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Full Length Research Paper

Influence of silencing the *MC4R* gene by lentivirus-mediated RNA interference in bovine fibroblast cells

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Melanocortin receptor 4 (MC4R) is a key element in the mechanisms used to regulate both aspects of keeping the balance between energy uptake and energy expenditure. *MC4R* was knocked down by lentivirus-mediated shRNA expressing plasmids, which were controlled by the U6 promoter in bovine fibroblast cells, and the expression of *MC4R* was examined by the real time-PCR and Western blot analysis. Real time-PCR analysis was used to characterize the expression of *Leptin, POMC, AGRP, MC3R* and *NPY* gene. The relative genes [*leptin,* proopiomelanocortin (*POMC*), agouti-related peptide (*AGRP*), *MC3R* and neuropeptide Y (*NPY*)] expression level seemed to be closely associated with the *MC4R* gene in bovine fibroblast cell lines (BFCs). The levels of both MC4R mRNA and protein were significantly reduced by RNA interference (RNAi) mediated knockdown of *MC4R* in BFCs cells transfected with plasmid-based MC4R-specific shRNAs. The finding of this study demonstrated that vector based siRNA expression systems were an efficient approach to the knockdown of the *MC4R* gene expression in bovine fibroblast cells and they provided a new molecular basis for understanding the relationship of *MC4R* and other genes, which were responsible for the regulation of energy homeostasis by the melanocortin system.

Key words: Melanocortin receptor 4 (MC4R), RNAi, bovine fibroblast cells, energy homeostasis.

INTRODUCTION

The melanocortin receptor 4 (MC4R), a G-protein coupled receptor, plays a pivotal role in controlling meal size and energy homeostasis in mammals (Govaerts et al., 2005; Adan et al., 2006). The accumulating evidence implicated the components of the CNS melanocortin system, which encompassed several receptors and their peptide ligands in the central regulation of feeding and energy balance (Lu et al., 1994; Fan et al., 1997). MC4R was one of the five melanocortin receptors (MCRs) and was shown to be widely distributed throughout numerous brain regions (Mountjoy and Wong, 1997). In spite of the evidence, presented about the role of several important factors in energy metabolism, the regulation and integration of the satiety pathway and the energy homeostasis have not been completely elucidated. These important factors are neuropeptide Y (NPY), leptin, proopiomelanocortin (POMC), Agouti-related peptide (AGRP) and their respective receptors (Pelleymounter et al., 1995; Stephens et al., 1995; Hu et al., 1996; Boston et al., 1997; Clement et al., 1998; Friedman and Halaas, 1998), whereas only few researches have been conducted on the relationship of *MC4R* with the expression of upstream regulatory genes in bovine.

RNA interference (RNAi) is a wide and powerful tool which will lead to sequence-specific gene silencing at the post-transcriptional level. In mammalian cells, synthetic siRNAs can be transferred into the cells to induce transient gene silencing, whereas short hairpin RNAs (shRNAs) could be driven by RNA polymerase III promoters

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Abbreviations: MC4R, Melanocortin receptor 4; **AGRP,** agouti-related protein.

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and are subsequently cleaved by the Dicer to produce siRNAs in order to introduce mRNA degradation; but there are lots of problems related to the method of delivery, especially low efficiency of transfection in primary cells. However, lentivirus can stably transfect to many cells, such as primary cells, suspension cells and stem cells, at high efficiency, and also sustain long-term gene expression by integrating itself into the host genome. Therefore, lentiviral vector is considered as the most suitable delivery method for this study.

In the recent studies (Chen et al., 2000; Ste et al., 2000), knockout of the *MC4R* gene was usually carried out *in vitro* or *in vivo* of mice. However, differences may exist between the bovine *MC4R* mRNA and the other species mRNA in terms of the length and spatial structure, so as to affect the effectiveness of shRNAs screening and relative regulation genes. Therefore, it is necessary to perform shRNAs screen in primary bovine cells which naturally knockdown the *MC4R* gene expression.

