MOLECULAR CHARACTERIZATION OF *MC3R* AND EVALUATION OF ITS POTENTIAL ROLE AS A MODIFIER OF LUNG FUNCTION IN CYSTIC FIBROSIS

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Abstract

Cystic fibrosis (CF), the most common lethal autosomal recessive disorder among Caucasians, affects approximately 30,000 individuals in the United States. CF is caused by loss of function mutations in the CF transmembrane conductance regulator (*CFTR*) gene. Once the disease-causing gene for CF was identified, numerous studies attempted to correlate *CFTR* mutations with specific CF phenotypes because genotype-phenotype correlations can predict a course of the disease and lead to the design of a genotype-specific therapeutic strategy. Unfortunately, distilling correlation has been challenging for several reasons. First, CF is a multi-system disease that involves different organs and its phenotype is analyzed in context of its various clinical components. Second, patients exhibit a wide range of disease severity although CF is considered a classic Mendelian disorder. In fact, the degree of variability observed in 293 individuals with the identical *CFTR* mutations suggests that factors other than the *CFTR* genotype contribute to lung function variation. Given that obstructive lung disease is the cause of death in 90% of CF patients, there has been continued interest in determining these factors that influence the severity of pulmonary disease in CF patients.

MC3R has been identified as a compelling candidate for modifying CF lung disease. Before exploring its role as a modifier, we aimed to understand the molecular organization of *MC3R*. Using 5' RACE, we discovered a novel upstream exon that extends the length of the 5' UTR in *MC3R* without changing the ORF. The full-length 5' UTR directs utilization of an evolutionarily conserved second in-frame ATG as the primary translation start site. MC3R synthesized from the second ATG is localized to apical membranes of polarized Madin Darby Canine Kidney (MDCK) cells, consistent

with its function as a cell surface mediator of melanocortin signaling. Expression of MC3R causes re-localization of a known accessory factor for MC2R, MRAP2, to the apical membrane, which is coincident with the location of MC3R. In contrast, protein synthesized from *MC3R* cDNAs lacking the 5' UTR displayed diffuse cytosolic distribution and no effect upon the distribution of MRAP2. Our findings demonstrate that a previously unannotated 5' exon directs translation of MC3R protein that localizes to apical membranes of polarized cells.

To define the mechanism of lung disease in CF that underlies the linkage signal at chr20q13.2, we evaluated *MC3R* as a potential modifier of lung function. Three rare variants in *MC3R* were significantly associated with lung function in individuals with CF, suggesting that variation in *MC3R* contibutes to CF lung disease severity. *In silico* prediction tools and *in vitro* studies were performed to understand the functional effect of the rare variants. We demonstrated that antagonist (siRNA) or agonist ($[D-Trp^8]-\gamma-MSH$) of MC3R can modulate pro-inflammatory cytokine expression. Evidence for the presence of MC3R in primary airway epithelial cells of the lungs further supported our hypothesis that MC3R is a modifier of lung function in CF. These studies support the role of MC3R as a mediator of the inflammatory response and a potential therapeutic target in the treatment of CF.

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In honor of my mother, who encouraged me to pursue my Ph.D.
and helped me believe that I could do anything

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Introduction and literature review

Cystic Fibrosis

Cystic fibrosis (CF) is the one of the most common lethal autosomal recessive disorder among Caucasians. CF affects approximately 30,000 Americans with 1,000 new cases reported annually in the US (1). Individuals with CF have obstructive and inflammatory disease that affects the lungs, pancreas, sweat glands, intestines, biliary tract, and male reproductive tract (Figure 1.1) (2). Pulmonary disease accounts for most of the morbidity and mortality in patients with CF (3). With significant advances in the diagnosis and treatment of CF, patients are experiencing better overall lung function and the median predicted age of survival has steadily risen from 28 years in 1988 to 41 years in 2012 (4).

CF is caused by loss of function mutations in the CF transmembrane conductance regulator (*CFTR*) gene (5). CFTR encodes a cAMP-regulated chloride channel that is expressed on the apical membrane of epithelial cells (6). The role of CFTR in epithelial cells extends beyond chloride permeability as it is known also to regulate transepithelial sodium channels (7), potassium channels (8), and bicarbonate transporters (9,10). Loss of chloride transport in pulmonary epithelium leads to an increase in intracellular salt concentration (11). This salt imbalance dehydrates the lumen, which impairs ciliary function. Abnormalities in submucosal gland fluid secretion affect the composition and quality of airway mucus, making easier for bacteria to be trapped (12). Defective bacterial clearance leads to chronic infection and persistent inflammation (13).

One of the hallmarks of CF is allelic heterogeneity. To date, there have been close to 2000 mutations found in *CFTR*. Some of the phenotypic variability in individuals with CF can be explained by the nature of the mutations in *CFTR*. However, genotype-

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Figure 1.1: CF is a highly variable, multi-system disease caused by mutations in

CFTR. The consequences of CF manifest in multiple organs, including the lungs,

pancreas, sweat gland, biliary tract, intestine and male reproductive tract.

Cystic Fibrosis is an autosomal recessive disorder with a highly variable phenotype

Sinopulmonary
Progressive Obstructive
Lung Disease
Rhinosinusitis

Sweat gland
[Cl$^-$] > 60 mmol/L (98%)

Pancreas
Exocrine Impairment (90%)
Diabetes (25%)

Intestinal Obstruction
Meconium ileus (15%)
DIOS (variable)

Male reproductive tract
Congenital Bilateral Absence of the
Vas Deferens (98%)

Aberrant fluid and ion transport across epithelial tissues

phenotype studies have revealed that variability in disease severity is influenced by factors other than the *CFTR* genotype (14-16). This chapter will discuss the clinical manifestations of CF, the classes of *CFTR* mutations, the correlations between genotype and phenotype, the environmental and genetic modifiers, and the role of melanocortin-3-receptor (*MC3R*) as a potential modifier of lung disease severity in CF.

Clinical Background

Obstructive lung disease is the cause of death in 90% of CF patients (17). However, patients exhibit a wide range of phenotypic presentations and severity. The classic form of CF is diagnosed if a patient has elevated sweat chloride and demonstrates clinical disease in one or more organs as described below (2).

Lung

The loss of CFTR chloride channels produces an abnormally high salt concentration in the airway surface fluid (18). Impaired transport of chloride and sodium across the respiratory epithelium impairs mucociliary clearance and causes a recurrent cough that becomes persistent. The chronic airway obstruction is followed by colonization with *P. aeruginosa* and *S. aureus* (19). Because these pathogens become well established within the airway surface, they are not effectively eradicated.

In addition to pulmonary obstruction, inflammation contributes to the decline in respiratory function in CF. Chronic bacterial infection in the airways results in inflammation and leads to progressive lung tissue damage. Elevated levels of pro-inflammatory cytokines, such as interleukin (IL)-8, and reduced levels of anti-

inflammatory cytokines have been found in the airways of patients with CF (20-22). Toll-like receptors are known to mediate inflammation in part by activation of the transcription factor NF-κB, which induces the production of inflammatory proteins and cytokines (23). Different therapies, such as antibiotics (24-26), hypertonic saline (27), and bronchodilators (28-30), have been found to slow the progressive respiratory failure. These therapies have been responsible for improvement in patient survival (31-33).

Pancreas

CF is associated with three different types of pancreatic disease: pancreatic insufficiency, pancreatitis, and CF-related diabetes (CFRD).

Pancreatic insufficiency (PI) is defined by abnormal exocrine function, and it occurs in over 80% of patients with CF. The damage begins in utero and can be identified in neonates on the basis of elevated serum immunoreactive trypsinogen (IRT). Hence, IRT serves as a marker for newborn screening for CF (34). While the *CFTR* genotype does not predict survival due to the complex interaction between genetic and environmental modifiers of lung disease, it is highly predictive of pancreatic exocrine status (35).

The defective chloride secretion in the pancreas causes progressive pancreatic damage, leading to pancreatitis. Pancreatitis occurs mainly during adolescence and young adulthood. While pancreatitis is reported for patients carrying a wide range of mutations, the incidence of pancreatitis is higher among patients with pancreatic sufficiency (PS) than those with PI (36).

Patients with exocrine pancreatic insufficiency often develop endocrine pancreatic dysfunction, which ultimately results in CFRD. CFRD illustrates features of both type I and type II diabetes, including decreased insulin production and insulin resistance (37). Approximately 25% of CF patients develop CFRD by 20 years of age, and the risk depends on the *CFTR* genotype (38). Several studies have shown that pulmonary function is worse in CF patients with CFRD compared with those without diabetes (39-41).

Intestine

Up to 20% of patients with CF are born with intestinal obstruction called meconium ileus (MI) (42). MI occurs in the distal ileum or proximal colon and is fatal unless surgically treated to relieve the obstruction (43). The pathogenesis of MI is unclear, but it is believed to result from the loss of CFTR-mediated chloride and/or bicarbonate transport by the intestinal epithelium (44,45). Nutritional evaluation of infants and children revealed an association of MI with malnutrition in CF, potentially due to the surgical treatment for MI and poor essential fatty acid status (46). It has been demonstrated that in a pig model of CF, intestinal expression of CFTR without pancreatic or hepatic correction can partially restore CFTR-mediated anion transport and rescue MI (47). The optimal medical treatment and nutritional intervention for CF patients with MI need to be further investigated.

Male reproductive tract

More than 98% of men with CF are infertile due to congenital bilateral absence of the vas deferens (CBAVD) (2). These men are able to produce sperm, but they lack the canal that carries sperm from the testis to the ejaculatory duct. In a guideline for the

appropriate use of genetic tests in infertile couples, screening for *CFTR* mutations

including the 5T allele is strongly recommended with a diagnosis of CBAVD (48). It is

important to note that some individuals with the 5T allele in *trans* with a severe *CFTR*

mutation may have nonclassic CF or male infertility due to CBAVD; in 40% of the cases,

they are healthy and fertile as a consequence of incomplete penetrance of the 5T variant

(49,50). It has been recently demonstrated that the number of TG repeats adjacent to 5T

determines disease penetrance (51). Hence, diagnosis and interpretation in the case of the

5T variant in intron 8 of the *CFTR* gene should be done in conjunction with TG repeating

testing.

CFTR Mutation Class

The *CFTR* gene is located on chromosome 7q31.2 and encodes a 6.5 kb mRNA

transcript with 27 exons. The CFTR protein is comprised of 1480 amino acids and

contains two six-pass transmembrane domains, two nucleotide binding domains and a

regulatory domain. CFTR is synthesized in the ER and N-linked glycosylated at amino

acid residues 894 and 900 (52). The mature CFTR protein is then transported to the cell

membrane under the guidance of cargo proteins, such as synthaxin 1A and SNAP-23, that

bind to the N-terminus, as well as NHERF, CAL, and synthaxin 6 that bind to the C-

terminal tail of CFTR (53-56). The fully glycosylated wildtype CFTR is located at the

plasma membrane (57) and is detected as 'C band' at 170 kD. The mutant CFTR, such as

F508del, is detected as 'B band' at 140 kD because it is not fully processed (58). As a

result, it is retained in the ER and subsequently degraded by a proteasome.

Close to 2000 variants in *CFTR* have been described to date, but one mutation – F508del – accounts for approximately 70% of CF chromosomes (59). Most of the *CFTR* mutations are extremely rare. Only six other mutations have a pan-ethnic frequency of greater than 1%: G542X, W1282X, G551D, 621+1G>T, N1303K, and R553X (60). The *CFTR* mutations can be classified according to the mechanisms by which mutations disrupt CFTR protein synthesis and its chloride channel function (Figure 1.2) (61,62). This classification, however, is deemed simplistic because mutations can affect multiple properties of CFTR, such as protein folding and gating function. The understanding of the molecular mechanism of each CFTR dysfunction allows mutation-specific pharmacotherapy to be developed (63).

Class I

Class I mutations lead to the creation of unstable, truncated mRNA transcript as a result of abnormal splicing or insertion of a premature stop codon. G542X is an example of a nonsense mutation in this class that significantly reduces the level of mutant CFTR compared to wildtype CFTR. There is evidence that null mutations can be overcome by aminoglycoside antibiotics, which suppress premature stop codons and permit translation to continue to synthesize full-length CFTR (64,65).

Class II

Class II mutations produce a misfolded protein that lacks a mature glycosylation pattern. As a result, the protein is retained and degraded in the ER by the quality control mechanisms. The most common mutation in *CFTR*, F508del, belongs to this category.

