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The Asp298Asn polymorphism of melanocortin-4 receptor (MC4R) in pigs: evidence for its potential effects on MC4R constitutive activity and cell surface expression

J. Zhang†, J. Li†, C. Wu, Z. Hu, L. An, Y. Wan, C. Fang, X. Zhang, J. Li* and Y. Wang*

Key Laboratory of Bio-resources and Eco-environment of Ministry of Education, College of Life Sciences, Sichuan University, Chengdu 610065, China.

Summary

In humans and mice, melanocortin receptor 4 (MC4R) and melanocortin receptor accessory protein 2 (MRAP2) can form a complex and control energy balance, thus regulating body weight and obesity. In pigs, a missense variant (p.Asp298Asn) of MC4R has been suggested to be associated with growth and fatness; however, the effect of Asp298Asn substitution on MC4R function is controversial, limiting its application in animal breeding. Here we examined the effect of this polymorphism on MC4R constitutive activity, cell surface expression and signaling, and its interaction with MRAP2 in pigs. We found that: (i) both pig $MC4R^{Asp}$ and $MC4R^{Asn}$ can be activated by its ligands (α-MSH and ACTH) and stimulate cAMP/PKA signaling pathway, as detected by pGL3–CRE–luciferase reporter assay, indicating that, like $pMC4R^{Asp}$, $pMC4R^{Asn}$ is coupled to the cAMP/PKA signaling pathway; (ii) compared with $pMC4R^{Asp}$, $pMC4R^{Asn}$ loses the basal constitutive activity and shows a decreased surface expression, as detected by dual-luciferase reporter assay and Nano-HiBiT system; (iii) as in other vertebrates, both $pMC4R^{Asp}$ and $pMC4R^{Asn}$ can interact with pMRAP2, thus decreasing receptor surface expression and enhancing ligand sensitivity, although, in contrast to $pMC4R^{Asp}$, the basal constitutive activity of $pMC4R^{Asn}$ cannot be affected by pMRAP2; and (iv) RNA-seq data analysis revealed a co-expression of *MC4R* and *MRAP2* in pig hypothalamus. Taken together, our data provide convincing evidence that Asp298Asn substitution decreases the constitutive activity and cell surface expression of MC4R or MC4R–MRAP2 complex, which may affect energy balance and be a valuable selection marker for breeding programs in pigs.

Keywords constitutive activity, MC4R polymorphism, MRAP2, pig, surface expression

Introduction

Melanocortin-4 receptor (MC4R), through binding to its ligands [e.g. α-melanocyte-stimulating hormone (α-MSH) and agouti-related protein (AgRP)], has been reported to play a pivotal role in the regulation of food intake, energy balance and fatness in mammals (Krude *et al.* 1998; Jiang *et al.* 2002; Cone 2005). Mapped on chromosome 1 (SSC1) which encompassed the largest number of economically important QTL in pigs (Kim *et al.* 2000b; Hu *et al.* 2005), the *MC4R* locus has been implicated with many economic traits such as backfat, food intake and daily bodyweight gain. For example, in pigs, a missense substitution (c.1426G>A, p.Asp298Asn) has been identified in the coding region of the *MC4R* ($MC4R^{Asn}$). Pigs with the homozygous variant allele ($MC4R^{Asn}$) have higher backfat thickness, higher daily bodyweight gain and higher food intake than animals with the homozygous genotype $MC4R^{Asp}$ in many pig breeds (Kim *et al.* 2000a; Hernandez-Sanchez *et al.* 2003; Houston *et al.* 2004; Xiao *et al.* 2006; Van den Maagdenberg *et al.* 2007; Schwab *et al.* 2009; Davoli *et al.* 2012; Choi *et al.* 2016; Melnikova *et al.* 2018).

Although the polymorphic variant of MC4R ($MC4R^{Asn}$) may affect many economic traits of pigs, it remains largely unknown how this polymorphism (Asp298Asn) alters receptor function and how it contributes to phenotypic changes in pigs. Kim *et al.* demonstrated that pig $MC4R^{Asn}$

*Address for correspondence

J. Li and Y. Wang, College of Life Sciences, Sichuan University, Chengdu, 610065, China.
E-mails: cdwyjhk@gmail.com (YW); lijuanscuhk@163.com (JL)

†These authors contributed equally to this work.

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is inactive, but does not impair its binding to DNP-αMSH (an analog of its endogenous ligand, α-MSH) in HEK293 cells. In 2008, Fan *et al*. further showed that MC4R^{Asn} can bind DNP-α-MSH, α-MSH and AgRP (an endogenous inverse agonist or antagonist of MC4R) with high affinity and produce cAMP *in vitro*, but displays little/no change in the basal activity when compared with pig/human MC4R^{Asp} (Fan *et al*. 2008). Similarly, Patten et al. found the human MC4R^{Asn} can stimulate cAMP formation without affecting ligand binding in HEK293 cells (Patten *et al*. 2007). As the effect of Asp298Asn substitution on MC4R function is uncertain, it casts doubt on whether Asp298Asn polymorphism of MC4R can be used as a selection marker in porcine breeding programs.

Recently it was reported that MC4R can interact with melanocortin receptor accessory protein 2 (MRAP2) and form a complex to modulate MC4R function in the hypothalamus (Chan *et al*. 2009; Asai *et al*. 2013; Baron *et al*. 2019). MRAP2 is a single transmembrane protein, which can regulate the cell surface expression, ligand sensitivity and signaling of MC4R (Chan *et al*. 2009; Asai *et al*. 2013; Liu *et al*. 2013; Sebag *et al*. 2013). In humans, MRAP2 can interact with MC4R and regulate the cell surface expression and trafficking of MC4R (Chan *et al*. 2009). In mice, MRAP2 can enhance the responsiveness of MC4R to α-MSH (Asai *et al*. 2013), and MRAP2 deletion causes obesity in mice (Asai *et al*. 2013). A recent study showed that loss-of-function *MRAP2* variants were pathogenic for monogenic hyperphagic obesity, hyperglycemia and hypertension (Baron *et al*. 2019). In addition, *MRAP2*-deficient mice have targeted deletion in *Sim1*-positive hypothalamic neurons, which express MC4R, develop severe obesity and have several phenotypic similarities with the MC4R-null mice (Novoselova *et al*. 2016). Further studies have also demonstrated that MRAP2 is able to modify the pharmacological properties of MC4R, which is involved in the regulation of energy balance in several vertebrate species, such as chicken (Zhang *et al*. 2017), cats (Habara *et al*. 2018), zebrafish (Josep Agulleiro *et al*. 2013; Sebag *et al*. 2013) and sea lampreys (Zhu *et al*. 2019). All of these findings support the hypothesis that, within the hypothalamus, MC4R and MRAP2 can form a functional complex and play a pivotal role in governing energy balance such as food intake, growth, fatness and obesity in mice and humans, and in other vertebrates (e.g. chicken, zebrafish) as well.

Considering the pivotal roles of MC4R–MRAP2 complex in controlling energy balance in vertebrates (e.g. food intake, growth, fatness, energy expenditure and obesity of humans and mice), our present study aimed to address whether the amino acid substitution (Asp298Asn) can affect the constitutive activity, surface expression and signaling of MC4R, and its interaction with pMRAP2. The results from present study may provide valuable insights into the roles of MC4R^{Asp} and MC4R^{Asn} in the control of food intake, fatness, growth rate and body weight of pigs and other farm animals.

Materials and methods

Chemicals, primers, peptides and antibodies

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) and restriction enzymes were obtained from TaKaRa (Shiga, Japan). All primers used were synthesized by Beijing Genome Institute (BGI, China) and are listed in Table S1. Pig (p-)ACTH$_{1-39}$, α-MSH (acetyl-α-MSH) were synthesized by GL Biochem Ltd (Shanghai, China). The purity of synthesized peptides was more than 95% (analyzed by HPLC) and their structures were verified by mass spectrometry. Anti-Flag Affinity Gel beads were purchased from Biomake Company (Biomake, Shanghai, China). Rabbit anti-Myc-tag polyclonal antibody (385052) and rabbit anti-MRAP2 (Center) polyclonal antibody (612249) were purchased from Zen Bioscience company (Zen Bioscience, Chengdu, China).