In this study, a shRNA-expressing lentiviral vector was constructed to target the bovine *MC4R* gene, and the resulting interference effect was analyzed in bovine fibroblast cells (BFCs). Subsequently, the resulting induction along with the expression of leptin, POMC, AGRP and NPY genes were studied.

MATERIALS AND METHODS

Isolation and culture of cell

Auris tissues, blocked from 20 month-old male local yellow bovine in the Animal Center of Institute of animal science – Chinese academy of agriculture science, were resected under aseptic conditions. Bovine fibroblast cells and human embryonic kidney 293 T cells (HEK-293T) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL, USA) and supplemented with 10% fetal bovine serum (FBS; Gibco BRL, USA) at 37°C under 5% CO_2. Cell cultures, at 80% confluence, were detached with trypsin-EDTA and plated into 12-well plates or 6-well plates at a density of 5 x 10^4 cells/cm^2 for transfection only.

MC4R silencing

pHelper 1.0, pHelper 2.0 and pGCSIL-GFP plasmids were purchased from Genechem Co. Ltd (Shanghai, China). The oligonucleotide sequence was as follows: 5'- GAGCTTCACGGTG GATATT-3' (Clone 1), 5'- GGTACTTCACTATCTTCTA -3' (Clone 2), 5'- GACTCCACATTAAGAGGAT -3' (Clone 3), 5'- CCTGAT ATTCTACATCTCT -3' (Clone 4) and 5'- 'TTTCTCCGAACGTGT CACGT -3' (Negative control). pGCSIL-GFP Vector (U6 promoter; polylinker: accggt CCGCAGGTATGCACGCGTgaattc; lower case: linker sequence) was digested by *Age* I and *Eco*R I and ligated by *T4* DNA ligase. The ligated DNA solution was transformed into *E. coli* DH5α and incubated on a LB plate (LB solid medium containing 50 μg L^{-1} ampicillin and 2% agarose gel) at 37°C for 16 h. The lentivirus was produced in 293T cells by transient co-transfection of three plasmids, including 20 μg pGCSIL-GFP vector (1-4, and NC), 15 μg packaging vector pHelper 1.0 and 10 μg VSVG expression plasmid pHelper2.0. Then, transfection was performed by Lipofectamine™ 2000 reagent (Invitrogen, USA) on the basis of the manufacturer's instructions. The viral particles were harvested from the medium at 48 h after transfection, and were named as Lvsh MC4R -1,-2,-3, or -4 (KD1-4), and Lvsh-NC (NC, non-silenced control lentivirus). Lvsh MC4R or Lvsh-NC were incubated with polybrene and BFCs cells at a multiplicity of infection (MOI = 50) for 12 h at 37°C and 5% CO_2, respectively. Then, the medium one was replaced with fresh DMEM medium containing 10% FBS and the incubation was continued for another 96 h before the cells were harvested.

Real-time PCR analysis

Total RNA was extracted by Tiangen RNeasy mini kit (Tiangen, China) according to the manufacturer's instructions. About 2 to 5 μg of the total RNA were reversely transcribed by Quant one step RT-PCR kit (Tiangen, China) with oligo$(dT)_{18}$ primers. Real-time RT-PCR was performed by an ABI Prism™ 7900HT Sequence Detection System (Applied Biosystems, USA). Then, each PCR reaction with a total volume of 25 μL contained 2 μL of cDNA, 0.5 μL of 10 μmol L^{-1} primers, 9.5 μl ddH_2O and 12.5 μl SYBR Premix *Ex Taq* (2×) (TaKaRa, China). The relative expression levels of all genes were normalized to 18S rRNA expression levels, respectively, and all primers are shown in Table 1.

Western blot analysis

The entire cellular protein was obtained with BCA Protein Assay Kit based on the manufacturer's instructions (Bestbio, China). The aliquots were separated on SDS-PAGE (10%) and transferred to nitrocellulose membranes. The MC4R antibody (SC6879-C19) was purchased from Santa Biotechnology (Santa Cruz Biotechnology Inc., USA).