Figure 1.2: CF is characterized by extensive allelic heterogeneity. CF mutations can be grouped into several categories or types based on how the mutations affect the CFTR protein. About half of the individuals with CF are homozygous for the most common *CFTR* mutation, F508del, which causes defective protein folding and processing in the ER, resulting in minimal amounts of CFTR at the cell surface. Several corrective agents are approaching clinical trials. Kalydeco™ is the first and only FDA-approved drug to treat individuals with the G551D mutation. (Figure from Herrmann *et al. Best Pract Res Clin Gastroenterol.* 2010) (71)

	Normal	Class I	Class II	Class III	Class IV	Class V
Biochemical phenotype		No CFTR synthesis	CFTR trafficking defect	Dysregulation of CFTR (diminished ATP binding and hydrolysis)	Defective chloride conductance or channel gating	Reduced *CFTR* transcription and synthesis
Types of mutations (examples)		Nonsense G542X, W1282X Frameshift 394delTT Splicing 711+1G>T, 1717-1G>A	Missense G85E, S549R, L1065P, N1303K Amino acid deletion ΔF508	Missense S492F, V520F, G551D, R553G, R560S, R560T	Missense R117C, L227R, R347P, A455E, D1152H	Splicing 1811+1.6kb A>G, 2789+5G>A, 3849+10kbC>T, IVS8-5T
Potential therapy (examples)		Gentamicin Ataluren (PTC 124) Gene transfer	Butyrates Curcumin Gentamicin Gene Transfer	Gentamicin Gene transfer	Milrinone Gene transfer	Ataluren (PTC 124) Gene transfer

Class II mutations result in a severe clinical phenotype as CFTR is virtually absent at the cell surface. It has been demonstrated that class II mutants, while misfolded, can conduct some chloride conductance (66,67). This reveals future opportunity to design therapies that can help mutant proteins escape ER-associated degradation and permit surface expression of the partially functioning chloride channel.

Class III

Class III mutations disrupt the activation and regulation of CFTR at the cell membrane. For instance, G551D is expressed at the cell surface but is unable to conduct chloride in response to elevated cAMP due to impaired ATP binding. In Jan. 2012, a drug called Ivacaftor (Kalydeco[TM]) was approved by FDA for CF patients who have at least one copy of the G551D mutation (68). The effectiveness of Kalydeco[TM] to increase the activity of defective CFTR protein represents a significant milestone in the development of treatments for CF.

Class IV

Class IV mutations give rise to functional proteins that localize to the apical surface but have reduced chloride conductance. One way to overcome this problem is by increasing the overall cell surface content of these mutant proteins to compensate for the relative reduction in conductance. CF patients with one or two copies of class IV mutations, such as R117H, usually have a milder CF phenotype (69). F508del is also a class IV mutation because the open time for chloride transport is 10% of wildtype levels.

Class V

Class V mutations, such as 3849+10kb C>T, generate properly and improperly spliced transcripts. These patients often demonstrate milder manifestation of the disease since their CFTR protein is active but reduced in number. The variable disease expression has been shown to have inverse correlation with the level of correctly spliced transcripts (70). This class of mutations emphasizes the role of splicing in regulation of disease severity.

Genotype-Phenotype Correlation

Once the disease-causing gene for CF was identified, numerous studies attempted to correlate *CFTR* mutations with specific CF phenotypes. Genotype-phenotype correlations can predict a course of the disease and lead to the design of a therapeutic strategy based on the specific genotype (72). Unfortunately, distilling correlation has been challenging for several reasons. First, CF is a multi-system disease that involves different organs and its phenotype is analyzed in context of its various clinical components, such as pancreatic exocrine function, pulmonary disease, sweat chloride level, vas deferens abnormalities in males (73). Second, patients exhibit a wide range of disease severity although CF is considered a classic Mendelian disorder. It is perhaps not surprising given the number of *CFTR* mutations that have been identified so far. Lastly, most *CFTR* mutations are extremely rare. Determining the functional consequence of each mutation is essential in assessing its disease liability, but it is infeasible to perform analysis of every mutation. Grouping mutations according to the mechanisms by which

mutations disrupt CFTR protein synthesis has improved our ability to uncover which specific phenotypes are strongly correlated with particular classes of mutations.

The highest degree of correlation between the *CFTR* genotype and the CF phenotype is found in the pancreatic status. Concordance of pancreatic function status among affected individuals within the same family suggested that genetic factors, such as the *CFTR* genotype, affects the degree of pancreatic disease (74). We now know that *CFTR* genotype is a predictor of pancreatic status because previous studies have demonstrated that patients with PS carry mutations (classes IV-V) that are different from those with PI (classes I-III) (73,75). In light of this observation, mutations that are definitively associated with PI are often categorized as severe mutations. In contrast, mutations that are associated with PS are labeled as mild mutations. Identification of patients carrying two severe alleles predictive of PI through newborn screening program allows early nutritional support and pancreatic enzyme supplements (76).

Unlike the strong association between the *CFTR* genotype and the pancreatic phenotype, the severity and course of pulmonary disease are not predicted by the genotype. One study has posited that the presence of class I or II mutations on both chromosomes is associated with worse respiratory disease and a lower probability of survival (77). Nonetheless, it is generally agreed that correlation between severity of pulmonary disease and genotype is difficult to discern (73,78). In a study of 293 patients homozygous for F508del mutation, considerable variability in pulmonary function was found at all ages (Figure 1.3) (74). The degree of variability observed in individuals with the identical *CFTR* genotypes reveals that factors other than *CFTR* contribute to the lung

Figure 1.3: The variability in lung function with an identical *CFTR* genotype is considerable. The airway flow measure, forced expiratory volume in one second (FEV$_1$), is plotted against age in individual patients who are homozygous for F508del mutation. The red and blue circles represent 17-year-old patients with 10% and 90% FEV$_1$ measures, respectively. The variability in pulmonary function indicates that there must be factors other than the *CFTR* genotype that affect the severity of lung disease. (Figure from Kerem *et al. NEJM.* 1990) (74)
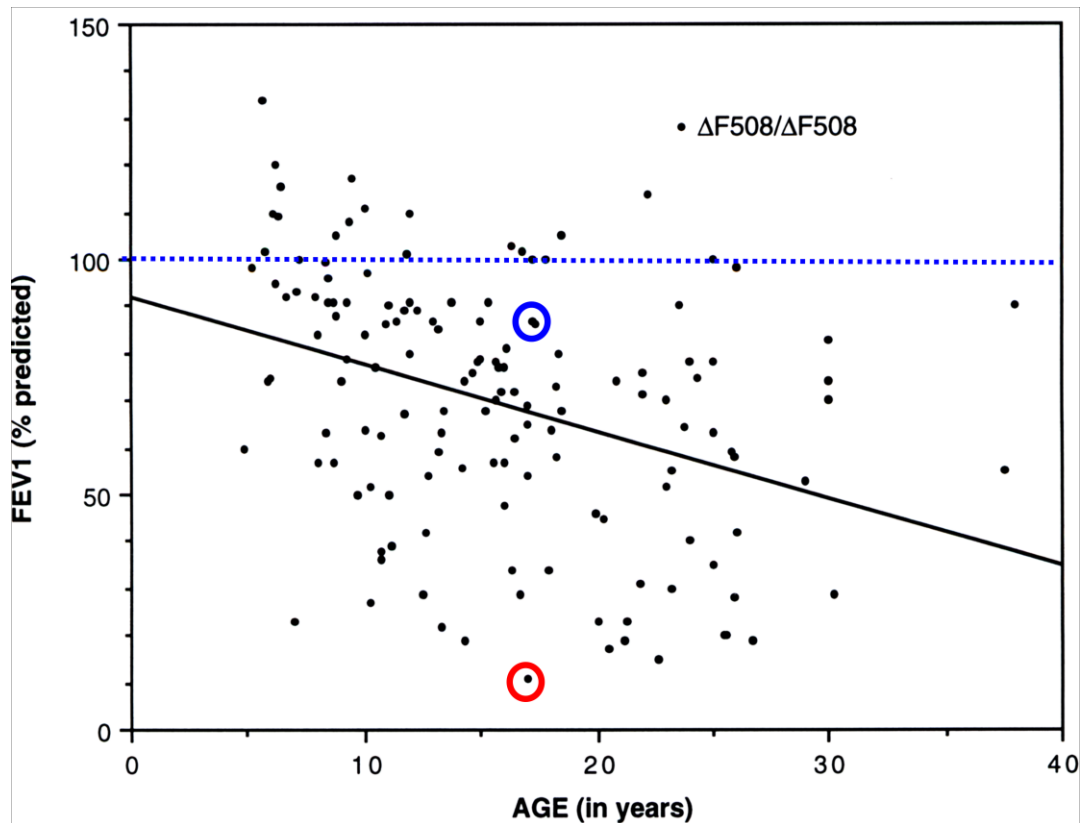
function variation, thus emphasizing the importance of determining these factors that influence the severity of pulmonary disease in each patient (79,80).

Modifiers of CF Phenotype

Environmental modifiers

Phenotypic variation in a monogenic disorder, such as CF, can be attributed to several sources, including different mutations in the disease-causing gene, genetic modifiers, environmental factors, random events, and interactions between any of these factors (Figure 1.4) (81). Since environmental factors account for 50% of the variation in CF lung function (82), identifying these factors can help in development of therapies and interventions to decrease the morbidity and mortality associated with the disease. So far, the following environmental and socioeconomic factors have shown to impact lung function in CF: secondhand smoke (83), health insurance (84), household income (85), air pollution (86), ambient temperature (87), and environmental allergens (88). Three factors listed above will be described in detail.

Secondhand smoke causes premature death and disease in non-smoking children and adults (89). Exposure to secondhand smoke has immediate adverse effects on the cardiovascular system and increases the risk for acute respiratory infections. Perhaps not surprisingly, any exposure to secondhand smoke has been shown to adversely affect cross-sectional as well as longitudinal measures of lung function in those with CF (90-92).

A growing body of evidence indicates that socioeconomic status (SES) is a strong predictor of health status in several chronic diseases. Improved health is associated with

Figure 1.4: Multiple factors interact to determine the CF phenotype. Phenotypic variation seen in individuals with CF can be attributed to several factors, such as the *CFTR* genotype, modifier genes, environmental factors and stochastic events. (Figure from Collaco and Cutting. *Curr Opin Pulm Med.* 2008) (81)
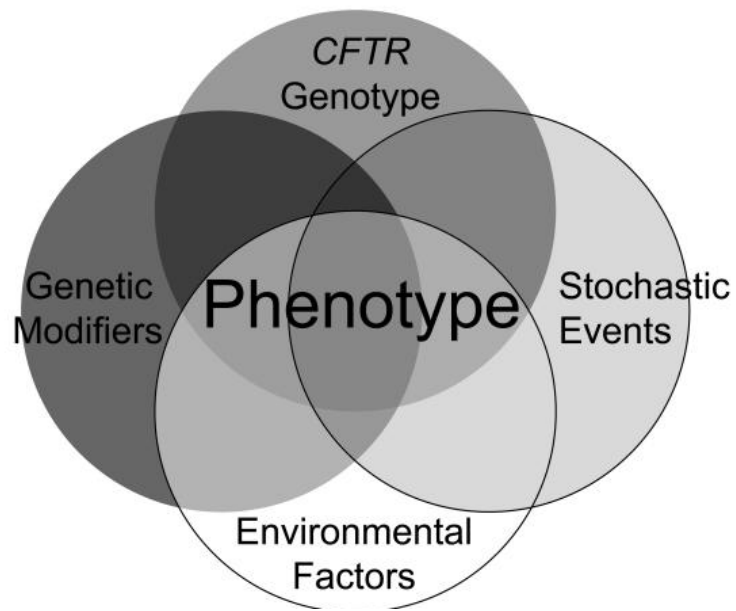
higher income, more education, better job and safer neighborhood. Using public assistance as a proxy for low SES, it has been demonstrated that Medicaid patients with CF have poorer lung function and require more treatments for pulmonary exacerbations than non-Medicaid patients (93). This difference may be explained by inadequate access to primary care or poor adherence to prescribed regimens. Interestingly, no significant relationship was found between SES and CF-related morbidity in the Canadian population (94). The authors speculate that universal access to health services in Canada that covers the cost of most CF medications and nutritional supplements may ameliorate previously documented SES disparities.

Lastly, the presence of environmental allergens has been associated with poorer respiratory outcomes in other obstructive lung diseases, such as asthma (95) and COPD (96). Similarly, environmental allergens were found to be associated with more rapid decline in lung function in CF (88). This suggests that management of environmental allergies can be a target for therapeutic interventions for individuals with CF.

In contrast to the environmental modifiers that have adverse effects on CF phenotype, there are therapeutic treatments that improve the quality of life of CF patients. Improved growth and nutritional status early in life has been associated with better pulmonary function later in life, which suggests that early intervention, such as nutritional counseling, can have a favorable impact on survival by slowing the rate of decline in pulmonary function (97-99). Pancreatic enzyme supplementation also has a significant impact on survival of CF patients (100). Since most patients have abnormal pancreatic exocrine function, pancreatic enzyme replacement therapy is utilized by about 90% of CF patients in Northern Europe where F508del mutation is commonly found

within the CF population (101). Lastly, the wide use of antibiotics for lung infections has contributed to improvement in survival (102). The long-term use of low dose azithromycin was demonstrated to significantly reduce the number of pulmonary exacerbations that required additional courses of antibiotics prior to infection with *P. aeruginosa* (103). Similarly, the administration of inhaled tobramycin in conjunction with standard therapy for CF improved lung function, decreased bacterial load in sputum, and reduced the need fewer hospitalizations (24).

