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Molecular and pharmacological characterization of poultry (*Gallus gallus*, *Anas platyrhynchos*, *Anser cygnoides domesticus*) and pig (*Sus scrofa domestica*) melanocortin-5 receptors and their mutants

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ABSTRACT

The melanocortin-5 receptor (MC5R) is a member of the G protein-coupled receptor superfamily that plays a critical role in lipid production, skeletal muscle fatty acid oxidation, and adipocyte lipolysis. Although multiple functions and important value of MC5R in human beings have been fully demonstrated, however, the potential molecular cloning, pharmacological characteristics and key amino acids in poultry and pig were still not fully understood. Herein, we successfully cloned *MC5R* genes from chicken (*Gallus gallus*, *cMC5R*), duck (*Anas platyrhynchos*, *dMC5R*), goose (*Anser cygnoides domesticus*, *gMC5R*) and pig (*Sus scrofa domestica*, *pMC5R*), and compared their genetic and protein difference with hMC5R through phylogenetic analysis and homology models. Besides, we constructed three alanine-substitution mutants for each of MC5Rs through homologous reorganization, including c/d/gMC5R-D119A/F254A/H257A and pMC5R-D204A/F339A/H342A. Subsequently, we focused our investigation on the pharmacological characterization of four wide-type MC5Rs and their mutants in HEK293T cells, including the intracellular cAMP generation and phosphorylation level of ERK1/2. The results showed that these mutants had decreased cAMP levels under the stimulation of ligands, in spite of enhanced basal activity for c/d/gF254A and pH342A, indicating their important roles in the location and activation of receptors. Notably, these MC5Rs and mutants displayed significant species-specific phenotypes in the activation of pERK1/2 with ligands, which was not completely consistent with hMC5R. These findings demonstrated that presence of interspecies differences for MC5Rs, particularly for the pERK1/2 pathway. Taken together, our study expands current knowledge about the molecular and pharmacological characterization of c/d/g/pMC5Rs, providing preliminary data for MC5R-targeted drug screening or genetic breeding of economic animals in the future.

1. Introduction

G protein-coupled receptors (GPCRs) are the largest family of membrane proteins and mediate most of our physiological responses to hormones, neurotransmitters and environmental stimulants, and thereby have great potential as therapeutic targets for a broad spectrum of diseases (Rosenbaum et al., 2009; Venkatakrishnan et al., 2013). Among the subgroups of GPCRs, melanocortin receptors (MCRs) belong to rhodopsin-like GPCRs with seven transmembrane domains (TMD) and thus far possess five sub-types (MC1R to MC5R) (Mountjoy et al., 1992). Previous evidence has proved that MCRs play an important role in pigmentation, lipolysis, stress, immunity, energy metabolism and cardiovascular function (Getting, 2006). Specifically, MC1R is associated with the production of epidermal melanin pigments (Herraiz et al., 2017); MC2R anticipates the regulation of adrenal function, and its dysfunction would lead to a potentially fatal disease-familial glucocorticoid deficiency (FGD) (Chhajlani et al., 1996). MC3R is produced in the central nervous system and some peripheral tissues (Gantz et al., 1993), which plays an important role in regulating body fat and accelerating diet-induced obesity (Chen et al., 2000). MC4R is mainly expressed in the hypothalamus and involved in regulating food intake and body weight (Krashes et al., 2016). MC4R stimulates hormones (α-MSH) by activating α-melanocytes. Mutations in MC4R are found in obese patients, representing the most common cause of unilateral obesity (Vaisse et al., 2000). In addition to hMC4R, studies on role of MC4R in regulation of food intake have been extended to other species,

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such as chicken (Wang et al., 2016a) and giant panda (Wang et al., 2016b).

Different from other four types of MCRs, MC5R is a protein that consists of 343 amino acids and expressed in a variety of forms and widely distributed in skeletal muscle and exocrine tissues (Konda, 1994). MC5R is involved in lipid production, skeletal muscle fatty acid oxidation, and adipocyte lipolysis. Previous studies showed that defects in MC5R could cause skeletal muscle fatty acid and fat cell metabolism, and exocrine gland dysfunction (Li et al., 2011). Mice with MC5R deficiency had accelerated lipolysis and reduced oil secretion (Chen et al., 1997). In humans, MC5R can increase the oxidation of carnitine transferase and fatty acids in a dose-dependent manner under the activation of the endogenous agonist α-MSH (Yang et al., 2013a). It has also been proved that some missense polymorphisms in hMC5R would affect its binding activity with ligands and downstream functions, particularly for conserved amino acids in transmembrane domains (TMD). For example, the mutations of E92 (TMD 2), D115/119 (TMD 3), W251, F254 and H257 (TMD 6) would result in decreased NDP-α-MSH binding affinity and potency with hMC5R (Yang et al., 2011). Upon stimulation by active ligands, MC5R couples to the stimulatory heterotrimeric G protein (Gs) and activates adenylyl cyclase to enhance the intracellular accumulation of cAMP (Kobayashi et al., 2016). Thus, the generation of cAMP is recognized as the important indicator to evaluate the receptors' response to stimulators. Although multiple functions and important value of MC5R in human beings have been fully demonstrated, however, the potential molecular cloning, pharmacological characteristics and key amino acids of MC5Rs in poultry and pig were still not fully understood. Accordingly, poultry and pig are critical economic animals worldwide, which provides cost-effective proteins and energy for human daily life. Most notably, MC5R mediated synthesis of fatty acid is critical for improving the production and benefit of economic animals. Therefore, a better understanding of molecular, pharmacological characteristics and related signal pathways during the interaction between receptors and ligands would provide preliminary data for MC5R-targeted drug screening or genetic breeding of poultry and pig in the future.