Then, the membranes were incubated with goat anti-MC4R protein antibody at room temperature for 2 h and stained by the secondary antibody mouse anti-goat (SC2354) immunoglobulin G, horseradish peroxidase (HRP) conjugate (Santa Cruz Biotechnology Inc., USA) for 1 h. Anti-GAPDH antibody was obtained from Sigma Biotechnology as a positive control (Sigma, USA).

Statistical analysis

Statistical analyses were performed by student's t-test using SPSS statistical software (SPSS Inc., USA). $P < 0.05$ was considered as statistically significant.

RESULTS

Construction and generation of MC4R shRNA lentivirus

The identities of the pGCSIL-GFP-shMC4R vectors were confirmed by the PCR and sequencing method, and the result indicated that the recombinant pGCSIL-GFP-shMC4R-1,-2,-3,-4 were successfully constructed (Figure 1a). Lentivirus targeting MC4R-1,-2,-3,-4 and –NC (Lvsh-MC4R-1,-2,-3,-4 and -NC) was produced by co-transfected packaging vector (pHelper1.0) and VSVG expression plasmid (pHelper2.0) into 293T cells. Then the viral titer was determined to be in the range of 1 x 10^8 TU ml^{-1} medium by GFP assay under the fluorescence microscope (Figure 1b).

Table 1. Parameters of oligo-nucleotide primer pairs for PCR primers.

Target gene	Primer sequence	Product size	Annealing temperature	Reference sequence
MC3R	F:CTTCCTCCACCTCGTCC R:AGGCTCCAGAAAGCATAG	137	60	FJ433881
MC4R	F:TCTCCCGAGGTGTTTGTG R: ATGGTTTCCGACCCGTTG	167	60	NM_174110.1
Leptin	F:AGACCATAACAGCAGACAG R:TCCAGGCAATTCACTTCC	175	60	NM_173928.2
POMC	F:AACGCCATCATCAAGAAC R:CTTCAGGGTCAACTTTCC	162	60	NM_174151
AGRP	F:GTCGCTGAAGAGGATAACGG R:TGCGGCAGTAGCAGAAGG	215	60	NM_173983.1
NPY	F:TGCGACACTACATCAATCTCATC R:GTTTCATTTCCCATCACCACATATG	222	60	NM_001014845
18S rRNA	F:TCTCCTTCGGGCTGGTCATC R:GCACACGCTGGCAACATTG	131	60	NR_036642.1

Detection of MC4R silencing of Lvsh-MC4R in FBCs

The Auris tissue block was minced into pieces and digested in the presence of type II collagenase. Then, the resulting pellet was cultured for 96 h, in which most of the cells were slender with fibroblast shape under microscope. Generated lentivirus was further used to infect FBCs cells to obtain the most effective target virus (Figure 2a). The total RNA was extracted after 72 h later, and the Real-time PCR results indicated that infection with Lvsh-MC4R-1,-2,-3 or -4 could inhibit *MC4R* mRNA expression by approximately 71, 8, 11 or 15%, respectively, in contrast to the infection with Lvsh-MC4R-NC (Figure 2b). As the transfection efficiency of the bovine fibroblast cells was around 80%, the result suggested that Lvsh-MC4R-1 markedly suppressed *MC4R* mRNA expression, while Lvsh-MC4R-2, -3 and -4 did not influence the levels of *MC4*R mRNA. Subsequently, the result from the western blot, when compared to the infection of LvshMC4R-NC (Figure 2c), further confirmed, that the Lvsh-MC4R-1 could significantly suppress the expression of *MC4R* at both levels of mRNA and protein, and maintain satisfactory silencing effect in BFCs.

Effect of Lvsh-MC4R on expression of regulatory genes

In order to observe the effect of *MC4R* silencing on the other genes of energy metabolism, Lvsh-MC4R1 was selected as the knockdown group (KD), which was compared with the negative control group (NC) to detect the expression level of the four upstream regulatory genes in all the samples. As shown in Figure 3, the *Leptin, POMC* and *NPY* genes expression in the KD group was detected to be appreciably lower than that of the NC group; but the expression of *MC3R* gene in NC group was found to be significantly lower than the KD group, especially, where it was found that the expression of *AGRP* gene in the KD group was much significantly higher than that of the NC group.

