Functional Characterization of Novel Naturally Occurring Mutations in Human Melanocortin-3 Receptor Gene

by

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Abstract

The prevalence of obesity is increasing in both developed and developing countries. Obesity is a disorder of energy homeostasis, associated with increased risk of diabetes mellitus, cardiovascular diseases and certain types of cancers. Hence, there is increasing interest in the molecular mechanisms of the pathogenesis of obesity. Although environmental factors play an important role in the pathogenesis of obesity, genetic factors confer an individual's susceptibility to obesity. Mutations in several genes such as those encoding leptin, leptin receptor, proopiomelanocortin (POMC), and melanocortin-4 receptor (MC4R), have been identified to cause monogenic obesity.

Melanocortin-3 receptor (MC3R), another member of the melanocortin receptor family, and MC4R, are both expressed in central nervous system at sites potentially involved in regulating energy homeostasis. Rodent genetic studies demonstrate that MC3R is involved in regulating energy metabolism. It may serve as an auto-inhibitory receptor on POMC neurons to modulate food intake, potentially explaining the observation that *Mc3r* knock out mice do not exhibit hyperphagia. Mice lacking *Mc3r* gene have increased fat mass and decreased lean body mass, suggesting that clinical research addressing energy homeostasis and MC3R should focus on adiposity rather than body weight. Up to now, 24 mutations in *MC3R* gene have been identified. Detailed functional data were lacking for most of these mutations.

In the present study, detailed functional studies of 18 naturally occurring *MC3R* mutations were performed. Receptor expression, ligand binding and signaling properties were investigated. Ten mutants (S69C, A70T, F82S, I87T, M134I, L249V, A260V, M275T, T280S and L297V) had significantly decreased cell surface expression, and mutants A260V, M275T and L297V had decreased total expression. S69C, F82S, D158Y, R257S, T280S and L299V displayed significant defects in both ligand binding and signaling. M134I and M275T had decreased maximal binding, but exhibited similar signaling as wild-type (WT) MC3R, indicating the presence of spare receptors. L249F and L285V had normal maximal binding with decreased maximal signaling, suggesting that these mutants were defective in signaling. Data from multiple mutations at T280 demonstrated the necessity of Thr in different aspects of MC3R functions.

In conclusion, in the present study, detailed functional data of these naturally occurring mutations were obtained. D158Y, T280S and L299V might be the pathogenic causes for human obesity. S69C, F82S, and R257S, defective in function, were identified from lean subjects, suggesting that mutations in *MC3R* are not 100% penetrant in causing human obesity. F82, D158, L249, T280 and L299 played an important role in MC3R functions. These findings contribute to a better understanding of the structure-function relationship of MC3R. Whether and how other mutants that had normal signaling could affect energy homeostasis in vivo requires further investigation. More detailed studies, including in vivo studies and cosegregation analysis are needed.

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List of Abbreviations

AC	Adenylate Cyclase
ACTH	Adrenocorticotrophic Hormone
AgRP	Agouti-related Protein
ARC	Arcuate Nucleus
BAT	Brown Adipose Tissue
Bmax	Apparent maximal binding
BMI	Body Mass Index
BSA	Bovine Serum Albumin
CART	Cocaine and Amphetamine Regulated Transcript
CNS	Central Nervous System
DMEM	Dulbecco's Modified Eagle's Medium
DMH	Dorsomedial Hypothalamus
EGFP	Enhanced Green Fluorescent Protein
ERK	Extracellular Signal-regulated Kinase
FOS-IR	FOS-immunoreactivity
GABA	γ-Aminobutyric Acid
GPCR	G Protein-coupled Receptor
GWAS	Genome-wide Association Study
HEK	Human Embryonic Kidney

IGF-1	Insulin-like Growth Factor 1
JAK	Janus Kinase
MAPK	Mitogen-activated Protein Kinase
MC3R	Melanocortin-3 Receptor
MC4R	Melanocortin-4 Receptor
MCR	Melanocortin Receptor
MSH	Melanocyte Stimulating Hormone
NHANES	National Health and Nutrition Examination Survey
NPY	Nuropeptide Y
ob	Obese
ObR	Leptin Receptor
PBS-IH	PBS for Immunohistochemistry
PH	Posterior Hypothalamic
PI3K	Phosphatidylinositol 3-Kinase
PKA	Protein Kinase A
POMC	Proopiomelanocortin
PTP1B	Protein-tyrosin Phosphatase 1B
PVN	Paraventricular Nucleus
Rmax	Maximal Response
SCN	Suprachiasmatic Hypothalamic Nuclei
SNP	Single Nucleotide Polymorphism
SOCS3	Suppressor of Cytokine Signaling 3
STAT	Signal Transducer and Activator of Transcription

UCP1	Uncoupling Protein 1
VMH	Ventromedial Hypothalamic Nucleus
VMN	Ventromedial
WHO	World Health Organization
WT	Wild Type

Chapter 1

Introduction

The prevalence of obesity is increasing rapidly and it has become an enormous economic and health burden worldwide. There is an urgent need to investigate the molecular mechanisms accounting for the development of obesity. Although the obesity trend is mainly attributed to the prevailing obesogenic environment and life style, genetic factors have also been demonstrated to be important in the pathogenesis of obesity. A number of genes have been identified to be associated with human energy regulation and obesity, including leptin, leptin receptor, proopiomelanocortin (POMC), and melanocortin-4 receptor (MC4R).

Melanocortin-3 receptor (MC3R) is another member of melanocortin receptors. Both MC3R and MC4R are expressed in brain at sites considered to be involved in regulating energy homeostasis. Several studies demonstrate that MC3R is involved in regulating different aspects of energy homeostasis. At least twenty-four naturally occurring mutations in *MC3R* gene have been identified. Detailed functional studies of these mutations will contribute to a better understanding of the structure-function relationship of MC3R and the potential mechanisms on how *MC3R* mutations cause human adiposity or obesity.

This chapter presents an overview of the obesity trends and the current strategies to study obesity. The role of leptin-melanocortin system in the regulation of energy homeostasis is discussed.

1.1 Obesity

1.1.1 Definition of obesity

Obesity is generally considered as a state that body weight, especially the adipose tissue, is increased and leads to multiple health deterioration and shortened life expectancy (Brodsky 1998; Spiegelman and Flier 2001). The body mass index (BMI) is commonly used to define obesity. BMI is calculated as weight in kilograms divided by the square of height in meters (kg/m^2). A BMI of less than 25 indicates normal weight, while between (including equal to) 25 and 29 implies overweight, and greater than or equal to 30 means obesity (1995; 2000). The severe obesity (BMI≥40) that will dramatically shorten patients' life expectancy if untreated is described as morbid obesity (Brodsky 1998). Table 1 shows the classification of adult obesity according to the cut-off points proposed by the World Health Organization (WHO). In 2002, a cooperating program held by the International Association for the Study of Obesity and WHO found that Asians are particularly sensitive to obesity-related diseases. Therefore, the recommended optimal BMI for Asians is set to 18.5~23 (Choo 2002; James, et al. 2002).

Table 1. Classification of adult obesity based on BMI proposed by WHO (2000).

Classification	BMI	Risk of comorbidities
Underweight	<18.50	Low (but risk of other clinical problems increased)
Normal range	18.50–24.99	Average
Overweight:	≥25.00	
Preobese	25.00–29.99	Increased
Obese class I	30.00–34.99	Moderate
Obese class II	35.00–39.99	Severe
Obese class III	≥40.00	Very severe

Until now, there is no agreement on the classification of obesity in children and adolescents (2000), because the weight and height of children are continuously changing. In some countries, BMI-for-age growth charts have been applied to assess obesity in children. In the United States, children and adolescents having a BMI greater than or equal to 95% on the BMI-for-age charts are regarded obese (Ogden, et al. 2012).

Based on the predominant localization of accumulated adipose tissues, there are two types of obesity. One is central-android obesity in which body fat is primarily in the upper body. Another one is peripheral-gynecoid obesity, which means adipose tissue is predominantly located in the thighs, hips and buttocks (Brodsky 1998). Generally, for any accumulation of body fat, males have the double amount of abdominal fat as in premenopausal females on average (2000). Because abdominal fat dramatically vary within a narrow range of BMI, body circumference indexes including waist circumference and waist-to-hip circumference ratio are applied to identify central-android obesity, which has higher risk of obesity-related diseases such as cardiovascular disease and coronary heart disease, independent on BMI (2000; Marik 2000). The waist-to-hip circumference ratio higher than 1.0 in men or higher than 0.85 in women is considered to imply accumulated abdominal fat mass (2000).

1.1.2 Obesity epidemiology

The prevalence of obesity rises rapidly and has become a worldwide health and economic burden. In the United States, the trends of obesity are obtained from the National Health and Nutrition Examination Survey (NHANES). NHANES is a serious of multistage, cross-sectional national health examination of noninstitutionalized population. From 1999, NHANES has become a continuous survey in a cycle of 2 years (Flegal, et al. 2012). In the United States, the

prevalence of adult obesity rose from 14.25% to 31% between 1978 and 2000 (Deitel 2003). Between 2000 and 2005, the prevalence of obesity (BMI≥30) increased by 24%, morbid obesity (BMI ≥ 40) prevalence increased by 52%, and the prevalence of super obesity (BMI ≥ 50) increased by 75% (Sturm 2007). In 2009~2010, NHANES data suggested a slightly increased, but still high prevalence of adult obesity, 35.5% in males and 35.8% in females (Flegal et al. 2012).

The increase in the prevalence of obesity has been observed globally, in both developed and developing countries. In UK, adult obesity prevalence rose from 6% in 1980 to 21% in 2001 in males, from 8% in 1980 to 23.5% in 2001 in females. Moreover, morbid obesity prevalence in UK had a threefold increase during the past decade (Deitel 2003). Studies from other countries like Brazil and Japan indicated that obesity prevalence progressively increases in the past years but at different rates (James, et al. 2001). In 2005, around 33% of adult population worldwide became obese or overweight. Although the obesity prevalence was higher in developed countries compared to developing countries (20.3% vs 6.7%) in 2005, it was estimated that developing regions would have a much higher increase in the prevalence of overweight and obesity in future, because of multiple factors including increasing population, population aging, urbanization and switch to western lifestyle (Kelly, et al. 2008).

The high prevalence of obesity is affecting children and adolescents worldwide. Between 2003 and 2007, the prevalence of obesity increased by 10% for children in the United States aged 10 to 17, with a 9% increase in overweight among girls. In 2007, about 17% of children in the United States were obese. (Singh, et al. 2010). In Japan, the prevalence of obese children aged from 6 to 14 rose from 5% in 1974 to 10% in 1993, and that of extreme obesity increased

from 1% to 2% (Deckelbaum and Williams 2001). In Northern Africa, although there were over 7% children that were under-nourished, the proportion of overweight children exceeded 8%. Similarly, in Eastern Asia, where there were 3.4% children that were under-nourished, 4.3% of children were overweight (Deckelbaum and Williams 2001).

1.1.3 Obesity and its associated diseases

Obesity is associated with many different diseases, including insulin resistance, diabetes, hypertension, cardiovascular diseases, stroke, gallbladder and liver diseases, colon diseases, arthritis of joints, many forms of cancers, and depression (Brodsky 1998; Deitel 2003). In the World Health Report 2002, 20 main global health risks were identified that affect the disease and death rate worldwide. Overweight and obesity are the 10^{th}. Some other risks on this list such as hypertension, high cholesterol and physical inactivity are also related to obesity (Deitel 2003).

Obesity in children is considered to be a predictor of adult overweight or obesity. Goran *et al.* summarized four longitudinal studies and concluded that children initially becoming obese at younger age will have higher obesity prevalence when they become adults (Deckelbaum and Williams 2001). Similarly, a Japanese study reported that about one-third of obese children became obese adults (Deckelbaum and Williams 2001). In a long-term study targeting the consequences of overweight adolescents followed for up to 50 years, there are increasing risks of morbidity associated with cardiovascular and other forms of chronic diseases even in the obese adolescents that had lost weight during adulthood,(Deckelbaum and Williams 2001), indicating that pediatric obesity may work as a trigger for the co-morbidities in adulthood.

Furthermore, co-morbidities related to overweight and obesity among children and adolescents are similar with those among adults. In a study summarizing 1027 consecutive

patients aged from birth to 19 from 1982 to 1995, the diagnosis of type 2 diabetes increased from about 4% before 1992 to 16% in 1994, accompanying the rise in adolescent obesity (Pinhas-Hamiel, et al. 1996). Among children who are moderately overweight, there is an increase in the levels of low-density lipoprotein as well. With the more marked degree of obesity, it is common to observe increased plasma triglyceride level and decreased high-density lipoprotein level. And with severe obesity, blood pressure elevation becomes more common (Deckelbaum and Williams 2001). According to data form Bogalusa Heart Study the majority of overweight children aged from 5 to 10 have an increased cardiovascular risk, such as hypertension, hyperlipidemia, or hyperinsulimia. Among the same cohort, more than 20% of overweight children had two or more such risk factors (Deckelbaum and Williams 2001).

Other forms of co-morbidities are also related to overweight or obesity in childhood, including skin fungal infections, and psychological problems such as depression, anxiety and negative self-esteem (Deckelbaum and Williams 2001). Therefore, a bundle of evidence suggests that overweight and obesity may significantly affect children and adolescents, physically and mentally. Many effects of pediatric obesity lead to increased risks of chronic diseases in adulthood, leading to significant health and economic burden worldwide.

1.1.4 Strategies to study obesity

It has long been considered that obesity is a disorder of energy balance. That is, obesity is due to either excessive energy intake or deficient energy expenditure (Spiegelman and Flier 2001; Walley, et al. 2009). Abnormal fat storage and/or mobilization are considered as another potential explanation to human obesity (Walley et al. 2009). Adipocyte population and size together determine the fat mass in human bodies. The adipocyte population increases

continuously throughout the childhood. During adulthood, however, the number of adipocytes is controlled at a constant value although there is annual turnover of about 10% cells. Thus, fat mass in adults is predominantly determined by adipocyte sizes (Spalding, et al. 2008). The same study found that in patients with early-onset obesity, there is a larger increase adipocyte numbers. Therefore, obesity means larger adipocyte population and/or larger adipocyte size compared to lean people (Spalding et al. 2008).

Although it is considered that the genetic changes do not account for the rapidly increased prevalence of obesity during the past several decades, it is hard to deny the fact that different individuals react differently to similar environments and diets, indicating the important roles of genetics in the pathogenesis of obesity. Some strategies to study the association between obesity and specific genes have been applied, providing a better understanding of obesity pathogenesis and potential ways to prevent or treat obesity.

1.1.4.1 Genome-wide linkage studies

Genome-wide linkage scan is a family-based approach to examine whether the markers, such as single nucleotide polymorphisms (SNPs), distributed throughout the genome, cosegregate with phenotypes related to specific diseases (Walley et al. 2009). It is useful to localize the causal genes for monogenic diseases. More than 60 whole genome scans have been performed for obesity and 250 quantitative trait loci linked with obesity-derived phenotypes have been identified by 2005 (Rankinen, et al. 2006). Some candidate genes have been identified by positional cloning based on linkage study results. However, it is difficult to replicate the linkage results. Indeed, it is hard to detect strong evidence of linkage for BMI at any locus. Linkage study succeeds when the variants of one gene cause a strong effect. The failure to replicate the

linkage results for obesity suggests that the variants of these candidate genes only exert small effects (Walley et al. 2009).

1.1.4.2 Candidate gene studies

Compared to genome-wide linkage study, the candidate gene approach is a short-range method and can be applied with unrelated individual subjects (Walley et al. 2009). Many genes considered to be involved in the regulation of energy homeostasis have been investigated, such as genes encoding leptin (Montague, et al. 1997), leptin receptor (Clement, et al. 1998), POMC (Krude, et al. 1998) and MC4R (Vaisse, et al. 1998). Abundant evidence has supported that missense mutations in these genes are associated with obesity. Although none of these genes reaches the genome-wide linkage significance as mentioned above, there is enough evidence and sufficient replications of the results to support their effects on obesity (Walley et al. 2009).

1.1.4.3 Genome-wide association studies (GWAS)

Different from candidate gene approach, GWAS is a hypothesis-free method, combining association and linkage in gene identification. It uses case-control study to compare how much all the genes of the genome vary between different individuals and to determine the association of genes with diseases (Walley et al. 2009). GWAS becomes applicable based on the HapMap, which is an international project aiming to determine the patterns of gene variations in the human genome (2003). GWA has so far successfully identified a set of obesity genes. A recent study analyzing 16,876 European individuals found strong association signal 188 kb downstream of *MC4R.* The signal is associated with BMI in both adults and children aged 7 to 11 (Loos, et al. 2008). This finding implies that the variants near *MC4R* affect fat mass and obesity probably due to modulating MC4R function and altering food intake (Walley et al. 2009).

GWAS is based on the hypothesis that the common diseases are resulted from some common gene variants, but not from a great amount of rare forms of gene variants (Walley et al. 2009). Current GWAS identify markers previously not known to associate with the diseases, and the results of GWAS have reported genes rarely overlapping with those identified by candidate gene approach (Walley et al. 2009). Thus, GWAS approach has increased the number of obesity-related genes significantly.

1.1.4.4 Summary of strategies to study obesity

Recently a considerable number of genes related to obesity have been identified, but together these genes only account for a small percentage of obesity. The possible reasons include: the current genetic studies do not specifically select obese samples; the main phenotype studied only focuses on BMI; and the GWAS approach still requires improvement (Walley et al. 2009). Many genetic studies were not initially targeted for obesity, but turned to provide a great amount of information about association of gene variants with BMI. This type of study helps to identify genes important for modest increase in obesity at the population level. However, specifically selecting extremely obese subjects may help identify genes of large effect in relatively small sample size (Walley et al. 2009).

Currently, BMI is the main phenotype studied in analysis of obesity-related genes. However, BMI is only one of many obesity-associated phenotypes and it cannot describe the detailed distribution of accumulated fat depots, for example visceral and central abdominal fat, in each individual. It has been noticed that abdominal fat volume varies independent of BMI and abdominal obesity has strong association with type 2 diabetes and cardiovascular diseases (2000; Marik 2000). In addition, BMI is not an optimal parameter for certain types of people, such as

athletes who have significant muscle mass (Walley et al. 2009). Thus, phenotypes concerning distribution of fat depots should be involved in the studies of obesity-related genes. Parameters such as waist-to-hip circumference should be considered at least as essential as BMI. On the other hand, phenotypes based on cellular and molecular levels should be investigated, providing the potential explanation to obesity (Walley et al. 2009).

There are limitations of GWAS studies, including the use of case-control study designs and the financial challenges presented by the analyses of large data pools (Walley et al. 2009). Case-control study designs are popular, but they are not very reliable when population substructure exists, and they have difficulty to control the gene-environment interactions. Prospective cohort studies, in which diseases can be estimated in ahead, and family-based studies should be applied to provide the insights into gene-environment interactions (Walley et al. 2009). In addition, it should be noted that current GWAS approaches have different results for some obesity-related gene variants among different areas and regions, thus, the large-scale GWAS covering different populations may highlight both the common obesity- related variants that influence all the populations and the variants specific to a certain ethnic population (Walley et al. 2009).

1.2 Leptin-melanocortin system and its role in human obesity

1.2.1 Leptin and leptin receptor

1.2.1.1 Leptin

Leptin is a 16-kDa hormone, primarily secreted by adipocytes of the white adipose tissue. It is released into circulation in proportion to the body fat mass content, transported across blood-brain barrier and targeting the hypothalamus, especially the arcuate nucleus (ARC) (Maffei, et al.

1995; Zhang, et al. 1994). Leptin was first discovered in mouse in 1994. It was observed that mice carrying a mutation in *obese* (*ob*) gene showed a complete lack of the encoded leptin and exhibited weight gain and severe obesity, as shown in *ob/ob* mouse (Zhang et al. 1994).

In 1997, leptin involvement in the regulation of energy balance in humans was confirmed. It was the first time that human congenital leptin deficiency was described with the phenotypes of hyperphagia and morbid obesity (Montague, et al. 1997). The leptin deficient infants were born with normal body weight, but then increased body weight rapidly by increasing food intake without any nutrient preference and developed early-onset obesity (Farooqi and O'Rahilly 2008; Oswal and Yeo 2007).