Animals and tissues

Yorkshire pigs ($n = 4$, genotype A/A = *MC4R* Asn/Asn) were obtained from the Sichuan Animal Science Academy (Chengdu, China). Animals were allowed access to feed and water *ad libitum* under normal conditions. Pigs were electrocuted and their hypothalamus tissue collected, immediately frozen in liquid nitrogen and stored at −80°C until use. All animal experimental protocols used in this study were approved by the Animal Ethics Committee of College of Life Sciences, Sichuan University (Chengdu, China).

Cloning the cDNA of MRAP2 and MC4R and constructing their expression plasmids

Total RNA was extracted from the hypothalamus with RNAzol Reagent (Molecular Research Center, Cincinnati, USA). Then cDNA was prepared from 1 μg of total RNA by reverse transcription (RT) using ReverTra Ace® (Toyobo, Osaka, Japan). The RT samples were then used in PCR amplification of target genes. The coding regions of pig *MRAP2* (pMRAP2), *RAMP3* and *MC4R* (pMC4R^{Asn}) were amplified using high-fidelity KOD DNA polymerase (Toyobo, Osaka, Japan) and specific primers under the following cycling conditions: 35 cycles of 94°C for 30 s, 64°C for 30 s and 68°C for 90 s. The PCR products were cloned into pcDNA3.1 (+) expression vector (Invitrogen, Carlsbad, CA, USA) and sequenced by Beijing Genome Institute (BGI, Beijing, China).

Based on the cDNA sequence of *MC4R* deposited in the GenBank (access no. NM_005912), a gene-specific primer pair (Table S1) was designed to amplify the complete coding regions of human *MC4R* (hMC4R^{Asp}) from HEK293 genomic DNA, which was extracted by a genome DNA

extraction kit (Tiangen Biotech, Beijing, China) following the manufacturer's instructions. Then, the PCR products were cloned into pcDNA3.1 (+) vector and sequenced. Expression plasmid of chicken *MRAP2* (*cMRAP2*) was constructed in our previous study (Zhang *et al.* 2017). In this experiment, the expression plasmids encoding pMC4R^{Asp} and hMC4R^{Asn} were also constructed using a Mut Express II Fast Mutagenesis Kit V2 (Vazyme, Nanjing, China) with specific primers (Table S1) following the manufacturer's instructions.

For HiBiT analysis, the four expression plasmids encoding pMC4R^{Asp}, pMC4R^{Asn}, hMC4R^{Asp} and hMC4R^{Asn} with HiBiT (VSGWRLFKKIS) tagged at their N-termini were also constructed in this study. All these constructs were verified by sequencing (BGI, Beijing, China).

Functional analysis of pig MC4R

According to our established method (Zhang *et al.* 2017), the functionality of MC4R was examined in Chinese hamster ovary (CHO) cells by a system of co-transfecting a cAMP-sensitive luciferase reporter construct (pGL3–CRE–luciferase) and a receptor expression plasmid [or corresponding empty pcDNA3.1 (+) vector, and/or pig MRAP2 plasmid]. In brief, CHO cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 in air. Growth media consisted of Dulbecco's modified Eagle's medium (Corning, NY, USA), 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) and 1% antibiotic/antimycotic solution (Gibco, Carlsbad, CA, USA). CHO cells were first cultured on six-well plates and grown for 24 h before transfection. Then, transfection was performed with 900 ng of DNA per well (700 ng of pGL3–CRE–luciferase plasmids and 200 ng of variant receptor plasmids) and 2 μl jetPRIME (Polyplus Transfection, Illkirch, France) in 200 μl buffer. Variant receptor ± MRAP2 (or empty pcDNA3.1 vector) was transfected at a ratio of 1:5. Transfected cells were incubated in fresh growth media for an additional 24 h. CHO cells were trypsinized and subcultured in a 96-well plate for an additional 24 h before peptide treatment. After removal of the medium, the cells were treated with 100 μl peptide-containing medium (or peptide-free medium). After 6 h of treatment, CHO cells were harvested with 1× passive lysis buffer (Promega, Madison, WI, USA). The luciferase activity was measured with a Multimode Microplate Reader (TriStar LB 941, EG&G Berthold, Bad Wildbad, Germany) according to the manufacturer's instructions.

Co-immunoprecipitation assay

To investigate the interaction of pig MC4R^{Asp}/MC4R^{Asn} with MRAP2, we first prepared pcDNA3.1(+) expression plasmids encoding an N-terminally Myc-tagged MRAP2 (Myc-MRAP2) and N-terminally 3 × Flag-tagged receptors (Flag-MC4R) by PCR, as described previously (Zhang *et al.* 2017). Then these expression plasmids of pMRAP2 (3000 ng) and pMC4R (600 ng) were used to co-transfect CHO cells cultured in a 60 mm dish (Nunc, Rochester, NY, USA) at 37°C by jetPRIME transfection reagent (Polyplus transfection, France) according to the manufacturer's instructions. After 24 h of transfection, CHO cells were lysed for 2 h on ice in a lysis buffer (containing 0.1% *n*-dodecyl-*β*-maltoside and protease inhibitors). The cell lysates were centrifuged at 1000 g for 1 min at 4°C and the supernatants were used for Western blot or Co-immunoprecipitation (Co-IP) assay. For Co-IP, the cell lysates were added to anti-Flag Affinity Gel beads and incubated overnight at 4°C. After incubation, the beads were washed with lysis buffer, the supernatant removed and 2× Laemmli sample buffer added. After boiling for 10 min, samples were run in SDS–polyacrylamide gel and either rabbit anti-Myc-tag antibodies (1:2000) or rabbit anti-MRAP2 antibodies (1:2000) were used in western blot.

Detection of the basal constitutive activity of pMC4R^{Asp} and pMC4R^{Asn}

The basal constitutive activity of pMC4R^{Asp} and pMC4R^{Asn} expressed in CHO cells was detected by dual-luciferase reporter assays (Promega, Madison, WI, USA) established in our previous studies (Zhang *et al.* 2017). In brief, CHO cells were cultured in a 48-well plate at a density of 1×10^5 cells per well before transfection. After 24 h of incubation, a mixture containing 720 ng of pGL3–CRE–luciferase reporter construct (which is capable of monitoring receptor-stimulated cAMP production), 60 ng of pRL–TK construct (used to normalize transfection efficiency), 120 ng of pMC4R^{Asp}/pMC4R^{Asn} expression plasmid, various amounts (0–600 ng) of pMRAP2/cMRAP2 [or empty pcDNA3.1(+)] expression plasmid and 3 μl of JetPRIME reagent, was prepared in transfection buffer and used to transfect CHO cells following the manufacturer's instructions. At 24 h later, culture medium was removed and 100 μl 1× passive lysis buffer (Promega) added to each well. Luciferase activities of 20 μl cellular lysates were measured using a Dual-Luciferase Reporter Assay Kit (Promega). The cells transfected with empty pcDNA3.1(+) vector were used as controls. The luciferase activity of CHO cells expressing pMC4R^{Asp}/pMC4R^{Asn} was normalized to Renilla luciferase activity derived from the pRL-TK vector and then expressed as relative fold increase as compared with the control [CHO cells transfected with the empty pcDNA3.1(+) vector]. The basal constitutive activity of human MC4R and pMRAP2 was detected using the same method.

Detection of cell surface expression of pMC4R^{Asp} and pMC4R^{Asn} by Nano-Glo® HiBiT detection system

To quantify the cell surface expression of pMC4R^{Asp}/pMC4R^{Asn}, the Nano-Glo® HiBiT Extracellular and Lytic

Detection System purchased from Promega Corporation (Promega) were used in this study. The Nano-Glo® HiBiT Extracellular Detection System can quantify HiBiT-tagged MC4R expressed on the cell membrane, whereas the Lytic Detection System can determine the total HiBiT-tagged MC4R levels in cultured cells.