DISCUSSION

In this study, lentiviral vectors are used to silence bovine *MC4R* gene expression not only in HEK-293T cells, butalso in bovine fibroblast cells. Human U6 promoter was used to drive siRNA expression from the DNA templates and four target sites were designed. Finally, it was only one effective target site (Lvsh-MC4R1) that could down-regulate the *MC4R* gene expression in bovine fibroblast cells. Lentiviral vectors were chosen as the shRNA delivery vehicle to suppress the *MC4R* gene in bovine fibroblast cells, for the reason that lentivirus can integrate itself into the genome of host cells and sustain long-term gene expressions (Gropp and Reubinoff, 2007). Generally, a lentiviral vector expression system consists of three plasmid vectors, which include lentiviral plasmid (transfer vector), packaging vector and VSVG

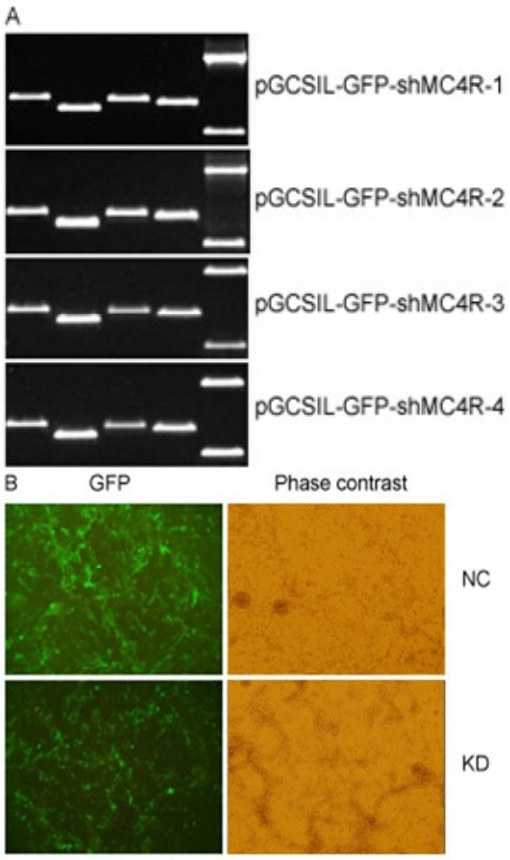

Fig. 1 The identities of pGCSIL-GFP-shMC4R vectors and LvshMC4R.
A. The recombinant pGCSIL-GFP-shMC4R-1,-2,-3,-4 plasmids were identified by PCR. Lane 1, 3, 4, PCR products of the recombinant pGCSIL-GFP-shMC4R-1,-2,-3,-4 plasmids, Lane 2, PCR products of the empty vector; Lane 5, DNA ladder (DL 2000), 500 bp (up) and 250 bp (down); the positive clones were 352bp and the negative clones were 318 bp. B. 293T cells were infected with Lv-shMC4R (KD) or -NC (NC), and phase contrast or GFP expression which was examined after 72 h.

expression plasmid, and a packaging cell line (pseudovirion producing cell line). In this study, there are four lentiviral plasmids that are successfully constructed to target *MC4R* and a negative control using the three plasmid expression system. The result, shown In Figure 2, suggested that the lentiviral vector expression system could effectively drive siRNA and transcribe it from the DNA templates into the bovine fibroblast cells.