Similar to *ob/ob* mice, in addition to hyperphagia and severe obesity, leptin deficiency in humans also correlate with physiological disorders in many other aspects, including hyperinsulinaemia, hypothalamic-pituitary thyroidal axis defects, decreased number of T-cells and defects in reproductive function (Farooqi, et al. 1999; Friedman and Halaas 1998; Ozata, et al. 1999; Strobel, et al. 1998), suggesting the role of leptin in regulating other physiological processes. Interestingly, there is gradual improvement of the phenotypes mentioned above in leptin deficient adults over decades, indicating that the regulatory role of leptin can be gradually compensated by other mechanisms (Ozata et al. 1999). The congenital leptin deficiency can be rescued by recombinant hormone replacement (Licinio, et al. 2004). In addition to restoring normal body weight, leptin administration also improves endocrine, metabolic and immunological dysfunctions (Licinio et al. 2004).

Leptin regulates energy balance by acting on both food intake and energy expenditure (Walley et al. 2009). The circulating levels of leptin are in proportion to body fat content. The

increase in adiposity will raise leptin concentration, induce satiety, and then inhibit food intake (Considine, et al. 1996; Maffei et al. 1995; Walley et al. 2009). Starvation or prolonged fasting will reduce the leptin level and trigger alterations in metabolism to maintain energy balance (Ahima, et al. 1996). In rodents, leptin increases energy expenditure through thermogenesis of brown adipose tissue (BAT). In mitochondria-rich BAT, thermogenesis occurs by disconnecting the oxidation from ATP generation, which is mediated by uncoupling protein 1 (UCP1) (Walley et al. 2009). It was observed that in fasting state, high level of leptin in central nervous system (CNS) could reverse the reduction in UCP1 mRNA expression and result in the sympathetic activation of BAT (Rahmouni and Morgan 2007; Sivitz, et al. 1999). In rodents, losing BAT function is associated with metabolic disorder and obesity (Lowell, et al. 1993), whereas, experimental induction of BAT causes lean phenotype (Ghorbani, et al. 1997).

Until now, frameshift or missense mutations in *leptin* gene have been identified (Gibson, et al. 2004; Ozata et al. 1999; Strobel et al. 1998). The frameshift mutations identified in Pakistani families led to truncated leptin, due to the deletion of a guanine residue at position 133 (Gibson et al. 2004). The homozygous missense mutation identified in a Turkish origin family was caused by substitution of arginine residue at position 105 with tryptophan (Strobel et al. 1998). These mutations result in the deficiency of leptin, and hence lead to phenotypes similar to *ob/ob* mice. Despite the similarities, there are still some differences between leptin deficient mice and humans. The elevated corticosterone levels in *ob/ob* mice (Friedman and Halaas 1998) are not observed in human cases. Leptin deficient humans do not have decreased energy expenditure as *ob/ob* mice (Farooqi and O'Rahilly 2006).

Although leptin has been proven to be effective in rescue of congenital leptin deficiency, its potential role in inducing weight loss for common forms of obesity has not been established yet (Oswal and Yeo 2007). These findings indicate that leptin serves as the sensor of body fat mass content, and the primary role of leptin in energy balance is to maintain the sufficient body fat depots in fasting situation, rather than diminish the excessive body weight when energy is adequate, because the evolutionary pressure induces the system to prefer body fat storage but not consumption (Ahima et al. 1996; Oswal and Yeo 2007).

1.2.1.2 Leptin receptor

The majority of leptin functions are mediated in CNS by leptin receptor (ObR). Shortly after the cloning of *obese* gene, *diabetes* gene was identified to encode leptin receptor (Chen, et al. 1996). There are different isoforms of leptin receptors, due to alternative splicing of the mRNA transcript (Lee, et al. 1996; Tartaglia 1997), named from ObRa, ObRb, ObRc, ObRd, ObRe and ObRf. Depending on the length of their intracellular domains, leptin receptors are classified as short and long. The long isoform ObRb is considered the fully functional leptin receptor, whereas other isoforms are all short and limited in signaling capacity (Bjorbaek, et al. 1997; Lee et al. 1996; Myers, et al. 2008).

Leptin receptor is a member of interleukin-6 receptors, with similar structure of class 1 cytokine receptor family (Farooqi and O'Rahilly 2008). ObRb has the highest mRNA expression in hypothalamus (Elmquist, et al. 1998), where the functions of leptin are mediated (Elmquist et al. 1998). It has one-transmemberane domain, extracellular domain that binds to leptin and intracellular domain that activates Janus kinase (JAK) and signal transducer and activator of transcription (STAT) (Bates, et al. 2003; Bjorbaek et al. 1997; Ghilardi and Skoda 1997). The

short forms of leptin receptor ObRa and ObRc are highly expressed in the cerebral microvessels that compose the blood-brain barrier, suggesting the role of these receptors in leptin transportation into CNS. Impaired leptin transportation across blood-brain barrier may contribute to leptin resistance (Hileman, et al. 2002).

Mice lacking leptin receptor (*db/db* mice) exhibit almost identical phenotypes as *ob/ob* mice (Farooqi and O'Rahilly 2008; Oswal and Yeo 2007). Mutations in *leptin receptor* are also identified in humans with severe early-onset obesity. It was first reported in three Algerian-origin patients with severe obesity that a homozygous mutation in *leptin receptor* caused truncated receptor, missing the transmembrane and intracellular domains (Clement et al. 1998). These individuals had similar phenotypes as leptin deficient patients, but they exhibit some additional endocrine disorders, such as abnormality in growth, impaired basal and stimulated levels of growth hormone and reduced insulin-like growth factor 1 (IGF-1) level (Clement et al. 1998). Lack of leptin receptor leads to more complicated consequences than congenital leptin deficiency, suggesting that leptin receptor is probably targeted by other ligands besides leptin (Oswal and Yeo 2007). In another study, five nonsense and four missense mutations in *leptin receptor* were reported, demonstrating up to 3% of patients with severe early-onset obesity associated with *leptin receptor* mutations (Farooqi, et al. 2007). These mutations caused either partial or complete loss of leptin receptor function by preventing signaling activity, as shown by abnormal STAT3 phosphorylation (Farooqi et al. 2007). Interestingly, the affected children in this study did not show abnormality in growth and IGF-1 levels (Farooqi et al. 2007). However, the lack of pubertal growth spurt, due to hypogonadotropic hypogonadism caused by loss of leptin receptor function, will lead to a decreased final height in adulthood (Farooqi et al. 2007).

1.2.1.3 Leptin signaling and obesity

Activation of long isoform leptin receptor (ObRb) transduces signals through JAK/STAT pathway. ObRb does not contain intrinsic tyrosin kinase activity. Therefore, once leptin binds to its receptor, cytoplasmic kinase JAK2 is recruited and phosphorylates numerous tyrosine residues on intracellular domain of leptin receptor (Ghilardi and Skoda 1997). Subsequently, the phosphorylated intracellular domain offers binding sites for STATs, which then become activated and translocate to the nucleus, serving as transcription factors to trigger downstream activities (Bates et al. 2003).

STAT3 is considered to play an important role in energy homeostasis (Bates et al. 2003; Vaisse, et al. 1996). Tyrosine residue at position 1138 of ObRb is a key residue essential for STAT3 binding to leptin receptor and activation. Transgenic mice with Y1138S exhibit hyperphagia, obesity and suppression of hypothalamic melanorcortin system activities, indicating that STAT3 is involved in the regulation of energy balance through melanocortin system (Bates et al. 2003). Mice carrying homozygous mutation Y1138S have similar phenotypes as *db/db* mice. However, *db/db* mice have more complicated syndromes, including infertility, abnormal growth, impaired glucose metabolism, hypothyroidism and impaired immune system, whereas, mice harboring Y1138S retain gonadal functions (Bates et al. 2003). These data suggest that STAT3 is essential for energy homeostasis, especially in controlling feeding behavior, but it is not required for other functions of leptin, such as reproductive function (Bates et al. 2003).

The leptin-JAK-STAT signaling pathway is under negative feedback regulation of some molecules (Bjorbaek, et al. 1999; Kaszubska, et al. 2002; Myers et al. 2008). Leptin stimulates

the suppressor of cytokine signaling 3 (SOCS3) mRNA expression in hypothalamus. SOCS3 then binds to JAK2 in a leptin-dependent manner and inhibits both JAK autophosphorylation and JAK-mediated leptin receptor phosphorylation (Bjorbaek et al. 1999; Myers et al. 2008). In fact, as a potent inhibitor of JAK-STAT signaling pathway, high expression level or overactivation of SOCS3 is considered as a sign of leptin resistance (Bjorbaek et al. 1999; Farooqi and O'Rahilly 2008). On the other hand, mice with neuron-specific deletion or haploinsufficiency for SOCS3 have enhanced STAT3 expression and are sensitive to leptin administration (Howard, et al. 2004; Kievit, et al. 2006).

Another protein behaving as a negative regulator of leptin-JAK-STAT signal pathway is protein tyrosine phosphatase 1B (PTP1B), also known as tyrosine-protein phosphatase nonreceptor type 1. PTP1B dephosphorylates JAK2 (Kaszubska et al. 2002) and hence inhibit the signal transduction in vitro. PTP1B knockout mice exhibit enhanced STAT3 expression, increased sensitivity to leptin, and increased resistance to diet-induced obesity (Klaman, et al. 2000).

1.2.2 The melanocortin system in regulation of energy homeostasis

1.2.2.1 The melanocortin system

The functionally active leptin receptor ObRb is highly expressed in hypothalamus (Elmquist et al. 1998), and the majority of leptin functions on regulating food intake is mediated by the melanocortin system (Oswal and Yeo 2007). After crossing the blood-brain barrier, leptin directly acts on two mutually opposing neurons within ARC: one type of neurons co-express the orexigenic peptides neuropeptide Y (NPY) and agouti-related protein (AgRP), while the other type of neurons co-express the anorexigenic peptides POMC and cocaine- and amphetamine-

regulated transcript (CART) (Cone 2005). Leptin binding inhibits the expression of the potent appetite stimulator NPY as well as AgRP which is an antagonist primarily antagonizing MC3R and MC4R, whereas leptin binding stimulates the expression of POMC and CART (Cone 2005; Farooqi and O'Rahilly 2008).

POMC undergoes post-translational cleavage in a tissue-specific manner, generating a set of small biologically active peptides, including melanocortins α□□□β□□□γ-melanocyte stimulating hormone (MSH), adrenocorticotrophic hormone (ACTH), β-lipotrophin and β-endorphin (Pritchard, et al. 2002). The determination of peptide generation depends on the specificity of the endoproteases expressed in the particular tissue (Pritchard et al. 2002). The melanocortins act through a family of G protein-coupled receptors (GPCRs), which are known as melanocortin receptors (MCRs). There are five members of this family, named MC1R to MC5R, according to the order of their cloning. MC1R is expressed on melanocytes and skin, controlling skin and hair pigmentation. MC2R, which is the ACTH receptor, is expressed in the adrenal gland and controls adrenal steroidogenesis. MC5R is expressed in exocrine glands and is essential in the generation of sebum. MC3R and MC4R are primarily expressed in CNS, and accumulating evidence suggests that they play a critical role in the regulation of energy homeostasis (Gantz and Fong 2003; Oswal and Yeo 2007; Tao 2005).

1.2.2.2 Melanocortin-4 receptor (MC4R)

The *MC4R* mutations have been considered as the most common cause for human monogenic obesity (Vaisse, et al. 1998; Yeo, et al. 1998).

MC4R is a member of family A rhodopsin-like GPCRs. Human *MC4R* (hMC4R) was cloned by two groups independently (Gantz, et al. 1993b; Mountjoy, et al. 1994). It was localized to chromosome 18q21.3 via fluorescent *in situ* hybridization, and it is an intronless gene, encoding a receptor of 332 amino acids (Gantz et al. 1993b; Magenis, et al. 1994). Northern blotting and *in situ* hybridization studies show that MC4R has wide distribution in brain, including the areas of cortex, striatum, thalamus, hypothalamus, hippocampus and brainstem (Gantz et al. 1993b; Kishi, et al. 2003; Liu, et al. 2003; Mountjoy et al. 1994). In addition, MC4R is also expressed in astrocytes (Caruso, et al. 2007).

MC4R can couple to all the different major classes of heterotrimeric G proteins, including Gs, Gi/o and Gq proteins, changing the levels of second messengers through different enzymes (Tao 2010a). Classically, binding of the endogenous ligands α-MSH and β-MSH leads to alteration of its three-dimensional structure, the binding of G protein to activated receptor, the exchange of GTP for GDP on Gα subunit, and dissociation of GTP-bound Gα subunit with Gβγ dimmer. GTP-Gαs subunit then moves to activate adenylate cyclase (AC) on cell membrane. The activated AC catalyzes the generation of cAMP from ATP, and subsequently cAMP activates protein kinase A (PKA) to regulate cell metabolism, including gene expression, cell membrane permeability and cell secretion.

In GT1-1 cells, a mouse hypothalamic cell line endogenously expressing MC4R, as well as in HEK293 cells stably expressing MC4R, it is observed that the activation of MC4R increases the intracellular calcium levels, indicating that the signaling of MC4R in such situations is mediated through Gq/phospholipase C-dependent pathway (Newman, et al. 2006; Nickolls, et al. 2005). In heterologous cells expressing MC4R and GT1-7 cells, which is another

mouse hypothalamic cell line expressing MC4R endogenously, the activation of MC4R stimulates pertussis toxin-sensitive GTPγS binding, suggesting that MC4R also couples to Gi/o proteins (Buch, et al. 2009). Agouti and AgRP are antagonists for MC4R (Butler 2006). It is found that AgRP also stimulates GTPγS binding, implicating that AgRP antagonizes MC4R by both preventing Gs activation and stimulating Gi/o activation (Buch et al. 2009). AgRP also serves as an inverse agonist for MC4R (Haskell-Luevano and Monck 2001; Tao, et al. 2010). It was first reported that the fragment of AgRP, AgRP (83-132), is an inverse agonist for human MC4R (Nijenhuis, et al. 2001). Later study showed that full length AgRP, AgRP (87-120), and two short peptides derived from AgRP also serve as inverse agonists for MC4R (Chai, et al. 2003). AgRP decreases basal activity of human MC4R, causing reduced basal cAMP level to the detection limit (Tao et al. 2010). There is also *in vivo* evidence showing that AgRP can regulate energy balance despite the absence of endogenous agonists in mice (Tolle and Low 2008).

In heterologous cells expressing MC4R, the peptide agonist-activated receptors undergo internalization through clathrin-coated pits that depends on GPCR kinase (GRK) 2, β-arrestin, and dynamin (Gao, et al. 2003; Shinyama, et al. 2003). Prolonged activation of MC4R will cause its translocation from endosome to lysosome, and then degradation in lysosome. It is rarely observed that the internalizaed MC4Rs are recycled to the cell surface (Gao et al. 2003). AgRP, although being an inverse agonist, also stimulates the association of MC4R with β-arrestin and internalization subsequently. Thus AgRP decreases the amount of MC4R on the cell membrane available for agonists (Breit, et al. 2006).

The fact that Agouti antagonizes the action of α□MSH not only at MC1R, but also at MC4R, and that *Agouti* mice are featured with obesity and hyperphagia in addition to hair color

alteration (Lu, et al. 1994), suggests the potential role of MC4R in control of food intake and energy balance. It is reported that homozygous *Mc4r* knockout mice display hyperphagia, obesity, accelerated linear growth, hyperinsulinemia and hyperglycemia (Huszar, et al. 1997). The heterozygous null *Mc4r* mice show intermediate effects on body weight compared to their WT and homozygous knockout littermates, indicating there is a gene dosage effect of MC4R (Huszar et al. 1997).

Rodent studies show that *Mc4r* deficiency is associated with dysfunction of energy expenditure. In young non-obese *Mc4r* knockout mice, the oxygen consumption decreases by 20% and there is also a reduction in locomotor activity compared to the weight-matched WT animals (Ste Marie, et al. 2000). When the *Mc4r* knockout mice are exposed to a high-fat diet, unlike WT mice, which transiently increase the caloric intake, but decrease the food consumption to retain the isocaloric intake, *Mc4r* knockout mice quickly develop obesity due to hyperphagia, a decrease in diet-induced thermogenesis, and the lack of motor activity (Butler, et al. 2001). It indicates that MC4R is essential to mediate the appropriate alteration in energy balance regulation in response to changes in calories in diets.

Interestingly, there is an anatomical diversity in the role of MC4R in energy balance (Balthasar, et al. 2005). Balthasar *et al.* reported that in *Mc4r* null mice that have MC4R expression specifically restored in the amygdala and paraventricular nucleus (PVN), about 60% of the obesity phenotype was ameliorated, but there was no influence on energy expenditure. This suggests that MC4R expression in the amygdala and PVN seems to be associated with the regulation of energy intake, and MC4R expressed elsewhere is probably involved in control of energy expenditure (Balthasar et al. 2005). The specific MC4R-expressing neurons controlling

energy expenditure are still unclear. The candidates comprise MC4R bearing neurons in raphe pallidus and the sympathetic preganglionic neurons of the intermediolateral nucleus in spinal cord (Balthasar et al. 2005).

As mentioned above, in 1998 two distinct groups reported the frameshift mutations in *MC4R* that are associated with dominantly inherited early-onset obesity in human for the first time (Vaisse et al. 1998; Yeo et al. 1998). Since then, over 150 mutations in *MC4R* have been reported in different ethnic populations. Totally at least 105 residues of MC4R have been reported to be mutated (Tao 2009). Mutations in *MC4R* account for between 0.5% and 6% of severe early-onset obesity cases, making it the most prevalent form of human monogenic obesity (Farooqi, et al. 2003).

The majority of the *MC4R* mutations reported are in the heterozygous state (Tao 2010a). The phenotypes observed in most heterozygous *MC4R* mutations are due to haploinsufficiency rather than dominant negative activity (Oswal and Yeo 2007). Rare *MC4R* mutations are reported to exert dominant negative activity, for example D90N (Biebermann, et al. 2003). The possible explanation is the heterodimerization of WT MC4R and D90N, causing the impairment of WT MC4R function (Biebermann et al. 2003).

Although most *MC4R* mutations are heterozygous, there are small portion of homozygous *MC4R* mutations reported (Tao 2010a). For example, Farooqi *et al.* reported six families in which the patients carried homozygous *MC4R* mutations (Farooqi et al. 2003). They found that all the carriers had a more severe phenotype than those carrying the heterozygous *MC4R* mutations. The phenotypes include increased body weight, increased height, raised bone mineral density, hyperinsulinaemia and increased food consumption in the *ad libitum* test meal

(Farooqi et al. 2003). Thus, Farooqi *et al.* demonstrated that the degree of the receptor dysfunction observed *in vitro* could predict the amount of food consumed by patients carrying the particular *MC4R* mutations during the same *ad libitum* test meal (Farooqi et al. 2003). Again, these results emphasize that the quantitative variation of MC4R expression greatly influences its function.

MC4R is constitutively active in vivo (Tao 2010a). *MC4R* mutants losing constitutive activity accounts for one explanation how the mutations in *MC4R* lead to obesity (Srinivasan, et al. 2004). Constitutively active *MC4R* mutants are expected to be associated with a lean phenotype, even anorexia nervosa. For example, mutant I251L, which has higher constitutive activity, is considered to be the potential reason that this variant is negatively associated with BMI (Xiang, et al. 2006). However, there are six constitutively active *MC4R* mutants identified from obese patients, including H76R, S127L, D146N, H158R, P240L and L250Q. The mechanism how these mutations associate with obesity still needs further investigation (Tao et al. 2010).

In addition to the classic cAMP pathway, MC4R also activates mitogen-activated protein kinase (MAPK) pathway, which has long-term effects on cellular activities (Fanelli and De Benedetti 2011; Ruiz-Gomez, et al. 2010). In CHO-K1 cells stably expressing MC4R, NDP-MSH binding with MC4R activates p42/p44 MAPK in a time- and dose-dependent pattern. Conversely, the activation of MAPK pathway can be abolished by MC4R antagonist SHU9119. The phosphatidylinositol 3-kinase (PI3K) inhibitors LY294002 and wortmannin block p42/p44 MAPK activation, but PKA inhibitor Rp-cAMPS does not affect the activation process, indicating that MC4R activates p42/p44 MAPK through PI3K-mediated pathway (Fanelli and De

Benedetti 2011). The naturally occurring mutations in *MC4R*, D90N, and laboratory-generated D298A were reported to impair both cAMP and MAPK pathways. Mutant D298N retained cAMP generation but had no MAPK signaling, further demonstrating that MC4R activates cAMP and MAPK pathway via different structural requirements (Ruiz-Gomez et al. 2010). Also the same group found that the AC inhibitor 2', 5'-dideoxyadenosine prevented MC4R-mediated p42/p44 MAPK activation (Ruiz-Gomez et al. 2010). Taken together, these results suggest that cAMP is essential for activation of p42/p44 MAPK.