In brief, CHO cells cultured on a 96-well plate were transfected with HiBiT-tagged receptor (HiBiT-MC4R) expression plasmid (or co-transfected with HiBiT-MC4R and MRAP2 plasmids, or RAMP3 plasmids) and incubated for an additional 24 h. To quantify the cell surface expression of HiBiT-MC4R, 40 μl Nano-Glo® HiBiT Extracellular Reagent, in which LgBiT protein can bind to HiBiT-MC4R expressed on the cell surface and generates luminescence, was added, and the luminescence values were measured by a Multimode Microplate Reader (TriStar LB 941, EG&G Berthold, Germany). To quantify the total expression levels of HiBiT-MC4R in cells, Nano-Glo® HiBiT Lytic Reagent was added to lyse cells, and the luminescence values were also measured according to the manufacturer's instruction. Finally, the relative cell surface expression level of MC4R (HiBiT-MC4R signal on cell membrane) was first normalized by the total HiBiT-MC4R signals in cells and then expressed as the percentage to the control group.

Tissue expression analysis of pig *POMC*, *MC4R* and *MRAP2* using RNA-Seq data deposited in public SRA database

To examine the tissue distribution of *POMC*, *MC4R* and *MRAP2* mRNA in pigs, RNA-Seq data for eight pig tissues (small intestine, liver, spleen, pituitary, thymus, hypothalamus, diaphragm, and longissimus muscle) were downloaded from the *SRA* database (accession no. PRJNA353772, Iowa State University, USDA-ARS). The quantification of reads was performed with Salmon version 0.8.2 (Patro *et al.* 2017) against the Ensembl Sscrofa11.1 database (www.ensembl.org/Sus_scrofa/). The transcripts per million values were used to estimate the abundance of *POMC*, *MC4R* and *MRAP2* mRNA transcript.

Data analysis

All statistical analysis was performed using GraphPad Prism 7 (Graph Pad Software Inc., San Diego, CA, USA). The dose–response curves were constructed using nonlinear regression models. Student's *t*-test was used to compare two groups; for more than two groups one-way ANOVA was performed followed by Dunnett's test. In all analyses, a two-tailed probability of <5% (i.e. $P < 0.05$) was considered statistically significant. All experiments were repeated at least twice to validate our results.

Results

Functional Analysis of pig MC4R^{Asp} and MC4R^{Asn}

To test whether, like pig MC4R^{Asp} (pMC4R^{Asp}), pig MC4R^{Asn} is functional and capable of transmitting a signal, we examined the functionality of pig MC4R^{Asn} expressed in CHO cells, using a pGL3–CRE–luciferase reporter system established in our laboratory (Zhang *et al.* 2017), which is capable of monitoring receptor-induced cAMP production. As shown in Fig. 1, both pMC4R^{Asp} and pMC4R^{Asn} could be potently activated by pig α-MSH/ACTH (Table 1) and stimulate luciferase activities of CHO cells in a dose-dependent manner. This finding indicates that, like pMC4R^{Asp}, pig MC4R^{Asn} is still functional and can bind its ligand, as previously reported (Fan *et al.* 2008). However, we found that both ACTH and α-MSH can induce a 6-fold increase in luciferase activity of CHO cells expressing pMC4R^{Asn}, whereas they can induce 3-fold increase in CHO cells expressing pMC4R^{Asp} under the same conditions. Moreover, pig α-MSH is about 7.2- and 2.7-fold more potent than pACTH in activating pMC4R^{Asp} and pMC4R^{Asn} respectively, estimated by their EC_{50} values (Table 1, Fig. 1).

To verify whether, like pMC4R^{Asp}, pMC4R^{Asn} is coupled to the intracellular AC/cAMP/PKA pathway, the pharmacological inhibitors of adenylate cyclase (AC)/protein kinase A inhibitor (PKA) were used to test whether they could inhibit MC4R^{Asn} signaling. As shown in Fig. 2, both MDL12330A (an AC inhibitor, 20 μM) and H89 (a PKA inhibitor, 10 μM) could inhibit α-MSH- and ACTH-induced luciferase activity of CHO cells expressing either pMC4R^{Asp} or pMC4R^{Asn}. This finding clearly indicates the functional coupling of pMC4R^{Asn} to the AC/cAMP/PKA signaling pathway.

Constitutive activity and cell surface expression of pig MC4R

It is clear that MC4R displays a strong basal constitutive activity in mice, birds and teleosts; therefore we further tested whether the polymorphism (Asp298Asn) could affect the basal constitutive activity of MC4R using a dual-luciferase reporter assay established in our laboratory (Zhang *et al.* 2017). As shown in Fig. 3a, we observed that pig MC4R^{Asp} expressed in CHO cells display a strong constitutive activity. Strikingly, pMC4R^{Asn} displays no constitutive activity under the same condition.

Using the Nano-Glo® HiBiT extracellular and lytic detection system, we further examined the relative cell surface expression level of pMC4R^{Asp} and pMC4R^{Asn} in CHO cells. As shown in fig. 3b, both pMC4R^{Asp} and pMC4R^{Asn} can be expressed on the cell surface. However, MC4R^{Asn} has a decreased cell surface expression when compared with pMC4R^{Asp}.

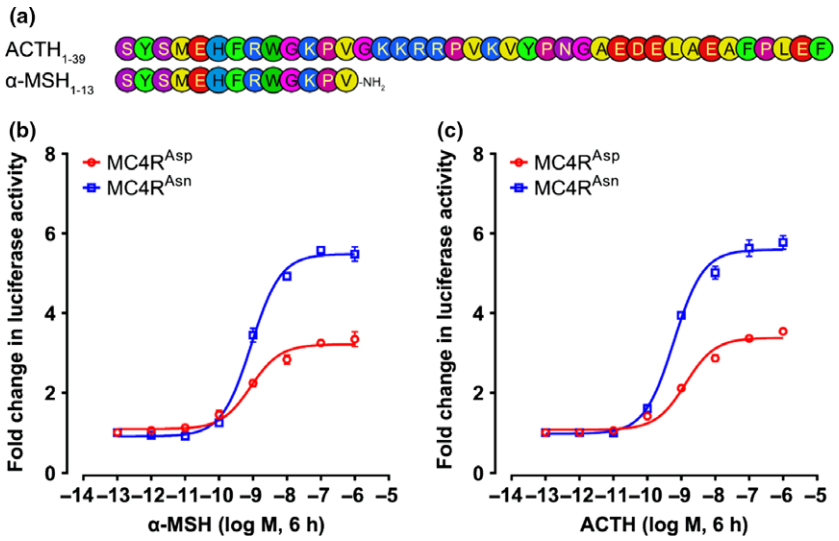

Figure 1 Effects of ACTH and α-MSH in activating pig MC4R^{Asp} and MC4R^{Asn}. (a) Amino acid sequences of pig ACTH and α-MSH (acetyl-α-MSH with an amidated C-terminus) used in this study. (b) Effect of α-MSH in activating pMC4R^{Asp} and pMC4R^{Asn} expressed in Chinese hamster ovary (CHO) cells, as monitored by the pGL3–CRE–luciferase reporter system. (c) Effect of ACTH in activating pig MC4R^{Asp} and MC4R^{Asn} expressed in CHO cells, as monitored by the pGL3–CRE–luciferase reporter system. Each data point represents the mean ± SEM of three replicates ($N = 3$) [each dose–response curve shows one representative experiment (repeated four times)].