In this study, we first cloned *MC5R* genes from chicken (*cMC5R*), duck (*dMC5R*), goose (*gMC5R*) and pig (*pMC5R*), and constructed eukaryotic expression vectors. Besides, three mutants in each of four species were constructed by single point mutation technology, including c/d/gMC5R-D119A/F254A/H257A and pMC5R-D204A/F339A/H342A. Then, we determined the intracellular cAMP levels of wide type and mutants in human embryonic kidney T cells (HEK293T) by dual-luciferase reporter genes assay, and the phosphorylation level of extracellular regulated protein kinases 1/2 (ERK1/2) induced by ligands through western blot analysis. Consequently, we found that the all alanine-substitution mutants showed decreased cAMP generation under the stimulation of ligands in spite of enhanced basal activity for c/d/gF254A and pH342A, indicating the important roles of these positions in location and activation of cAMP signaling. Meanwhile, under the activation of agonist NDP-α-MSH or antagonist AgRP, only partial receptors and mutants showed similar pERK1/2 expression compared with hMC5R. These results demonstrated MC5Rs from different species and their mutants displayed various actions under the interaction with ligands, suggesting the presence of interspecies differences for MC5Rs.

2. Materials and methods

2.1. Materials and reagents

Fast mutagenesis system kit was purchased from Transgen Biotech (Beijing, China). ClonExpress®II one step cloning kit and ExFect 2000 transfection reagent was obtained from Vazyme Biotech (Nanjing, China). DMEM/F-12 1:1 medium was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Newborn calf serum was purchased from Biological Industries (Kibbutz Beit Haemek, Israel). Reporter gene plasmids pGL4.29-[luc2P/CRE/Hygro] and pGMLR-TK were purchased from Promega (Madison, USA).

2.2. Molecular cloning of c/d/g/pMC5R

Total DNAs from chicken, duck and goose blood were isolated using the TIANamp Blood DNA Kit (TianGen Biotech, Beijing, China) following the manufacturer instructions. Total RNA from pig liver was isolated using the RNAsimple Total RNA Kit (TianGen Biotech). The coding sequence (CDS) of *c/d/g/pMC5R* were amplified by PCR or reverse transcription PCR (RT-PCR) using the specific primers (Table S1) that designed in accordance with the predicted sequence in GeneBank. The PCR amplification products of MC5Rs were digested with *Hind* III and *Xho* I restriction enzymes, then were cloned in the pcDNA3.1(+) vector through using the ClonExpress®II one step cloning kit (Vazyme Biotech). The pcDNA3.1(+)-c/d/g/pMC5R recombinant plasmids were subsequently sequenced to ensure that no errors were induced by PCR reactions.

2.3. Phylogenetic analysis and homology models

The cloned *c/d/g/pMC5Rs* as described above and other related genes from NCBI database, including from human (accession number: NM_005913), monkey (NM_001193897), sheep (NM_001078656), chimpanzee (NM_001009119), cattle (NM_001015542), norway rat (NM_013182) and mouse (L27081). Multiple alignment of selected sequences was conducted by ClustalX 2.1. Then, a maximum likelihood tree was produced by MEGA 6.0. The reliability of the resulting tress was evaluated by bootstrap method with 1000 replications. Lastly, phylogenetic tress was visualized by iTOL. For homology models of MC5Rs, SWISS-MODEL was used to perform protein 3D structure prediction. The structural figures were polished and generated by PyMOL.

2.4. Site-directed mutagenesis

Each three mutations of four MC5Rs (12 mutant plasmids in total) were introduced into the MC5R with the Fast Mutagenesis System Kit (Transgen Biotech). Subsequently, the nucleotide sequences of the mutated MC5Rs were sequenced to ensure no errors during amplification. The isolation of pure plasmids was performed using the EndoFree Maxi Plasmid Kit V2 (TianGen Biotech) and these DNAs were used on next transfection experiments.

2.5. Cell culture and transfection

HEK293T cells were cultured in DMEM/F-12 medium supplemented with 10% newborn calf serum in 5% CO_2 atmosphere at 37 °C. Cells were harvested until 60–80% confluency reached. For transient expression of receptors, MC5Rs alone or with reporter gene plasmids, about 12 μg/plate in total (MC5R: pGL4.29: pGMLR-TK = 1: 10: 2), were transiently transfected into HEK293T cells with by using ExFect®2000 transfection reagent (Vazyme Biotech) as recommended by manufacturer instructions. Then, the transfection mixtures were replaced with serum-free culture medium for 24 h.

2.6. cAMP generation by dual-reported genes method

The transiently transfected cells that expressing MC5Rs or mutants with reporter gene plasmids were treated with different concentrations ($10^{-11} \sim 10^{-6}$ M) of α-MSH, NDP-α-MSH, SHU9119 or AgRP (diluted with OG-BSA) at 37 °C for 6 h. The supernatant was removed and cells were immediately washed with DPBS and lysed by cell lysis buffer (in kit). Intracellular cAMP generations were quantified following the manufacturer instructions using the dual luciferase reporter gene assay kit (Beyotime Biotech). Supernatant of each sample were detected for 1 s by GloMax-Multi (Promega).