Genetic modifiers

Family-based studies have been used to estimate genetic and nongenetic contributions to disease severity in CF. The role of genetic modifiers was affirmed when pulmonary function was found to be more similar among monozygotic twins than dizygotic twins and sibling pairs (82). Furthermore, heritability of lung function severity has been estimated to be 0.54-1 using cross-sectional and longitudinal measures of lung function in affected twins and siblings (104). The higher concordance of lung function in monozygotic twin pairs and the high estimate of heritability for the FEV_1 measures in affected siblings validate the importance of searching for CF modifier genes. Identification of the modifier genes will help us understand the pathogenesis of CF and lead to a more rational use of conventional treatments. A number of candidate genes have been studied in two or more separate populations of individuals with CF. Three modifier genes – TGFβ1 (105,106), IFRD1 (107), and MSRA (15,108) – will be discussed in detail.

Genetic variation in *TGFβ1* (transforming growth factor β1) gene as well as the upstream region has been found to modify lung disease severity in CF. TGFβ1 is a cytokine that plays an important role in regulating the immune system. Dysfunction in TGFβ1 signaling has been associated with numerous diseases, such as Alzheimer's disease (109), inflammatory bowel disease (110) and congenital heart disease (111). Using family-based transmission analysis, polymorphisms in *TGFβ1* was found to modify CF lung function, but not nutritional status. Interestingly, non-F508del homozygous patients carrying at least one C-T-C haplotype had better lung function than patients carrying the T-C-C haplotype, suggesting that association of *TGFβ1* alleles with lung function variation was dependent upon *CFTR* genotype (106).

Polymorphisms in *IFRD1* (interferon-related developmental regulator 1), histone-deacetylase-dependent transcriptional co-regulator that is expressed during terminal neutrophil differentiation, have been shown to contribute to lung function variation in CF independent of *CFTR* genotype. In particular, the rs7817 polymorphism is significantly associated with cross-sectional and longitudinal measures of lung function. The follow-up analysis confirmed that the heterozygote genotype (CT) of *IFRD1* is associated with poorer lung function than either homozygote (CC or TT). Functional studies using *Ifrd*$^{-/-}$ mice showed delayed bacterial clearance but ameliorated disease with less weight loss and less neutrophilic inflammation upon challenging the mice with *P. aeruginosa*. Analysis of human neutrophils showed a significant association with *IFRD1* polymorphisms with neutrophil effector function. Though neutrophils are not the only CF-relevant cells influenced by *IFRD1* polymorphisms, these data suggest that IFRD1

affects the pathogenesis of CF lung disease through the modulation of neutrophil effector activity.

Variations in *MSRA* (methionine sulfoxide reductase A) gene are associated with MI, the intestinal obstruction phenotype of CF. MI, like lung disease severity, is a highly heritable trait, further supporting that genetic modifiers are probably responsible for this manifestation. Functional studies using two CF mouse models – null model with high rates of obstruction and p.Arg117His model with lower rates of obstruction – revealed that loss of *Msra* provides protection from intestinal obstruction during the time of weaning. Like IFRD1, MSRA modifies MI independently of variation in *CFTR*. Discovery of *MSRA* as a modifier of MI offers new insight into the mechanism of intestinal complication of CF.

Twin-Sib Study for Investigation of CF Lung Disease Severity

In addition to *TGFβ1* and *IFRD1*, genome-wide association and linkage studies identified modifier loci of lung disease severity in CF at 11p13 and 20q13.2 (112). FEV_1, the most useful measure of lung disease severity and a well-established predictor of survival (113), was used in this study to compare lung function measures. This thesis will focus on 20q13.2, the locus of interest that contains a potential modifier gene, *MC3R*.

Non-parametric linkage analysis of 486 sibling pairs from the family-based study discovered a genome-wide significant locus (LOD = 5.03) on 20q13.2 (Figure 1.5). This locus is responsible for as much as 50% of the variation in lung function in the sibling pairs with CF. This result was followed by SNP association testing in the linked region using unrelated individuals with two severe *CFTR* alleles from other studies. In this 1.3

Figure 1.5: 20q13 contains a modifier for lung disease severity in CF. Genome-wide

linkage scan for the lung phenotype of 486 sibling pairs with CF identified a locus on

chromosome 20q13 that contains a modifier for lung function. There are five genes found

in the 1.3 Mb region of maximum linkage, one of which is *MC3R*. The bottom figure

shows the LocusZoom plot of association in the critical linkage region. (Figures from

Wright *et al. Nature Genetics.* 2011) (112)
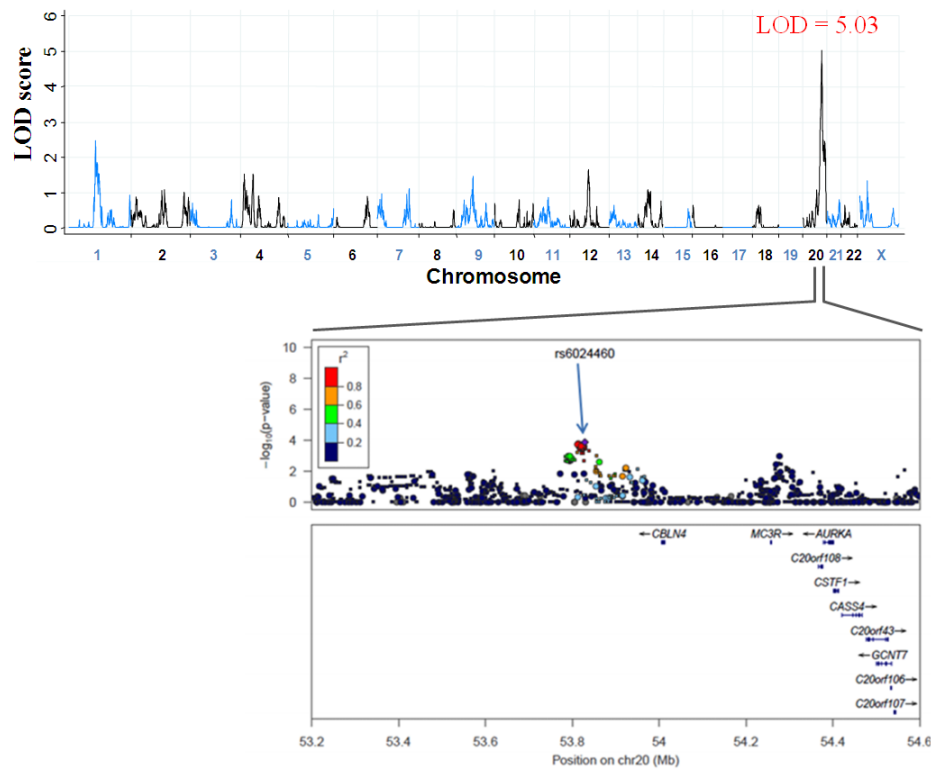
Mb linked region, demarcated by 1 LOD unit below the maximum, there were two clusters of SNPs. A 16-kb cluster of SNPs in high LD within the 1 LOD support interval was located is a gene desert and are centromeric to five genes: *CBLN4*, *MC3R*, *AURKA*, *CSTF1* and *CASS4*. One SNP within this cluster (rs6024460; LOD = 3.87) exceeded the threshold for regional significance. Notably, a second smaller cluster of SNPs covering approximately 8 kb was identified near *MC3R*. The SNP with the highest LOD score in the second cluster (rs1326022; LOD = 2.94) was located 20 kb downstream of *MC3R*, raising the possibility that common variants in or near this gene may influence lung function.

MC3R as a Potential Modifier of Lung Function in CF

None of the SNPs in the 16-kb cluster lies within a gene, but this does not exclude the possibility that this region regulates expression of the downstream genes. However, the second smaller cluster of SNPs is directly over *MC3R*, a promising candidate gene for modifying lung function in CF (114). Various mechanisms by which MC3R affects CF lung disease are explored here.

There are several reasons why MC3R is a biologically plausible candidate for modifying CF lung disease. First, MC3R and the family of melanocortin receptors have known roles in inflammation in the brain and outside of central nervous system (115-117). Individuals with CF have an increased level of NF-κB-regulated proinflammatory cytokines, such as IL-8 (118,119). Since inflammation is a key feature of CF lung disease, altering the heightened baseline level of inflammation or modulating repetitive cycles of inflammation due to infection could reduce lung injury in CF patients (120).

Along these lines, Getting *et al.* reported that melanocortin peptides inhibit leukocyte accumulation in allergic and non-allergic mouse models of lung inflammation (115). It has been demonstrated that binding of melanocortin to melanocortin receptors leads to an inhibition of the NF-κB signaling in macrophages as well as other inflammatory cells (121), and the same mechanism has been shown to be operant for MC3R in bronchial airway epithelial cells (116). Investigation of MC3R's effect on the NF-κB signaling and the inflammatory response could lead to therapeutic interventions for modulating lung inflammation.

Alternatively, MC3R may be involved in regulation of lung function by influencing energy homeostasis. Nutritional status has a significant impact on lung disease in CF patients (122). In the general population, both common and rare variants in *MC3R* are associated with weight gain in children (123-125). Furthermore, *Mc3r*$^{-/-}$ mice have increased fat mass, reduced lean mass and higher feed efficiency compared to the wildtype littermates while eating less and maintaining normal metabolic rates (126). Since elevated resting energy expenditure is highly correlated with lung tissue damage in people with CF, prevention of negative energy balance could reduce pulmonary exacerbations (127). MC3R might contribute to the complex interaction between lung inflammation and malnutrition.

Lastly, MC3R may directly influence CFTR expression. The idea that MC3R may increase the functional activity and cell surface expression of CFTR was derived from the result from an empiric screen where siRNAs targeting MC2R and MC3R partially restored CFTR function (128). This result was described in a patent filed by a Belgium company, Galapagos, in which they explained a method for identifying compounds that

increase the functional activity and cell surface expression of CFTR. The patent revealed

that reduction of certain target genes, including MC2R, lead to proper post-translational

modification of CFTR. CFTR correction was shown by a shift from B-band, representing

the immature CFTR protein, to C-band, representing mature form of CFTR. The

company validated this finding by correcting trans-epithelial chloride transport in primary

bronchial epithelial cell cultures derived from a CF patient homozygous for F508del

mutation (CFBE41o- cells). Table 1.1 includes the final list of 19 genes that show

expression of increased cell surface expression of F508del CFTR upon reduction of a

target mediated by at least two shRNAs. Interestingly, MC3R was on the preliminary list

of 315 hits that restored CFTR-dependent halide flux activity but failed to be on the final

list of hits because its effect was not recapitulated with a completely independent shRNA

targeting *MC3R* through a difference sequence (David Fischer, personal communication,

2010). Since MC3R and MC2R are in the same receptor family and they have high

sequence homology, MC3R is a reasonable candidate for examination of its role in

regulation of CFTR function.

Melanocortin Receptor Family

Overview

 MC3R is a G-protein-coupled receptor (GPCR) for melanocyte-stimulating

hormone (MSH) (Figure 1.6). It belongs to the melanocortin family that consists of five

GPCRs, which mediate key physiological functions, such as pigmentation,

steroidogenesis, energy balance, and food intake (Table 1.2) (129). They are labeled

MC1R to *MC5R* according to the order in which each was identified (130). To date,

Table 1.1: Efficacy of the restoration of chloride transport in primary CF bronchial epithelial cell culture. Whereas the baseline chloride flux in the CF culture is usually less than 2% of non-CF control culture, reduction of each target in this table, including MC2R, led to a significant increase in chloride transport across the epithelial monolayer.