Table 1 EC_{50} values (nM) of α-MSH and ACTH in activating pig MC4R^{Asp} and MC4R^{Asn} expressed in Chinese hamster ovary (CHO) cells in the absence or presence of pMRAP2

	α-MSH		ACTH	
	Receptor	+pMRAP2	Receptor	+pMRAP2
pMC4R^{Asp}	10.06 ± 1.78	2.81 ± 0.39	72.86 ± 12.37	1.08 ± 0.18
pMC4R^{Asn}	6.32 ± 1.39	1.82 ± 0.43	16.91 ± 2.39	3.62 ± 0.63

pMC4R^{Asp}/MC4R^{Asn} can interact with pMRAP2 in vitro

It is reported that MC4R can interact with MRAP2 and form a functional unit to regulate food intake in the hypothalamus of humans, mice, chickens and zebrafish (Asai *et al.* 2013; Josep Agulleiro et al. 2013; Sebag *et al.* 2013; Zhang *et al.* 2017; Bruschetta *et al.* 2018). In this study, to reveal the interaction of pMC4R and pMRAP2, we first cloned *MRAP2* cDNA from pig hypothalamus (MG016710). Pig *MRAP2* cDNA is predicted to encode a protein of 206 amino acids, which shares relatively high amino acid sequence identity with that of humans (93%), mice (86%) and chickens (75%) (Fig. 4a). Like human MRAP2, pig MRAP2 contains a putative glycosylation site (NRT/S) at the N-terminal, a transmembrane domain, a putative domain (LKAHKYS) crucial for the formation of antiparallel homodimers (Sebag & Hinkle 2009) and a long C-terminal tail with many conserved residues (or regions) observed among vertebrates (Fig. 4a).

To verify the interaction between pMC4R^{Asp}/pMC4R^{Asn} and pMRAP2, Co-IP assay was performed to monitor their interaction in CHO cells co-transfected with Myc-pMRAP2 and Flag-pMC4R^{Asp}/Flag-pMC4R^{Asn} expression plasmids. As shown in Fig. 4b, pMRAP2 could interact with pig MC4R^{Asp} or MC4R^{Asn} (Fig. 4), which could be easily observed by the detection of a single clear pMRAP2 band (~30 kDa) in co-immunoprecipitated samples, using either anti-pMRAP2 (Fig. 4b) or anti-Myc-tag antibody. This finding indicates that, like pMC4R^{Asp}, pMC4R^{Asn} can interact and form a complex with pMRAP2 in pigs.

The pharmacological property of pMC4R^{Asp} and pMC4R^{Asn} is modified by pMRAP2

To determine whether the pharmacological property of pig pMC4R^{Asp}/pMC4R^{Asn} could be modified by MRAP2, we tested the responsiveness of pMC4R^{Asp}/pMC4R^{Asn} to their putative ligands (α-MSH/ACTH) in the presence of pMRAP2 (Fig. 5) using pGL3–CRE–luciferase reporter assay. When co-expressed with pMRAP2, either pMC4R^{Asp} or pMC4R^{Asn} showed an increased (3.6-fold/3.5-fold) sensitivity to α-MSH, and the EC_{50} values of α-MSH in activating pMC4R^{Asp}/pMC4R^{Asn} are 2.81 ± 0.39 nM/1.82 ± 0.43 nM respectively (Table 1). Interestingly, in the presence of pMRAP2, the EC_{50} values of pACTH in activating pMC4R^{Asp} (1.08 ± 0.18 nM)/pMC4R^{Asn} (3.62 ± 0.63 nM) change dramatically (67-fold/4.7-fold) (Table 1), indicating that they can function as an ACTH receptor, as previously reported and discussed in zebrafish and chickens (Josep Agulleiro *et al.* 2013; Zhang *et al.* 2017).

Taken together, pMRAP2 can increase the sensitivity of pMC4R^{Asp}/pMC4R^{Asn} to α-MSH and ACTH, particularly toward ACTH. In addition, we also found that both α-MSH

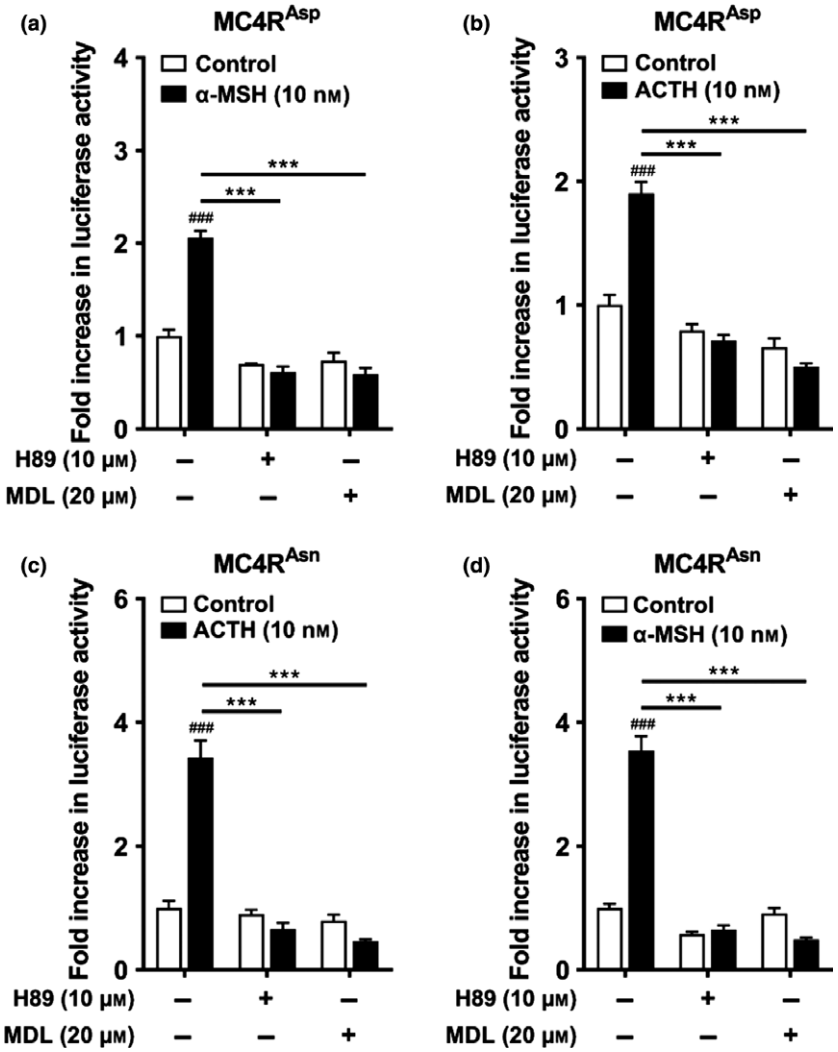

Figure 2 Effects of AC/PKA inhibitors in ACTH/α-MSH-induced cAMP production in CHO cells transfected with pMC4R^{Asp} or pMC4R^{Asn}. (a, b) Effect of H89 (10 μM) and MDL-12330A (20 μM) on α-MSH (a)/ACTH (b)-induced luciferase activity of CHO cells expressing pMC4R^{Asp}, as monitored by the pGL3–CRE–luciferase reporter system. Each inhibitor was added 1 h before peptide treatment. (c, d) Effects of H89 (10 μM) and MDL-12330A (20 μM) on α-MSH (c)/ACTH (d)-induced luciferase activity of CHO cells expressing pMC4R^{Asn}, as monitored by pGL3–CRE–luciferase reporter system. Each inhibitor was added 1 h before peptide treatment. Each data point represents the mean ± SEM of four replicates ($N = 4$) (each figure shows one representative experiment repeated three times). ### $P < 0.001$ vs. control (in the absence of drug); *** $P < 0.001$ between two groups.

and ACTH caused an approximately 6-fold increase in the luciferase activity of CHO cells expressing pMC4R^{Asn}, whereas both ligands induce an approximately 3-fold increase in the luciferase activity of CHO cells expressing pMC4R^{Asp}, in the presence of MRAP2 (Fig. 5).