2.7. Western-blotting analysis of pERK1/2

HEK293T cells were treated for 5 min in serum free medium with 50 nM NDP-α-MSH or 50 nM AgRP or ligand diluent OG-BSA as control. The medium was removed and cells were immediately washed with ice-cold DPBS and lysed with cell lysis buffer adding PMSF and protein phosphatase inhibitors (Solarbio Life Science). Cellular lysates were collected by centrifugation at 12,000 rpm at 4 °C for 8 min, and protein concentrations were quantified by a BCA kit (Beyotime Biotech). Protein (100 μg) were separated by a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (PVDF, Millipore) using a Mini trans-blot cell and system (Bio-Rad). The membranes were blocked with 5% non-fat dried milk in TBST (tris buffer saline with 0.1% Tween-20) for 2 h, then incubated with phospho-p44/42 MAPK (ERK1/2) rabbit mAb and α-tubulin rabbit mAb (1:1000 dilution, cell signaling technology) at 4 °C for overnight. After thorough washing with TBST, the anti-pERK1/2 and α-tubulin primary antibodies were incubated with anti-rabbit IgG HRP-linked antibody (1:5000 dilution) for 1.5 h at room temperature. The bound antibodies were detected by FluorChemM (ProteinSimple) using ECL reagent (New Cell & Molecular Biotech).

2.8. Statistical analysis

Digital images of Western-blots were analyzed by densitometry using Image J and the dose–response analysis was performed using GraphPad Prism 6 software. All data were presented as means ± SD. Unpaired *t*-test between two groups or one-way ANOVA among multiple groups were used to calculate *P*-values ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

3. Results and discussions

3.1. Phylogenetic and protein structures analysis

Based on the putative MC5Rs genes from NCBI database (cMC5R, KF670718; dMC5R, XM_005018192; gMC5R, XM_013179517; pMC5R, XM_021097606.1), we designed favorable forward and reverse primers (Table S1) to amplify the full-length sequences from DNA extracts of poultry blood and pig liver. As shown in Fig. 1A, targeted genes including *c/d/gMC5Rs* (978 bp) and *pMC5R* (1233 bp) were successfully amplified. Additional first-generation sequencing results showed that *c/d/gMC5Rs* have 100% identity with the putative sequences, whereas *pMC5R* displays an excess sequence of 108–164 bp at *N*-terminal. Consistently, the DNA sequence of cloned *cMC5R* shows 100% identity with a previous report on melanocortin receptor genes in the chicken (Takeuchi and Takahashi, 1998). To investigate the genetic characterization of *MC5R genes*, we conducted phylogenetic analysis from 11 species. We found that *MC5R genes* of chicken, duck and goose were clustered in one subgroup, however, the *pMC5R* showed higher homology with livestock such as sheep and cattle (Fig. 1B). Subsequently, we compared their homology at amino acids level. Comparison analysis showed that the human MC5R shared 77.8%, 78.2%, 78.2% and 63.2% identity with chicken, duck, goose and pig, suggesting the significant difference between human and other animals (Fig. 1C). Consistent with the genetic alignment analysis, the c/d/gMC5Rs have high identity more than 95% with others, but only have 56.1–57.1% identity with pMC5Rs. Of note, the dMC5R and gMC5R displayed 100% identity. To fully compare the difference between h/c/d/g/pMC5Rs, we predicted their protein 3D structures by homology model analysis. Our results showed that hMC5R possesses seven transmembrane domains, which is consistent with previous study (Yang et al., 2013b). Notably, c/d/g/pMC5Rs also have seven transmembrane domains that seem to similar with hMC5R. Nevertheless, we still observed several differences between five MC5Rs, particularly for non-transmembrane domains. As marked parts by green circle, h/pMC5Rs have one more helix during the coil region compared with c/d/gMC5Rs. In addition, compared with hMC5R, other four MC5Rs also have one more helix at *C*-terminal (marked by purple circle). Collectively, these results demonstrated the genetic and protein difference between all five MC5Rs, suggesting that these MC5Rs from poultry and pig may possess different functions both *in vitro* and *in vivo*.