It is known that α-MSH potentiates leptin-induced STAT3 activation (Fredholm, et al. 2007). It is also known that both ObRb and MC4R activate MAPK pathway (Fanelli and De Benedetti 2011; Ferre, et al. 2008; Ruiz-Gomez et al. 2010) and that MAPK pathway is involved in STAT3 activation (Kuroki and O'Flaherty 1999). In cells co-expressing ObRb and MC4R, PD98095, which is the specific MEK inhibitor, weakens α-MSH-potentiated STAT3 activation, suggesting that α-MSH-stimulated MC4R potentiates STAT3 activation through MAPK pathway. This finding highlights the cooperation of ObRb and melanocortin receptor system in signaling, indicating the potential biological implications of these two systems not restricted to obesity (Zhang, et al. 2009).

1.2.2.3 Melanocortin-3 receptor (MC3R)

Unlike MC4R, the specific role of MC3R in energy homeostasis is still controversial.

MC3R also belongs to family A GPCRs. It has homology with MC4R, with 76% similarity and 58% identity (Gantz et al. 1993b; Magenis et al. 1994). *MC3R* was cloned from rat (Roselli-Rehfuss, et al. 1993) and human (Gantz, et al. 1993a) in 1993 by two independent

groups. *MC3R* gene is intronless, encoding a typical seven-transmembrane-spanning protein consisted of 361 amino acids (Gantz et al. 1993a). MC3R has wide distribution in human body. It is mainly expressed in the hypothalamus, particularly in the ARC, the posterior hypothalamic area (PH) and the ventromedial hypothalamic nucleus (VMH) (Jegou, et al. 2000). In addition to the CNS, MC3R is also expressed peripherally. MC3R was observed to be expressed in placenta (Gantz et al. 1993a), renal tissues (Chandramohan, et al. 2009), immune cells (Getting, et al. 2008), and GI tract including stomach, duodenum and pancreas (Gantz et al. 1993a). Consistent with its wide distribution, MC3R has broad functions, such as modulation of arterial pressure in response to high-salt diet (Chandramohan et al. 2009), in the modulation of anti-inflammatory activity (Getting et al. 2008), and participating in the regulation of energy homeostasis.

MC3R and MC4R are both expressed in the brain and share the endogenous ligands α-MSH and β-MSH (Fan, et al. 2008; Schioth, et al. 1996), suggesting potentially overlapping roles of these two receptors. Furthermore, *MC3R* gene is localized on chromosome 20q13.2 by fluorescence *in situ* hybridization (Gantz, et al. 1993c; Magenis et al. 1994), a locus associated with obesity and type 2 diabetes (Ji, et al. 1997; Lembertas, et al. 1997; Zouali, et al. 1997), indicating that MC3R is involved in regulation of energy balance.

The mouse genetic studies showed that unlike *Mc4r*-knockout mice, which are hyperphagic and obese, *Mc3r* knockout mice are hypophagic on regular chow and have normal energy expenditure without apparent increase in body weight (Butler, et al. 2000; Chen, et al. 2000). When switched to high fat diet, *Mc3r* knockout mice still do not have hyperphagia despite the slightly increased body weight (Butler et al. 2000; Chen et al. 2000). It was also observed that *Mc3r* knockout mice have increased fat mass and decreased lean mass, together with

reduced spontaneous locomotor activity and energy expenditure as well as increased respiratory quotient when fed with high fat diet (Butler et al. 2000; Chen et al. 2000). In a study using mouse strain Black Swiss; 129, which is resistant to obesity and sensitive to leptin, it was observed that *Mc3r* knockout mice were 20% heavier than wild-type (WT) mice with 2.4-fold fat mass at comparable ages when switched to high fat diet, which had comparable adiposity as *Mc4r* knockout mice (Zhang, et al. 2005). Collectively, these studies indicate that the weight gain of *Mc3r* knockout mice is entirely dependent on the increased fat mass and that MC3R is involved in the regulation of feed efficiency and nutrient partition rather than food intake.

However, it is reported that both peripheral and central administration of the selective MC3R agonist D-Trp^{8}-γ-MSH can stimulate food intake (Lee, et al. 2008; Marks, et al. 2006). Furthermore, central administration of a low dose of MC3R antagonist PG-932 leads to transient suppression of food intake and modest decrease in body weight (Lee et al. 2008).

The co-expression of MC3R on POMC neurons within the ARC may account for the mechanism how MC3R regulates food intake. The MC3R mRNA is co-expressed in half of the POMC neurons, and this is a unique feature of MC3R but not MC4R (Cowley, et al. 2001; Jegou et al. 2000). It is hypothesized that MC3R also functions as an auto-inhibitory receptor. The auto-inhibition of MC3R might be mediated through γ-aminobutyric acid (GABA)-regulated inhibitory tone on POMC neurons or via direct inhibition on POMC neurons by MC3R activation (Cowley et al. 2001). Thus, activation of MC3R suppresses melanocortin tone, leading to stimulated food intake, and deletion of MC3R strengthens POMC functions, resulting in the observed relative hypophagia and reduction in lean body mass. MC3R might serve as a 'brake' on MC4R-mediated decrease in food intake and prevent cachexia (Marks et al. 2006). In addition,

mice lacking both MC3R and MC4R are more obese than the mice lacking MC3R or MC4R alone (Chen et al. 2000), suggesting the two receptors serve complementary and non-redundant roles in energy homeostasis.

Recent studies demonstrate that MC3R also serves as a mediator of the entrainment of food intake (Sutton, et al. 2010; Sutton, et al. 2008). Restricted, scheduled feeding can induce food anticipatory behaviors. Unlike WT mice, *Mc3r* knockout mice do not display increased spontaneous activity while on restricted feeding and had altered oscillation patterns of clock gene expression for example Bmal1, Npas2 and Per2 when *ad libitum* fed (Sutton et al. 2008). Further studies investigated hypothalamic FOS-immunoreactivity (FOS-IR) associated with food restriction. In WT mice feeding dramatically increases FOS-IR in dorsomedial hypothalamus (DMH), but not in ventromedial (VMN) or suprachiasmatic hypothalamic nuclei (SCN) compared to *ad libitum* feeding. *Mc3r* knockout mice show a significant decrease in FOS-IR in DMH during feeding. The results further suggest that MC3R is involved in the regulation of food restriction adjustment and MC3R may participate in the regulation of neurons in DMH (Begriche, et al. 2012). Collectively, MC3R plays multiple roles in the regulation of energy homeostasis.

Only 24 non-synonymous mutations in *MC3R* gene have been identified in either obese patients or humans with normal body weight (Calton, et al. 2009; Lee, et al. 2007; Lee, et al. 2002; Mencarelli, et al. 2011; Zegers, et al. 2011). T6K and V81I are two polymorphic variants (Hani, et al. 2001). I183N, the first potential pathogenic mutation was discovered in two patients (father and daughter) in Singapore (Lee et al. 2002). Several functional studies found that this mutation led to impairment in receptor functions (Rached, et al. 2004; Tao and Segaloff 2004).

Also, I183N was demonstrated to cosegregate with childhood obesity (Lee et al. 2007). These discoveries all together indicate that I183N is a pathogenic mutation.

In an Italian cohort three *MC3R* mutations were identified to cosegregate with obesity in the families studied, including A293T, I335S and X361S (Mencarelli, et al. 2008). A293T and X361S had similar ligand binding and signaling properties as WT MC3R, whereas I335S totally lost ligand binding and signaling due to intracellular retention, suggesting that I335S may contribute to human obesity (Tao 2007). The impact of A293T and X361S in the pathogenesis of obesity requires further study.

Not all the mutations in *MC3R* gene lead to impairment in receptor functions. However, not all the aspects of receptor functions, including expression, ligand binding, signaling, or activation of calcium or MAPK signaling, receptor internalizaiton, desensitization and recycling were investigated in individual studies. Further investigation, including applying neuronal cells as expression system, is still required. In addition, like *MC4R* mutations, not all the loss-of-function mutations in *MC3R* will lead to obesity (Calton et al. 2009; Tao and Segaloff 2005), suggesting that *MC3R* mutations may not be fully penetrant in the pathogenesis of obesity.

As mentioned above, *Mc3r* knockout mice do not exhibit significantly increased body weight compared to WT mice, however, they have increased fat mass and decreased lean body mass. This discovery indicates that clinical research addressing human energy homeostasis and *MC3R* mutations should pay more attention to adiposity, rather than the criterion like BMI. Recently, the need for ‘deep phenotyping’ in human genetic studies of obesity is highlighted (Muller, et al. 2010). Put together, the specific role of MC3R in human homeostasis is still

controversial. More detailed studies, including *in-vivo* studies applying transgenic animals or cosegregation studies, are still required.

Summary

Obesity has become an alarming worldwide epidemic. It is associated with insulin resistance, hyperglycemia, hypertension and dyslipidemia and therefore results in diabetes mellitus and cardiovascular disease. It has tremendous health, economic and social burdens. Hence, there is enormous interest in understanding the molecular mechanisms accounting for the regulation of energy homeostasis and the pathogenesis of human obesity.

It is well recognized that obesity is the result of interactions of environmental and genetic factors. The fact that people in the similar environment have variable susceptibility to obesity indicates the important role of genetic factors in pathogenesis of obesity. The genetic determinants of body weight are still poorly studied, but a number of genes have been identified leading to obesity when mutated, including genes encoding leptin, leptin receptor, POMC, and MC4R. MC3R, however, although having the similar distribution in brain as MC4R, needs further investigation of its role in the pathogenesis of human adiposity.

The present study performed detailed functional studies of novel naturally occurring mutations in human *MC3R* gene *in vitro*, to obtain a better understanding of structure-function relationship of MC3R, and to lay the groundwork for future investigation of the pathogenic role of *MC3R* in human obesity or adiposity.

Chapter 2

2.1 Introduction

Obesity, due to imbalance of energy intake and energy expenditure, has become an alarming epidemic in the western world. The causes of obesity include environmental, psychosocial, and genetic factors (Spiegelman and Flier 2001). Through the studies of identifying genetic factors of obesity, mutations in several genes encoding proteins involved in regulation of energy homeostasis have been found to be associated with obesity (Clement et al. 1998; Farooqi et al. 2003; Hinney, et al. 2010; Krude, et al. 1998).

The melanocortin-3 receptor (MC3R) is considered a potential regulator of energy homeostasis (Butler et al. 2000; Chen et al. 2000; Zhang et al. 2005). It is a member of family A (rhodopsin-like) G protein-coupled receptors (GPCRs) (Gantz et al. 1993a; Roselli-Rehfuss et al. 1993). The principal endogenous ligands of MC3R are the small peptides cleaved from the proopiomelanocortin (POMC), including α- and γ-melanocyte-stimulating hormones (MSH). The endogenous antagonist for MC3R is Agouti-related protein (Ellacott and Cone 2006; Gantz and Fong 2003), which we showed to be also an inverse agonist for the MC3R (Tao et al. 2010). MC3R is expressed in brain, placenta, stomach, duodenum, pancreas (Gantz et al. 1993a), kidney (Humphreys 2004), as well as macrophages (Getting et al. 2008). The highest density of MC3R mRNA has been found in hypothalamus, especially the ventromedial hypothalamic nucleus, the arcuate nucleus and the posterior hypothalamic area (Jegou et al. 2000). Since hypothalamus

plays a key role in controlling energy homeostasis (Cone 1999; Gantz and Fong 2003), it was hypothesized that MC3R is involved in the regulation of energy homeostasis.

Genetic studies in rodents showed that in the absence of *Mc3r*, mice did not exhibit hyperphagia (Butler et al. 2000; Chen et al. 2000). Due to a higher feed efficiency and alterations in nutrient partitioning, *Mc3r* knockout mice have increased fat mass, reduced lean mass, and normal metabolism with normal or even slightly decreased food intake (Butler et al. 2000; Chen et al. 2000; Zhang et al. 2005). In situ hybridization indicated that MC3R mRNA is distributed similarly with that of POMC mRNA in the arcuate nucleus, suggesting that MC3R also served as an inhibitory autoreceptor on POMC neurons and therefore stimulate food intake (Marks et al. 2006). Recently, it is also reported that MC3R plays a critical role in entrainment to restricted feeding (Sutton et al. 2010; Sutton et al. 2008).

The role of *MC3R* mutation in human obesity is controversial. Unlike MC4R, where over 150 mutations have been identified (Tao 2010a), relatively few *MC3R* mutations have been reported (Calton et al. 2009; Feng, et al. 2005; Lee et al. 2007; Lee et al. 2002; Mencarelli et al. 2011; Mencarelli et al. 2008; Zegers et al. 2011) (reviewed in (Tao 2010b)). Recently, nine novel naturally occurring *MC3R* mutations were reported in Singaporean patients, two North American cohorts, and a Finnish cohort (Calton et al. 2009; Lee et al. 2007; Valli-Jaakola 2007) (Fig. II-1), but the functions of these mutations were incompletely characterized. In this study, we performed detailed functional analysis of these mutations in vitro.

2.2 Materials and Methods

2.2.1 Plasmid and peptides

Wild-type (WT) human MC3R cDNA tagged with 3×HA tags at the N-terminus inserted in vector pcDNA3.1 was obtained from Missouri S&T cDNA Resource Center (http://www.cDNA.org/, Rolla, MO). [Nle^4, D-Phe^7]-α-MSH (NDP-MSH) was purchased from Bachem (King of Prussia, PA). α-MSH was purchased from Phoenix Pharmaceuticals (Belmont, CA). D-Trp^8-γ-MSH was obtained from Peptides International (Louisville, KY). ^{125}I-NDP-MSH was obtained from the Peptide Radioiodination Service Center at the University of Mississippi (University, MS).

2.2.2 Site-directed mutagenesis of human MC3R

Human *MC3R* mutations were generated by QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). Plasmids were prepared using IsoPure DNA purification kits from Denville Scientific (Metuchen, NJ). Automated DNA sequencing was performed by the DNA Sequencing Facility of University of Chicago Cancer Research Center (Chicago, IL) to confirm that the correct mutation was generated and no errors were introduced during the cycling reactions.

2.2.3 Cells and transfections

Human Embryonic Kidney (HEK) 293T cells, obtained from American Type Culture Collection (Manassas, VA), were grown at 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 50 μg/ml of gentamicin, 100 IU/ml of penicillin, 100 μg/ml of streptomycin, 20μg/ml of amphotericin B, 10 mM Hepes and 10% newborn calf serum. Cells were plated on gelatin-coated 35mm 6-well clusters from Corning (Corning, NY). When the cells reached 50-70% confluency, transient transfection was performed with the calcium

precipitation method (Chen and Okayama 1987). One microgram of plasmid in 2ml media was used per 35mm well. Cells were incubated for approximately 48h after transfection before used to measure ligand binding and ligand-stimulated cAMP generation.

2.2.4 Ligand binding to intact cells

Forty-eight hours after transfection, cells were washed twice with warm Waymouth's MB752/1 media (Sigma-Aldrich, St. Louis, MO) containing 1mg/ml bovine serum albumin (BSA) (referred to as Waymouth/BSA). Fresh Waymouth/BSA was added to each well, incubated with 50 μl of ^{125}I-NDP-MSH (~100,000 cpm) with or without different concentrations of unlabeled NDP-MSH, α-MSH or D-Trp^{8}-γ-MSH at 37 C for 1h. The total volume in each well was 1 ml. The final concentrations of unlabeled ligands are indicated in the figures. After incubation, cells were washed twice with cold Hank's balanced salt solution (Sigma-Aldrich) containing 1 mg/ml BSA to terminate the reaction. Then cells of each well were solubilized with 100μl of 0.5M NaOH. Cell lysates were collected with cotton swabs and counted in a gamma counter. All determinations were performed in duplicate and the experiment was performed at least three times. Apparent maximal binding capacity (Bmax) and concentrations that lead to 50% inhibition (IC_{50}) values were calculated using GraphPad Prism 4.0 (San Diego, CA).

2.2.5 Ligand-stimulated cAMP generation

After 48h incubation, transfected HEK-293T cells were washed twice with warm Waymouth/BSA. Then fresh Waymouth/BSA containing 0.5mM isobutylmethylxanthine (Sigma-Aldrich) was added to each well. After 15 min incubation at 37 C, either Waymouth/BSA alone or different concentrations of ligands were added. The total volume in each well was 1 ml. The final concentrations of the ligands are indicated in the figures. After 1h

incubation at 37 C, cells were then placed on ice to terminate the reaction, media were aspirated and intracellular cAMP were extracted with 0.5N percholoric acid containing 180μg/ml theophylline. The cAMP concentrations were measured by radioimmunoassay (Fan et al. 2008; Tao et al. 2010). All determinations were performed in triplicate and the experiment was performed at least three times. Maximal responses (Rmax) and EC_{50} values were calculated by GraphPad Prism 4.0.

2.2.6 Localization of MC3R expression by confocal microscopy

HEK293 cells stably transfected with WT or mutant hMC3Rs containing 3×HA tags at the N-terminus were plated onto poly-D-lysine-coated slides (Biocoat cellware from Falcon) and were immunostained with Alexa Flour 488-conjugated anti-HA.11 antibody on the third day using a protocol based on our previous report (Tao and Segaloff 2003). Briefly, on the day of experiment, cells were washed with filtered phosphate buffered saline for immunohistochemistry (PBS-IH) for three times and then were fixed with 4% paraformaldehyde in PBS-IH for 30 min. After three more washes with PBS-IH, cells were incubated with blocking solution (5% BSA in PBS-IH) for 1h at room temperature, and incubated with Alexa Fluor 488-labeled anti-HA.11 antibody (Covance, Berkeley, CA) diluted 1:100 in PBS-IH containing 0.5% BSA for 1h at room temperature in the dark. Then the cells were washed five times with PBS-IH and covered with VectaShield Mounting Media (Vector Laboratories) and a glass coverslip. Images were collected with a Bio-Rad laser scanning confocal microscope with excitation by 488nm argon laser and detected with a 530- to 560-nm filter.

2.2.7 Quantification of MC3R expression by flow cytometry

Flow cytometry was performed as described previously (Fan and Tao 2009; Wang, et al. 2008; Wang and Tao 2011). Briefly, HEK293 cells were plated on gelatin-coated 35mm 6-well clusters and transfected with 4 micrograms of plasmid in 2ml media for each well. After 48h incubation, cells were washed once with filtered PBS for immunohistochemistry (PBS-IH), detached, and then precipitated by centrifugation at 500 × g. Cells were fixed by 4% paraformaldehyde in PBS-IH for 30 min, and for measuring total expression, cells were permeabilized with 1% Triton X-100 in PBS-IH for 4 min (this step was omitted for measuring cell surface expression). Cells were then blocked with PBS-IH containing 5% BSA for 1h. The cells were then stained with primary anti-HA.11 antibody (Covance) diluted 1:50 in PBS-IH containing 0.5% BSA for 1h, and washed with PBS-IH containing 0.5% BSA. The secondary Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, CA) diluted 1:2000 in PBS-IH containing 0.5% BSA was added under low light. After 1h incubation, cells were washed and resuspended in PBS-IH with 0.5% BSA. An Accuri flow cytometer with a 488-nm wavelength laser was applied to quantify hMC3R mutant expression in 15,000 cells from each transfection. Cells transfected with pcDNA3.1 empty vector were used as control for background staining. All the incubations and washes were performed at room temperature. The expression levels of the mutants were calculated as percentage of WT hMC3R expression as described before using the formula: [mutant – pcDNA3]/[WT – pcDNA3] x100% (Fan and Tao 2009; Wang et al. 2008; Wang and Tao 2011). Samples of cells transfected with pcDNA3.1 empty vector or WT hMC3R were included in each experiment and processed together with the samples transfected with mutant hMC3Rs.