In contrast to all other hormone systems known so far, the melanocortin system was the only one that was not only regulated by agonistic peptides, but also by two endogenously occurring neuropeptides that were blocked from ligand-induced MCR signalling: agouti and “agouti-related protein” (AGRP). Agouti binds to almost all MCR with high affinity, whereas AGRP exhibits rather selective binding towards the MC3R and MC4R (Ollmann et al., 1997). This study indicated that AGRP impaired the appetite controlled in a melanocortin-independent manner, and it was defined as a classic competitive antagonist, since binding of AGRP prevented or displaced the binding of melanocortins to the MC3R or MC4R (Tolle and Low, 2008; Wu et al., 2008). However, this study herein found out that the expression of *AGRP* gene, presented significantly, was high after *MC4R* gene was silenced. This result supports the suggestion that mechanisms, other than competitive antagonism of MC4R signaling, should also be considered when discussing the physiological effects of AGRP on the molecular level. As the receptors for NPY and MC3R are known, the members of the G-protein-coupled receptor family were down regulated in hypothalamic regions under conditions that were known by enhancing the local NPY release (Widdowson et al., 1997). In this study, mRNA level of *NPY* gene was down regulated, and oppositely, that of *MC3R* gene was up regulated, suggesting that MC3R and MC4R genes were competitively bound with NPY. Adipose tissue-derived hormones, such as leptin, increase POMC expression in α-MSH-releasing neurons located in the arcuate nucleus of the hypothalamus (Shimizu et al., 2007). Various peripheral and central factors could signal energy balance to the POMC neurons, as ARC POMC neurons express the functional OB-Rb leptin receptor, while leptin apparently stimulates these neurons (Cheung et al., 1997; Schwartz et al., 1997). However, the expression level of leptin and POMC went down as the MC4R was knocked down in this study, suggesting that they might be the medium in the signalling pathway of the melanocortin system.

In summary, one out of the four designed target sites reduced the MC4R gene expression by lentivirus mediated in bovine fibroblast cells, and the expressions of a series of genes of the CNS melanocortin system were detected. The results of this study provided a new molecular basis for understanding the mechanism of energy homeostasis, and also laid the foundation for further study of the biological functions of *MC4R* gene and other related genes.

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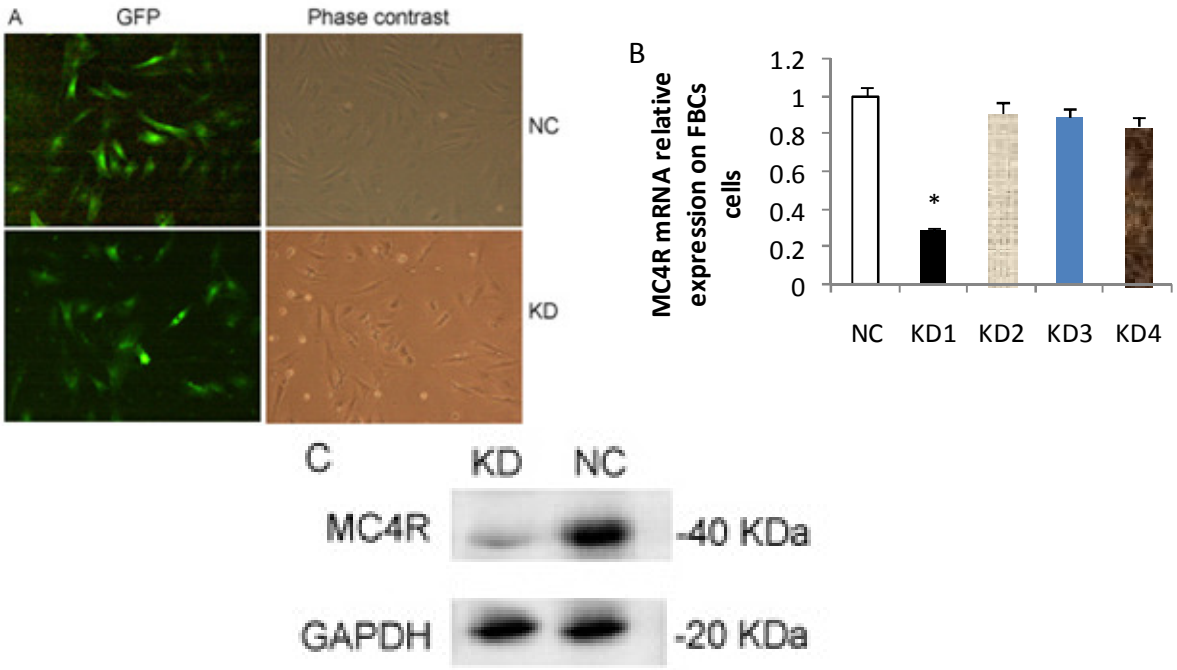

