > γ-MSH. Again, hMC3R has the same rank order for these ligands. Both pMC3R and hMC3R bind to AgRP with high affinity, with IC_{50} of 0·67 and 2·35 nM respectively.

Previously, the MC3Rs have been cloned from a number of species, including human (Gantz *et al.* 1993*a*), rat (Roselli-Rehfuss *et al.* 1993), mouse (Desarnaud *et al.* 1994), and elephant (Rompler *et al.* 2006). In lower vertebrates, the MC3Rs have been cloned from the chicken (Takeuchi & Takahashi 1999) and zebrafish (Logan *et al.* 2003) but was found to be absent in some other fishes such as the pufferfishes (Klovins *et al.* 2004*a*). The numbers we reported herein for pMC3R are roughly in line with previous studies. For example, with NDP-MSH, the most commonly used analog for MCR studies, the IC_{50} reported here for pMC3R is 4·36 nM. Previously, the K_d for rat MC3R was reported to be 3·78 nM (Kim *et al.* 2002), and the Ki for human MC3R was 5·6 nM (Chen *et al.* 2006), and for the dogfish MC3R was 0·93 nM (Klovins *et al.* 2004*b*).

In the original publication describing the discovery of D-Trp^8-γ-MSH as a potent and selective MC3R agonist (Grieco *et al.* 2000), an IC_{50} of 6·7 nM and EC_{50} of 0·33 nM were reported for hMC3R. Our data for hMC3R are somewhat higher than those reported before (IC_{50} of 90·74 nM and EC_{50} of 1·95 nM). These differences might be due to the use of different cell lines and different methods in the two studies. For example, for binding studies, Grieco *et al.* used membranes prepared from L cells stably expressing hMC3R, whereas we used live HEK293T cells transiently expressing hMC3R (only cell surface receptor binding is detected). However, our data for pMC3R (IC_{50} of 10·65 nM and EC_{50} of 1·24 nM) are very close to the numbers reported for hMC3R by Grieco *et al.* (2000).

It should be pointed out that the ranking in terms of affinity is not the same as that in terms of potency. For example, for pMC3R, NDP-MSH has higher binding affinity than D-Trp^8-γ-MSH. However, it is not as potent in eliciting activation of signaling pathways as the latter. This may be due to the difference in their relative efficacies. Both affinity and efficacy can independently affect the potency of a particular ligand (Besse & Furchgott 1976).

The data presented herein suggest that D-Trp^8-γ-MSH is a superpotent agonist for the pMC3R. Since it does not activate the MC4R efficiently (Grieco *et al.* 2000), it is an excellent tool for *in vivo* studies directed towards dissecting the role(s) of the MC3R in regulating pig energy balance. However, the *in vitro* data described herein should only be used as the starting point for further *in vivo* studies. Previous studies showed that pMC4Rs respond to the natural (AgRP) and artificial (SHU9119) antagonists normally *in vitro*. However, *in vivo*, these antagonists do not increase food intake as observed in rodents (Barb *et al.* 2004). It was suggested that the MC4R might not play a critical role in regulating feed intake in the pig (Barb *et al.* 2004). Similarly, two naturally occurring mutations (R236H and D298N) in the pMC4R were identified in different strains of pigs that are associated with differences in performance characteristics such as average daily gain and average daily intake as well as carcass fat (Kim *et al.* 2000, Meidtner *et al.* 2006). In the case of D298N, not all studies could confirm the original associations (Park *et al.* 2002, Stachowiak *et al.* 2006). Similarly, our experiments *in vitro* could not replicate the original functional studies (Kim *et al.* 2004, Fan *et al.* 2007). Therefore, the data on the naturally occurring mutations in the pMC4R are also much less convincing than those on human MC4R (reviewed in Tao (2005, 2006)). All these point to the importance of using caution when extrapolating *in vitro* data as well as the potential difference of the relevance of the melanocortin system in regulating energy homeostasis in the pig.

In summary, we have cloned the pMC3R. Expression of the cloned pMC3R in HEK293T cells revealed that indeed it was functional. Pharmacological characterizations using a series of ligands revealed that the pMC3R and hMC3R have the same rank orders for these ligands. We showed that D-Trp^8-γ-MSH was the most potent ligand (and selective for the MC3R), therefore, best suited for further *in vivo* studies to elucidate the functional importance of the MC3R in energy homeostasis in the pig.

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