2.2.8 Statistical analyses

Student's *t* test was used to determine the significance of differences in the expression, ligand binding and signaling parameters between WT and mutant hMC3Rs. Statistical analysis was carried out using GraphPad Prism 4.0.

2.3 Results

2.3.1 Signaling properties of the mutant hMC3Rs in response to NDP-MSH, D-Trp^8-γ-MSH or α-MSH stimulation

In order to investigate whether the missense mutations in human *MC3R* cause any alterations in the function of the receptor, the levels of intracellular cAMP generated in response to the agonists were measured.

We first studied the signaling response to NDP-MSH stimulation. NDP-MSH is a superpotent analog of MCRs except MC2R (Sawyer, et al. 1980). As expected, NDP-MSH caused dose-dependent increases in intracellular cAMP levels in cells transfected with WT hMC3R. Fig. II-2A showed the results of a typical experiment. In response to NDP-MSH, all the mutants showed similar EC_{50} as that of WT hMC3R. S69C and T280S displayed significant reduction in the maximal levels of cAMP generation compared to that of WT hMC3R (Table 2.1). The other mutants had apparent normal signaling.

Then the signaling property was tested with D-Trp^8-γ-MSH (Fig. II-3A), which is a superpotent and more selective analog of γ-MSH (Grieco, et al. 2000). γ-MSH is a potent endogenous MC3R agonist. With the stimulation of D-Trp^8-γ-MSH, similar results to NDP-MSH

stimulation were obtained (Table 2.2). Only S69C and T280S had significant decreases in maximal signaling compared to WT hMC3R (Table 2.2).

The signaling property was also investigated with the stimulation of α-MSH, an endogenous agonist (Fig. II-4A). The EC_{50} of WT hMC3R for α-MSH was about 5-fold higher than that for NDP-MSH. S69C and T280S displayed significantly increased EC_{50} and significantly decreased maximal signaling (Table 2.3). All the other mutants had normal signaling properties compared to WT hMC3R.

Unlike the human MC4R, which has significant constitutive activity that can easily be measured, the WT hMC3R has little constitutive activity (Tao 2007). The basal cAMP levels in cells transfected with WT hMC3R were similar to the basal cAMP levels in cells transfected with empty vector pcDNA3 (Tao 2007). In the current study, none of the mutants had increased basal activity compared to WT hMC3R (Fig. II-5). S69C and T280S had small but statistically significant decreases in basal activities (Fig. II-5).

2.3.2 Ligand binding of the mutant hMC3Rs

To investigate whether the mutants had normal binding activity, competitive ligand binding assays were performed. The three ligands used above were used as the competitors. When NDP-MSH was used as ligand (Fig. II-2B), S69C and T280S showed significantly decreased IC_{50}s (Table 2.1). All the other mutants had similar IC_{50}s as the WT hMC3R. When D-Trp^8-γ-MSH was used as the competitor (Fig. II-3B), only T280S showed significantly reduced IC_{50} (Table 2.2) whereas the other mutants had similar IC_{50}s as the WT hMC3R. When α-MSH

was used as the competitor (Fig. II-4B), three mutants (S69C, I87T and T280S) had significantly decreased IC_{50}s whereas the other mutants had similar IC_{50}s as the WT hMC3R (Table 2.3).

As shown in Fig. II-6, four mutants (S69C, M134I, M275T and T280S) showed significantly reduced maximal binding. All the other mutants had relatively normal maximal binding.

2.3.3 Localization studies of mutant hMC3Rs by confocal microscopy

To investigate the localization of these mutants, especially the mutants that displayed functional defects, confocal microscopy was performed. The Alexa-Fluor labeled HA antibody, which selectively binds to the three-tandem HA epitope tags at the N-terminus of hMC3R, revealed the localization of hMC3R. As shown in Fig. II-7, every mutant was expressed at the cell surface, including the mutants that had significant defects in ligand binding and signaling properties, such as S69C and T280S.

2.3.4 Quantification of mutant hMC3R expression by flow cytometry

To quantitate mutant hMC3R expression, flow cytometry was performed. The data in non-permeabilized cells showed that all nine mutants had significantly decreased cell surface expression compared to WT hMC3R (Fig. II-8A). As shown in Fig. II-8B, the results in permeabilized cells showed that A260V, M275T, and L297V had small, but statistically significant decreases in total expression levels. The other six mutants had similar total expression levels as that of WT hMC3R.

2.3.5 Co-expression studies of hMC3R mutations

To investigate whether the mutant hMC3Rs had any dominant negative activity on WT hMC3R signaling, co-expression experiments were performed. WT and empty vector or mutant hMC3Rs were co-transfected into HEK293T cells with the ratio of 1:1. As shown in Fig. II-9, L297V exhibited slight inhibition on WT hMC3R signaling. All the other mutants had no dominant negative activity on WT hMC3R signaling (Fig. II-9 and Table 2.4).

2.3.6 Multiple mutagenesis of T280

Although retaining some binding activity, T280S was almost devoid of signaling, which attracted our attention. The only difference between threonine and serine is that threonine has an extra methyl group. To further investigate the importance of T280 in hMC3R, multiple mutations were generated, changing T280 to hydrophobic (Ala, Leu and Tyr), polar (Cys), negatively charged (Asp), or positively charged (Arg) residues. The ligand binding and signaling properties of these mutants were measured using NDP-MSH as the ligand (Fig. II-10 and Table 2.5). The results showed that no ligand binding could be detected for T280A, T280C, T280R and T280Y. The maximal binding for T280L was significantly decreased, whereas T280D had similar maximal binding as the WT hMC3R. T280D and T280L had similar IC_{50} to that of WT hMC3R. Signaling experiments showed that T280A, T280C, T280D, T280R, and T280Y had no response to NDP-MSH stimulation. T280L had similar EC_{50} as WT hMC3R, but the maximal signaling was significantly decreased (Fig. II-10 and Table 2.5).

The results of flow cytometry showed that in non-permeabilized cells, all mutants were expressed at the cell surface, but at significantly lower levels compared to the WT hMC3R (Fig.

II-8A). In permeabilized cells, all mutants had similar total expression levels as the WT hMC3R (Fig. II-8B).

2.4 Discussion

In this study, we performed detailed functional analysis of nine naturally occurring *MC3R* mutations that have not been studied in detail before. Among these nine mutations, S69C was identified in a lean person, and the other mutations were found in obese patients (Calton et al. 2009; Lee et al. 2007; Valli-Jaakola 2007). All mutants had decreased cell surface expression. Six mutants had normal total protein expression. A260V, M275T, and L297V had small, although statistically significant, decreases in total protein expression. Four mutants (S69C, M134I, M275T and T280S) had significantly decreased maximal binding and two mutants (S69C and T280S) had significant defects in signaling.

To investigate whether the mutant hMC3Rs were mislocalized, we performed confocal microscopy and flow cytometry. Confocal microscopy in non-permeabilized cells showed that all mutants were expressed on the cell surface (Fig. II-7) but flow cytometry showed that cell surface expression levels were significantly decreased to about 50~75% of WT hMC3R (Fig. II-8A). Flow cytometry in permeabilized cells showed that six mutants had normal total protein expression levels whereas A260V, M275T, and L297V had small, although statistically significant, decreases in total protein expression levels (Fig. II-8B). These results suggest that six mutants, including S69C, A70T, I87T, M134I, L249V, and T280S, were partially retained intracellularly due to misfolding. The majority of GPCR mutations causing human diseases belong to this class (Tao 2006) and intracellular retention comprises the majority of MC4R mutations causing obesity (Tao 2005).

A260V, M275T, and L297V had small decreases in total protein expression levels (Fig. II-8B). Premature termination and frameshift mutations are predicted to cause decreased protein levels, partly due to nonsense-mediated mRNA decay (Tao 2006). However, only a few examples were reported for missense mutations that result in decreased total protein expression. A naturally occurring synonymous mutation of human dopamine D2 receptor was reported to markedly change mRNA stability through changing mRNA secondary structure and therefore caused decreased protein expression (Duan, et al. 2003). Whether similar mechanism accounts for the decreased total expression for the three hMC3R mutations in the present study remains to be studied. We reported recently that several missense mutations in the *MC4R* gene also cause decreased total expression levels (Fan and Tao 2009; Wang and Tao 2011). It should be emphasized that total expression levels were not measured in many of the functional studies on naturally occurring mutations in GPCRs that cause human diseases.

Mutant T280S exhibited dramatic functional defects in both binding and ligand-stimulated intracellular cAMP generation (Figs. II-2~II-6 and Tables 2.1-2.3). T280S also had significantly reduced cell surface expression (Figs. II-7 and II-8). The functional defects of T280S might be due to, at least partially, the decrease in cell surface expression. Comparing binding and signaling data suggested that it was also defective in signaling *per se*. $T280^{6.36}$ sits deeply in the transmembrane domain 6 (TM6), near the intracellular surface (6.36 is a nomenclature system illustrating the position of a residue in TM, (Ballesteros and Weinstein 1995)). TM6 plays an important role in GPCR signaling mechanism (Millar and Newton 2010). Therefore, we generated additional mutations at codon 280 changing Thr to different classes of amino acids and performed detailed functional analysis (Fig. II-10). The results showed that all these mutants were expressed at the cell surface, but had significantly decreased cell surface

expression levels (Fig. II-8A), although the total expression levels were normal (Fig. II-8B). No binding or signaling could be detected for T280A, T280C, T280R and T280Y. Therefore these mutants were defective in ligand binding. T280D was a signaling defective mutant with normal ligand binding. T280L displayed similar EC_{50} and IC_{50} as WT hMC3R, but had significantly decreased maximal binding and signaling (Fig. II-10 and Table 2.5). We suggested that for T280L, due to decreased cell surface expression, maximal binding and signaling were decreased. Our data demonstrated the importance of T280 in different aspects of hMC3R functions including receptor expression, ligand binding and signaling. Changing Thr to Ser that has similar structure as Thr caused significant functional defects; however, mutating the polar Thr to the negatively charged Asp or hydrophobic nonpolar Leu maintained partial receptor function. These results demonstrated that it is not reliable to predict the functional ramification of mutations simply based on their amino acid characteristics. Instead detailed functional studies are necessary.

Mutants M134I and M275T had decreased maximal ligand binding (Fig. II-6), consistent with their reduced cell surface expression. But they had normal signaling (Tables 2.1-2.3), suggesting the presence of spare receptor (Tao 2005). A70T and M134I were identified in two unrelated Asian children and their obese mothers. Both mother and child carrying M134I had type 2 diabetes,(Lee et al. 2007) indicating that decreased cell surface expression might cause decreased signaling in vivo.

Previously, functional studies were reported for several naturally occurring *MC3R* mutations. For I183N, first reported in 2002 (Lee et al. 2002), three studies showed that the mutation causes a total loss of signaling (Lee et al. 2007; Rached et al. 2004; Tao and Segaloff

2004). Three mutations, A293T, I335S, and X361S, were identified from an Italian cohort (Mencarelli et al. 2008). Two groups independently showed that I335S is a loss of function mutation, whereas no significant defect could be identified for A293T and X361S (Mencarelli et al. 2008; Tao 2007). Recent genetic studies in Europe reported new *MC3R* mutations in obese patients causing dramatic functional defects (Mencarelli et al. 2011; Zegers et al. 2011). Of the three mutations identified from an obese cohort in Belgium (N128S, V211I, and L299V), functional studies showed that L299V has impaired function due to decreased cell surface expression resulting in defective signaling, whereas although N128S and V211I are expressed on the cell surface, N128S has increased EC_{50} and V211I has decreased maximal response in response to α-MSH stimulation (Zegers et al. 2011). Another study showed that the prevalence of functionally relevant mutations is significantly higher in the obese group in Italian and French cohorts (Mencarelli et al. 2011).

Because the mutations were identified in heterozygous state, we performed co-transfection experiments to investigate whether the mutant receptor had dominant negative effect on the WT receptor signaling. Our data showed that most of the mutants have no dominant negative effect (Fig. II-9 and Table 2.4). Only L297V had small, although significant, effect on WT hMC3R signaling (Fig. II-9 and Table 2.4). These data were consistent with previous studies demonstrating lack of dominant negative effect on the other *MC3R* mutations (Tao 2007; Tao and Segaloff 2004), similar to the *MC4R* mutations (Cone 2000; Roth, et al. 2009). It has been reported that MC3R homodimerize (Mandrika, et al. 2005) and dimerization is considered to be responsible for dominant negative activity exerted by some mutant GPCRs (Tao and Segaloff 2004) including MC4R (Biebermann et al. 2003; Mandrika et al. 2005). Whether the mutant receptors studied herein can heterodimerize with the WT receptor remains to be investigated.

In the related MC4R, more than 150 distinct mutations have been reported and mutations in the MC4R are widely accepted to be the most common monogenic form of obesity and an important contributor to polygenic obesity (reviewed in (Tao 2010a)). Genetic, clinical, and pharmacological data from numerous studies were supportive of this hypothesis. However, relatively few mutations in the MC3R have been identified in humans and their relevance in obesity pathogenesis is still hotly debated (reviewed in (Tao 2010b)). At least twenty-four mutations in the MC3R have been identified in humans. In a recent editorial, we suggested that because of the phenotype of Mc3r knockout mice (increased fat mass with normal body weight), human genetic studies on the MC3R mutation and disorders in energy balance should pay special attention to the body fat (adiposity) instead of simple body weight (for example, using body mass index) (Tao 2010b), the so-called "deep phenotyping" (Muller et al. 2010). Large-scale molecular genetic studies (quantitatively or case-control, population-based or extreme groups), with thorough phenotyping, combined with detailed functional studies as presented in this manuscript, will likely help to clarify the potential pathogenic role of MC3R mutation in human energy balance disorder (Tao 2010b).

In summary, we obtained detailed functional data of nine naturally occurring *MC3R* mutations. We demonstrated that T280S might be a possible pathogenic cause for human obesity and T280 played an important role in MC3R expression, binding and signaling, contributing to a better understanding of structure-function relationship of MC3R. Whether and how the mutants that did not affect signaling are related to human adiposity remains to be further studied, preferably vetted in vivo. Their decreased cell surface expression likely will have physiological consequence. The accumulating literature supports the hypothesis that *MC3R* mutations are associated with human obesity or adiposity, predisposing the carrier to obesity in the current

obesogenic environment. This predisposition is most likely due to haploinsufficiency rather than dominant negative activity.

Table 2.1 Binding and signaling properties of WT and mutant hMC3Rs with NDP-MSH as the ligand.

hMC3R	n	NDP-MSH binding	NDP-MSH-stimulated cAMP	
		IC_{50} (nM)	EC_{50} (nM)	*R*max (% WT)
WT	8	2.06±0.37	0.36±0.02	100.00
S69C	3	0.81±0.04[b]	0.39±0.07	54.67±8.49[a]
A70T	3	1.88±0.56	0.37±0.13	103.96±2.18
I87T	3	1.08±0.11[a]	0.29±0.09	91.05±10.14
M134I	3	1.94±0.49	0.51±0.08	80.31±12.26
L249V	3	1.98±0.41	0.43±0.12	84.98±18.29
A260V	3	1.53±0.03	0.52±0.17	91.96±18.09
M275T	3	2.22±0.65	0.46±0.04	89.72±7.27
T280S	3	1.07±0.13[a]	0.29±0.15	12.86±5.64[b]
L297V	3	2.30±0.66	0.49±0.23	106.87±14.46

[a] Significantly different from the WT hMC3R, $p < 0.05$.

[b] Significantly different from the WT hMC3R, $p < 0.01$.

The data are expressed as the mean ± SEM for the mutant hMC3Rs. The maximal responses (*R*max) were 2142 ± 507.5 pmol cAMP/10^6 cells for WT hMC3R.

Table 2.2 Binding and signaling properties of WT and mutant hMC3Rs with D-Trp^8-γ-MSH as the ligand.

hMC3R	n	D-Trp^8-γ-MSH binding	D-Trp^8-γ-MSH-stimulated cAMP	
		IC_{50} (nM)	EC_{50} (nM)	*R*max (% WT)
WT	15	9.86±2.47	0.76±0.08	100.00
S69C	3	8.20±2.38	0.57±0.11	42.59±1.40[c]
A70T	3	4.38±1.27	0.95±0.24	145.97±40.50
I87T	3	4.49±1.52	0.38±0.10[b]	82.38±13.93
M134I	3	18.98±1.26	1.26±0.13	128.96±31.04
L249V	3	6.19±1.25	0.52±0.07	111.78±7.54
A260V	3	4.99±1.49	1.01±0.17	99.85±33.38
M275T	3	4.49±0.21	0.45±0.04	119.87±29.30
T280S	3	0.52±0.05[a]	2.17±0.48	3.76±1.00[b]
L297V	3	4.53±1.51	0.46±0.02	137.78±19.95

[a] Significantly different from the WT hMC3R, $p < 0.05$.

[b] Significantly different from the WT hMC3R, $p < 0.01$.

[c] Significantly different from the WT hMC3R, $p < 0.001$.

The data are expressed as the mean ± SEM for the mutant hMC3Rs. The maximal responses (*R*max) were 1747.31 ± 433.39 pmol cAMP/10^6 cells for WT hMC3R.

Table 2.3 Binding and signaling properties of WT and mutant hMC3Rs with α-MSH as the ligand.

hMC3R	n	α-MSH binding	α-MSH-stimulated cAMP	
		IC_{50} (nM)	EC_{50} (nM)	*R*max (% WT)
WT	13	36.68±6.50	1.68±0.19	100.00
S69C	4	6.75±1.57[a]	4.42±0.59[a]	36.34±0.89[c]
A70T	3	30.39±9.27	2.42±0.09	194.46±79.36
I87T	3	6.60±1.75[a]	1.96±0.52	107.42±22.47
M134I	3	31.1±13.28	7.48±2.05	129.69±39.45
L249V	3	27.73±1.81	3.69±0.94	133.33±28.31
A260V	3	26.00±3.69	2.07±0.51	97.42±21.32
M275T	3	24.76±8.99	3.64±0.87	71.97±18.10
T280S	3	3.44±0.60[a]	22.39±5.72[a]	4.20±3.32[b]
L297V	3	32.31±10.73	2.37±0.37	145.61±31.40

[a] Significantly different from the WT hMC3R, $p < 0.05$.

[b] Significantly different from the WT hMC3R, $p < 0.01$.

[c] Significantly different from the WT hMC3R, $p < 0.001$.

The data are expressed as the mean ± SEM for the mutant hMC3Rs. The maximal responses (*R*max) were 1407.77 ± 411.25 pmol cAMP/10^6 cells for WT hMC3R.

Table 2.4 NDP-MSH-stimulated signaling in cells co-transfected with WT and/or mutant hMC3Rs.

hMC3R	n	NDP-MSH-stimulated cAMP	
		EC_{50} (nM)	*R*max (% WT)
WT + pcDNA	5	1.67±0.41	100.00
WT + S69C	3	1.66±0.71	94.05±6.76
WT + A70T	3	3.13±1.96	121.06±34.59
WT + I87T	3	1.41±0.47	95.62±13.69
WT + M134I	3	0.55±0.14	80.74±6.23
WT + L249V	3	1.04±0.18	86.39±8.48
WT + A260V	3	1.42±0.31	85.63±9.52
WT + M275T	3	0.48±0.09	92.66±17.08
WT + T280S	4	1. 50±0.23	81.20±22.68
WT+ L297V	3	0.90±0.35	78.89±2.32[a]

[a] Significantly different from WT hMC3R + pcDNA, $p < 0.05$.

The data are expressed as the mean ± SEM for the mutant hMC3Rs. The maximal responses (*R*max) were 2452.40 ±308.11 pmol cAMP/10^6 cells for WT hMC3R + pcDNA.

Table 2.5 Binding and signaling properties of multiple mutations at T280 with NDP-MSH as the ligand.

hMC3R	n	NDP-MSH binding		n	NDP-MSH-stimulated cAMP	
		IC_{50} (nM)	*B*max (% WT)		EC_{50} (nM)	*R*max (% WT)
WT	3	4.56±2.24	100.00	3	0.63±0.41	100.00
T280A	3	ND	ND	3	ND	ND
T280C	3	ND	ND	3	ND	ND
T280D	3	3.91±1.68	115.97±10.06	3	ND	ND
T280L	3	4.06±2.26	37.78±8.48[a]	3	1.47±1.18	26.35±3.15[b]
T280R	3	ND	ND	3	ND	ND
T280Y	3	ND	ND	3	ND	ND

[a] Significantly different from the WT hMC3R, $p < 0.05$.