3.2. Construction of mutants and induced basal activity change in HEK293T cells

Previous study has demonstrated that the substitution of charged amino acid residue Glutamic acid (D) 119 in TMD3, aromatic amino acids phenylalanine (F) 254 in TMD6 and histidine (H) 257 in TMD7 with alanine significantly decreased NDP-α-MSH binding and receptor activity, implying that these amino acid positions are critical for ligands-hMC5R interaction (Yang et al., 2013a). To investigate whether these specific amino acids residues in conserved region have a similar effect on the function of cloned c/d/g/pMC5Rs, we replaced three amino acid residues by alanine, the simplest chiral amino acids, for each MC5Rs by reasonable homologous recombination (Table S1), respectively. Further sequencing results showed that we successfully obtained 12 alanine-substitution mutants for four MC5Rs, including c/d/gMC5R-D119A/F254A/H257A and pMC5R-D204A/F339A/H342A (Fig. 1C and S1). In 1989, *Costa* et al. found that δ-opioid receptors still exhibit some activity in the absence of ligand stimulation, which was then termed receptor intrinsic or basal activity (Costa and Herz, 1989). In addition, basal activity of receptors plays an important regulatory role in tuning signal conversion process (Orozco-Cabal et al., 2006). Given this phenomenon, we first determined the basal activity of c/d/g/pMC5Rs and their mutants in HEK293T cells through measuring the cAMP levels. Notably, a dual-luciferase reporter genes assay was applied for this measurement, which has lower limit of detection and higher sensitive (10 to 100-fold) than other methods such as enzyme-linked immune-sorbent assay (ELISA) to directly detect cAMP. Interestingly, we observed that the F254A mutants in c/d/gMC5Rs displayed significant increased basal activity compared with the wide type, especially for cMC5R with about 15-fold change (Fig. 2). No significant changes were found in the D119A/H257A mutants of c/d/gMC5Rs. For pMC5R, only the H342 mutant showed enhanced basal activity. These results demonstrated that MC5Rs can be successfully expressed and have certain activities without the stimulation of ligands after being transfected into HEK293T cells. Meanwhile, the mutations at certain amino acid positions would affect its basal activity such as F254A in c/d/gMC5Rs and H342A in pMC5R.

3.3. Signaling properties of four MC5Rs and their mutants with ligands

Having shown that c/d/g/pMC5Rs and their specific mutants demonstrated different-levels basal activity in the absence of ligands, we next sought to investigate whether the cloned four MC5Rs and their mutants could respond to stimulation of ligands. To that end, we determined the generation of intracellular cAMP in the presence of increasing ligands. As shown in Fig. 3 and Table 1, the mutants c/d/gMC5R-D119A and pMC5R-D204A completely lost reactivity against all three agonists (α-MSH/NDP-α-MSH/SHU9119) and one antagonist (AgRP), suggesting the acidic amino acid Glu (D) at position of 119 in c/d/gMC5R and 204 in pMC5R plays an irreplaceable role in the intracellular cellular signaling. Besides, under the stimulation of agonist α-MSH, the reactivity of c/d/gMC5R-F254A/ H257A and pMC5R-F339A/H342A significantly decreased. The activity of their mutants could be improved by NDP-α-MSH with EC_{50} from 10^{-10} to 10^{-11} mol/L, a more potent synthetic agonist than natural agonist (α-MSH) for hMC1R/MC3R/MC4R/MC5R (Bednarek et al., 1999; Handl et al., 2007). There results demonstrated that NDP-α-MSH has selective potent agonism not only for most subtypes of MCRs (except MC2R) (Wang and Tao, 2011, 2013; Yang and Harmon, 2017), but also for MC5Rs

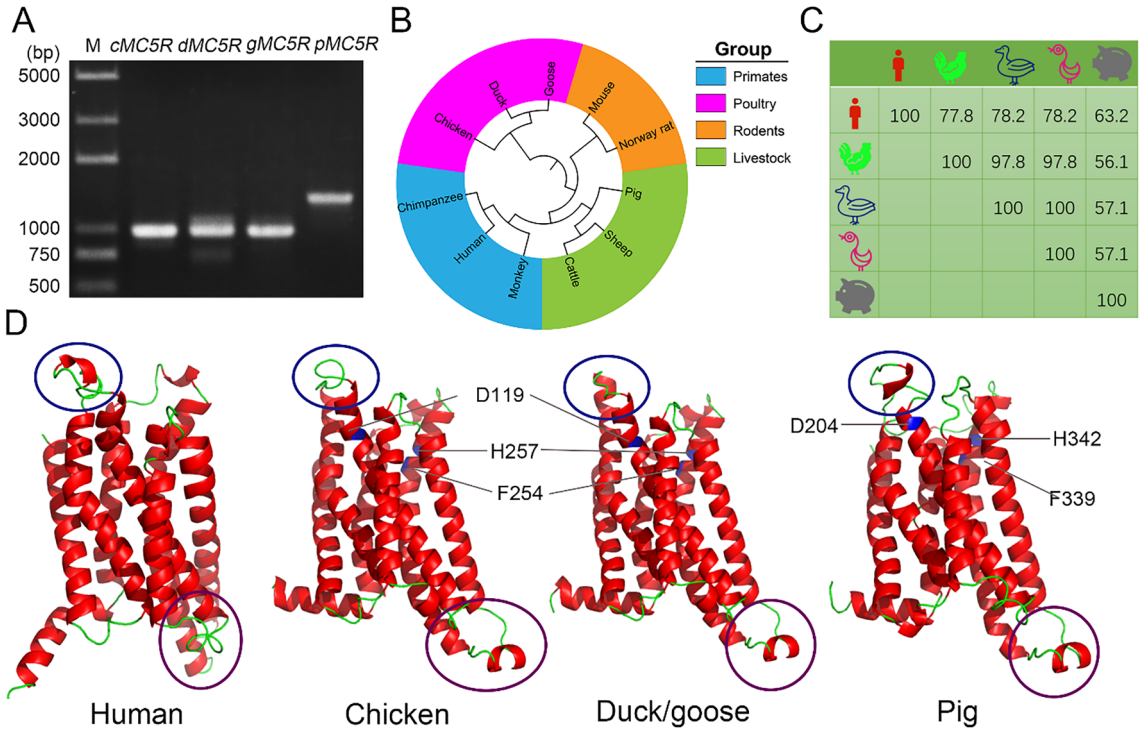

Fig. 1. Genetic and protein characterization of MC5Rs from different sources. (A) PCR amplification product of MC5Rs gene from chicken (*cMC5R*), duck (*dMC5R*), goose (*gMC5R*) and pig (*pMC5R*); (B) Phylogenetic analysis of MC5R DNA sequences from different species; (C) Identity comparison of MC5Rs amino acid sequences from human, chicken, duck, goose and pig; (D) Homology models of four MC5R proteins. Protein backbones were shown as a cartoon with the helices (red) and strands (green). The significant difference of second structures were labeled with blue or purple circles. Meanwhile, the key amino acids for subsequent site-directed mutations were marked.