TARGET Gene Symbol	SEQ ID NO: DNA	Forskolin-Genistein induced chloride flux in CF primary epithelial cells compared to control cells (non-CF)
UGT3A2	2	22.9%
PHGDH	3	6.6%
B3GNT3	4	4.7%
PPIH	5	9.1%
CELSR3	6	11.8%
MC2R	7	6.8%
MAS1L	8	6.6%
LRRK2	9	15.9%
NLRP1	10, 11, 12, 13, 14	9.0%
PMS1	15, 16, 17	9.3%
MAK	18	9.5%
CPD	19	14.8%

Figure 1.6: Schematic presentation of the general structure of GPCR. MC3R is a GPCR that contains an extracellular N-terminus, followed by seven transmembrane domains, and an intracellular C-terminus.
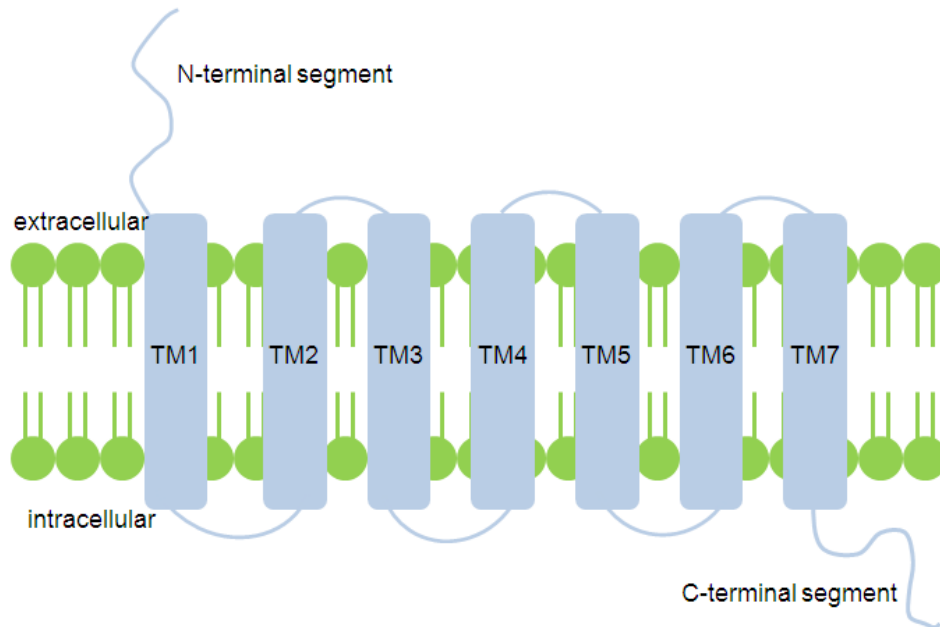
Table 1.2: Biological distributions and functions of melanocortin receptor family.

	MC1R	MC2R	MC3R	MC4R	MC5R
Function	Pigmentation regulation	Adrenal steroidogenesis	Energy homeostasis, inflammation	Energy homeostasis	Regulation of exocrine gland secretion
Ligand	ACTH and MSH	ACTH	ACTH and MSH	ACTH and MSH	ACTH and MSH
Mutation / Disease	Polymorphism in the *MC1R* is associated with red hair, fair skin, freckling, and skin cancer risk in the Caucasian population	Inactivating mutations of the MC2R as a cause of familial glucocorticoid deficiency	Mutations in this gene increase body mass and feeding efficiency in humans and mice	Mutations in this gene have been associated with 1–5% of early-onset human obesity	Candidate for dyslexia susceptibility genes
Tissue	Melanocytes, melanoma, macrophages, adipose	Adrenal, adipose	Brain, placenta, stomach, duodenum, pancreas, kidney, macrophages	Brain, spinal cord	Ubiquitously expressed, including brain, exocrine glands, skeletal muscle, etc.

MC2R is the only member of this family that has known accessory factors: melanocortin-2-receptor accessory protein (*MRAP*) and its paralogue *MRAP2* (129,131). Inactivating mutations of *MC2R* and *MRAP* cause of familial glucocorticoid deficiency type 1 and 2, respectively (131). Mutations in *MC4R* are associated with 1-5% of early-onset human obesity (132).

Mechanism of Action

Binding of melanocortin peptides to the melanocortin receptors stimulates adenylyl cyclase, elevates cAMP and leads to activation of PKA (133,134) . Interestingly, melanocortin peptides have been shown to mediate anti-inflammatory effects (121). Understanding the mechanism of action of melanocortin receptors is crucial in designing synthetic melanocortin agonist for appropriate receptors. The anti-inflammatory signaling pathway of melanocortin receptors will be described here.

The melanocortin peptides have been reported to interact with cells of the immune system and down-regulate the production of pro-inflammatory cytokines (135,136). It is believed that melanocortins regulate inflammation by inhibiting activation of the transcription factor NF- κB (137-140). MC3R is the only melanocortin receptor that is specifically activated by γ-MSH (141). NF-κB induces transcription of many molecules involved in inflammation, including cytokines. When bound to IκB inhibitory proteins, NF-κB is retained in an inactive form in the cytoplasm. Once IκB is phosphorylated and subsequently ubiquitinated, NF-κB dissociates from its inhibitory proteins and translocates to the nucleus where it induces the transcription of inflammatory response genes (Figure 1.7). It has been demonstrated in several cell types that α-MSH prevents

Figure 1.7: Anti-inflammatory effects of melanocortins. The activation of melanocortin receptors prevents NF-κB translocation to the nucleus and inhibits production of a wide range of inflammatory mediators.
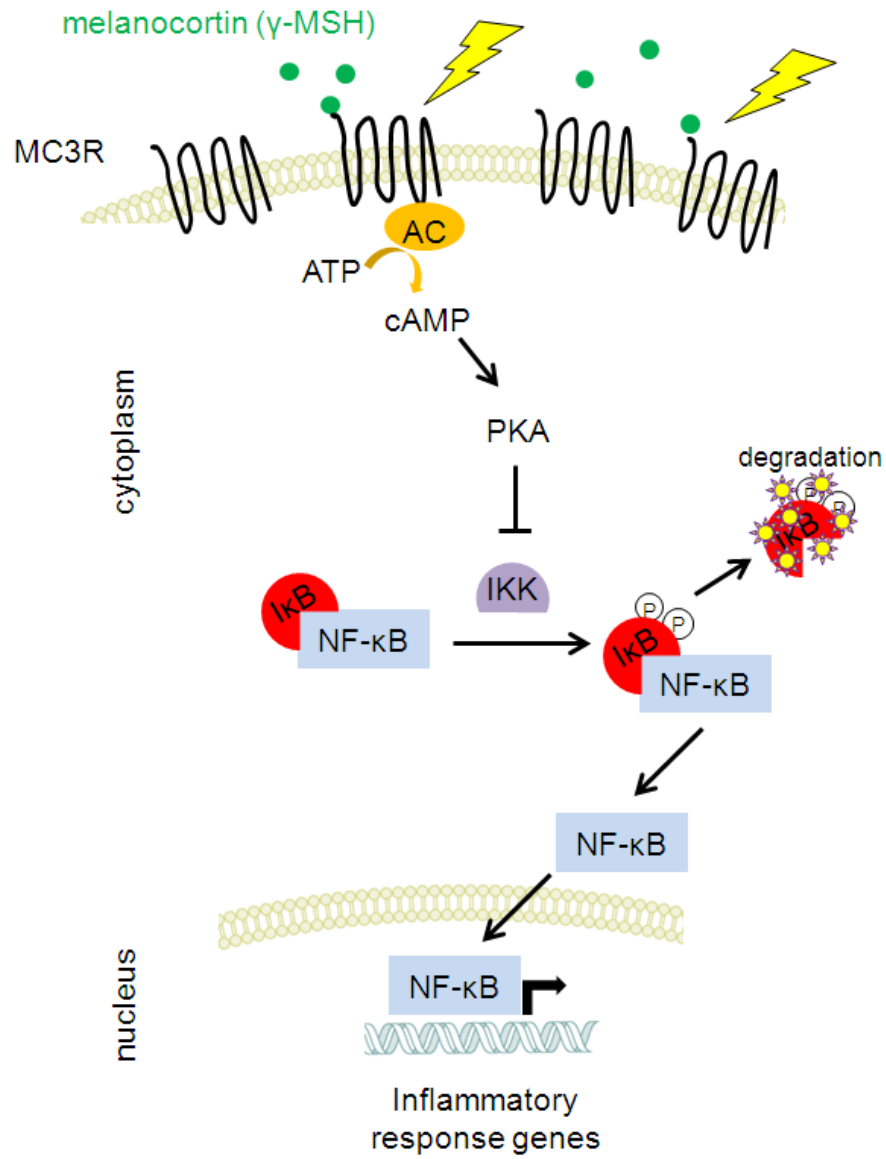
NF-κB translocation upon being induced by inflammatory stimuli. NF-κB inhibition results from cAMP generation by α-MSH, activation of PKA, and prevention of IκB from phosphorylation (142,143). Hence, the melanocortins provide important targets for developing pharmacological agents that can potentially be used to modulate the inflammatory process (144).

Localization of MC3R

As a member of a family of proteins that signal through the GPCR pathway, MC3R should be translocated across the ER and localized at the cell surface to enable binding to its ligands. Several studies have investigated the localization of MC3R in different cell types (Table 1.3) (123,145,146). For example, one study indicated that HA-MC3R, a fusion protein made from a commercially available plasmid that is forced to initiate MC3R synthesis at the first in-frame ATG, shows cell surface as well as intracellular expression in HEK293 cells (145). In contrast, other studies concluded that MC3R-EGFP and GFP-MC3R are expressed at the plasma membrane of HEK293 and COS-7 cells, respectively. In each case, intracellular expression is evident in the provided photomicrographs (123,146). One of the limitations of the previous studies is that the expression was only studied in non-polarized cells. Such cell types do not represent the true physiological state of MC3R because it is known to be predominantly expressed in the brain where polarized neurons have a distinct distribution of membrane proteins (147). CF is caused by dysfunction of CFTR in polarized airway epithelial cells of the lung. Asymmetric distribution of proteins involved in ion and water transport is

Table 1.3: Localization of MCR expression by confocal microscopy.

	MC3R (example 1)	MC3R (example 2)	MC3R (example 3)
Plasmid	N' terminal 3xHA-MC3R	N' terminal GFP-MC3R	MC3R-C' terminal GFP
Cell	HEK293	COS-7	HEK293
Fluorescence	Green – anti-HA	Green – GFP Red – nucleus	Green – GFP
Conclusion	WT MC3R was expressed on the cell surface as well as intracellularly (145)	White arrows indicate the presence of fluorescence on the cytoplasmic membranes (146)	By confocal microscopy, EGFP-tagged MC3Rs were expressed in the plasma membrane (123)
Confocal images			

critical to their ability to maintain airway surface liquid (148,149). Thus, our interest in

MC3R as a genetic modifier of lung function in CF requires investigation of its

expression in the lung and localization in polarized cells.

Interaction between the accessory proteins and melanocortin receptors

MRAP and MRAP2 are hypothesized to have other functions in addition to being

accessory proteins for MC2R (150). Investigation of such roles of MRAPs can reveal

their interactions with other melanocortin receptors and elucidate various mechanisms

that control biologically relevant processes (Figure 1.8). All melanocortin receptors,

including MC3R, have been demonstrated to functionally interact with MRAP and

MRAP2 (131). In one study, MRAP enhanced MC3R coupling to adenylyl cyclase in

response to stimulation with α-MSH in HEK293 cells (151). MRAP was shown to

colocalize with MC3R in the perinuclear region, cytoplasm and plasma membrane (151).

Colocalization of MRAP and MC3R indicates that these proteins may interact with each

other and are trafficked to the cell membrane together. Further studies are needed to

determine if and how MRAP stabilizes MC3R at the cell surface to decrease receptor

degradation and increase receptor recycling. These effects of MRAP suggest that MRAP

could be an integral modulator of MC3R.

Present studies of MC3R as a modifier of lung disease in CF

The work presented here represents an on-going study that will attempt to

elucidate the mechanism of CF lung disease reflected by the linkage signal at chr20q13.2.

The purpose of the research in this thesis is to characterize *MC3R* and evaluate its role in

Figure 1.8: Effect of MRAP on MC2R and MC3R in a signaling pathway. MC2R is retained in the ER in the absence of MRAP. MRAP assists MC2R trafficking to the cell surface and enhances MC2R signaling in the adrenal gland. MRAP has been found to significantly reduce MC3R signaling.
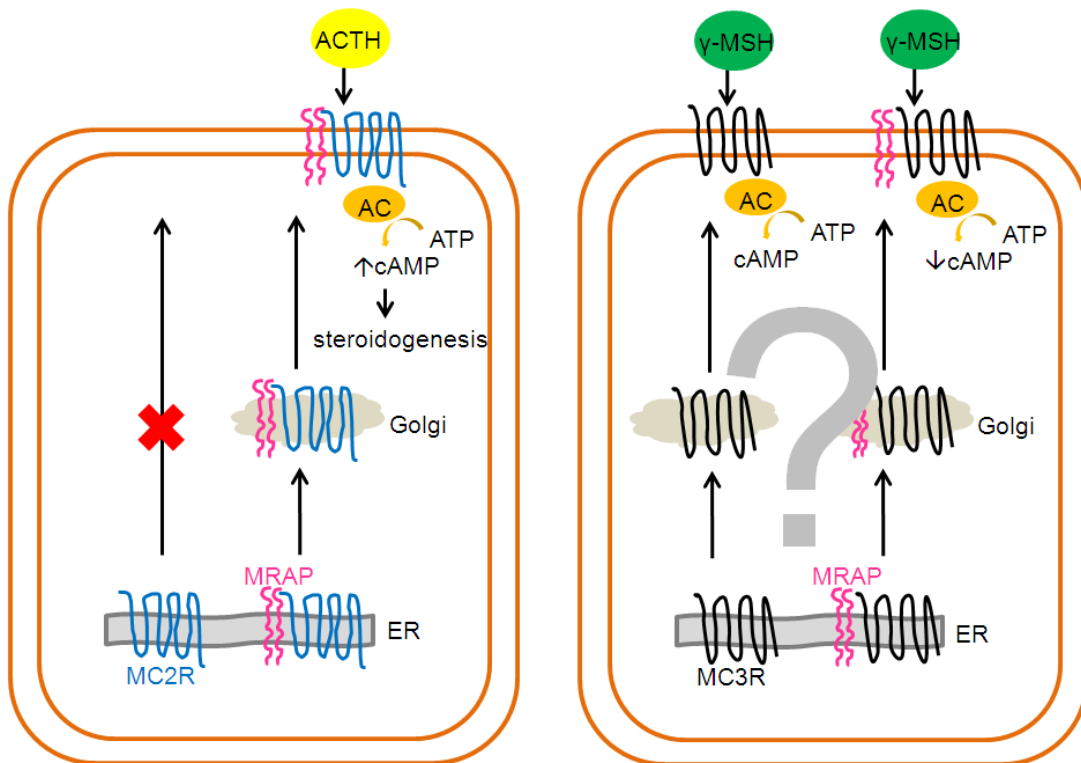
modifying CF lung disease. Given that MC3R is known to be involved in lung inflammation and contributes to energy homeostasis, we selected *MC3R* as the most compelling candidate under the linkage peak. Ultimately, our goal is to provide insight from this research in the development of new therapies that will alleviate the longitudinal decline in lung function of CF patients.