Fig. 2 The effect of infection with Lv-shMC4R and LvshMC4R- NC in BFCs.
A. BFCs were infected with Lv-shMC4R (KD) and LvshMC4R- NC (NC), and representative phase contrast or GFP expression was examined after 72 h. B. BFCs were infected with Lv-shMC4R or Lvsh- NC, and the level of MC4R mRNA was detected by Real-time PCR 72h later. mRNA levels in NC were taken as 1.0. C. MC4R protein levels were detected by western blot 72h later. Each treatment was established in duplicate and each sample was examined in duplicate. All data were expressed as means ± SEs

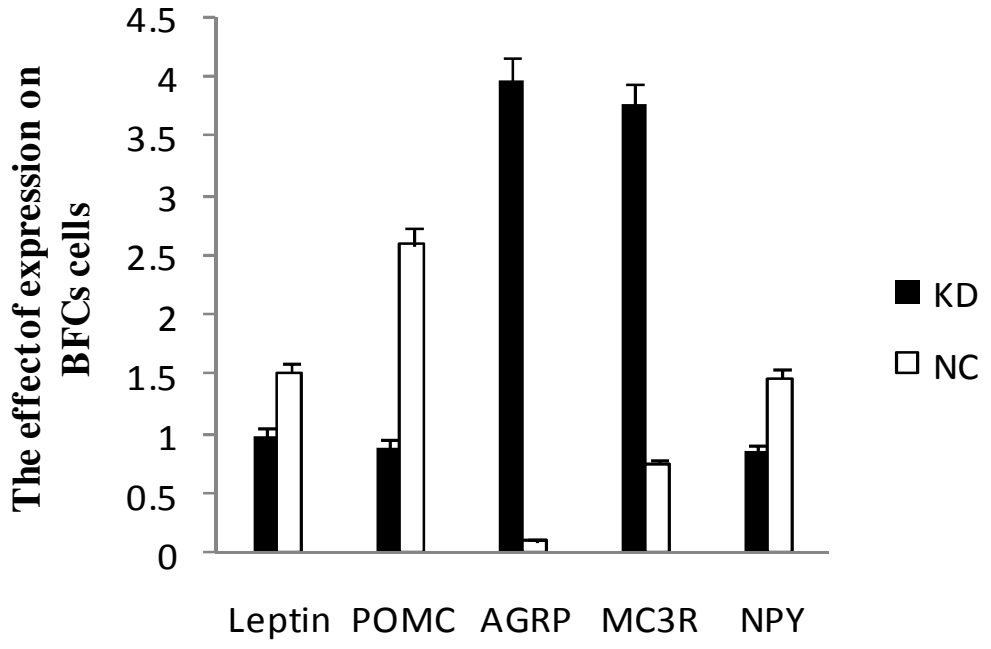

Fig. 3 Expression of relative genes in BFCs infected with Lv-shMC4R (KD) and LvshMC4R-NC (NC) were examined at day 3 using Real-time PCR.
The mRNA levels in KD of Leptin were taken as 1.0. All samples were examined in duplicate, and data were presented as means ± SEs.

REFERENCES

Adan RA, Tiesjema B, Hillebrand JJ, la Fleur SE, Kas MJ de Krom M (2006). The MC4 receptor and control of appetite. Br. J. Pharmacol. 149: 815-827.

Boston BA, Blaydon KM, Varnerin J, Cone RD (1997). Independent and additive effects of central POMC and leptin pathways on murine obesity. Science, 278: 1641-164.

Chen AS, Marsh DJ, Trumbauer ME, Frazier EG, Guan XM, Yu H, Rosenblum CI, Vongs A, Feng Y, Cao L, Metzger JM, Strack AM, Camacho RE, Mellin TN, Nunes CN, Min W, Fisher J, Gopal-Truter S, MacIntyre DE, Chen HY, Van der Ploeg LH (2000). Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. Nat. Genet. 26: 97-102.