MRAP2 actions on the constitutive activity and surface expression of pMC4R^{Asp} and pMC4R^{Asn}

In pigs, Asp298Asn substitution can decrease the constitutive activity and cell surface expression of MC4R (Fig. 3). Similarly, using the same approaches, we also demonstrated that Asp298Asn substitution can decrease the constitutive activity and cell surface expression of human MC4R (hMC4R) (Fig. 6c and Fig. 7c). These consistent findings led us to further investigate whether these receptor functions could be modified by pMRAP2. As shown in Fig. 6a, the basal constitutive activity of pMC4R^{Asp} could be enhanced by pMRAP2 dose dependently. In sharp contrast, the basal constitutive activity of pMC4R^{Asn} was not altered by pMRAP2. As our finding in pigs are different from those of previous studies in humans (Gillyard *et al.* 2019), mice (Asai *et al.* 2013), chickens (Zhang *et al.* 2017), orange-spotted grouper (Rao *et al.* 2019) and zebrafish (Sebag *et al.* 2013), in which the basal constitutive activity of MC4R could be inhibited by MRAP2, we further tested the effects of chicken MRAP2 on the pMC4R^{Asp}/pMC4R^{Asn}. As shown in Fig. 6b, like pMRAP2, chicken MRAP2 can also stimulate the basal constitutive activity of pMC4R^{Asp}, but not pMC4R^{Asn}. Moreover, we also found that pig MARP2 can dose-dependently inhibit the basal activity of human MC4R^{Asp}, but has no effect on the basal activity of human MC4R^{Asn} (Fig. 6c). These findings, taken together, indicate that pMC4R^{Asp} differs from human MC4R^{Asp} and displays an enhanced basal activity in the presence of pMRAP2, but the basal activity of pMC4R^{Asn}/hMC4R^{Asn} cannot be enhanced by pMRAP2.

Using Nano-Glo® HiBiT Detection System, we also investigated whether the cell surface expression of pMC4R^{Asp}/pMC4R^{Asn} could be affected by pMRAP2. As

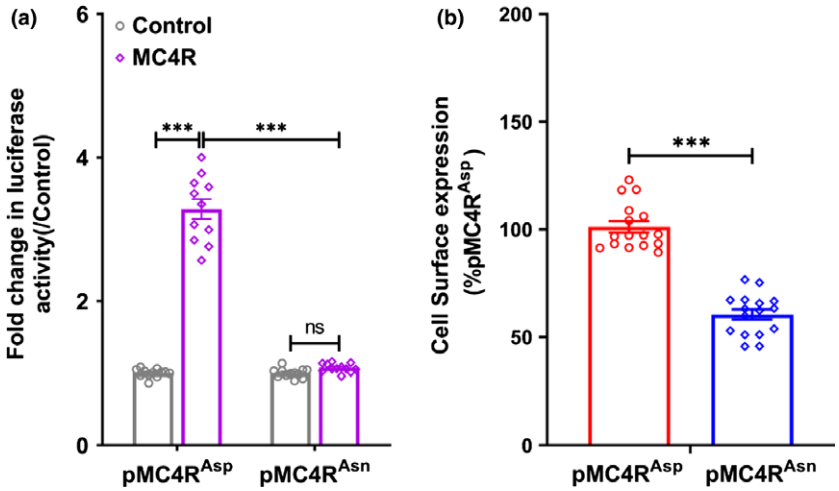

Figure 3 The basal constitutive activity and surface expression of pMC4R^{Asp} and pMC4R^{Asn}. (a) pMC4R^{Asp} expressed in CHO cells displays a strong basal constitutive activity detected by dual luciferase reporter assay, whereas pig MC4R^{Asn} shows no basal activity under the same conditions [CHO cells transfected with empty pcDNA3.1(+) vector was used as a control]. (b) Cell surface expression of pig MC4R^{Asp} and MC4R^{Asn} was measured by the HiBiT-tagging extracellular detection system (Promega) in CHO cells transfected with pMC4Rs, and the total expression of HiBiT-pMC4R measured by HiBiT lytic assay was used as an internal control to normalize transfection efficiencies. Data are shown as mean ± SEM and analyzed by two-tailed *t*-test. ***, $P < 0.001$. ns, No significant difference.

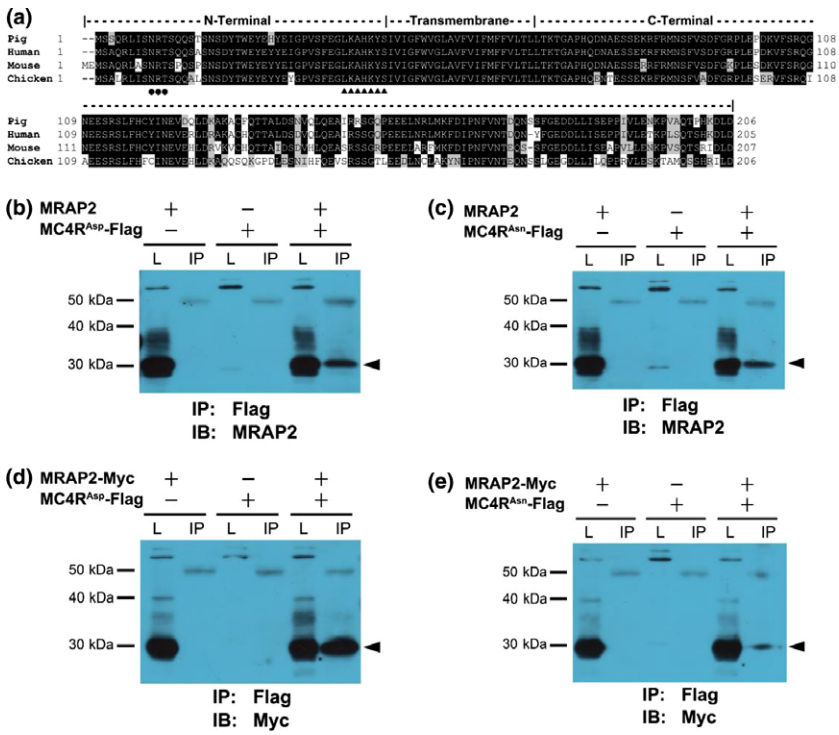

Figure 4 Interaction between pig MRAP2 and MC4R^{Asp} or MC4R^{Asn}. (a) Amino acid sequence alignment of pig MRAP2 (*Sus scrofa*, MG016710) with MRAP2 of humans (*Homo sapiens*, NP_612418), mice (*Mus musculus*, NM_001359955) and chickens (*Gallus gallus*, KT183012). Identical amino acids are shaded in black, and similar amino acids shaded in gray. A predicted *N*-linked glycosylation site is indicated by a black circle. The domain (LKAHKYS) required for the formation of antiparallel MRAP2 homodimer is indicated by arrows. (b–e) Co-IP assays show the interactions between pMRAP2 and pMC4R^{Asp} (b, d) or pMC4R^{Asn} (c, e) in CHO cells detected by different antibodies (anti-MRAP2/anti-Myc-tag). Arrowheads indicate the positive pMRAP2 band (~30 kDa) in immunoprecipitated samples (IP) detected by the anti-MRAP2 or anti-Myc-tag antibody. The ratio of MC4R to MRAP2 plasmid used in these experiments is 1:5 (w/w). L, cell lysates; IP, immunoprecipitated samples (Anti-Flag antibody used); IB, immunoblotting (anti-MRAP2 or anti-Myc-tag antibody used).