Chapter 2 describes the molecular and cellular characterization of the human *MC3R* gene. Previously, *MC3R* was predicted to be a single-exon gene with unknown translation start site. My study revealed that *MC3R* contains an upstream exon that extends the length of 5' untranslated region (UTR). The presence of 5' UTR was necessary for initiation of translation from the second in-frame ATG. I also explored localization of full-length (exons 1 and 2) as well as partial (truncated exon 2) MC3R in polarized MDCK cells. Furthermore, I investigated the interaction between MC3R and MRAPs to determine how MC3R may be modulated.

In Chapter 3, I present data that supports *MC3R* as a potential modifier of CF lung disease. My work identified three rare coding variants in *MC3R* that associate with lung disease severity. I further expanded the role of *MC3R* as a lung modifier by examining how modulation of MC3R alters pro-inflammatory signaling. In addition, I inspected the expression of MC3R in primary airway epithelial cells of the lungs to support my hypothesis that MC3R modifies lung function in CF.

Taken together, this body of work suggests that *MC3R* acts as a modifier of CF lung disease by modulating inflammatory pathways in the airways. The work presented in this thesis can be utilized in designing new targets for therapies against pulmonary disease in CF.

Chapter 2:

Molecular and cellular characterization of human melanocortin-3-receptor (*MC3R*)

Introduction

The melanocortin receptor family, consisting of five G-protein coupled receptors (GPCRs), mediate key physiological functions such as pigmentation, steroidogenesis, energy balance, and food intake (129). Variation in the coding regions of melanocortin receptors has been associated with a number of medically important diseases and traits. For example, inactivating mutations of the melanocortin-2-receptor (*MC2R*) or MC2R-accessory-protein (*MRAP*) are known to cause familial glucocorticoid deficiency (131,152), and mutations in *MC4R* are responsible for 1-5% of early-onset human obesity (132,153-157). Like *MC4R, MC3R* has been found to play a role in energy metabolism. Both common and rare variants in *MC3R* have demonstrated association with obesity, but results have been inconsistent (123,125,158-162). *MC3R* has also been associated with hypertension, tuberculosis susceptibility and inflammation phenotypes (115,163-165). We are interested in studying MC3R as a potential modifier of lung disease in cystic fibrosis (CF) (112). Before exploring its role as a modifier, we aimed to understand the molecular organization of *MC3R* by obtaining the full-length mRNA transcript, determining the native protein length, and verifying its cellular distribution.

The NCBI Reference Sequence Database predicts *MC3R* to be a single exon gene and it is unclear if translation begins at the first or second in-frame ATG. While the second ATG is conserved across vertebrates, the first ATG is only conserved in non-human primates (www.genome.ucsc.edu). Prior studies indicate that both ATGs can function as translation initiation codons and that the sequence between the first and second ATG is not critical for the ligand binding of the receptor (166,167). Recently, it has been shown that the second ATG is preferentially used as the translation initiation

site in a truncated form of MC3R (168). Lack of information regarding the composition of the full-length mRNA transcript as well as conflicting results on translation initiation prompted us to identify native transcription and translation start sites to gain a more complete understanding of the MC3R protein.

Characterizing the localization of MC3R in a proper cell type is vital in deciphering its role in regulation of energy homeostasis in the general population and in modulation of lung function in tuberculosis and CF. As a member of a family of proteins that signal through the GPCR pathway, MC3R should be localized to the plasma membrane to enable binding to its ligands. Several studies have reported cytoplasmic and membrane localization of MC3R in different cell types (123,145,146). One of the limitations of prior studies is that localization was assessed in non-polarized cells. Such cell types do not represent the true physiological state of MC3R because it is known to be expressed in tissues, such as the brain, where polarized neurons have a distinct distribution of membrane proteins (147). Furthermore, as we investigate the role of MC3R as a genetic modifier of lung function in CF, we are interested in studying its localization in polarized cells derived from airway epithelia of the lungs. Finally, all melanocortin receptors, including MC3R, demonstrate functional interaction with accessory proteins MRAP and its paralogue MRAP2 (131). In this study, we provide evidence that MC3R and MRAP2 interact and colocalize in polarized MDCK cells. Molecular characterization of MC3R provides information essential to resolving its role in obesity, inflammation, and lung disease in humans.

Results

The human *MC3R* gene has two exons.

The NCBI Reference Sequence Database predicts *MC3R* to be a single exon gene and its transcript as intronless (NM_019888). The reference sequence of *MC3R* places the first in-frame ATG at base pairs (bp) 2 to 4, while the second and the third in-frame ATGs are located 37 and 109 codons downstream, respectively (Fig. 2.1A). Because none of these ATGs has the characteristics of a typical translation start site (i.e. Kozak consensus sequence), we performed 5' and 3' rapid amplification of cDNA ends (RACE) to determine the full-length of the *MC3R* mRNA transcript. As we are exploring the role of MC3R in human lung diseases, we performed RACE upon mRNA extracted from bronchial epithelial cells from a CF patient (CFBE41o- cells).

The 5' RACE mapped the start site of transcription approximately 527 bp 5' of the reference sequence of *MC3R*. The full-length transcript contains an additional 5' exon of ~252 bp, labeled now as exon 1, and an intron of 248 bp (Fig. 2.1B). The original exon, re-labeled as exon 2, is 27 bp longer than previously predicted. The 3' RACE verified that *MC3R* does not have any downstream exon and that a poly(A) tail is added 116 bp downstream of the stop codon. The highly conserved canonical poly(A) signal AAUAAA was not found in the 3' UTR. However, two potential poly(A) signals, AAGAAA and UAUAAA, were found 46 bp and 21 bp from the poly(A) tail, respectively.

To confirm the RACE results, reverse-transcription PCR (RT-PCR) was performed using primers positioned in exons 1 and 2 of *MC3R*. DNA fragments of the expected size were obtained upon amplification of genomic DNA (gDNA) from human

Figure 2.1: Mapping of the 5' transcription start site and the gene structure of *MC3R.* (A) The nucleotide locations of the first three in-frame start codons in the reference sequence of *MC3R* (NM_019888) are annotated using the Feb. 2009 (hg19) assembly of the human genome. The underlined sequence around each start codon demarcates the region inspected for correspondence to the Kozak consensus sequence (GCCGCC**ATG**G). (B) This diagram summarizes the gene structure of *MC3R* deduced from 5' and 3' RACE experiments. 5' RACE found a novel exon upstream of the reference sequence of *MC3R*. A start codon was not detected in-frame. The original exon is 27 bp longer than previously predicted. The 252+ bp upstream exon (labeled now as exon 1), 248 bp intron, and 1111 bp original exon (re-labeled as exon 2) all contained splicing consensus sequences. The diagram also illustrates the positions of the 3 pairs of *MC3R* primers used in RT-PCR. (C) The image shows agarose gel electrophoresis of ethidium bromide stained gDNA and cDNA fragments amplified by PCR. RT-PCR demonstrated that *MC3R* mRNAs from human brain tissue, HBE cells, and CFBE41o- cells include sequences from exon 1 and exon 2. The label on the top of each lane of the gel represents different primers that were used to amplify the product. Prior to RT, RNA was treated with DNase to eliminate the contaminating gDNA. The *TBP* primers were designed from two different exons with an intervening intron that is short enough to be amplified during elongation. The size of *TBP* band indicated that there is no gDNA contamination in the cDNA samples (797 bp in gDNA, 116 bp in cDNA).
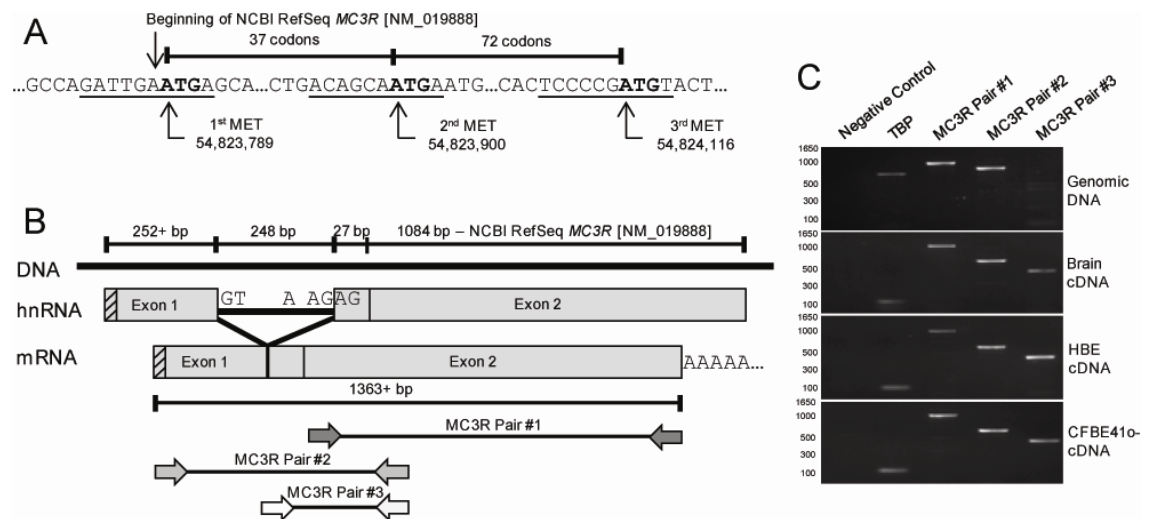
lymphocytes and cDNA synthesized from primary human brain tissue (temporal lobe) mRNA (Fig. 2.1C). In gDNA and brain cDNA, the *MC3R* primer pair #1 amplified a fragment of 1084 bp that corresponded to the length expected from exon 2. Primer pair #2 amplified a 921 bp gDNA fragment and a 673 bp brain cDNA fragment that were extended from exon 1 to exon 2 consistent with the presence or excision, respectively, of the 248 bp intron. Primer pair #3 amplified a 476 bp brain cDNA fragment that extended from the exon 1 - exon 2 junction to exon 2 indicative of intron splicing. Primer pair #3 generated only non-specific products upon amplification of gDNA, as expected. These results show that *MC3R* contains an upstream intron that is excised in brain mRNA. The cDNAs synthesized from human bronchial epithelial (HBE) and CFBE41o- cell RNA were also studied. Each cell line generated amplification products identical to those obtained from amplification of brain cDNA (Fig. 2.1C). Thus, *MC3R* is composed of two exons that are spliced to form an mRNA of approximately 1363 bp in primary brain tissue and in pulmonary airway epithelial cells.

Translation is initiated at the second in-frame ATG of *MC3R*.

To determine if translation initiated at the first or second in-frame ATG (or elsewhere), we created a plasmid containing a full-length *MC3R* cDNA and then mutated the first two ATG codons to GCG. The full-length *MC3R* cDNA was created by joining exon 1 and exon 2 of *MC3R* via fusion PCR and was fused in-frame to a c-Myc epitope at the C-terminus to facilitate detection of translated protein. After the introduction of mutations to the first and/or second ATG and sequence verification, Madin Darby Canine Kidney (MDCK) cells were transiently transfected with each plasmid and expressed

protein was detected with an anti-c-Myc antibody. As a control for transfection, a

plasmid encoding the MC2R-accessory-protein (*MRAP*) with a C-terminal FLAG epitope

was co-transfected with each MC3R construct.