[b] Significantly different from the WT hMC3R, $p < 0.01$.

ND, could not be determined.

The data are expressed as the mean ± SEM for the mutant hMC3Rs. The maximal responses (*R*max) were 3220.33 ±720.98 pmol cAMP/10^6 cells for WT hMC3R.

Figure II-1. **Schematic model of human MC3R with the mutations studied in Chapter 2 highlighted.**

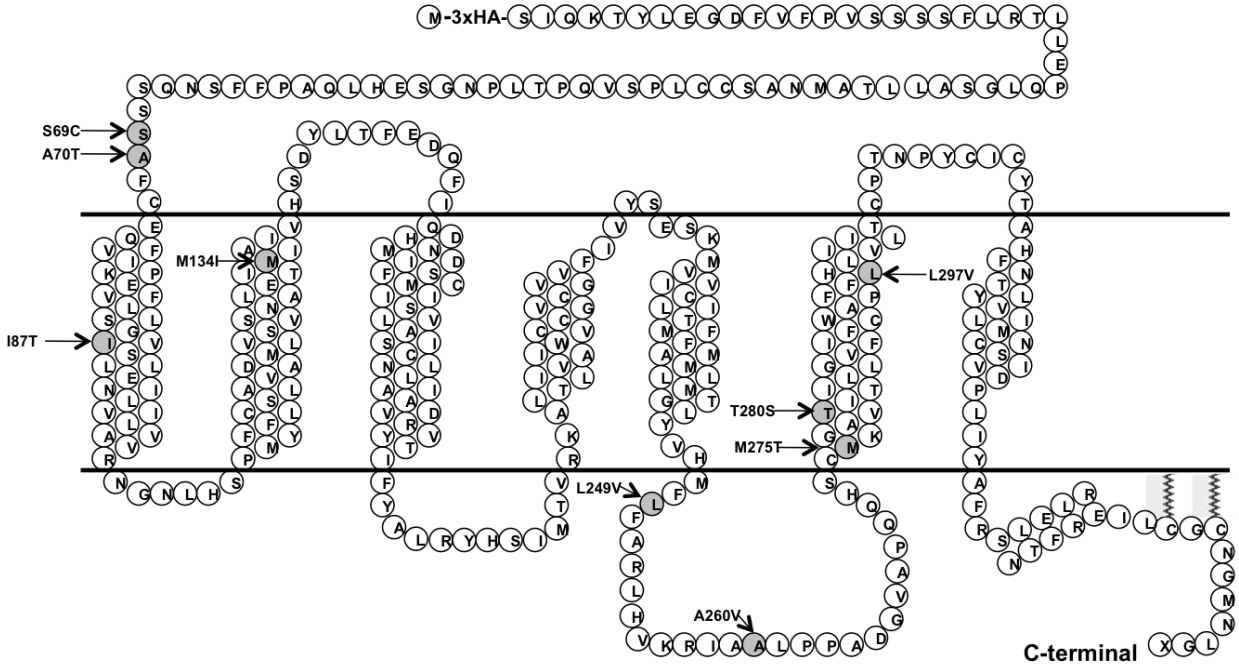

M-3xHA-
S69C
A70T
M134I
I87T
T280S
M275T
L297V
L249V
A260V
C-terminal

Figure II-2. Signaling and ligand binding properties of WT and mutant hMC3Rs with NDP-MSH as the ligand. HEK293T cells were transiently transfected with the indicated hMC3R constructs and binding and signaling of the hMC3Rs were measured as described in Materials and Methods. In (A), HEK293T cells transiently transfected with the indicated hMC3R constructs were stimulated with different concentrations of NDP-MSH. Intracellular cAMP levels were measured using RIA. Results are expressed as the mean ± SEM of triplicate determinations within one experiment. In (B), different concentrations of unlabeled NDP-MSH were used to displace the binding of ^{125}I-NDP-MSH to hMC3Rs on intact cells. Results shown are expressed as % of WT binding ± range from duplicate determinations within one experiment. All experiments were performed at least three times.

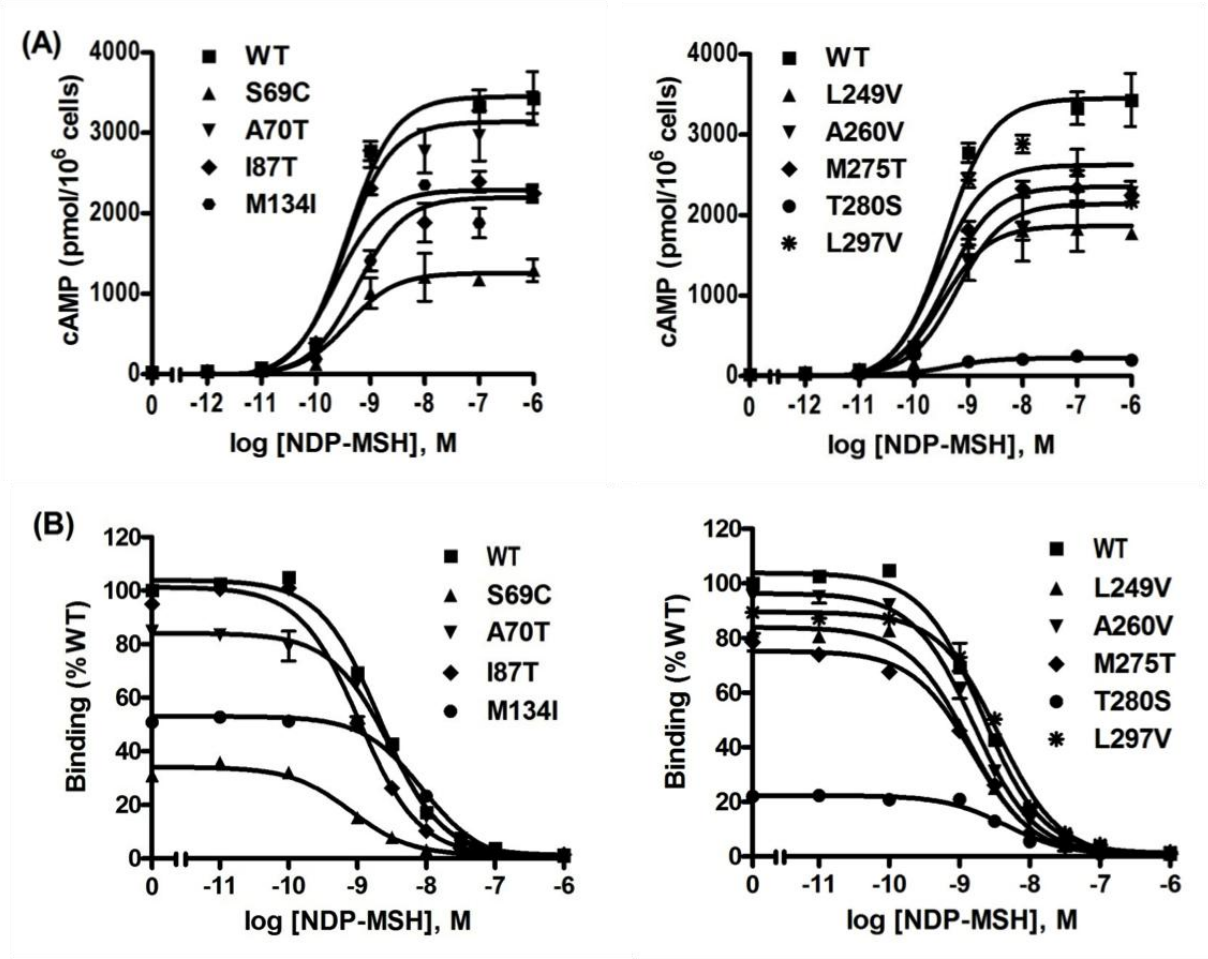

(A)
cAMP (pmol/10^6 cells)
4000
3000
2000
1000
0
WT
S69C
A70T
I87T
M134I
0 -12 -11 -10 -9 -8 -7 -6
log [NDP-MSH], M
cAMP (pmol/10^6 cells)
WT
L249V
A260V
M275T
T280S
L297V
log [NDP-MSH], M
(B)
Binding (% WT)
120
100
80
60
40
20
0
WT
S69C
A70T
I87T
M134I
0 -11 -10 -9 -8 -7 -6
log [NDP-MSH], M
Binding (% WT)
WT
L249V
A260V
M275T
T280S
L297V
log [NDP-MSH], M

Figure II-3. **Signaling and ligand binding properties of WT and mutant hMC3Rs with D-Trp^{8}-γ-MSH as the ligand.** See the legend to Fig. II-2 for details. The only difference is that D-Trp^{8}-γ-MSH was used as the ligand instead of NDP-MSH.

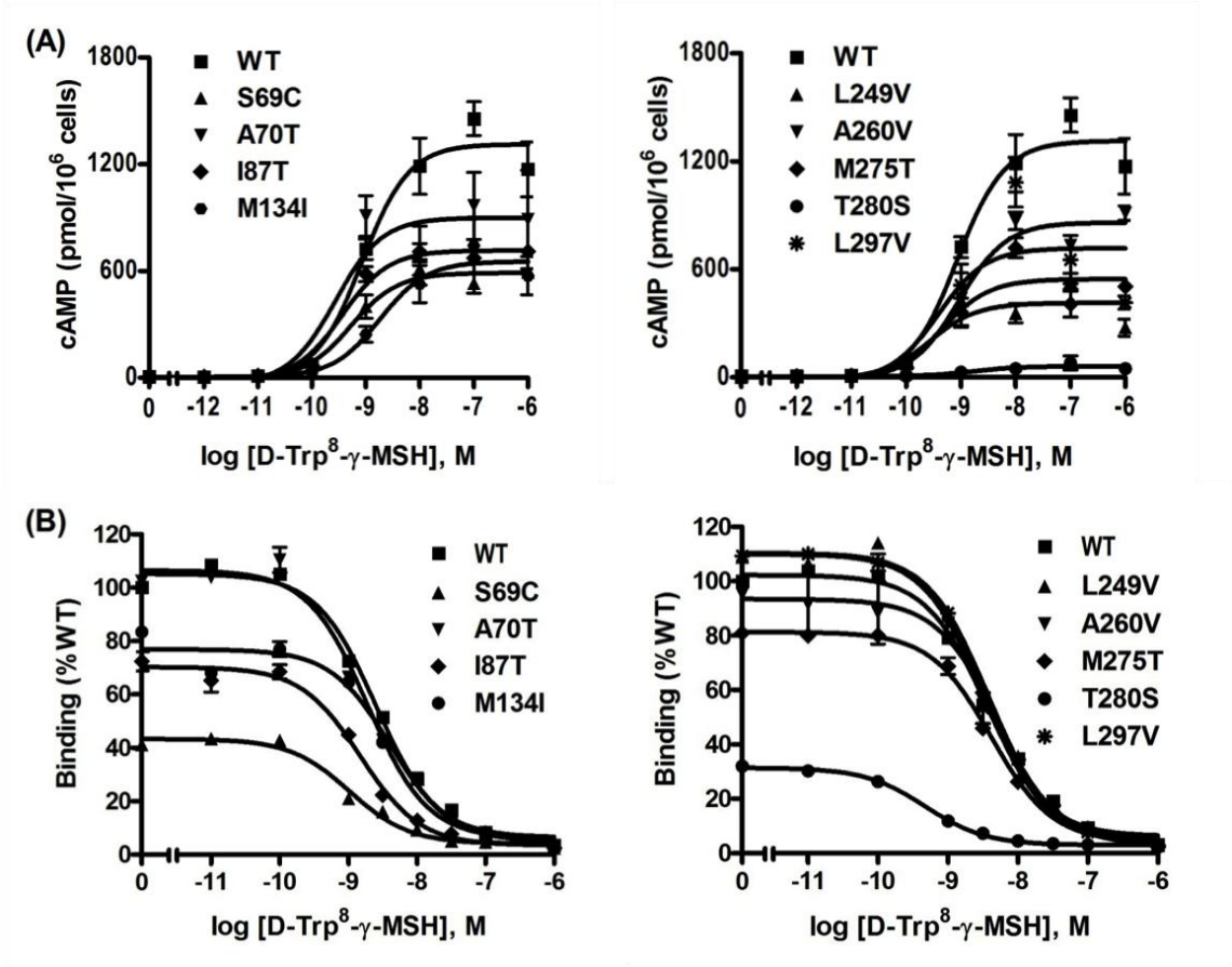

(A)
cAMP (pmol/10^6 cells)
1800
1200
600
0
WT
S69C
A70T
I87T
M134I
0 -12 -11 -10 -9 -8 -7 -6
log [D-Trp^8-γ-MSH], M
WT
L249V
A260V
M275T
T280S
L297V
(B)
Binding (%WT)
120
100
80
60
40
20
0
0 -11 -10 -9 -8 -7 -6

Figure II-4. **Signaling and ligand binding properties of WT and mutant hMC3Rs with α-MSH as the ligand.** See the legend to Fig. II-2 for details. The only difference is that α-MSH was used as the ligand instead of NDP-MSH.

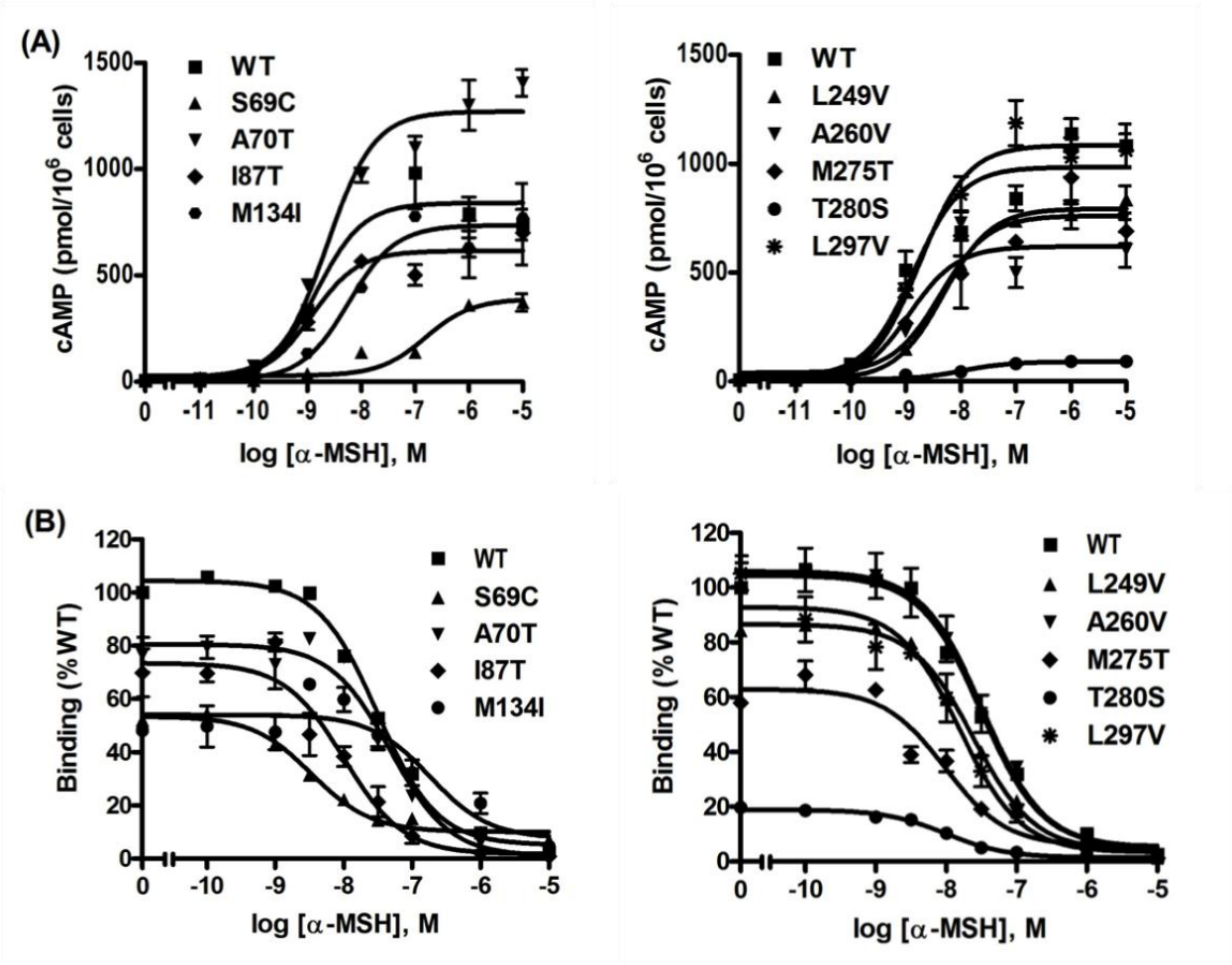

(A)
cAMP (pmol/10^6 cells)
1500
1000
500
0
WT
S69C
A70T
I87T
M134I
0 -11 -10 -9 -8 -7 -6 -5
log [α-MSH], M
cAMP (pmol/10^6 cells)
WT
L249V
A260V
M275T
T280S
L297V
log [α-MSH], M
(B)
Binding (%WT)
120
100
80
60
40
20
0
WT
S69C
A70T
I87T
M134I
0 -10 -9 -8 -7 -6 -5
log [α-MSH], M
Binding (%WT)
WT
L249V
A260V
M275T
T280S
L297V
log [α-MSH], M

Figure II-5. Basal activities of WT and mutant hMC3Rs. The results are expressed as % of WT basal signaling. Shown are mean ± SEM of 5-8 experiments. The basal cAMP levels in HEK293T cells expressing WT hMC3R were 9.68 ± 1.47 pmol/10^6 cells (mean ± SEM). Star (*) indicates significantly different from WT hMC3R.

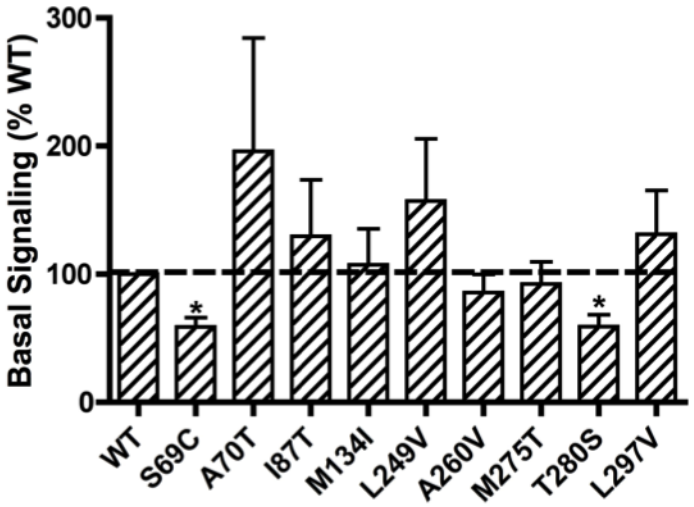

Basal Signaling (% WT)
300
200
100
0
*
*
WT
S69C
A70T
I87T
M134I
L249V
A260V
M275T
T280S
L297V

Figure II-6. Maximal binding of WT and mutant hMC3Rs. The results are expressed as % of WT maximal binding. Shown are mean ± SEM of all the binding experiments. Star (*) indicates significantly different from WT hMC3R.