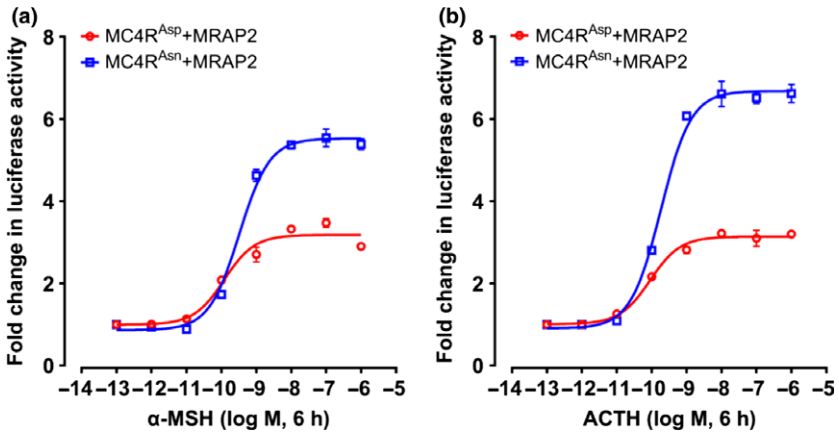

Figure 5 Effects of α-MSH and ACTH in activating pMC4R^{Asp}/MC4R^{Asn} in the presence of pMRAP2. (a) Effect of α-MSH in activating pMC4R^{Asp}/pMC4R^{Asn} expressed in CHO cells co-transfected with pMRAP2 expression plasmid, as monitored by a pGL3–CRE–luciferase reporter system. (b) Effect of ACTH in activating pMC4R^{Asp}/pMC4R^{Asn} expressed in CHO cells co-transfected with pMRAP2 expression plasmid, as monitored by a pGL3–CRE–luciferase reporter system. Each data point represents the mean ± SEM of replicates ($N = 3$) [each dose-responsive curve shows one representative experiment (repeated four times)].

shown in Fig. 7a and c, Asp298Asn substitution could decrease the cell surface expression of MC4R^{Asn} in pigs and humans, when compared with that of MC4R^{Asp}. Moreover, in the presence of pMRAP2, the surface expression of MC4R^{Asp}/MC4R^{Asn} could be further suppressed by pMRAP2 (Fig. 7a and c). As an internal control, RAMP3, another accessory protein for calcitonin receptor (Parameswaran & Spielman 2006) failed to alter the surface expression of both pMC4R^{Asp} and pMC4R^{Asn} (Fig. 7b). This finding clearly indicates that the cell surface expression of pMC4R^{Asp}/pMC4R^{Asn} (or hMC4R^{Asp}/hMC4R^{Asn}) is specifically regulated by MRAP2.

Tissue expression of pig *MC4R* and *MRAP2*

Using RNA-seq data, we analyzed the mRNA expression level of pig *POMC* [encoding the endogenous ligands (α-MSH/ACTH) for MC4R], *MC4R* and *MRAP2* in eight pig tissues, comprising the liver, spleen, thymus, hypothalamus, diaphragm, small intestine, pituitary and longissimus muscle. We found that pig *MC4R* mRNA (Fig. 8a) is highly expressed in the hypothalamus, *pMRAP2* (Fig. 8b) is highly expressed in the hypothalamus and anterior pituitary and weakly in the spleen and thymus, whereas *POMC* (Fig. 8c) is highly expressed in the hypothalamus and anterior pituitary. The co-expression of pig MC4R with MRAP2 within the hypothalamus support the interactions of MRAP2 and MC4R within the hypothalamus in pigs, as previously reported in other vertebrates.

Discussion

The MC4R polymorphic variant (MC4R^{Asn}) has long been suggested to be associated with economically important traits in pigs, such as food intake, backfat thickness, growth rate and carcass composition; however, the effect of this polymorphism (Asp298Asn) on MC4R function remains unclear, casting doubts on whether MC4R^{Asn} can be used as a selection marker in breeding programs. In this study, we investigated the functional difference between pMC4R^{Asp} and pMC4R^{Asn}. We demonstrated that pMC4R^{Asn} is functional, but displays a decrease in the basal constitutive activity and cell surface expression, when compared with pMC4R^{Asp}. Further studies proved that, like pMC4R^{Asp}, pMC4R^{Asn} can interact with MRAP2, and thus increase ligand sensitivity and decrease cell surface expression. However, the basal constitutive activity of pMC4R^{Asp}, and not pMC4R^{Asn}, can be significantly enhanced by pMRAP2. These findings, together with the co-expression of *MC4R* and *MRAP2* in pig hypothalamus, for the first time reveal the functional difference between pMC4R^{Asp} and pMC4R^{Asn}, which supports how they can interact with MRAP2 to regulate energy balance differentially, thus being involved in the regulation of phenotypic traits in pigs, as described in the literature.

Although the functional difference between MC4R^{Asp} and MC4R^{Asn} in pigs and humans has been evaluated in previous studies (Kim *et al.* 2004; Patten *et al.* 2007; Fan *et al.* 2008), the action of this polymorphism (Asp298Asn) on MC4R function remains controversial. In this study, we found that both MC4R^{Asp} and MC4R^{Asn} can be activated by α-MSH and ACTH and stimulate the cAMP/PKA signaling pathway. This finding clearly indicates that, like pMC4R^{Asp}, pMC4R^{Asn} is functional and capable of stimulating the Gs-AC/cAMP/PKA signaling pathway upon ligand binding. Our findings contrast with an early report in pigs, in which pMC4R^{Asn} could bind NDP-α-MSH with high affinity, but had a total loss of capability in cAMP signaling (Kim *et al.* 2004). However, our finding is partially supported by the latter two studies in humans and pigs. In humans, MC4R^{Asn}

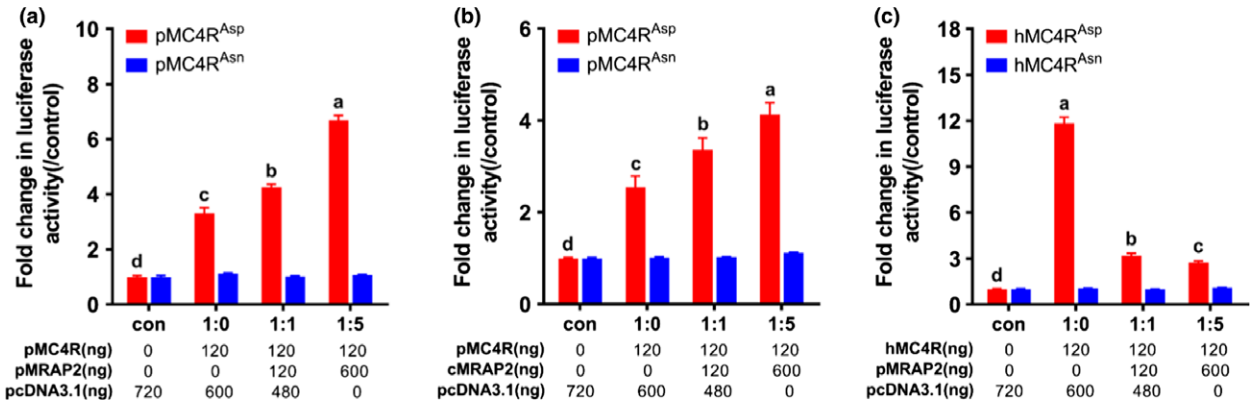

Figure 6 The action of MRAP2 on the constitutive activity of pig/human MC4R^{Asp} and MC4R^{Asn}, as detected by dual-luciferase reporter assay. (a, b) the constitutive activity of pig MC4R^{Asp} was further enhanced either by pig MRAP2 (a, pMRAP2) or by chicken MRAP2 (b, cMRAP2) dose-dependently. In contrast, pig MC4R^{Asn} shows no basal activity under the same conditions. (c) Human MC4R^{Asp} shows a strong constitutive activity and its basal activity could be inhibited by pMRAP2 dose dependently. In contrast, human MC4R^{Asn} shows no basal activity. CHO cells transfected with empty pcDNA3.1(+) vector was used as the control. Different letters indicate statistical difference between two groups ($P < 0.01$), as determined by one-way ANOVA and Tukey's test. Each data point represents the mean ± SEM of 4 replicates ($N = 4$) (each figure shows one representative experiment repeated three times).