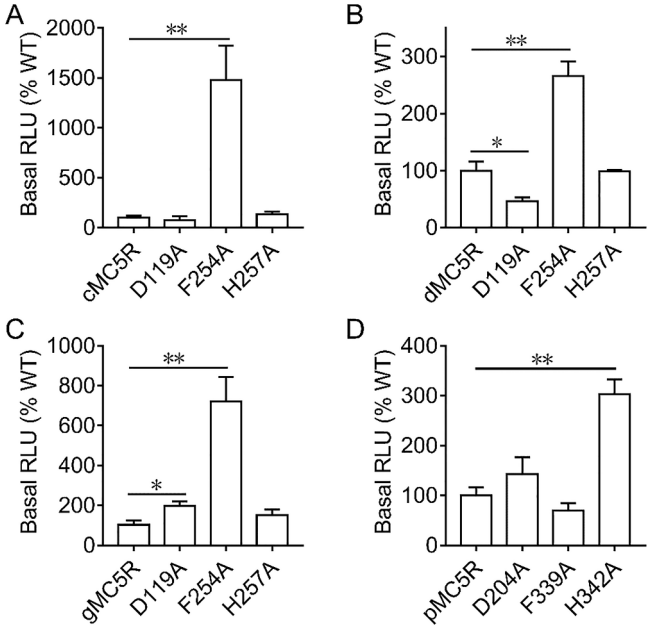

Fig. 2. Basal cAMP levels of c/d/g/pMC5Rs and alanine-substitution mutants. Basal cAMP levels of cMC5R (A), dMC5R (B), gMC5R (C) and pMC5R (D) were measured by dual-luciferase reporter genes method. All experiments were performed three independent experiments, and the mean ± SD is shown. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, determined by non-parametric one-way ANOVA.

from different species (poultry and livestock). Besides these two ligands, it has been suggested that melanocortin receptors from chicken such as cMC1R, cMC3R, cMC4R, cMC5R had higher affinity with ATCH than α-MSH (1–24). Because avian pituitary lacks an intermediate lobe and thus ACTH is recognized as the primary melanocortin ligand released by the avian pituitary (Chrétien, 2013; Takahashi and Mizusawa, 2013). To better understand the pharmacology of cMC5R, we compared its response to the stimulation of ACTH(1–24) with NDP-α-MSH. We found that cMC5R showed comparable affinity for these two ligands with an EC_{50} of appropriate 10^{-11} mol/L (Thomas et al., 2018). Consistently, a similar result was observed for red stingray (*Dasyatis akajei*) melanocortin receptors (Dores et al., 2018). In addition, a recent study demonstrated that co-expression of cMC5R with the accessory proteins cMRAP1 increased sensitivity for ACTH(1–24) by approximately 365-fold. However, co-expressing of cMRAP2 with cMC5R had no effect on its sensitivity to hACTH(1–24) (Thomas et al., 2018). It is conceivable that the cMRAP1 supplementation may further enhance the cMC5R reactivity with ligands.

SHU9119, a synthetic MSH analogue, has been proved as an antagonist of hMC3R and hMC4R (Grieco et al., 2007; Yang et al., 2002). However, we found that SHU9119 had a stronger agonist effect on MC5R than α-MSH in four animals. Besides, we found that the signal window of c/d/gMC5R-H257A to SHU9119 significantly increased, but pMC5R-H342A showed a decrease in signal window and an increased EC_{50} with SHU9119 than poultry, indicating that SHU9119 had a higher affinity for poultry MC5R than pig (Yang et al., 2013a). Consistent with our results, SHU9119 has been reported to enhance the fatty acid oxidation mediated by NDP-α-MSH in primary muscle cells (Juan Ji et al., 2007). *In vitro* studies have shown that AgRP is an effective selective antagonist of hMC3R and hMC5R (Yang et al., 1999), whereas we could not detect the response activity of four animal MC5R wild types to AgRP, and only the reactivity of c/d/gMC5R-F254A and pMC5R-F339A mutants could be detected. The significant enhanced basal activity of c/d/gMC5R-F254A may contribute to the determination of the antagonistic activity. However, pMC5R-F339A has a comparable activity with wide type and displayed decreased cAMP