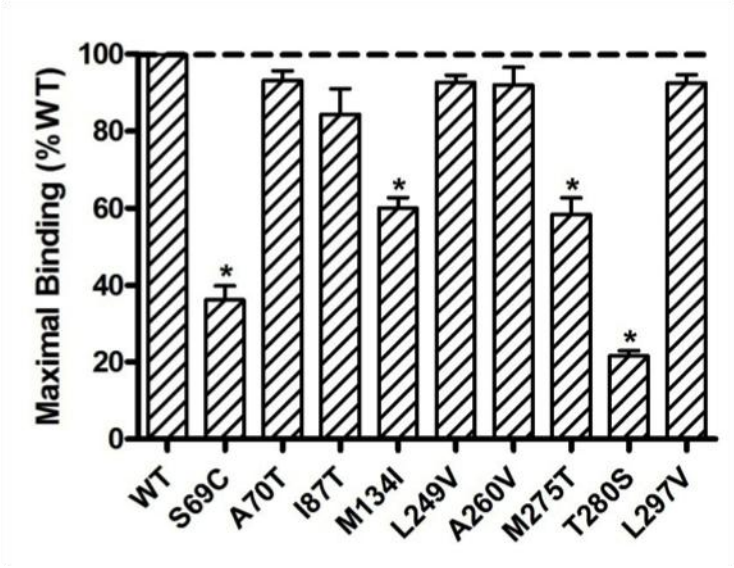

Maximal Binding (% WT)
100
80
60
40
20
0
*
*
*
WT
S69C
A70T
I87T
M134I
L249V
A260V
M275T
T280S
L297V

Figure II-7. Confocal imaging of cell surface expression of WT and mutant hMC3Rs. The WT or mutant 3HA-MC3Rs stably expressed in HEK293 cells were stained with Alexa Flour-conjugated anti-HA monoclonal antibody and imaged by confocal microscopy. This experiment was done twice with similar results.

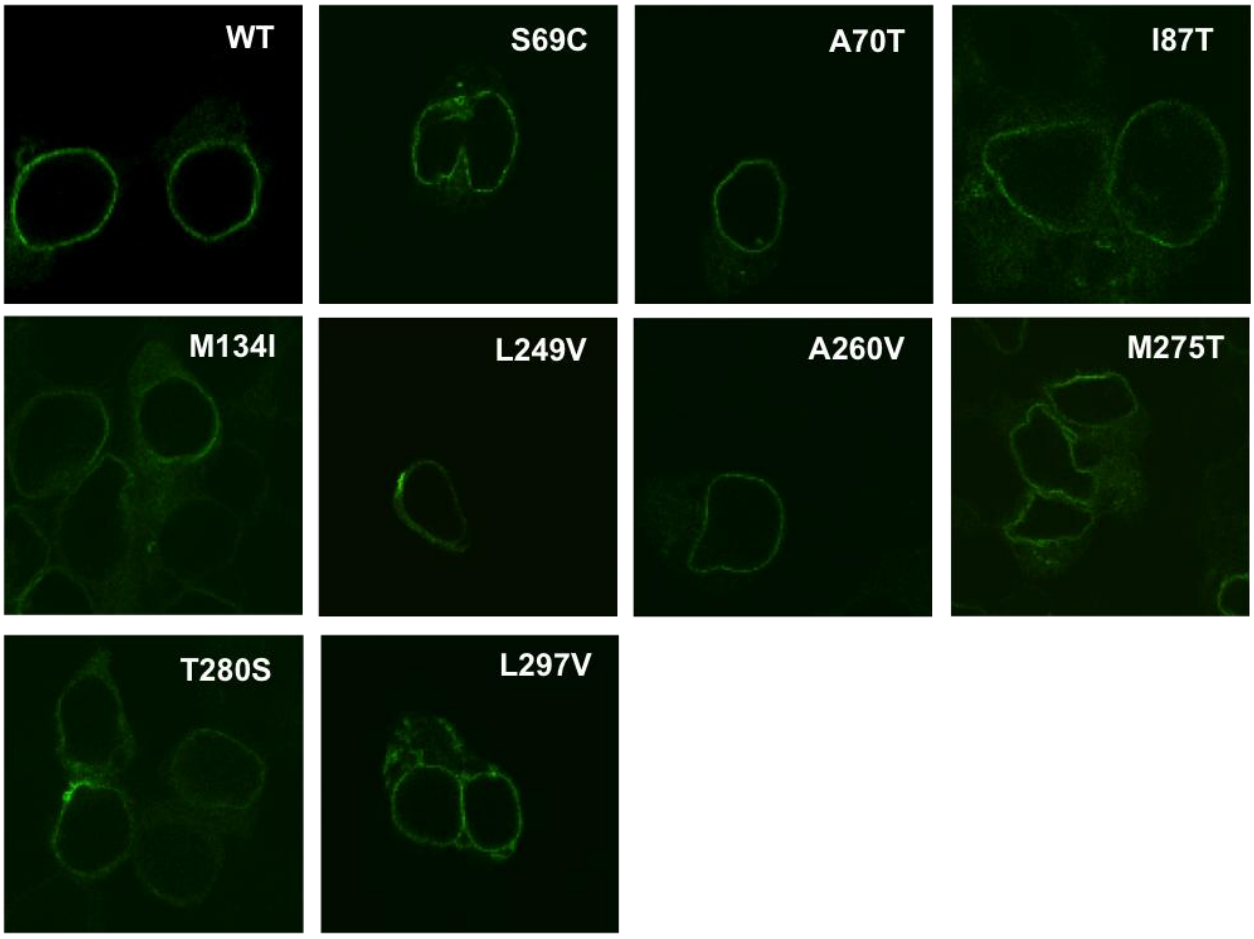

WT
S69C
A70T
I87T
M134I
L249V
A260V
M275T
T280S
L297V

Figure II-8. Quantitative measurement of cell surface (A) or total (B) expression of WT and mutant hMC3Rs by flow cytometry. In (A), cell surface expression of the WT and mutant hMC3Rs were measured by flow cytometry. The results were expressed as % of WT cell surface expression levels after correction of the nonspecific staining in cells transiently transfected with the empty vector as described in Materials and Methods. Data were mean ± SEM of 8-9 experiments. Star (*) indicates significantly different from WT hMC3R. In (B), total expression of the WT and mutant hMC3R were measured by flow cytometry. The results were expressed as % of WT total expression levels after correction of the nonspecific staining in cells transiently transfected with the empty vector as described in Materials and Methods. Data were mean ± SEM of 6-7 experiments. Star (*) indicates significantly different from WT hMC3R.

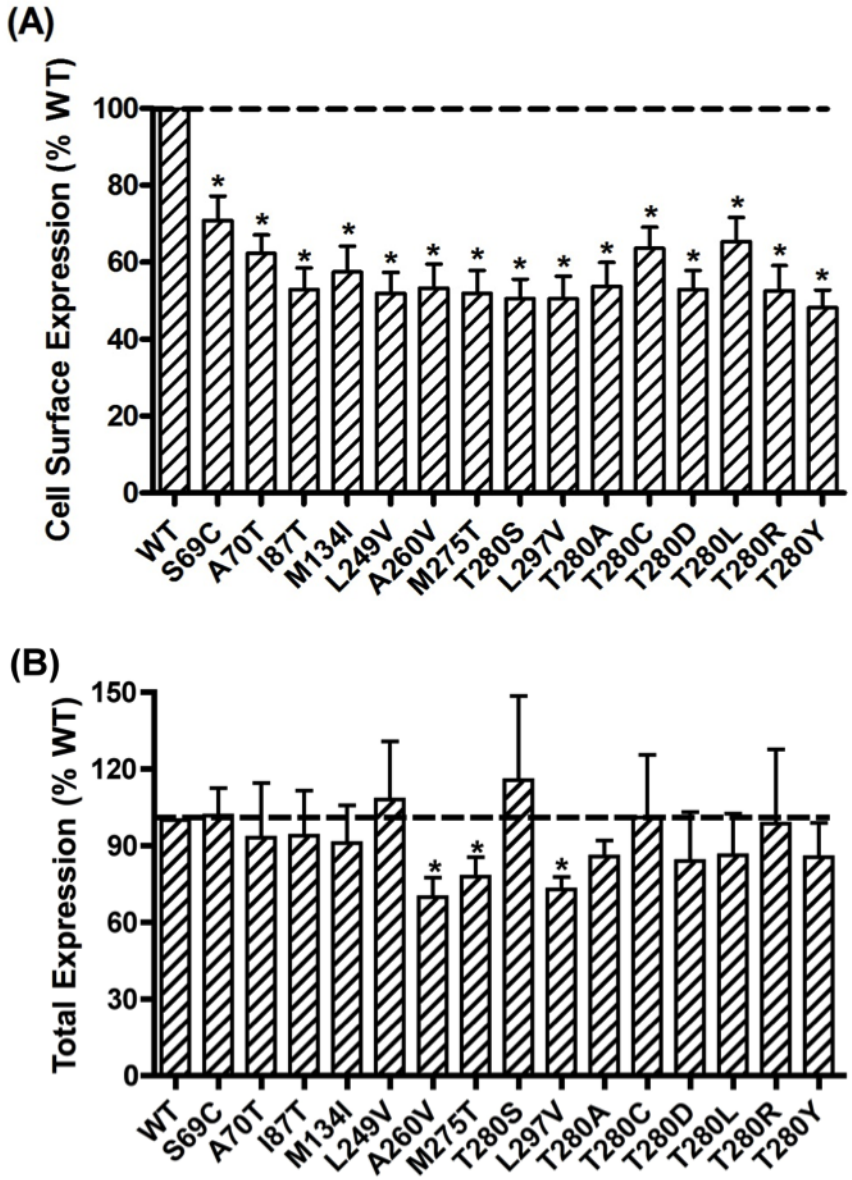
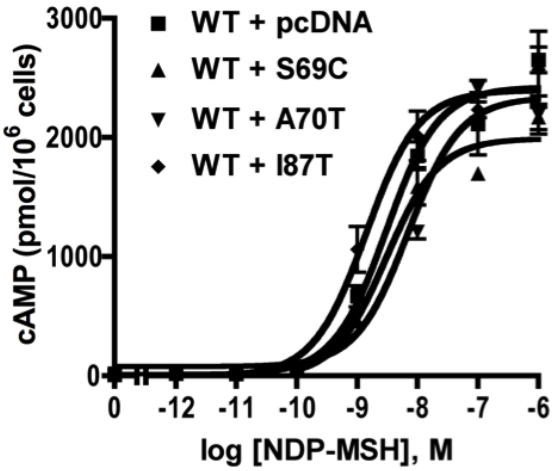
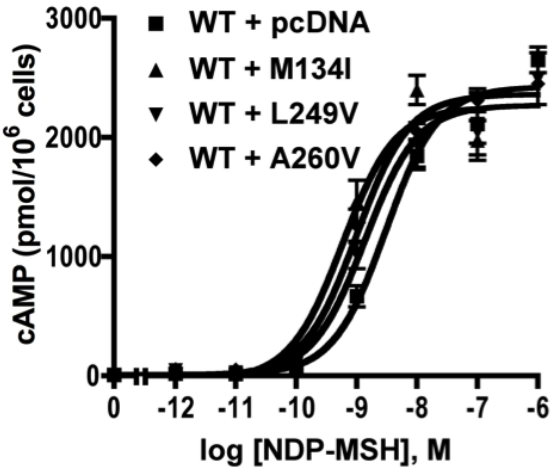

(A)
Cell Surface Expression (% WT)
100
80
60
40
20
0
WT
S69C
A70T
I87T
M134I
L249V
A260V
M275T
T280S
L297V
T280A
T280C
T280D
T280L
T280R
T280Y
(B)
Total Expression (% WT)
150
120
90
60
30
0
WT
S69C
A70T
I87T
M134I
L249V
A260V
M275T
T280S
L297V
T280A
T280C
T280D
T280L
T280R
T280Y

Figure II-9. Accumulation of intracellular cAMP in cells expressing WT and/or mutant hMC3Rs. HEK293T cells were transiently transfected with the indicated MC3R constructs, and intracellular cAMP levels were measured by RIA. The same amount of WT hMC3R plasmid was present in all groups. Empty vector was added to keep plasmid concentrations constant among the different groups. Results are expressed as the mean ± SEM of triplicate determinations within one experiment. All experiments were performed at least three times.


WT + pcDNA
WT + S69C
WT + A70T
WT + I87T
cAMP (pmol/10^6 cells)
3000
2000
1000
0
0 -12 -11 -10 -9 -8 -7 -6
log [NDP-MSH], M


WT + pcDNA
WT + M134I
WT + L249V
WT + A260V
cAMP (pmol/10^6 cells)
3000
2000
1000
0
0 -12 -11 -10 -9 -8 -7 -6
log [NDP-MSH], M

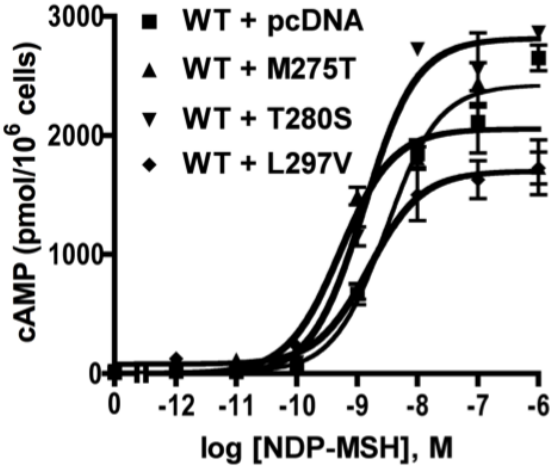
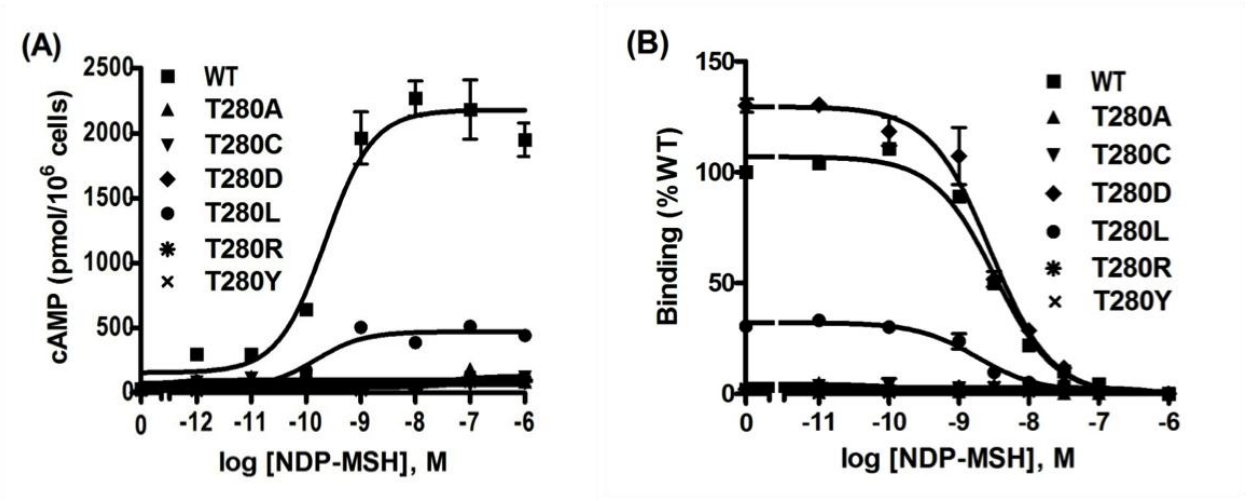

WT + pcDNA
WT + M275T
WT + T280S
WT + L297V
cAMP (pmol/10^6 cells)
3000
2000
1000
0
0 -12 -11 -10 -9 -8 -7 -6
log [NDP-MSH], M

Figure II-10. Multiple mutagenesis of T280. T280 was mutated to Ala, Cys, Leu, Asp, Arg, and Tyr. The signaling (A) and ligand binding (B) properties of these mutants were measured using NDP-MSH as the ligand.


(A)
cAMP (pmol/10^6 cells)
2500
2000
1500
1000
500
0
WT
T280A
T280C
T280D
T280L
T280R
T280Y
0
-12
-11
-10
-9
-8
-7
-6
log [NDP-MSH], M
(B)
Binding (% WT)
150
100
50
0
WT
T280A
T280C
T280D
T280L
T280R
T280Y
0
-11
-10
-9
-8
-7
-6
log [NDP-MSH], M

Chapter 3

3.1 Introduction

The rapidly increased prevalence of obesity draws more and more attention, as obesity raises the health and economic burden in both Western and developing countries. Although the rapidly increasing trend is mainly driven by environmental factors, it is recognized that genetic factors determine individual's susceptibility of obesity. Genome-wide association studies have identified some variants associated with the development of obesity (Clement et al. 1998; Krude et al. 1998; Loos, et al. 2008; Scuteri, et al. 2007).

It is well known that some hypothalamic circuits are involved in the regulation of food intake and energy homeostasis, especially the arcuate nucleus (ARC) and the paraventricular nucleus (PVN) (Gantz and Fong 2003). The melanocortin system plays an important role in the regulation of energy homeostasis herein (Gantz and Fong 2003). Melanocortin-3 receptor (MC3R) was the third cloned member of melanocortin receptor family. Together with its related receptor MC4R, it is primarily expressed in brain, especially in the hypothalamus that is involved in the regulation of energy homeostasis (Gantz et al. 1993a; Roselli-Rehfuss et al. 1993).

The potential role of MC3R in energy homeostasis is studied by *Mc3r* knockout mice model. The *Mc3r* knockout mice exhibit increased body fat and decreased lean body mass, with normal appetite or even hypophagia, suggesting increased feed efficiency (Butler et al. 2000; Chen et al. 2000). When mouse strain Black Swiss; 129 (which is responsive to leptin and

resistant to obesity) is used to generate the homozygous knockout mice, *Mc3r* knockout mice are 20% heavier than wild-type (WT), at a comparable degree as *Mc4r* knockout mice (Zhang et al. 2005). Mice lacking both *Mc3r* and *Mc4r* are more obese than mice only lacking only *Mc3r*, indicating that these two receptors play different, but complementary roles in the regulation of energy homeostasis (Chen et al. 2000). More proof has been provided to demonstrate that MC3R can stimulate food intake (Lee et al. 2008; Marks et al. 2006). This is possibly due to the co-expression of MC3R and POMC within the ARC (Bagnol, et al. 1999; Cowley et al. 2001; Jegou et al. 2000). MC3R may serve as an auto-inhibitory receptor on POMC neurons so that activation of MC3R suppresses melanocortin tone, causing increased food intake. MC3R may supplement MC4R-mediated reduction in food intake and prevent cachexia (Marks et al. 2006). Recent studies report that MC3R works as a mediator of the entrainment pattern of food intake to restricted feeding (Sutton et al. 2010; Sutton et al. 2008).

The loss-of-function mutations in *MC4R* gene are considered as pathogenic causes for human monogenic obesity (Vaisse et al. 1998; Yeo et al. 1998). Rare mutations in *MC3R* gene have been identified (Calton, et al. 2009; Feng et al. 2005; Lee et al. 2007; Lee, et al. 2002; Mencarelli, et al. 2011; Mencarelli et al. 2008; Zegers et al. 2011) (reviewed in (Tao 2010b)). The influence of these *MC3R* mutations on human obesity is controversial. The first *MC3R* mutation identified in two obese patients in Singapore is I183N, which is totally dysfunctional (Lee et al. 2002; Rached et al. 2004; Tao and Segaloff 2004) and cosegregate with childhood obesity (Lee et al. 2007). Therefore, I183N is considered as a pathogenic mutation. Calton *et al*. reported that *MC3R* mutations are not associated with severe obesity in the two North American cohorts they studied, because the prevalence of *MC3R* mutations in obese subjects is not significantly different from that in control groups (Calton et al. 2009). However, Mencarelli *et al.*

reported several *MC3R* mutations with impaired functions, having significantly higher prevalence in obese subjects of Italian and French origin (Mencarelli et al. 2011). Recently, we reported that mutation T280S, identified in an obese patient from Northern American cohorts, leads to significant decrease in cell surface expression, ligand binding and maximal signaling, and thus may serve as the potential pathogenic cause to human obesity (Yang and Tao 2012). Taken togehter, the pathogenic role of MC3R in the development of obesity still needs further investigation.

In this study, detailed functional analyses were performed on *MC3R* mutations identified in North American cohorts (Calton et al. 2009), Italian and French subjects (Mencarelli et al. 2011), children and adolescents in Belgium (Zegers et al. 2011) (Fig. III-1).

3.2 Materials and methods

3.2.1 Plasmid and peptides

Human MC3R cDNA tagged with 3×HA tags at the N-terminus inserted in vector pcDNA3.1 was obtained from Missouri S&T cDNA Resource Center (http://www.cDNA.org/, Rolla, MO). [Nle^4, D-Phe^7]-α-MSH (NDP-MSH) was purchased from Bachem (King of Prussia, PA). α-MSH was purchased from Phoenix Pharmaceuticals (Belmont, CA). ^{125}I-NDP-MSH was prepared by a modified chloramine-T method as described previously (Xiang et al. 2006).