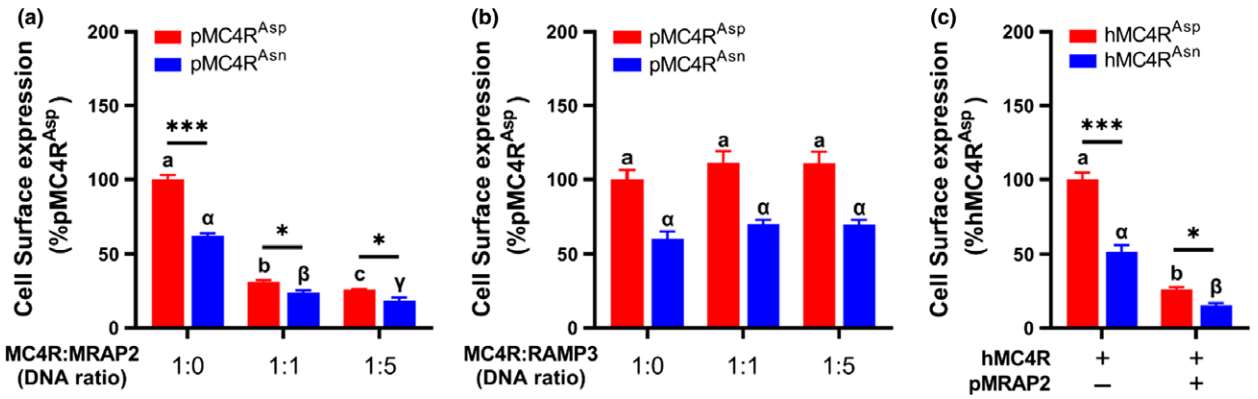

Figure 7 MRAP2 regulation of MC4R surface expression. (a–c) Surface expressions of pig MC4R^{Asp}/MC4R^{Asn} (a, b), human MC4R^{Asp}/pMC4R^{Asn} (c) in CHO cells transfected with MC4R and MRAP2 (or RAMP3, which is used as a negative control) at the indicated ratio, were measured by the HiBiT-tagging extracellular detection system (Promega) and the total expression levels of HiBiT-MC4R measured by HiBiT lytic assay were used as an internal control to normalize transfection efficiencies. Data are shown as mean ± SEM of four replicates ($N = 4$) and analyzed by two-tailed *t*-test. *, $P < 0.05$; ***, $P < 0.001$. Conditions marked by different letters (a, b, c) or (α, β, γ) are significantly different ($P < 0.01$) as determined by two-tailed *t*-test (each figure shows one representative experiment repeated three times).

expressed in HEK 293 cells can bind NDP-α-MSH with high affinity and stimulate cAMP accumulation. In pigs, MC4R^{Asn} can bind NDP-α-MSH and AgRP with high affinity and regulate cAMP accumulation, similarly to pMC4R^{Asp}. These findings tend to support the hypothesis that both pMC4R^{Asp} and pMC4R^{Asn} are functionally coupled to the cAMP/PKA signaling pathway, thus still being capable of mediating the anorexic action of α-MSH/ACTH in pigs.

It is well documented that MC4R has a strong basal constitutive activity in vertebrates (Tao 2008). In this study, we also proved that pig MC4R^{Asp} displays a strong constitutive activity. In contrast, pig MC4R^{Asn} has lost its constitutive activity. Likewise, we also found that, like pig MC4R^{Asn}, human MC4R^{Asn} also lost its constitutive activity under the same conditions. Our finding contrasts with a previous report, in which Asp298Asn substitution cannot affect the basal activity of human MC4R, or only decrease the constitutive activity of pig MC4R slightly (Fan *et al.* 2008). The discrepancy between the present and previous studies is most likely due to the sensitivity of different experimental approaches used. In addition, we also noted that Asp298Asn substitution can decrease the cell surface expression of MC4R detected by the Nano-HiBiT system. Clearly, our data provide the first proof that Asp298Asn substitution can affect MC4R function via inhibiting the basal constitutive activity and decreasing cell surface expression of MC4R in pigs and humans.

There is growing evidence that MC4R can interact with MRAP2 in vertebrates. To verify whether MC4R^{Asn} can interact with MRAP2 in pigs, we cloned pig MRAP2 and

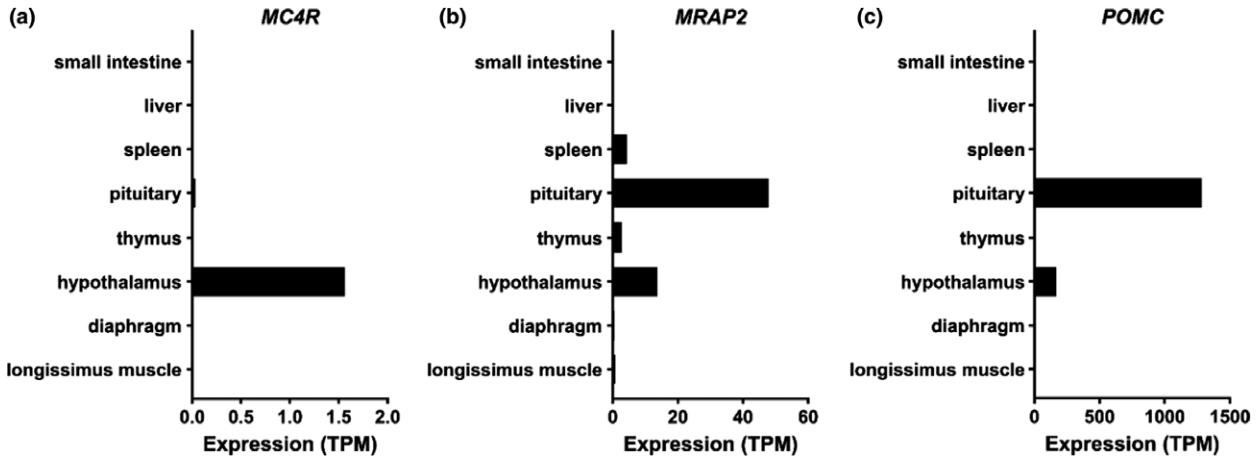

Figure 8 RNA-seq analysis of *POMC*, *MC4R* and *MRAP2* expression in pig tissues. The expression levels of *MC4R* (a) and *MRAP2* (b) *and POMC* (c), mRNA were estimated using RNA-Seq data (PRJNA353772, Iowa State University, USDA-ARS) from a single Yorkshire–Landrace–Duroc cross-bred pig including the small intestine, liver, spleen, pituitary, thymus, hypothalamus, diaphragm and longissimus dorsal. All data were calculated as transcripts per million.

found that MRAP2 is highly conserved between pigs and other vertebrate species including human and chickens (amino acid identity >75%). Moreover, a LKAHKYS domain known to be crucial for the formation of antiparallel MRAP2 homodimers and a single transmembrane domain were also noted in pig MRAP2 (Sebag & Hinkle 2009; Rouault *et al.* 2017). These conserved motifs suggest that pig MRAP2 may be able to modulate MC4R function in pigs. This hypothesis is supported by Co-IP assays, in which either pMC4R^{Asp} or pMC4R^{Asn} can interact with pMRAP2. This finding clearly indicates that either pMC4R^{Asp} or pMC4R^{Asn} can form the MC4R–MRAP2 complex.

It is reported that MC4R functions, such as the constitutive activity, surface expression, ligand responsiveness and cAMP signaling, can be modified by MRAP2. The interaction between pMC4R^{Asp}/pMC4R^{Asn} and pMRAP2 promotes us to further compare the functional difference between pMC4R^{Asp} and pMC4R^{Asn} in the presence of MRAP2. Interestingly, we demonstrated that co-expression of pMC4R^{Asp}/pMC4R^{Asn} with pMRAP2 in CHO cells can significantly increase the sensitivity of both pMC4R^{Asp} and pMC4R^{Asn} toward α-MSH and ACTH, especially toward ACTH with a 70-fold decrease in EC_{50} values. This finding is consistent with recent reports in chickens (Zhang *et al.* 2017) and zebrafish (Josep Agulleiro et al. 2013), indicating that both α-MSH and ACTH can function as the high-affinity ligands of MC4R and exert its actions via the MC4R–MRAP2 complex in pigs. In addition, we proved that pMRAP2 can not only decrease the surface expression of pMC4R^{Asp} as previously reported in mice, but also decrease the surface expression of pMC4R^{Asn} further. Similarly, we also showed that the surface expression of both human MC4R^{Asp} and MC4R^{Asn} can be suppressed by the co-expression of pMRAP2. All of these findings suggest that like pMC4R^{Asp}, pMC4R^{Asn} surface expression and ligand sensitivity and selectivity are probably modified by pMRAP2.