Cheung CC, Clifton DK, Steiner RA (1997). Proopiomelanocortin neurons are direct targets for leptin in the hypothalamus. Endocrinology, 138: 4489-92.

Clement K, Vaisse C, Lahlou N, Cabrol S, Pelloux V, Cassuto D, Gourmelen M, Dina C, Chambaz J, Lacorte JM, Basdevant A, Bougneres P, Lebouc Y, Froguel P, Guy-Grand B (1998). A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. Nature, 392: 398-401.

Fan W, Boston BA, Kesterson RA, Hruby VJ (1997). Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. Nature, 385: 165-168.

Friedman JM, Halaas JL (1998). Leptin and the regulation of body weight in mammals. Nature 395: 763-70.

Govaerts C, Srinivasan S, Shapiro A, Zhang S, Picard F, Clement K, Lubrano-Berthelier C, Vaisse C (2005). Obesity-associated mutations in the melanocortin 4 receptor provide novel insights into its function. Peptides, 26: 1909-1919.

Gropp M, Reubinoff BE (2007). Lentiviral-RNA-interference system mediating homogenous and monitored level of gene silencing in human embryonic stem cells. Cloning Stem Cells, 9: 339-345.

Hu Y, Bloomquist BT, Cornfield LJ, DeCarr LB, Flores-Riveros JR, Friedman L, Jiang P, Lewis-Higgins L, Sadlowski Y, Schaefer J, Velazquez N, McCaleb ML (1996). Identification of a novel hypothalamic neuropeptide Y receptor associated with feeding behavior. J. Biol. Chem. 271: 26315-26319.

Lu D, Willard D, Patel IR, Kadwell S, Overton L, Kost T, Luther M, Chen W, Woychik RP, Wilkison WO, Et A (1994). Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. Nature, 371: 799-802.

Mountjoy KG, Wong J (1997). Obesity, diabetes and functions for proopiomelanocortin-derived peptides. Mol Cell Endocrinol 128: 171-177.

Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen Y, Gantz I, GS Barsh GS (1997). Antagonism of central melanocortin receptors *in vitro* and *in vivo* by agouti-related protein. Science, 278: 135-138.

Pelleymounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T, Collins F (1995). Effects of the obese gene product on body weight regulation in ob/ob mice. Science, 269: 540-543.

Schwartz MW, Seeley RJ, Woods SC, Weigle DS, Campfield LA, Burn P, Baskin DG (1997). Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus. Diabetes 46: 2119-2123.

Shimizu H, Inoue K, Mori M (2007). The leptin-dependent and-independent melanocortin signaling system: regulation of feeding and energy expenditure. J. Endocrinol. 193: 1-9.

Ste ML, Miura GL, Marsh DJ, Yagaloff K, Palmiter RD (2000). A metabolic defect promotes obesity in mice lacking melanocortin-4 receptors. Proc. Natl. Acad. Sci. USA. 97: 12339-12344.

Stephens TW, Basinski M, Bristow PK, Bue-Valleskey JM, Burgett SG, Craft L, Hale J, Hoffmann J, Hsiung HM, Kriauciunas AE (1995). The role of neuropeptide Y in the antiobesity action of the obese gene product. Nature, 377: 530-532.

Tolle V, Low MJ (2008). *In vivo* evidence for inverse agonism of Agouti-related peptide in the central nervous system of proopiomelanocortin-deficient mice. Diabetes 57: 86-94.

Widdowson PS, Upton R, Henderson L, Buckingham R, Wilson S, Williams G (1997). Reciprocal regional changes in brain NPY receptor density during dietary restriction and dietary-induced obesity in the rat. Brain Res. 774: 1-10.

Wu Q, Howell MP, Cowley MA, Palmiter RD (2008). Starvation after AgRP neuron ablation is independent of melanocortin signaling. Proc. Natl. Acad. Sci. USA. 105: 2687-2892.