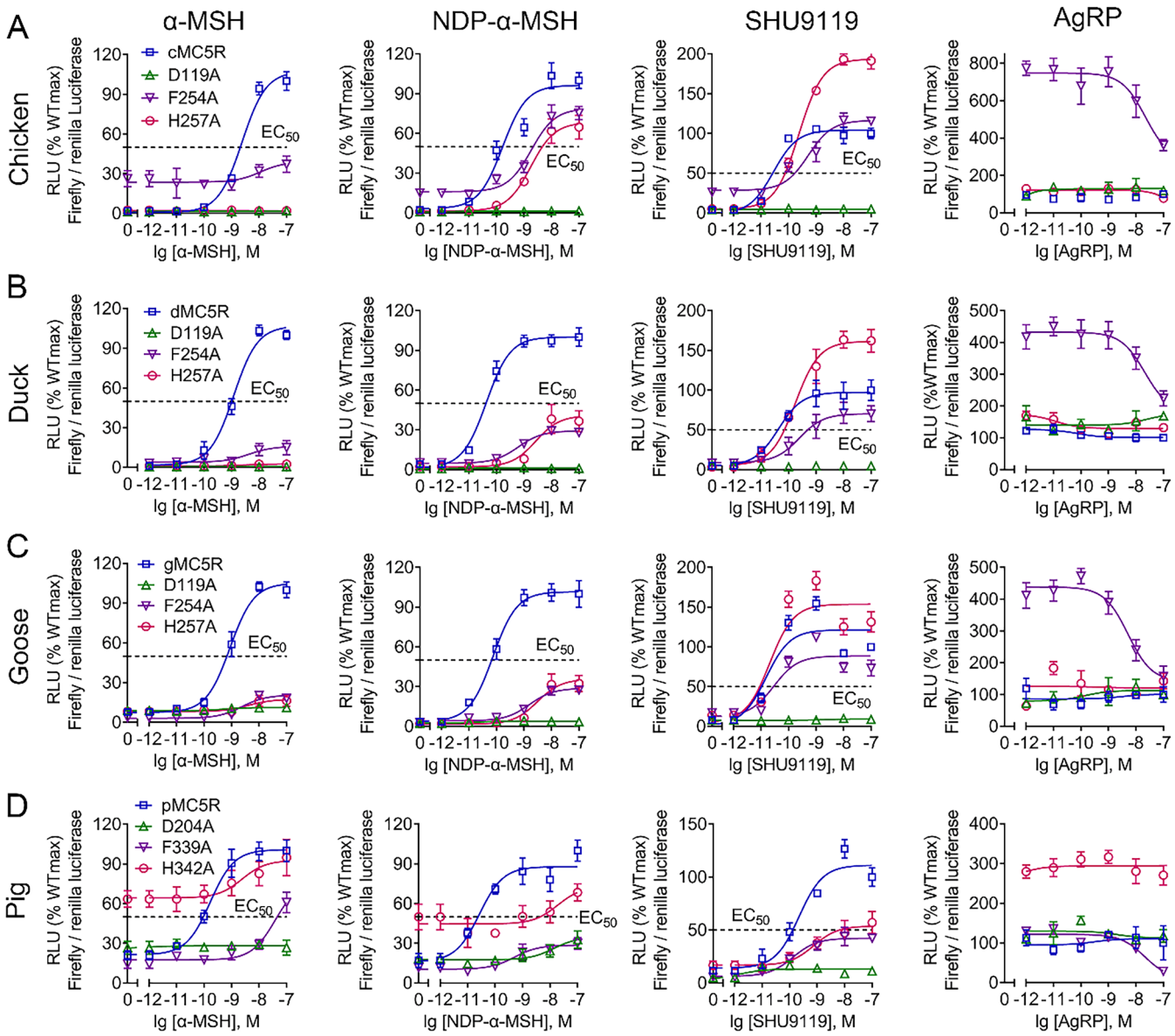

Fig. 3. Intracellular cAMP levels of c/d/g/pMC5Rs and alanine-substitution mutants stimulated by different ligands. Relative fluorescence intensities (RLU) of firefly/renilla luciferase of cMC5R (A), dMC5R (B), gMC5R (C) and pMC5R (D) under the stimulation of increasing concentration of four ligands were determined, and the percent were normalized with MC5Rs-WTmax at 100%. 50% effective concentrations (EC_{50}) for each combination were labeled.

generation. Therefore, we speculated that this is mainly because these mutations alter the spatial structure of proteins and corresponding response to AgRP. However, at the current stage, the specific reasons warrant more evidence.

In previous studies, to assess putative antagonist of GPCR by ELISA or radioisotope labeling, increased basal cAMP level is required by using the agonist of adenylate cyclase (AC) such as forskolin due to relatively low AC activity and cAMP level (Seamon et al., 1981). However, in turn, the exogenous addition of forskolin would result in increased uncertainties and errors (Gilissen et al., 2015; Hill et al., 2010). The application of dual-luciferase reporter method in our study could effectively overcome these obstacles. Interestingly, we found that although the basal cAMP level of pMC5R-F339A was unchanged compared with the significantly increased pMC5R-H342A, a dose-dependent decrease signal caused by AgRP in pMC5R-F339A was also detected. These results demonstrated that high basal activity of receptors is not sufficient for the identification of antagonists.

3.4. pERK1/2 levels of four MC5Rs and their mutants with two ligands

To further evaluate the signaling properties of c/d/g/pMC5R and their mutants, we measured the efficacy of two ligands in mitogen-activated protein kinases (MAPK) signaling (Johnson and Lapadat, 2002; Robinson and Cobb, 1997). During four types of MAPK cascade pathways, the extracellular signal-regulated kinases 1 and 2 (ERK1/2) are the most widely reported, which play an important role in normal physiological activities of human and animals (Kehat et al., 2011; Kehat and Molkentin, 2010). Previous studies showed that the hMC5R would activate the PKA signaling pathway and increase the phosphorylation level of ERK1/2 in HEK293 cells with the agonists (Rodrigues et al., 2009). Despite of these efforts, the phosphorylation levels of ERK1/2 in c/d/g/pMC5R and their mutants in the absence or presence of ligands are still unknown.