The most intriguing finding of the present study is that the basal constitutive activity of pMC4R^{Asp} could be further enhanced by the co-expression of pMRAP2. This finding contrasts with the previous reports in mice (Asai *et al.* 2013), chickens (Zhang *et al.* 2017) and humans (Gillyard *et al.* 2019), in which the basal activity of MC4R^{Asp} could be inhibited by MRAP2. These finding hints that, in the presence of MRAP2, the functional difference of MC4R^{Asp} between pigs and humans/chickens/mice is most likely due to the structural change in pig MC4R during speciation, thus conferring an enhanced basal constitutive activity of MC4R^{Asp} when forming a complex with pMRAP2. Unlike pMC4R^{Asp}, the basal activity of pMC4R^{Asn} cannot be enhanced by pMRAP2. The striking difference in the basal constitutive activity noted between pMC4R^{Asp} (high activity) and pMC4R^{Asn} (no/low activity), or between pMC4R^{Asp}–MRAP2 complex (high activity) and pMC4R^{Asn}–MRAP2 complex (no/low activity), strongly suggests a critical role of Asp298Asn substitution in the basal constitutive activity of MC4R/MC4R–MRAP2 complex in pigs. Presumably, the lack of constitutive activity of pMC4R^{Asn} may partially explain the greater magnitude (~6 fold) in response of pMC4R^{Asn}/pMC4R^{Asn}–MRAP2 to its ligands than that of pMC4R^{Asp}/pMC4R^{Asp}–MRAP2 with high basal activity (Fig. 5).

In humans, loss of constitutive activity of MC4R mutants causes obesity. It is reported that several MC4R mutations (R7H, T11A/S and R18C/H/L in the extracellular N-terminal domain) identified from obese patients have decreased basal constitutive activities, but have no changes in other functions, when compared with wt MC4R (hMC4R^{Asp}) (Srinivasan *et al.* 2004). In another study, two mutations (I102S and A154D) of MC4R found in obese

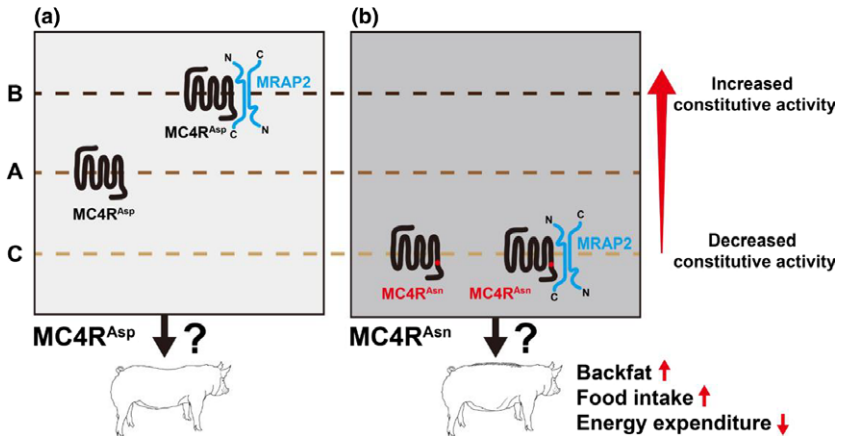

Figure 9 Proposed actions of Asp298Asn substitution on pig MC4R function and energy balance. (a) MC4R^{Asp} or MC4R^{Asp}–MRAP2 complex has a high constitutive activity, which is the critical set-point for energy balance (e.g. food intake and energy expenditure). (b) Unlike pMC4R^{Asp}, pMC4R^{Asn} has a decreased constitutive activity, regardless of the absence or presence of pMRAP2, thus altering the set-point for energy balance, which may favor increased backfat, food intake and growth rate and the decreased energy expenditure. Dashed lines A, B, and C indicate the proposed set-points for energy balance in different pig breeds, which are probably associated with pMC4R constitutive activity. However, this hypothesis (marked by a question mark) needs further verification.

subjects have also been reported to have decreased basal activities (Tao & Segaloff 2005). It is suggested that the basal constitutive activity of MC4R may act as a tonic satiety signal, which may be required for maintaining long-term energy homeostasis in humans (Srinivasan *et al.* 2004). In this study, we found that pMC4R^{Asp} or pMC4R^{Asp}/MRAP2 complex possesses a high constitutive activity; in contrast, MC4R^{Asn} or MC4R^{Asn}–MRAP2 complex has no such activity. Our findings led us to propose that the high basal constitutive activity of MC4R^{Asp}–MRAP2 complex (or MC4R^{Asp}) may be sufficient to maintain the satiety signal, leading to decreased food intake and increased energy expenditure (Fig. 9). Moreover, co-expression of MC4R and MRAP2 in pig hypothalamus also supports a potential role for the MC4R–MRAP2 complex in the central regulation of energy balance (e.g. food intake, energy expenditure) in pigs, as for those proposed in mice, chickens and zebrafish (Asai *et al.* 2013; Sebag *et al.* 2013; Zhang *et al.* 2017). In contrast, MC4R^{Asn} or MC4R^{Asn}–MRAP2 complex shows no constitutive activity, and together with its reduced surface expression, may increase food intake and decrease energy expenditure *in vivo*, thus leading to a significantly higher backfat thickness and growth rate in pig breeds. Taken together, we depicted a simplified model here (Fig. 9): the constitutive activity of MC4R^{Asp}–MRAP2 complex (or MC4R^{Asp}) may determine the basal set-point for energy balance, whereas Asp298Asn substitution causes the loss of basal constitutive activity of MC4R^{Asn}–MRAP2 complex (or MC4R^{Asn}) and reduced MC4R^{Asn} surface expression, thus reducing intracellular cAMP signaling and altering the set-point for energy balance, which consequently increases food intake, growth rate and backfat thickness.

Although the available data on the association of this polymorphism (Asp298Asn) with fatness are not totally consistent, a significant effect of the MC4R polymorphism on growth rates, fat deposition and feed consumption in a number of breeds and lines of pigs has been identified. It is clear that the frequency of the c.1426A allele shows a significant increase over the 20-year-long selection period in the Italian Large White pig breed (Fontanesi *et al.* 2015). The MC4R c.1426A allele showed intermediate frequencies (0.30–0.70) in many European local pig breeds (Munoz *et al.* 2018). This finding, together with our data showing that Asp298Asn substitution can decrease the constitutive activity and cell surface expression of MC4R, clearly indicates that MC4R^{Asn} is a valuable selection marker in breeding programs.

In addition, we found that pig MRAP2 can decrease surface expression, increase the ligand sensitivity and selectivity of pMC4R^{Asp}/pMC4R^{Asn}, and enhance the basal constitutive activity of pMC4R^{Asp}. These findings hint that MRAP2, through interaction with MC4R, can control the energy balance of pigs. In humans, four heterozygous variants in *MRAP2* gene were identified in patients with early-onset obesity (Asai *et al.* 2013). With large-scale sequencing of *MRAP2* in 9418 people, 23 rare heterozygous variants were identified associated with increased obesity risk in both adults and children (Baron *et al.* 2019). These findings also imply that, as a partner of MC4R, MRAP2 may also regulate fatness, growth and body composition in pigs, which may also make it a valuable candidate gene for animal breeding or gene editing (similar to MC4R), in the future. However, further study is needed to gain insight into the association of MRAP2 polymorphism with growth and fatness in pigs and other farm animals.

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Disclosure

The authors have nothing to disclose.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.
Table S1. Primers used in this study[a]