Confocal microscopy revealed that MC3R protein is detectable in similar numbers

of cells when the first ATG is intact or mutated (Fig. 2.2A and B). Most of the cells

expressing wildtype MC3R or MC3R with the first ATG mutated also expressed MRAP

(see merge panels). However, protein was not detected when the second ATG was

mutated (Fig. 2.2C) or when both ATGs were mutated (Fig. 2.2D). Detection of cells

expressing MRAP indicated that failure to detect MC3R protein when the second ATG

was mutated was not due to transfection issues. These results indicate that translation of

MC3R is initiated at the second ATG and the full-length protein encompasses 323 amino

acids. This conclusion is consistent with cross-species conservation of the second but not

the first ATG of *MC3R* and with studies of truncated 5' *MC3R* fused to reporter protein

that demonstrated preferential initiation from the second ATG (168).

MC3R displays discrete apical and sub-apical localization in polarized MDCK cells.

Expression of MC3R in non-polarized cells revealed membrane localization, as

expected for a transmembrane signaling protein (123,145,152). MC3R is abundantly

expressed in the neurons of adult rat brain (169), which are polarized cells that rely on

discrete localization of specific membrane proteins (i.e. axonal vs. dentritic). Polarized

MDCK cells distribute proteins in a pattern consistent with polarized neuronal cells as

both cell types share many common mechanisms of protein targeting (i.e. apical/axonal

and basolateral/dentritic) (170-172). To assess whether MC3R localizes to specific

Figure 2.2: *MC3R* **translation begins at the second methionine.** Fluorescent

photomicrographs of MDCK cells in x-y plane. Cells were transiently transfected with

MC3R wildtype plasmid or plasmid that has the first and/or second ATG mutated to

GCG and co-transfected with MRAP-FLAG as a transfection control. Cells were stained

with anti-c-Myc antibody and secondary antibody linked to Cy3 (red) to detect MC3R

and with anti-FLAG antibody and secondary antibody linked to Alexa Fluor 488 (green)

to detect MRAP. The green fluorescence indicated that MRAP was expressed in

approximately 5% of cells in each transfection. In the merge row, red and green

fluorescence were combined and DAPI was used to stain cell nuclei. (A) Wildtype MC3R

is detected in most cells that express MRAP. (B) When the first ATG is mutated, MC3R

is detected in about 90% of cells expressing MRAP. (C) MC3R is not detected when the

second ATG is mutated. (D) When both the first and second ATG are mutated, MC3R is

not detected. Scale bar: 20 μm.
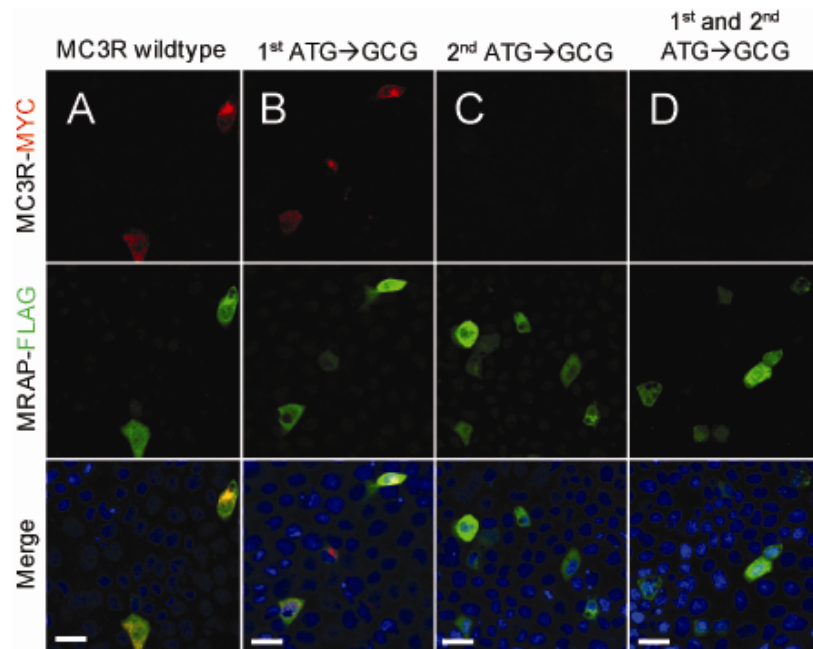
membrane compartments, the location of MC3R was investigated by confocal microscopy of polarized MDCK cells.

Polarization of MDCK cells was verified by staining with anti-ZO-1 antibody, a marker for tight junctions, or anti-Na$^+$/K$^+$ ATPase antibody, a marker for basolateral membrane surface. To mark the location of the apical membrane, the cells were co-transfected with a plasmid expressing the CF transmembrane conductance regulator tagged with GFP at the N- terminus (GFP-WT-CFTR), a protein known to localize to the apical surface of MDCK cells (57,173,174). Confocal imaging of these cells revealed that the proteins were present in their expected locations (Fig. 2.3A and B). In this set of experiments, the cDNA encoding native *MC3R* was used to avoid aberrant localization due to the presence of an epitope tag. MC3R protein was detected with anti-MC3R polyclonal antisera directed against an 88-aa peptide from the N-terminus of MC3R (Santa Cruz). In 11 of 16 cells examined, MC3R displayed localization that was coincident with CFTR. The overlap of two proteins resulted in yellow fluorescence in the merge, demonstrating that MC3R is localized at the apical surface (Fig. 2.3C). In the remaining 5 cells, MC3R demonstrated localization to regions near the apical membrane; however, the distribution did not overlap with CFTR (Fig. 2.3D). Thus, MC3R primarily appears to be apically located in polarized cells either in the same region as CFTR or less often in a sub-apical compartment, suggesting axonal distribution in neuronal cells.

Absence of the 5' UTR results in translation initiation at the first in-frame ATG and synthesis of protein that is aberrantly localized in polarized MDCK cells.

Figure 2.3: MC3R exhibits apical localization in polarized MDCK cells. Confocal

microscopy images of MDCK cells taken in the x-z plane with the bottom row in each

panel representing the composite scan and the panels above showing red or green

fluorescence. In the merge row, cells were counterstained with DAPI to detect the nuclei.

(A) The cells were immunostained with anti-ZO-1 antibody, a tight junction marker, in

red to demonstrate that apical-basolateral polarity is present. (B) Immunostaining of the

MDCK cells for Na^+/K^+ ATPase showed staining consistent with localization to

basolateral membranes. (C) Localization of MC3R overlapped with that of CFTR in the

majority of cells examined. (D) Localization of MC3R near the apical membrane did not

overlap with CFTR in the minority of cells examined. AP = apical. BL = basolateral.

Scale bar: 10 μm.
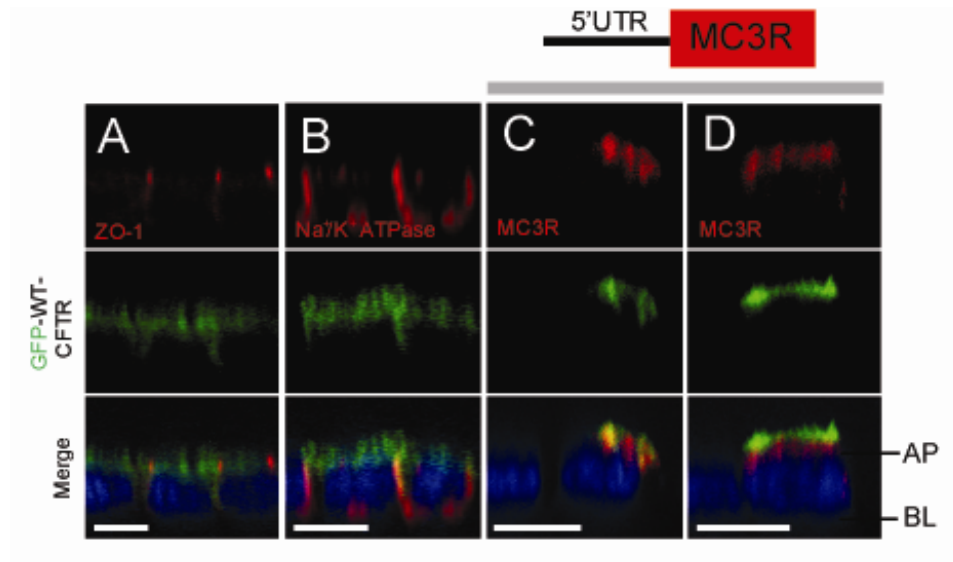
To investigate whether the newly discovered 5' UTR was responsible for the preferential use of the second in-frame ATG for translation initiation, exon 1 and 27 bp from the 5' of exon 2 were removed to create a partial version of *MC3R* (*parMC3R*). The partial version corresponds precisely to the 1084 bp single exon of *MC3R* that is annotated by NCBI RefSeq Database (NM_019888). The *parMC3R* cDNA was subcloned into a vector with a C-terminal c-Myc tag to enable detection in transfected cells. The protein translated from the *parMC3R* cDNA displayed a cytoplasmic distribution in polarized MDCK cells (Fig. 2.4A) that was distinctly different from protein translated from the full-length cDNA (Fig. 2.3C and D). The detection of protein synthesized from the *parMC3R* cDNA by both the N-terminal antisera and the C-terminal epitope tag indicated that translation initiated somewhere within the first 88 aa and continued to the native C-terminus. As only the first and second in-frame ATGs lie within the region recognized by the N-terminal antisera, we mutated each ATG to assess where translation was initiated in the *parMC3R* cDNA. When the first ATG of this partial construct was mutated to GCG, the translated protein localized to the apical regions of the polarized MDCK cells (Fig. 2.4B), as noted for protein translated from second ATG of the full-length *MC3R* cDNA (Fig. 2.3C and D). When the second ATG of the partial construct was mutated to GCG, the resulting protein displayed a cytoplasmic distribution (Fig. 2.4C). From these results, we conclude that translation of *parMC3R* cDNA is initiated from the first ATG, generating a protein that does not localize to the cell membrane.

Further studies were performed to exclude alternative explanations for the cytoplasmic location of MC3R protein translated from the first ATG. To test if the C-

Figure 2.4. In absence of the 5' UTR, MC3R is mislocalized to the cytoplasm of polarized cells. Confocal microscopy images of MDCK cells taken in the x-z plane. (A) MDCK cells were immunostained with anti-MC3R antisera in red and anti-c-Myc antibodies in green to detect the expression of a fusion protein, parMC3R-MYC. Protein synthesized from the partial exon 2 showed diffuse cytoplasmic distribution in polarized MDCK cells. Fluorescence from the anti-MC3R and anti-c-Myc staining overlapped almost completely (see merge panel). (B) When the first ATG of the partial construct was mutated to GCG, this led to translation initiation from the second ATG. Immunostaining with anti-ZO-1 antibody illustrated that this form of MC3R protein has discrete apical localization. (C) A form of MC3R is detected when the second ATG of the partial construct is mutated to GCG, but this protein displays diffuse cytoplasmic localization. (D) Staining with WGA outlined the plasma membrane. Immunostaining with anti-MC3R antisera revealed that the untagged parMC3R has cytoplasmic localization under the cell membrane. (E) MDCK cells stably expressing GFP-WT-CFTR were immunostained with anti-GFP antibody to detect CFTR and with anti-HA antibody to detect HA-parMC3R. GFP-WT-CFTR protein was used as a marker for apical surface expression. The commercially available HA-parMC3R plasmid is missing the 5' UTR and has the partial exon 2 of *MC3R* fused in-frame to the HA tag at the N-terminus, thus forcing the inclusion of the amino acids from the first to the second ATG. A diffuse cytoplasmic stain was observed for HA-parMC3R. These experiments reveal that the partial exon 2 of *MC3R* encodes aberrant forms of the protein that are distributed in the cytoplasm of polarized cells, which is distinctly different from the localization of the

protein encoded from the full-length *MC3R*. AP = apical. BL = basolateral. Scale bar: 10

μm.
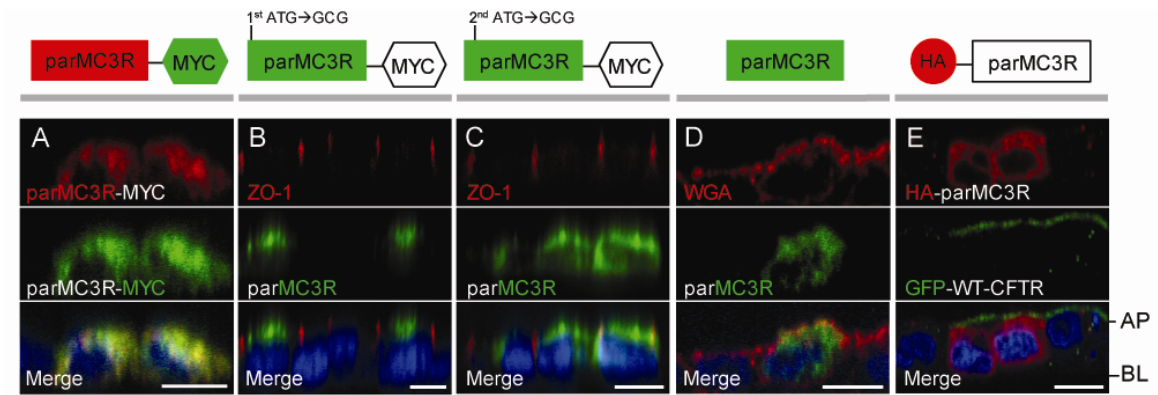
terminal tag interfered with protein biogenesis, the tag was removed. MDCK cells transfected with the untagged parMC3R plasmid were stained with WGA, a plasma membrane marker, and with the N-terminal anti-MC3R antisera. Protein encoded by the untagged *parMC3R* cDNA was cytoplasmic and did not co-localize with the cell membrane marker (Fig. 2.4D). We then transfected MDCK cells with a commercially available construct, labeled HA-parMC3R, that contains the partial exon 2 of *MC3R* fused in-frame to the HA epitope at the N-terminus (www.cdna.org). This construct was selected as it has been used in a number of functional studies of MC3R (131,145,151,175,176). Translated products from this vector include amino acids from the first ATG to the second ATG that we predict are not present in native MC3R. Staining of transfected cells with the anti-HA antibody revealed a diffuse cytoplasmic distribution in polarized MDCK cells that was distinct from apically located GFP-WT-CFTR (Fig. 2.4E). Together, these studies indicate that the 5' UTR from exon 1 and the 5' of exon 2 are essential for directing translation of a membrane localizing form of MC3R that begins at the second in-frame ATG.