After preliminary experiments, we chose 5 min as the stimulation duration with 50 nmol/L NDP-MSH or AgRP for our next test due to a better performance. We first compared the basal phosphorylation level of ERK1/2 between wide type and mutants in the absence of ligand

Table 1
Ligands potencies at the MC5Rs and mutants.

MC5Rs		α-MSH		NDP-α-MSH		SHU9119		AgRP	
		EC_{50} (nM)	Rmax (% WT)	EC_{50} (nM)	Rmax (% WT)	EC_{50} (nM)	Rmax (% WT)	EC_{50} (nM)	Rmax (% WT)
cMC5R	WT	2.1 ± 0.83	100	0.16 ± 0.02	100	0.03 ± 0.0032	100	ND	100
	D119A	ND	1.06 ± 0.92**	ND	2.73 ± 2.08**	ND	4.08 ± 0.71**	ND	125.5 ± 12.6*
	F254A	8.19 ± 4.62	41 ± 8.41**	1.89 ± 0.07**	95.28 ± 21.98	0.41 ± 0.13**	91.03 ± 37.45	20.33 ± 6.11	796.67 ± 86.34**
	H257A	ND	2.52 ± 1.14**	1.76 ± 0.24**	63.65 ± 9.38*	0.18 ± 0.1	161.09 ± 35.02*	ND	134.36 ± 6.89*
dMC5R	WT	1.04 ± 0.31	100 ± 0	0.05 ± 0.02	100	0.07 ± 0.04	100	ND	100
	D119A	ND	2.43 ± 3.03**	ND	1.74 ± 1.04**	ND	1.89 ± 2.06**	ND	142.2 ± 28.01*
	F254A	5.2 ± 0.89**	18.04 ± 5.92**	0.93 ± 0.54*	34.87 ± 9.11**	0.16 ± 0.11	52.81 ± 31.42	26.67 ± 5.23	406.55 ± 19.78**
	H257A	ND	3.42 ± 1.46**	2.68 ± 0.17**	41.58 ± 0.89**	0.12 ± 0.07	145.34 ± 31.19*	ND	134.74 ± 6.47*
gMC5R	WT	1.22 ± 0.36	100	0.04 ± 0.03	100	0.02 ± 0.0023	100	ND	100
	D119A	ND	4.24 ± 5.57**	ND	1.58 ± 1.97**	ND	5.71 ± 1.68**	ND	103.04 ± 11.25
	F254A	3.03 ± 1.05*	34.28 ± 38.4**	1.23 ± 0.32**	43.68 ± 13.3**	0.14 ± 0.1	66.97 ± 5.53	6.13 ± 1.19	405.89 ± 41.36**
	H257A	ND	7 ± 8.3**	2.56 ± 0.46**	56.12 ± 18.19*	0.06 ± 0.03	125.98 ± 5.06	ND	114.27 ± 30.51
pMC5R	WT	0.19 ± 0.04	100	0.04 ± 0.02	100	0.2 ± 0.03	100	ND	100
	D204A	ND	26.79 ± 4.3**	ND	47.35 ± 11.56*	ND	12.1 ± 0.15**	ND	139.95 ± 32.04
	F339A	30.58 ± 11.86**	69.85 ± 10.77**	0.5 ± 0.13**	32.92 ± 0.45**	0.12 ± 0.01*	52.45 ± 12.48**	16.29 ± 7.68	146.64 ± 33.06*
	H342A	4.52 ± 3.43	90.06 ± 3.83	21.65 ± 8.02*	82.82 ± 0.86	0.25 ± 0.43	61.19 ± 11.13**	ND	310.4 ± 45.97**

EC_{50}: 50% effective concentration; Rmax: maximal response; ND: not detected.
*P < 0.05, ** P < 0.01, *** P < 0.001, determined by Student's *t* test.