3.2.2 Site-directed mutagenesis of the human MC3R

Mutations in human *MC3R* were generated by QuikChange^{TM} site-directed mutagenesis kit (Stratagene, La Jolla, CA) with WT human 3×HA-MC3R as template. Plasmids were

prepared using IsoPure DNA purification kits from Denville Scientific (Metuchen, NJ). Automated DNA sequencing was performed by the DNA Sequencing Facility of University of Chicago Cancer Research Center (Chicago, IL) to confirm that the intended mutations were generated and introduced into the constructs correctly.

3.2.3 Cells and transfections

Human Embryonic Kidney (HEK) 293T cells, obtained from American Type Culture Collection (Manassas, VA), were maintained at 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 50 μg/ml of gentamicin, 100 units/ml of penicillin-streptomycin, 20μg/ml of amphotericin B, 10mM Hepes and 10% newborn calf serum. Cells were plated on gelatin-coated 35mm 6-well clusters (Corning, NY) and transient transfected with the calcium precipitation method (Chen and Okayama 1987) when the cells reached 50-70% confluency. One microgram of plasmid in 2ml media was used per 35mm well. After transfection, cells were incubated approximately 48h before used to measure ligand binding and ligand-stimulated cAMP generation.

3.2.4 Ligand binding to intact cells

48h after transfection, cells were washed twice with warm Waymouth's MB752/1 media (Sigma, St. Louis, MO) containing 1mg/ml bovine serum albumin (BSA) (referred to as Waymouth/BSA). Fresh Waymouth/BSA was added to each well, incubated with 50 μl of ^{125}I-NDP-MSH (~100,000 cpm) with or without different concentrations of unlabeled NDP-MSH or α-MSH at 37 C for 1h. The total volume was 1 ml in each well. The final concentrations of unlabeled ligands are indicated in the figures. After 1h incubation, cells were washed twice with

cold Hank's balanced salt solution (Sigma-Aldrich) containing 1 mg/ml BSA. Then cells were solubilized with 100µl of 0.5M NaOH. Cell lysates were collected with cotton swabs and counted in a gamma counter. All determinations were performed in duplicate. Apparent maximal binding capacity (Bmax) and concentrations that result in 50% inhibition (IC_{50}) were calculated using GraphPad Prism 4.0 (San Diego, CA).

3.2.5 Ligand-stimulated cAMP generation

After 48h incubation, transfected HEK-293T cells were washed twice with warm Waymouth/BSA. Then fresh Waymouth/BSA containing 0.5mM isobutylmethylxanthine (Sigma-Aldrich) was added to each well for 15 min incubation at 37 C. Then, either Waymouth/BSA alone or different concentrations of ligands were added. The total volume was 1 ml in each well. The final concentrations of the ligands are indicated in the figures. After 1h incubation at 37 C, cells were solubilized with cold 0.5M percholoric acid containing 180µg/ml theophylline. The cAMP concentrations were measured by radioimmunoassay (Tao et al. 2010). All determinations were performed in triplicate. Maximal response (Rmax) and concnetrations that result in 50% maximal response (EC_{50}) were calculated by GraphPad Prism 4.0.

3.2.6 Quantification of MC3R expression by flow cytometry

Flow cytometry was performed as described previously (Fan and Tao 2009; Wang, et al. 2008; Wang and Tao 2011). Briefly, HEK293 cells were plated on gelatin-coated 35mm 6-well clusters and transfected with 4 micrograms of plasmid in 2ml media for each well. After 48h incubation, cells were washed once with filtered PBS-IH, detached, and then precipitated by centrifugation at 500 × g. Cells were fixed by 4% paraformaldehyde in PBS-IH for 30 min and

then blocked with PBS-IH containing 5% BSA for 1h. The cells were then stained with primary anti-HA.11 antibody (Covance) diluted 1:50 in PBS-IH containing 0.5% BSA for 1h, and washed with PBS-IH containing 0.5% BSA. The secondary Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, CA) diluted 1:2000 in PBS-IH containing 0.5% BSA was added under low light. After 1h incubation, cells were washed and resuspended in PBS-IH with 0.5% BSA. An Accuri flow cytometer with a 488-nm wavelength laser was applied to quantify hMC3R mutant expression in 15,000 cells from each transfection. Cells transfected with pcDNA3.1 empty vector were used as control for background staining. All the incubations and washes were performed at room temperature. The expression levels of the mutants were calculated as percentage of WT hMC3R expression as described before, using the following formula: [mutant – pcDNA3]/[WT – pcDNA3] x100% (Fan and Tao 2009; Wang et al. 2008; Wang and Tao 2011).

3.2.7 Statistical analyses

Student's t test was used to determine the significance of differences in the expression, ligand binding and signaling parameters between WT and mutant hMC3Rs. Statistical analysis was carried out using GraphPad Prism 4.0.

3.3 Results

3.3.1 Ligand binding of the mutant hMC3Rs

Competitive ligand binding assays were performed with the superpotent analog NDP-MSH (Sawyer, et al. 1980) and the endogenous agonist α-MSH, respectively. When NDP-MSH was used, mutants F82S, D158Y and L299V showed little or no specific binding to radiolabeled

NDP-MSH (Fig. III-2A). All the other mutants had similar IC_{50} as WT hMC3R (Table 3.1). When α-MSH was used as the competitor (Fig. III-3A), mutants S17T, L249F and L285V had significantly increased IC_{50}. Mutants V177I and R257S had similar IC_{50} as that of WT hMC3R (Table 3.2). A previous study in which 25 amino acids were deleted from the N-terminus of human MC3R showed that the truncated MC3R has similar ligand binding as WT hMC3R, suggesting that N-terminus does not play an important role in ligand binding of MC3R (Schiöth, et al. 1997).

The maximal binding capacity was estimated (Tansky, et al. 2007; Wang et al. 2008) and data are presented in Fig III-4. F82S, D158Y and L299V had little binding. R257S had significantly decreased maximal binding. All the other mutants had normal maximal binding as WT hMC3R.

3.3.2 Signaling activity of the mutant hMC3Rs in response to NDP-MSH or α-MSH stimulation

To investigate whether and how these missense mutations would affect MC3R function, the intracellular cAMP levels stimulated by different ligands were measured, using radioimmunoassay.

First, signaling properties in response to NDP-MSH stimulation was investigated. Fig 3III-2B showed the results of one representative experiment. In response to NDP-MSH stimulation, except for S17T and V177I, the other six mutants had either reduced or no maximal signaling. D158Y had no signaling. F82S retained some signaling activity. However, the EC_{50} value could not be accurately calculated. Of the six mutants that we could calculate EC_{50} values,

only L299V had increased EC_{50}. The other five mutants had similar EC_{50} as the WT hMC3R (Table 3.1).

The signaling property was also investigated with the endogenous agonist, α-MSH (Fig. III-3B). D158Y was totally devoid of signaling. Five mutants (F82S, L249F, R257S, L285V and L299V) exhibited decreased maximal signaling compared to WT hMC3R. The other two mutants, S17T and V177I, had normal signaling in both EC_{50} and Rmax as that of WT hMC3R (Table 3.2).

Different from the human MC4R, the WT hMC3R has little constitutive activity (Abbracchio, et al. 2006). In the current study, none of the mutants caused increased basal activity. Four mutants (D158Y, V177I, L249F and L285V) had significantly decreased basal activity compared to WT hMC3R (Fig. III-5).

3.3.3 Quantification of mutant hMC3R expression with flow cytometry

To investigate the localization of these mutant hMC3Rs and to quantitate their expression, flow cytometry was performed. The data of non-permeabilized cells showed that all the mutants had similar cell surface expression as WT hMC3R except F82S, which had significantly decreased cell surface expression to about 60% of WT hMC3R (Fig. III-6A). In permeabilized cells, all the mutants had normal total protein expression (Fig. III-6B).

3.4 Discussion

In this study, we performed functional characterization of eight naturally occurring mutations in *MC3R* gene. Among these mutations, S17T, D158Y, V177I, and L299V were

identified in unrelated obese carriers, whereas F82S, R257S, and L285V were identified in normal weight subjects. L249F was identified in both obese and normal weight subjects (Calton et al. 2009; Mencarelli et al. 2011; Zegers et al. 2011). All these mutants were expressed on the cell surface, but F82S had significantly decreased cell surface expression (Fig. III-6A). All the mutants had normal total expression (Fig. III-6B). Four mutants (F82S, D158Y, R257S and L299V) had significantly decreased maximal ligand binding (Fig. III-4) and six mutants (F82S, D158Y, L249F, R257S, L285V and L299V) had significantly reduced maximal signaling (Tables 3.1 and 3.2).

To study whether the mutant hMC3Rs were mislocalized, flow cytometry was performed. Flow cytometry in permeabilized cells showed that all the mutants had normal total protein expression (Fig. III-6B) and flow cytometry in non-permeablized cells indicated that all the mutants except F82S had normal cell surface expression (Fig. III-6A). These results showed that F82S was partially intracellularly retained due to misfolding, which is the common cause for the majority of GPCR mutations leading to human diseases (Tao 2006), and most *MC4R* mutations belong to this class (Tao 2005).

Mutant F82S also had significant defects in both ligand binding and agonist-stimulated cAMP generation, likely due to the intracellular retention and decreased cell surface expression. A naturally occurring mutation in *MC4R* gene identified from obese patients with binge eating disorder substitutes the cognate Phe with Leu at codon 51, which leads to normal ligand binding and signaling with normal basal activity (Tao and Segaloff 2005). Changing Phe to Leu, which has small change in size, does not affect the receptor function (Tao and Segaloff 2005), whereas changing hydrophobic Phe to Ser with polar uncharged side chain significantly impaired the

function of MC3R, suggesting that the hydrophobic feature of codon 82 might be of importance for MC3R expression, trafficking, ligand binding and signaling.

Previously, it was found that D126 in MC4R is important for ligand receptor interaction (Yang, et al. 2000). Our previous study demonstrated that the corresponding Asp in MC3R, D158, plays an important role in multiple aspects of receptor function (Wang et al. 2008). Mutating Asp to Glu or Gln at codon 158 results in undetectable ligand binding and signaling. D158Q is expressed normally at the cell surface, whereas, D158E has significantly decreased cell surface expression (Wang et al. 2008). In the current study, mutant D158Y, despite normal cell surface expression, was almost totally devoid of ligand binding and signaling (Figs. III-2~III-4 and Tables 3.1 and 3.2). Combined with the results in the present study, D158 is important for receptor expression, ligand binding and signaling. D158Y might be the pathogenic cause to obesity for the patient carrying it.

Mutants L249F and L285V had normal cell surface and total expression (Fig. III-6) and similar maximal binding compared to WT hMC3R (Fig. III-4), with significantly decreased maximal signaling (Figs. III-2 and III-3, Tables 3.1 and 3.2), suggesting that these two mutants were partially defective in signaling. In a previous study, the functional investigation of a naturally occurring mutation at codon 249 was performed (Yang and Tao 2012). Mutating Leu to Val at codon 249 led to decreased cell surface expression, but did not affect ligand binding and signaling, suggesting the presence of spare receptors (Yang and Tao 2012). Altogether, the combined results indicated that Leu at codon 249 is important to MC3R expression and signaling. Mutant R257S had significantly decreased maximal binding and signaling (Figs. III-2 and III-3,

Tables 3.1 and 3.2) with normal cell surface and total protein expression (Fig. III-6). It might be defective in binding, resulting in corresponding decrease in signaling.

Since *Mc3r* konckout mice had increased fat mass and decreased lean body mass, with slight change in body weight (Butler et al. 2000; Chen et al. 2000), human genetic study of MC3R in energy homeostasis should focus on adiposity rather than body weight. Mutations F82S, R257S and L285V were identified in subjects with normal body weight, while L249F was identified in both obese and normal subjects. Whether the impaired signaling caused by these mutations will result in the human adiposity requires further investigation.

Mutant L299V exhibited normal cell surface and total expression (Fig. III-6) and significantly reduced ligand binding as well as signaling (Figs. III-2~III-4, Tables 3.1 and 3.2). The original report by Zegers and coworkers suggested that L299V has decreased cell surface expression and therefore they hypothesized that the impaired function of this mutant might be due to intracellular retention (Zegers et al. 2011). That observation is different from the results in the present study. Zegers *et al.* used enhanced green fluorescent protein (EGFP)-MC3R fusion proteins to investigate the localization (Zegers et al. 2011). However, there is evidence indicating that the addition of GFP can interrupt the intracellular trafficking of receptors (Tao 2010b). This might be the possible explanation for the abnormal cell surface expression of mutant L299V in the original publication.

According to the alignment of the amino acid sequences of MCRs, the Leu at codon 299 in MC3R is a conserved residue among MCRs, except MC2R. This residue corresponds to L265 in MC4R (Tao 2010a). The substitution of L265 in MC4R will dramatically alter the affinity and potencies of NDP-MSH and two other agonists (Pogozheva, et al. 2005). That might also be a

possible reason for the decreased ligand binding and signaling of mutant L299V in the present study.

Another possible reason for the reduced cAMP generation of mutant L299V is due to the important influence of the neighboring His at codon 298 (Zegers et al. 2011). H298 is also a conserved residue among MCRs (Tao 2010a). The cognate His residue in MC4R is considered to play an important role in MC4R activation (Oosterom, et al. 1999). Therefore, mutant L299V might lead to sterical hindrance for H298 and thus, the conformational change caused the dysfunction of MC3R (Zegers et al. 2011). Mutant L299V then might be the pathogenic cause for the increased adiposity or obesity in the child carrying it.

In the earlier study, we suggested a classification scheme to identify the naturally occurring mutations in *MC4R* (Tao 2005). Class I mutants are not expressed due to defects in protein synthesis or accelerated degradation; Class II mutants are retained intracellularly due to misfolding; Class III mutants are defective in ligand binding per se with expression on cell surface; Class IV mutants are defective in signaling although they have cell surface expression and bind to ligands; Class V mutants have no apparent defects in functions (Tao 2005). According to this scheme, F82S belongs to Class II; D158Y and L299V belong to Class III; L249F, R257S and L285V belong to Class IV; and S17T and V177I belong to Class V.

In the present study, the cAMP signaling of the mutant hMC3Rs has been investigated. However, it has been reported that MC3R also activates the MAPK pathway (Chai, et al. 2007). It is found that NDP-MSH stimulation induces ERK1/2 phosphorylation in a dose-dependent manner. The MC3R antagonist SHU9119 blocked ERK1/2 phosphorylation. On the other hand, the treatment of PKA inhibitor does not affect the phosphorylation of ERK1/2, suggesting that

cAMP signaling is not critical to MAPK signaling. MC3R activation induces MAPK pathway through PI3 kinase (Chai et al. 2007). Whether the mutant hMC3Rs could lead to defects in this signaling pathway and had any pathogenic effects requires further investigation.

In summary, functional studies of eight naturally occurring mutations in *MC3R* gene were performed. We showed that D158Y and L299V could potentially be a pathogenic cause for human obesity. F82, D158, L249 and L299 played important roles in different aspects of MC3R functions, contributing to a better understanding of the structure-function relationship of MC3R. Future work is still required to investigate the specific role of these mutants *in vivo* and whether these mutants will cause any defects in other signaling pathways.

Table 3.1 Binding and signaling properties of WT and mutant hMC3Rs with NDP-MSH as the ligand.

hMC3R	n	NDP-MSH binding	NDP-MSH-stimulated cAMP	
		IC_{50} (nM)	EC_{50} (nM)	*R*max (% WT)
WT	6	6.45 ±0.87	1.28±0.49	100.00
S17T	3	5.73±0.48	1.24±0.68	81.87±17.35
F82S	3	ND	ND	22.99±2.26[c]
D158Y	3	ND	ND	ND
V177I	3	4.33±1.61	0.62±0.14	61.81±10.02
L249F	3	7.74±1.99	4.98±2.54	50.02±2.74 [b]
R257S	3	5.88±0.12	3.26±1.62	44.05±12.03[a]
L285V	3	6.07±1.02	5.76±2.64	29.71±3.22[b]
L299V	3	ND	14.84±4.29[a]	38.53±5.31[b]

[a] Significantly different from the WT hMC3R, $p < 0.05$.

[b] Significantly different from the WT hMC3R, $p < 0.01$.

[c] Significantly different from the WT hMC3R, $p < 0.001$.

The data are expressed as the mean ± SEM for the mutant hMC3Rs. The maximal responses (*R*max) were 2526.00 ± 502.11 pmol cAMP/10^6 cells for WT hMC3R.

Table 3.2 Binding and signaling properties of WT and mutant hMC3Rs with α-MSH as the ligand.

hMC3R	n	α-MSH binding	α-MSH-stimulated cAMP	
		IC_{50} (nM)	EC_{50} (nM)	*R*max (% WT)
WT	5	28.49±2.86	2.90±1.43	100.00
S17T	3	50.89±4.58[a]	13.92±11.42	102.74±10.19
F82S	3	ND	ND	21.18±6.31[b]
D158Y	3	ND	ND	ND
V177I	3	51.28±10.25	3.10±1.96	80.18±11.26
L249F	3	58.66±2.57[b]	1.31±0.55	55.99±13.71
R257S	3	40.81±3.79	3.62±0.35	61.78±16.15
L285V	3	124.86±20.05[b]	18.90±10.64	40.95±11.19[a]
L299V	3	ND	115.70±50.16	29.05±8.74[a]

[a] Significantly different from the WT hMC3R, $p < 0.05$.

[b] Significantly different from the WT hMC3R, $p < 0.01$.

The data are expressed as the mean ± SEM for the mutant hMC3Rs. The maximal responses (*R*max) were 2826.50 ±33.50 pmol cAMP/10^6 cells for WT hMC3R.

Figure III-1. **Schematic model of human MC3R with the mutations studied in Chapter 3 highlighted.**

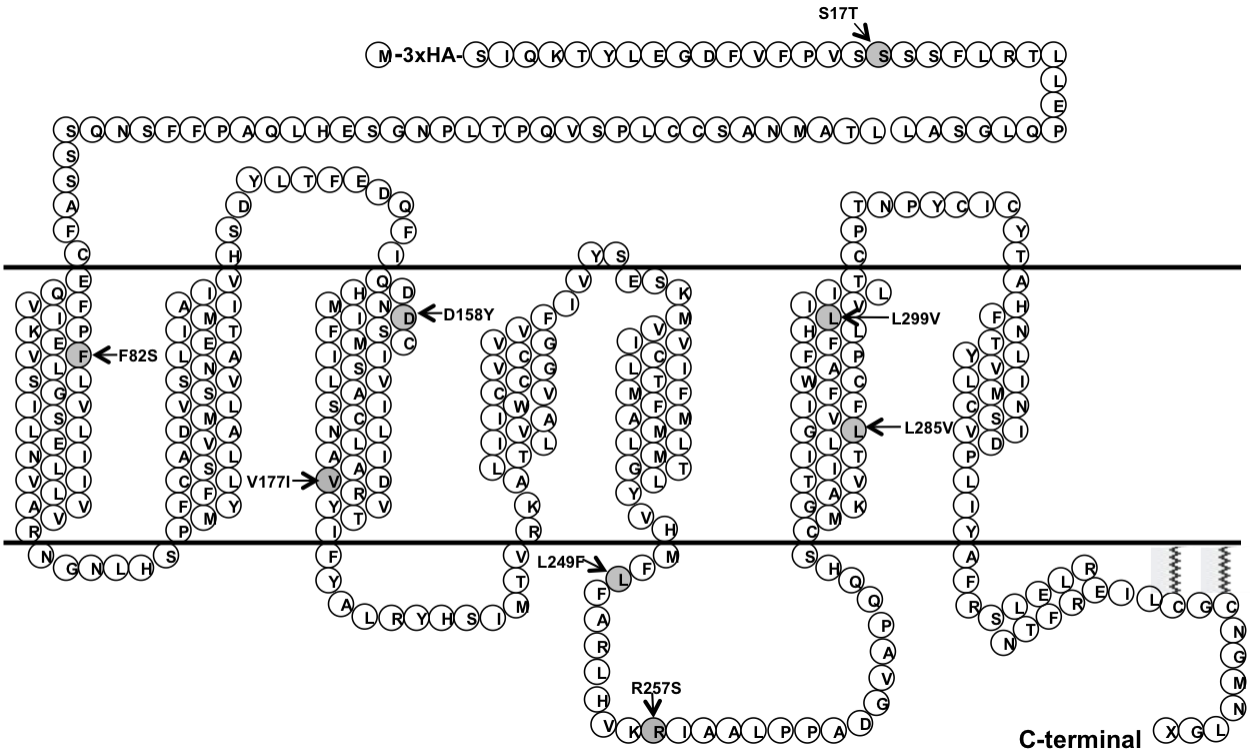

S17T
M-3xHA-
F82S
V177I
D158Y
L249F
L299V
L285V
R257S
C-terminal

Figure III-2. Ligand binding and signaling properties of WT and mutant hMC3Rs with NDP-MSH as the ligand. HEK293T cells were transiently transfected with the indicated hMC3R constructs and binding and signaling of the hMC3Rs were measured as described in Materials and Methods. In (A), different concentrations of unlabeled NDP-MSH were used to displace the binding of ^{125}I-NDP-MSH to hMC3Rs on intact cells. Results shown are expressed as % of WT binding ± range from duplicate determinations within one experiment. In (B), HEK293T cells transiently transfected with the indicated hMC3R constructs were stimulated with different concentrations of NDP-MSH. Intracellular cAMP levels were measured using RIA. Results are expressed as the mean ± SEM of triplicate determinations within one experiment. All experiments were performed at least three times.