MRAP2 colocalizes with MC3R and MC2R in polarized cells.

It has been shown that accessory proteins, MRAP and MRAP2, support cell surface expression of MC2R in non-polarized cells and interact with melanocortin receptors 1-5 to modulate signaling to intracellular second messengers (131). These single-pass transmembrane accessory proteins have been shown to localize at the cell surface as well as to the ER (177,178). However, the location of MRAP and MRAP2 in polarized cells has not been established. Also, it is not known if the accessory proteins

colocalize with melanocortin receptors in polarized cells. To address this issue, MRAP and MRAP2 were expressed in polarized MDCK cells by themselves and in the presence of MC3R, HA-parMC3R and HA-MC2R. The HA tagged version of MC3R was used as it localizes to a different cellular compartment than native MC3R.

MRAP and MRAP2 displayed diffuse cytosolic distribution in polarized MDCK cells (Fig. 2.5A, B). The localization of MC3R remained the same in the presence of the accessory proteins, as it was observed at or near the apical surface of the cell (Fig. 2.5C-E). Interestingly, the distribution of MRAP2 was substantially different when co-expressed with MC3R whereas MRAP distribution was unchanged (Fig. 2.5D and E). MRAP2 was found at discrete locations that partially overlapped with MC3R in the apical membrane (see merged x-z image in Fig. 2.5E). On the other hand, HA-parMC3R did not affect the localization of MRAP2. MRAP and MRAP2 remained cytoplasmic when co-expressed with HA-parMC3R (Fig. 2.5G-H). When expressed alone, MC2R was localized in the cytoplasm of polarized MDCK cells (Fig. 2.5I). MC2R localization was unaltered when co-expressed with MRAP (Fig. 2.5J). However, the cellular distribution of both MC2R and MRAP2 was dramatically altered when co-expressed. Both proteins were localized to membranes with overlapping expression in the lateral membrane and primarily MC2R expression in the apical compartment (Fig. 2.5K). These results suggest that translation of MC3R from the second ATG facilitates interaction between MC3R and MRAP2.

Figure 2.5. MRAP2 is re-distributed in the presence of MC3R and MC2R. Confocal microscopy images of MDCK cells taken in the x-y and x-z planes. Each photomicrograph shows immunostained cells after transfection with the plasmid listed on the top (none, MRAP-FLAG, or MRAP2-FLAG) and the plasmid listed across the left (none, MC3R, HA-parMC3R, HA-MC2R). The epitopes (HA and FLAG), Na^+/K^+ ATPase, ZO-1 and MC3R along the top row and left column are labeled according to the color of the fluorescence. Nuclei were counterstained with DAPI in blue. For detection of proteins in the apical compartment, x-y sections were above the nucleus. For detection of cytoplasmic or basolateral proteins, x-y sections transected through the nucleus. (A) MRAP alone displays a diffuse cytoplasmic distribution. Red staining in a 'chicken wire' pattern corresponds to the staining of ZO-1 in tight junctions formed by polarized cells. (B) MRAP2 alone displays diffuse cytoplasmic staining in polarized MDCK cells. (C) MC3R is localized near the apical membrane and is distinct from the localization of the basolateral protein Na^+/K^+ ATPase. (D) MRAP displays diffuse cytoplasmic distribution while co-expressed MC3R is localized near the apical membrane. (E) MRAP2 is localized to the plasma membrane in the presence of MC3R. (F) HA-parMC3R is expressed in the cytoplasm of polarized MDCK cells. (G) MRAP displays diffuse cytoplasmic distribution when co-expressed with HA-parMC3R. (H) MRAP2 localization is unchanged in the presence of HA-parMC3R. (I) MC2R is in the cytoplasm of polarized MDCK cells. (J) MRAP and MC2R show partial overlap in the cytoplasm. (K) MRAP2 displays localization to the plasma membrane when co-expressed with MC2R. MC2R is found in apical and lateral membranes while MRAP2 is primarily localized to lateral membranes. AP = apical. BL = basolateral. Scale bar: 10 μm.
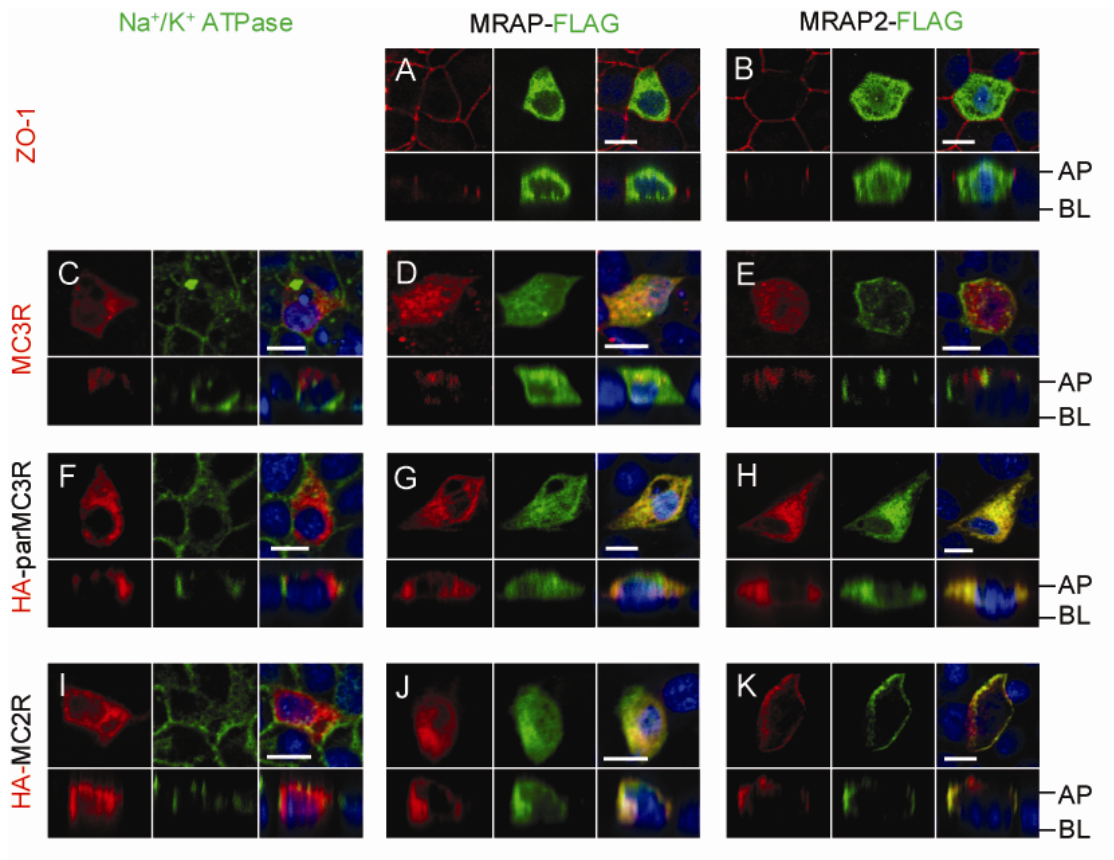
Discussion

In this study, we characterized the transcription and translation start sites of human *MC3R* and determined its localization in the presence or absence of MRAPs in polarized cells. Our results indicate that *MC3R* is a two-exon gene that requires a long 5' UTR for translation initiation from the second in-frame ATG. Furthermore, MC3R translated from the second ATG is apically located while protein that initiates at the first ATG remains cytoplasmic in polarized cells.

Colocalization of MC3R and MRAP2 at the cell membrane raises the possibility that MRAP2, in addition to being an accessory factor for MC2R, can affect the function of MC3R and influence human phenotypes. As several variants in *MC3R* have already been implicated in obesity, hypertension, tuberculosis susceptibility, lung inflammation, and pulmonary variation in CF (112,115,123,125,158-165), elucidation of *MC3R* gene structure and protein localization is a critical step in interpreting its role in human diseases and conditions.

Incomplete annotation of the 5' regions of human as well as mouse genomes illustrates an urgent need to experimentally validate the gene architecture (179-183). Experimental examination of transcriptional start sites of 106 mouse genes by 5' RACE revealed that more than half of the genes produced sequences that were longer than the predicted annotation (179). In the case of *MC3R*, we identified a novel upstream exon that extends the length of the 5' UTR without changing the ORF. The discovery of a noncoding first exon of *MC3R* should not be a surprise since approximately 40% of the known human genes have noncoding first exons (184). Determining the transcription start site of *MC3R,* and therefore its promoter region, is of great interest since *MC3R* is

involved in the regulation of numerous physiological processes. Though it is known that both enhancer and suppressor elements can be found tens of thousands of bases upstream or downstream from the transcription start site (185), most essential control elements are usually present within the proximal promoter (186). Moreover, UTRs have been known to regulate the synthesis of a protein by affecting mRNA stability (187), translation efficiency (188), and protein trafficking (189). Changes in the length or the sequence of the 5' UTR have been implicated in various human diseases, such as hereditary thrombocythemia (190) and X-linked Charcot-Marie-Tooth disease (191). Identification of native transcription start site is essential in defining the location of the promoter and exploring *cis*-regulatory elements that control gene expression.

Prior to the discovery of this upstream exon, the first ATG was an unlikely translation start site because the 5' UTR, consisting of one nucleotide, would not provide enough space for binding of various *trans*-acting assembly factors. While evolutionarily conserved across vertebrates, the second ATG is followed by a poor Kozak sequence and lacks an optimal context for ribosome recognition. In Feb. 2011, Ensembl listed two transcripts under *MC3R* (http://feb2011.archive.ensembl.org/), but the second transcript was removed in Apr. 2011 (http://apr2011.archive.ensembl.org/). Interestingly, both *MC3R* transcripts were predicted to contain only one exon but differed in their translation start sites. The *MC3R* transcript that is currently listed on Ensembl labels the second in-frame ATG as the translation start site based on cross-species conservation. In spite of the current annotation, most of the previous literature on MC3R assumes that translation begins at the first ATG and therefore the SNPs are named by counting from the first methionine. For example, two common *MC3R* variants, rs3746619 and rs3827103, that

have been frequently investigated are referred to as T6K or V81I variant, respectively (125,159,192-195). As our results confirm that proper translation of MC3R begins at the second ATG, located 37 amino acids downstream of the first ATG, rs3746619 (ie T6K) should be considered as a SNP in the 5' UTR that has variable association with obesity, not a missense variant.

While it is thought that translation initiates at the first ATG in most eukaryotic genes, upstream ATGs are found in 15% to 50% of the 5' UTRs depending on the organism (196). This reveals that deviations from the "first-ATG rule" may be more common than we may have appreciated. There are several possible reasons for why translation initiates at the second ATG in MC3R. The sequence context around the first ATG may be far from optimal for initiating translation, resulting in leaking scanning of the ribosome (197). A hairpin secondary structure downstream of the first ATG may stall the ribosome and increase the likelihood of recognizing the second ATG (198). The upstream ATG found in the 5' UTR of *MC3R* may be in involved in ensuring low basal translational level, as it has been demonstrated that cDNAs with long 5' UTRs with several upstream ATGs have a weak start context (199,200). Moreover, upstream ORFs have been correlated with significantly reduced protein expression of the downstream ORF (201). Given that variants that create a polymorphic upstream ORF, which alters cellular expression of the downstream protein, have already been identified in *MC2R* (201), discovery of such variants in *MC3R* can potentially explain the mechanism that affects protein expression and perhaps phenotypic variation.