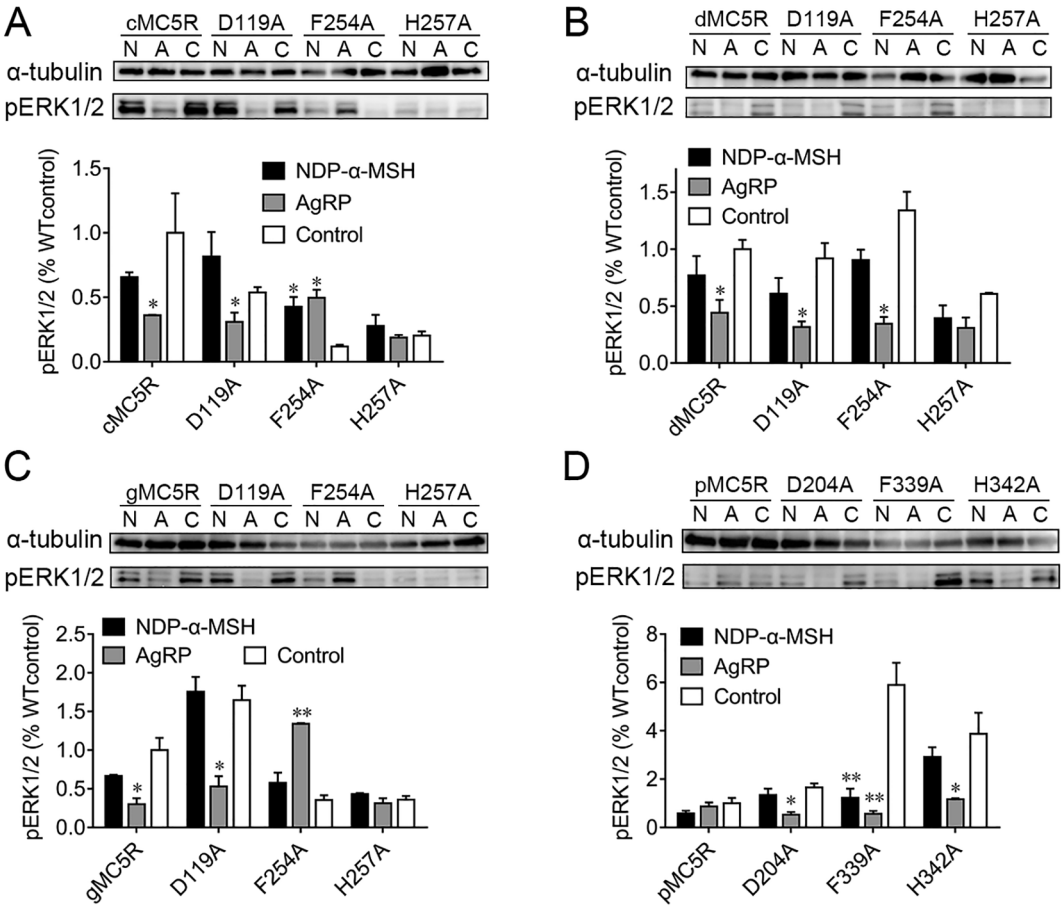

Fig. 4. ERK1/2 signaling of the cloned c/d/g/pMC3R and mutants in HEK293T cells. Western blots (upper panel) and grey value analysis of protein expression (lower panel) of cMC5R (A), dMC5R (B), gMC5R (C), pMC5R (D) and their mutants under the stimulation of NDP-α-MSH (N, 50 nm) or AgRP (A, 50 nm) or solvent (OG-BSA) as control. Grey values were quantified by Image J. All experiments were performed three times, and the mean ± SD is shown. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, determined by non-parametric one-way ANOVA.

stimulation by western blot and grey values analysis. The results showed that the pERK basal levels of c/gMC5R-F254A/H257A decreased, but the pERK basal levels of pMC5R-F339A/H342A were significantly increased (Fig. S2). We found that the pERK basal levels and cAMP levels of c/d/g/pMC5R mutants are not consistent, which were in agreement with previous observation that cAMP production and ERK1/2 activation are not necessarily related (Yang and Tao, 2016).

We next tested the level of pERK1/2 under the stimulations of

ligands. Under the activation of agonist NDP-α-MSH, the pERK1/2 level of cMC5R-F254A increased, and the pERK1/2 level of pMC5R-F339A decreased, while the pERK1/2 levels of other receptors and mutants showed no significant change. Meanwhile, under the stimulation of the antagonist AgRP, there were no significant changes in the pERK1/2 levels of c/d/gMC5R-H257A and pMC5R, but cMC5R-F254A showed increased pERK1/2 level, and the other receptors and mutants showed decreased pERK1/2 level (Fig. 4). Interestingly, the F254A mutagenesis from cMC5R and dMC5R showed opposite pERK1/2 response to AgRP despite of their consistent amino acid sequences. We speculated that this mutation may affect the location of receptors in cell surface and thereby result in this difference. However, the specific causes are still not clear at current stage and more investigations is warranted. Taken together, these MC5Rs and mutants display significant species-specific phenotypes, which was inconsistent with hMC5R, suggesting that presence of interspecies differences would affect the cellular signaling of MC5Rs. Coupled with the cAMP analysis, we found that D119A in c/d/gMC5Rs and D204A in pMC5R have no effect on the basal activity of cAMP and pERK1/2, but loss reactivity to all four ligands, indicating the key role of these two amino acid residues in cellular signaling. Importantly, only pMC5R-F339A showed decreased cAMP and pERK1/2 to AgRP stimulation, and thus can be employed for the screening of novel antagonists. In addition, the basal cAMP and pERK1/2 signals of pMC5R-H342A are increased, and the antagonistic activity induced by AgRP is weakened, suggesting its highly potential as a continuous activation mutant (CAM) for future genetic breeding.

4. Conclusion

In conclusion, we successfully cloned *c/d/g/pMC5Rs* and described their genetic and protein characters by comparing with hMC5R. To further investigate the functions of specific amino acids, 12 alanine-substitutions mutants were constructed. These cloned genes and their mutations were successfully expressed in HEK293T cells. Subsequent pharmacological experiments including cAMP and pERK1/2 analysis revealed that some specific mutations would affect the basal levels or response to ligands stimulation, which would contribute to the development of novel ligands and continuous activation mutant. Taken together, these finding demonstrated molecular and pharmacological characterization of poultry and pig melanocortin-5 receptors and their mutants, providing a new insight into further investigation on their physiological and pharmacological functions.

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Appendix A. Supplementary data

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