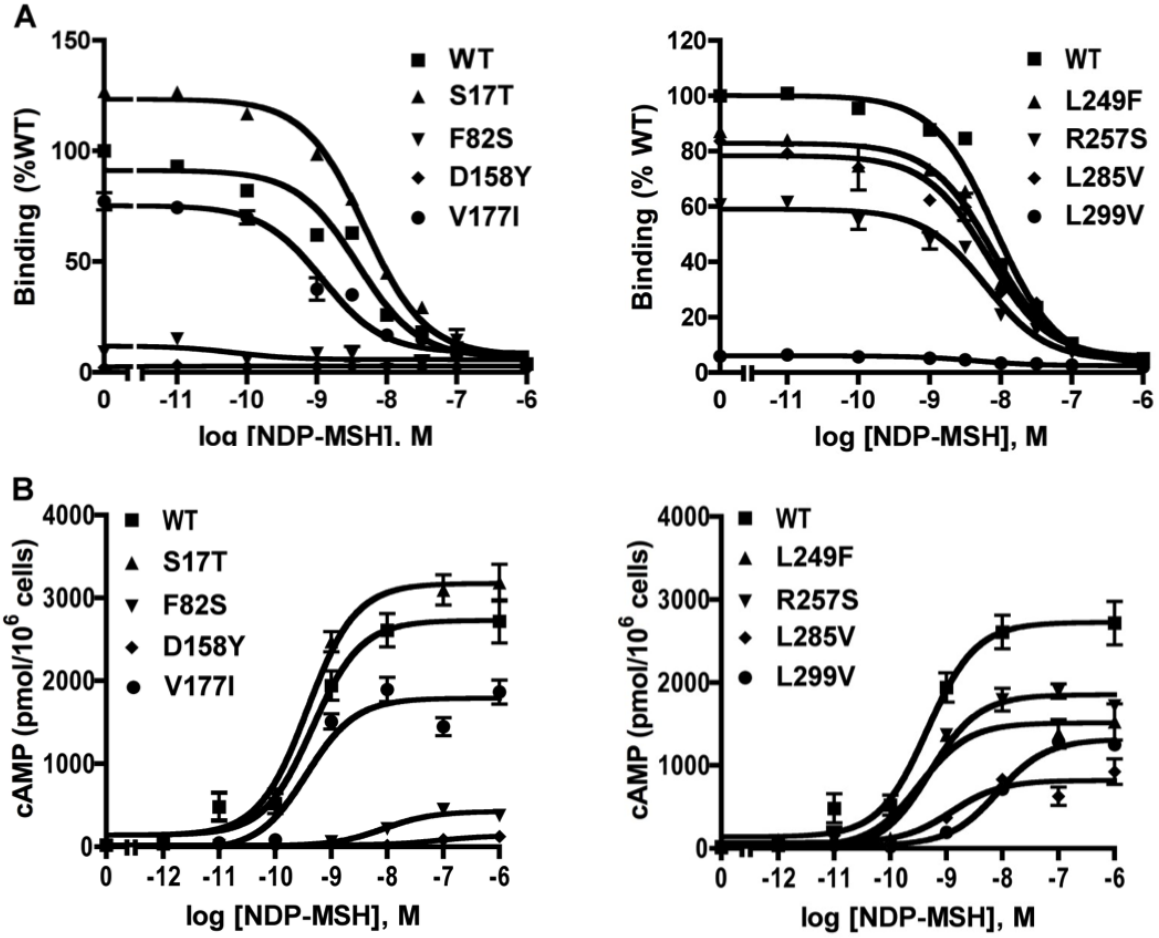

A
Binding (%WT)
150
100
50
0
0 -11 -10 -9 -8 -7 -6
log [NDP-MSH], M
WT
S17T
F82S
D158Y
V177I
Binding (% WT)
120
100
80
60
40
20
0
0 -11 -10 -9 -8 -7 -6
log [NDP-MSH], M
WT
L249F
R257S
L285V
L299V
B
cAMP (pmol/10^6 cells)
4000
3000
2000
1000
0
0 -12 -11 -10 -9 -8 -7 -6
log [NDP-MSH], M
WT
S17T
F82S
D158Y
V177I
cAMP (pmol/10^6 cells)
4000
3000
2000
1000
0
0 -12 -11 -10 -9 -8 -7 -6
log [NDP-MSH], M
WT
L249F
R257S
L285V
L299V

Figure III-3. **Ligand binding and signaling properties of WT and mutant hMC3Rs with α-MSH as the ligand.** See the legend to Fig. III-2 for details. The only difference is that α-MSH was used as the ligand instead of NDP-MSH.

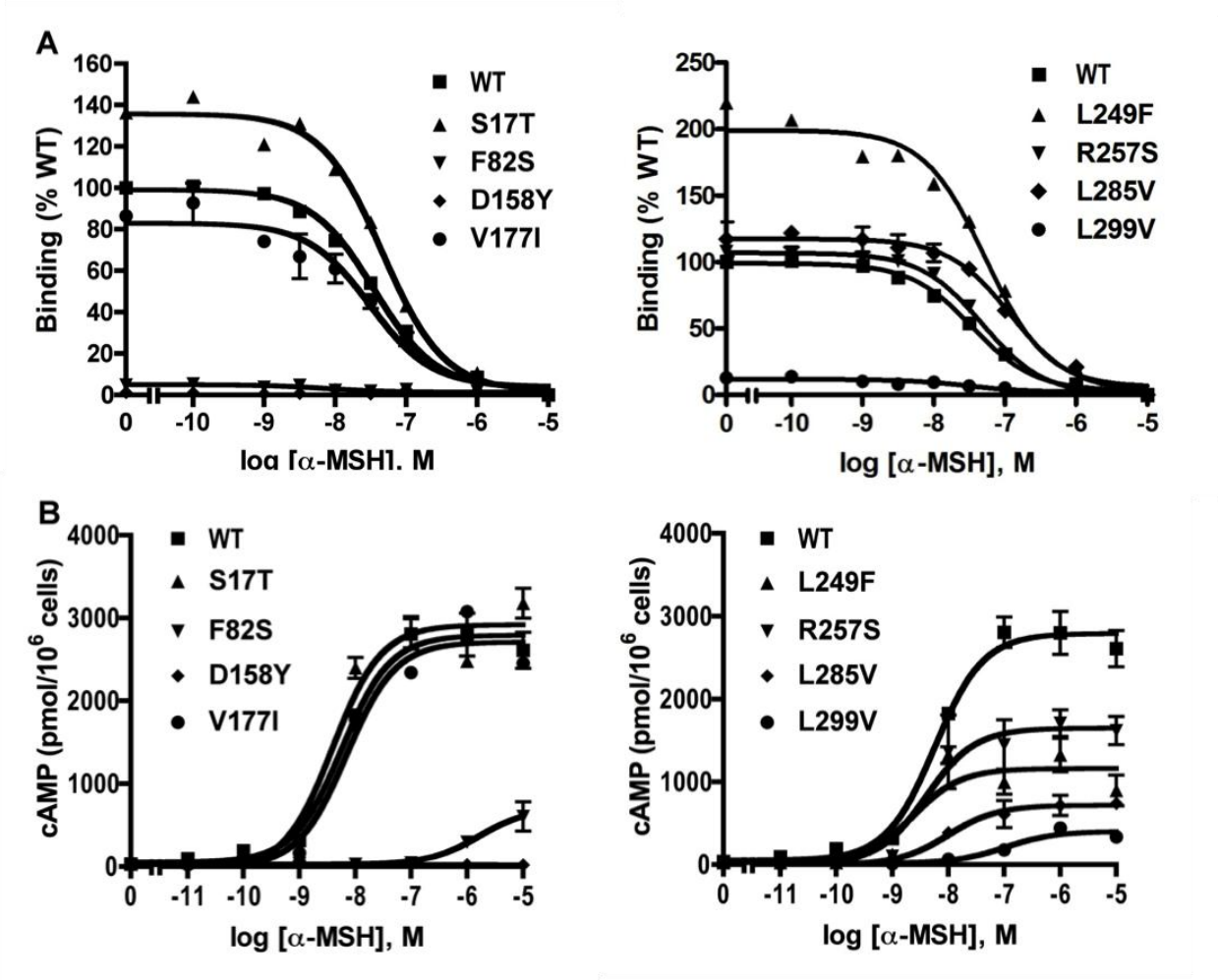

A
Binding (% WT)
160
140
120
100
80
60
40
20
0
0 -10 -9 -8 -7 -6 -5
log [α-MSH], M
WT
S17T
F82S
D158Y
V177I
Binding (% WT)
250
200
150
100
50
0
0 -10 -9 -8 -7 -6 -5
log [α-MSH], M
WT
L249F
R257S
L285V
L299V
B
cAMP (pmol/10^6 cells)
4000
3000
2000
1000
0
0 -11 -10 -9 -8 -7 -6 -5
log [α-MSH], M
WT
S17T
F82S
D158Y
V177I
cAMP (pmol/10^6 cells)
4000
3000
2000
1000
0
0 -11 -10 -9 -8 -7 -6 -5
log [α-MSH], M
WT
L249F
R257S
L285V
L299V

Figure III-4. Maximal binding of WT and mutant hMC3Rs. The results are expressed as % of WT maximal binding. Shown are mean ± SEM of all the binding experiments. Star (*) indicates significantly different from WT hMC3R.

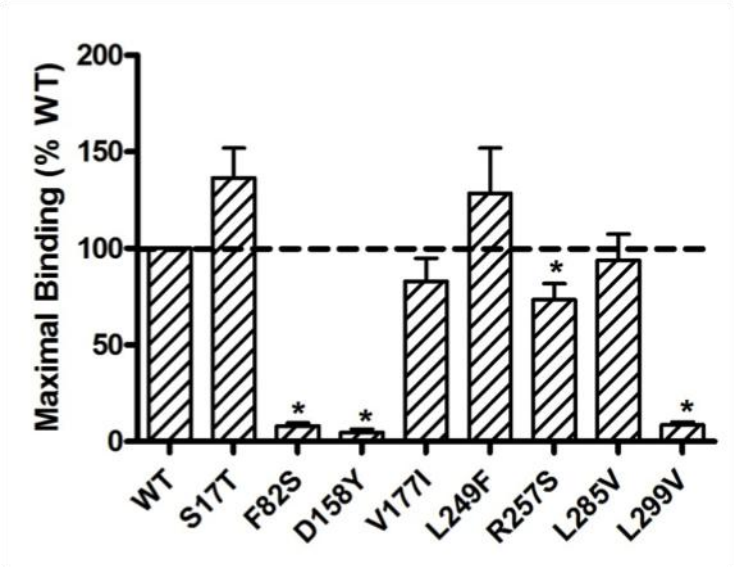

Maximal Binding (% WT)
200
150
100
50
0
*
*
*
WT
S17T
F82S
D158Y
V177I
L249F
R257S
L285V
L299V

Figure III-5. Basal activities of WT and mutant hMC3Rs. The results are expressed as % of WT basal signaling. Shown are mean ± SEM of 6-9 experiments. The basal cAMP levels in HEK293T cells expressing WT hMC3R were 40.15 ± 5.48 pmol/10^6 cells (mean ± SEM). Star (*) indicates significantly different from WT hMC3R.

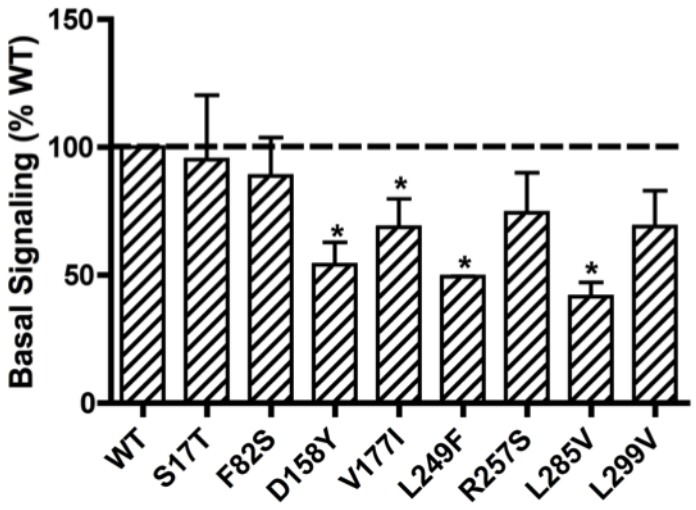
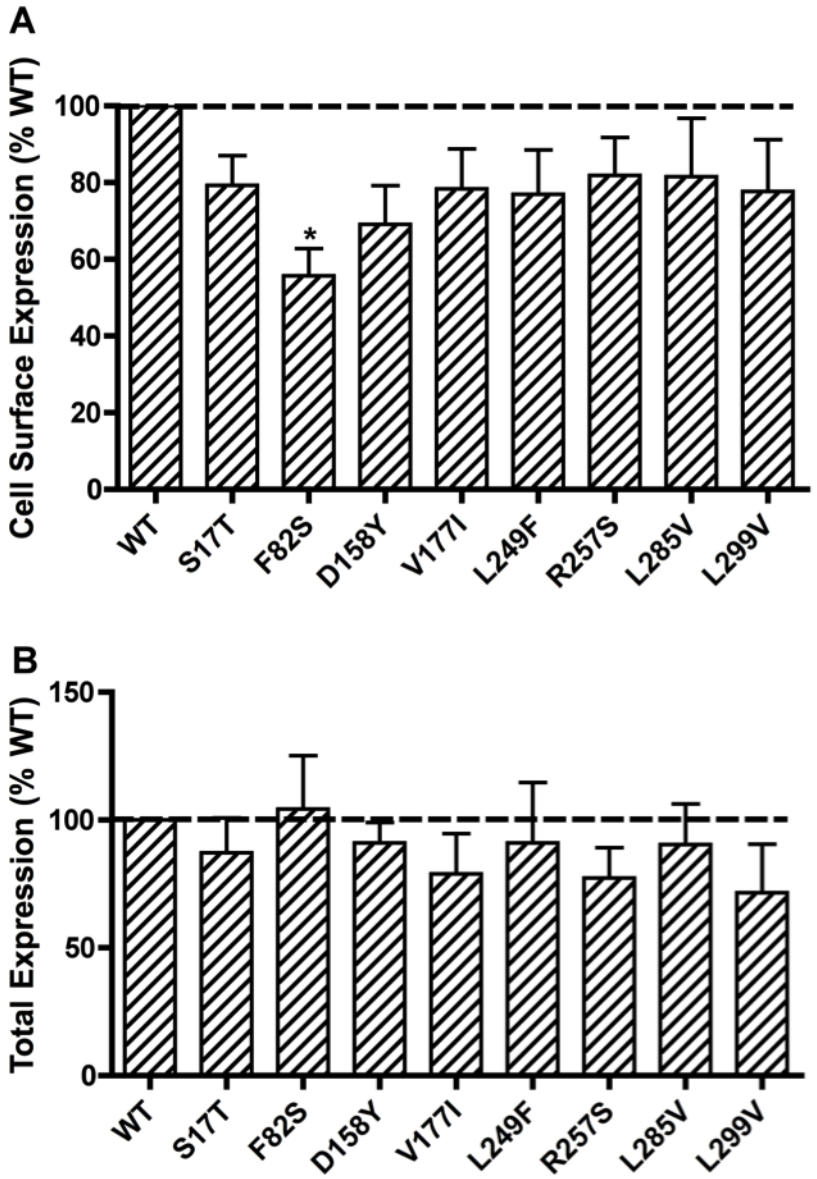

Basal Signaling (% WT)
150
100
50
0
*
*
*
*
WT
S17T
F82S
D158Y
V177I
L249F
R257S
L285V
L299V

Figure III-6. Quantitative measurement of cell surface (A) or total (B) expression of WT and mutant hMC3Rs by flow cytometry. In (A), cell surface expression of the WT and mutant hMC3Rs were measured by flow cytometry. The results were expressed as % of WT cell surface expression levels after correction of the nonspecific staining in cells transiently transfected with the empty vector as described in Materials and Methods. Data were mean ± SEM of 4-8 experiments. Star (*) indicates significantly different from WT hMC3R. In (B), total expression of the WT and mutant hMC3R were measured by flow cytometry. The results were expressed as % of WT total expression levels after correction of the nonspecific staining in cells transiently transfected with the empty vector as described in Materials and Methods. Data were mean ± SEM of 4-8 experiments. Star (*) indicates significantly different from WT hMC3R.


A
Cell Surface Expression (% WT)
100
80
60
40
20
0
*
WT
S17T
F82S
D158Y
V177I
L249F
R257S
L285V
L299V
B
Total Expression (% WT)
150
100
50
0
WT
S17T
F82S
D158Y
V177I
L249F
R257S
L285V
L299V

Conclusions and Future Prospectives

During the past decades there has been remarkable achievement in the field of obesity research. Obesity is the complex outcome of interactions of environmental and genetic factors. The identification of rare monogenic disorders in human genes from obese patients, which encode proteins of the leptin-melanocortin system, has provided substantial data demonstrating that the monogenic obesity exists.

Mutations in gene encoding leptin were first reported to be associated with severe early-onset obesity in human (Montague et al. 1997). Then, mutations in additional genes of leptin-melanocortin system were identified from obese patients, including leptin receptor (LEPR) (Clement et al. 1998), proopiomelanocortin (POMC) (Krude et al. 1998), prohormone convertase 1 (PC1) (Jackson, et al. 1997) and melanocortin-4 receptor (MC4R) (Vaisse et al. 1998; Yeo et al. 1998) (Reviewed in (Walley et al. 2009)). The mutations in MC4R account for about 6% of the pathogenesis of obesity, and therefore are considered to be the most common cause for the monogenic obesity (Tao 2010a). The functional studies of obese gene mutants have become a valuable tool to figure out the potential effects of gene alterations.

Melanocortin-3 receptor (MC3R) and MC4R are both expressed in brain. They also share the same sets of agonists and antagonists. Compared to MC4R, relatively few mutations in *MC3R* have been identified so far (Tao 2010b). Whether the mutations in *MC3R* are associated with obesity has drawn increasing attention. In this thesis, functional study of a total of 17 naturally occurring mutations in human *MC3R* was performed. Mutants D158Y, T280S and

L299V were shown to be potential pathogenic causes for the severe human obesity. The residues F82, D158, L249, T280 and L299 were observed to be critical in receptor expression, ligand binding and cAMP signaling, contributing to a better understanding of the structure-function relationship of MC3R. The co-expression experiments indicated that the majority of mutant hMC3Rs had haploinsufficiency, rather than dominant negative activity. Whether and how the mutants that did not have defects in receptor activation affect the adiposity of carriers still requires further investigation.

MC3R has been reported to activate the MAPK pathway via PI3 kinase (Chai et al. 2007). Whether the mutant hMC3Rs affect the MAPK pathway and subsequently are associated with human adiposity or obesity deserves future investigation. It may provide a better understanding of gene-phenotype relationship of MC3R in energy homeostasis.

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