The significance of the 5' UTR in *MC3R* goes beyond regulating its translation initiation because our data indicate that protein localization is also affected by the

methionine that is used to initiate translation. To investigate the localization of MC3R, we used polarized MDCK cells that have been extensively used as a model for studying the sorting of membrane proteins (170,202,203). Our study demonstrates that MC3R is localized at or near the apical surface in polarized MDCK cells. In contrast, MC3R produced from partial constructs that lack the 5' UTR displays cytoplasmic distribution, indicating that the protein is unable to efficiently localize to the cell surface. Previous studies that used the parMC3R constructs as well as the commercially available HA-parMC3R plasmid to study localization in non-polarized cell types concluded that this form of MC3R displays cell surface as well as intracellular expression (123,145,146). We have demonstrated that the absence of the 5' UTR causes incorrect usage of the first ATG for translation initiation. Therefore, we conclude that the partial constructs and the HA-parMC3R plasmid synthesize a protein that incorrectly includes the sequence between the two start codons, resulting in a distinctively different localization pattern as compared to native MC3R. While epitope tagging can be powerful and convenient in rapid analysis of protein function, it can interfere with the function or cellular processing of the tagged protein. For example, it has been shown that GFP-tagging of human acetylcholine receptor alters the channel property in Xenopus oocytes (204) and glycosylation of angiotensin type II receptor were affected by epitope tagging in HEK293 cells (205). This emphasizes the importance of understanding the potential impact of epitope tagging on protein expression and use of constructs that represent the true context of the native protein.

The signaling and trafficking properties of GPCRs are regulated by the receptor-interacting proteins that are differentially expressed in distinct cell types (206). Our study

reveals that MC3R may interact with MRAP2, suggesting that MRAP2 has unknown roles besides being an accessory protein for MC2R. This result is consistent with a previous study, which demonstrated that MRAPs can downregulate the expression and signaling of MC3R (131). These studies raise the questions of how and why MRAP2 interacts with other melanocortin receptors. It is possible that this interaction may vary in a cell-type specific manner and in response to changes in the environment. In contrast to MRAP2, MRAP does not colocalize with MC3R at the plasma membrane and is unable to assist in functional expression of MC2R in MDCK cells. Since it has been noted that functional expression of MC2R is challenging as it can only be achieved in cells of adrenal origin (207), the failure of MC2R to reach the plasma membrane in the presence of MRAP in our study may be due to the cell type that was used. Additional factors, such as unidentified accessory proteins, may be required to facilitate trafficking of MC2R or MC3R and MRAP to the cell surface in this specific polarized cell system. As our data indicates that MRAP2 could be an accessory protein that influences functional expression of MC3R, provided that these proteins are co-expressed *in vivo*, further work will be needed to investigate the mechanism by which MRAP2 modulates the function of MC3R.

In summary, our results indicate that *MC3R* is a two-exon gene that requires its 5' UTR for proper translation, localization, and potential interaction with MRAP2. Our study defines the native gene structure and the protein expression of MC3R, an important member of the melanocortin family that plays an integral role in numerous physiological processes and is implicated as a risk factor for important common conditions.

Materials and Methods

Cell culture

Human bronchial epithelial (HBE) cells and CF bronchial epithelial (CFBE41o-) cells carrying homozygous F508del *CFTR* mutation were maintained in Minimum Essential Medium (Gibco). Madin Darby Canine Kidney (MDCK) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Cellgro). MDCK cells stably expressing GFP-WT-CFTR were maintained in DMEM with 100 µg/ml hygromycin (Invitrogen). All media were supplemented with 10% FBS (Cellgro) and 1% penicillin-streptomycin (Gibco) at 37°C in an atmosphere of 5% CO_2.

RNA isolation

Total RNA was isolated from human brain tissue, HBE cells, and CFBE41o- cells by using RNeasy Mini Kit (Qiagen) as described by manufacturer's protocol. It was then treated with DNA-*free* DNase (Ambion) to remove contaminating DNA as described by manufacturer's protocol.

Rapid amplification of 5' and 3' cDNA ends (RACE)

5' and 3' RACE were performed using SMARTer^{TM} RACE cDNA Amplification Kit (Clontech), as described by manufacturer's protocol. The mRNA extracted from CFBE41o- cells was used for RACE. The gene-specific primers (GSP) were designed on the plus and minus strands of the reference sequence of *MC3R* (NM_019888). *MC3R* cDNA was amplified with a universal primer and *MC3R*-specific primer. Nested PCR amplification was subsequently performed with the nested universal primer and the

nested *MC3R*-specific primer to confirm the specificity of the primary PCR products. The

nested PCR products were gel purified and sequenced with Applied Biosystems 3730xl

DNA Analyzer.

Reverse transcription PCR (RT-PCR)

DNase-treated RNA was reverse-transcribed using iScript™ cDNA Synthesis Kit (Bio-

Rad). PCR was performed using *Taq* DNA Polymerase (Invitrogen). Following a 6-min

denaturation at 95°C, 40 cycles of 95°C for 45 s, 59°C for 45 s and 72°C for 1 min were

performed, followed by a 10-min extension at 72°C. RT-PCR products were visualized

by agarose gel electrophoresis with ethidium bromide staining.

Plasmids

The full-length MC3R plasmid was generated by amplifying exon 1 and exon 2 of *MC3R*

from genomic DNA. The 260-bp exon 1 band and 1131-bp exon 2 band were extracted

from the gel and re-amplified via fusion PCR to connect the two overhanging products.

The 1391-bp fusion amplicon was then subcloned into a StrataClone Mammalian

Expression Vector, pCMV-SC-CM, that contains a C-terminal c-Myc epitope tag vector

(Agilent). The parMC3R-MYC plasmid was created by subcloning the known reference

sequence of *MC3R* (NM_019888) into a StrataClone Mammalian Expression Vector with

a C-terminal c-Myc tag, pCMV-SC-CM (Agilent). The sequences of each construct were

verified by Sanger sequencing. The GFP-WT-CFTR plasmid was generated by removing

full-length cDNA from the existing peGFP-CFTR plasmid by enzyme digestion and

ligating into the multiple cloning sequence of the pcDNA5/FRT vector (Invitrogen). The

pcDNA3.1 plasmids encoding MC2R or partial MC3R with a 3× HA tag on the N terminus (HA-MC2R and HA-parMC3R) were purchased from Missouri S&T cDNA Resource Center (www.cdna.org). MRAP-FLAG and MRAP2-FLAG plasmids were generous gifts from Dr. Li Chan (Centre for Endocrinology, William Harvey Research Institute).

Site-directed mutagenesis

The first and/or second in-frame ATG was changed to GCG using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent), as described by manufacturer's protocol. To prevent the c-Myc tag of parMC3R-MYC plasmid from being expressed, nonsense mutations were introduced at the end of the partial *MC3R* sequence.

Transfection

MDCK cells were plated on the glass coverslips inside 12-well plates. When the cells were nearly 90% confluent, they were transiently transfected with 3.2 μg of a single plasmid or co-transfected with 1.6 μg of each of the two plasmids along with 4.8 μl Lipofectamine 2000 (Invitrogen), as described by manufacturer's protocol. Cells were fixed two days after transfection.

Immunocytochemistry and confocal microscopy

Two days after transient transfection, cells were fixed in 3.7% formaldehyde for 20 min. Staining with WGA (Molecular Probes W11262) was performed prior to permeabilization by incubating the cells in diluted WGA (1:100) for 5 min. Staining with

antibodies was performed after the cells were permeabilized with 0.5% Triton X-100 for 5 min and blocked with 2.5% goat serum for 30 min. The following primary antibodies were used for various proteins: HA (Sigma), c-Myc (Sigma), FLAG (Sigma), MC3R (Santa Cruz), GFP (Molecular Probes), ZO-1 (Invitrogen), and Na^+/K^+ ATPase (Millipore). The secondary antibodies were respectively conjugated to anti-mouse or anti-rabbit Alexa Flour 488 (Invitrogen) or Cy3 (Sigma). The primary and secondary antibodies were diluted to 1:200 and 1:50, respectively, in blocking buffer. The cells were incubated with primary antibodies for an hour, washed 3 times for 5 min each, incubated with secondary antibodies for an hour, and washed 4 times for 15 min each. ProLong Gold Antifade Reagent with DAPI (Molecular Probes) was used to stain the nuclei and mount the cells. Fluorescence was imaged using a Zeiss LSM510 confocal microscope at Johns Hopkins Microscope Facility.

Chapter 3:

Evaluation of MC3R's potential role as a modifier of lung function in cystic fibrosis

Introduction

Family-based studies have been used to estimate genetic and nongenetic contributions to disease severity in CF. Using affected twins and siblings, heritability of lung function severity has been estimated to be 0.54-1 using cross-sectional and longitudinal measures of lung function in CF (104). The higher concordance of lung function in monozygotic twin pairs and the high estimate for heritability of lung function in affected siblings validate the importance of searching for CF modifier genes (82). Identification of the modifier genes and their encoded products will help us better understand the pathogenesis of CF and could lead to potential therapeutic targets.

To identify genetic modifiers, both genome-wide association and linkage approaches have been used (107,208-210). Using 486 affected sibling pairs enrolled in the family-based CF Twin and Sibling Study (TSS), genome-wide association and linkage studies identified a significant modifier locus of lung disease severity at chr20q13.2 (LOD = 5.03) (112). This result was followed by SNP association testing in the linked region using unrelated individuals with two severe *CFTR* alleles from the Genetic Modifier Study and the Canadian Consortium for Genetic Studies (112). In the 1.3 Mb region of linkage, as demarcated by 1 LOD unit below the maximum, two clusters of SNPs met regional association. A 16-kb cluster of SNPs in high LD was located in a gene desert centromeric to five genes: *CBLN4*, *MC3R*, *AURKA*, *CSTF1* and *CASS4*. A second smaller cluster of SNPs was identified over *MC3R*. Of the five genes within the 1.3 Mb region of maximum linkage, *MC3R* was prioritized for follow-up based on the association evidence, its known role in the inflammatory pathway (115,116), its association with tuberculosis susceptibility (163,164), and its contribution to energy

metabolism (161,211,212). Furthermore, an empiric screen for new CF therapies using siRNAs revealed that reduction of MC3R resulted in restoration of chloride channel activity in cells expressing CFTR bearing the most common CF mutation, F508del (128).

To define the mechanism of lung disease in CF that underlies the linkage signal at chr20q13.2, *MC3R* was evaluated as a potential modifier of lung function in CF. We first searched for variants and SNP haplotypes in *MC3R* that associate with lung disease severity. Pulmonary function in CF was defined by age- and sex- specific forced expiratory volume in one second (FEV_1), a surrogate for survival in CF (113,213). The consequences of MC3R variants demonstrating association with lung function were assessed by *in silico* prediction tools. *In vitro* functional studies were also performed to determine the levels of *MC3R* mRNA transcripts carrying rare variants. Moreover, we evaluated whether or not the pro-inflammatory signaling pathway, which is altered in CF patients, can be mediated by MC3R agonist or siRNA. Lastly, we confirmed the presence of MC3R in primary airway epithelial cells of the lungs, further promoting the potential of MC3R to be a modifier of lung function in CF. The results of these approaches indicate that MC3R modulates the inflammatory pathway and affects lung function severity in CF. Gaining a mechanistic understanding of the cross-talk between MC3R and pro-inflammatory cytokines may be critical to developing new therapies to modulate lung inflammation in patients with CF.

Results

Rare variants in *MC3R* associate with lung function in CF siblings

To identify common and rare variants, a 1.8 kb region of genomic sequence that includes both exons and the intervening intron of the *MC3R* gene was analyzed in 151 sibling pairs with CF by Sanger sequencing. The siblings were selected from the family-based Twin and Sibling Study (TSS) (104,112). We selected sibling pairs who have 0 or 2 copies of the alleles identical by descent (IBD=0 or 2) at *MC3R*, because we hypothesized that a sibling pair that shares both alleles at *MC3R* will have a greater probability of sharing variants that contribute to lung functions than sibling pairs that share no alleles. The siblings carried at least one copy of F508del allele, the most common *CFTR* mutation, in order to minimize the effect of *CFTR* heterogeneity on lung function.

A total of eight variants were identified in *MC3R* (Fig. 3.1). Three variants are located in the 5' UTR of *MC3R* and one variant is found in the intron (Fig. 3.1A). Four variants are found in the coding region of *MC3R* (Fig. 3.1A). Of the eight variants, four variants have minor allele frequencies (MAF) less than 1%. Two of the four rare variants – g.54823323T>G and g.54824368A>C (p.T157P) – are novel. Seven individuals (2 pairs of IBD=2 siblings and 3 unrelated IBD=0 individuals) have one heterozygous rare mutation (Fig. 3.1B). The relative MAF of the variants in *MC3R* met the requirements of Hardy-Weinberg equilibrium and did not differ from those reported in public databases of the European population.

Figure 3.1: Characteristics of common and rare variants in *MC3R*. (A) Diagram of human *MC3R* gene and the approximate positions of the variants. The amino acid change of each applicable variant is shown. The location of ATG represents the translation start site. (B) Summary of variants identified in *MC3R*. The change in nucleotide and/or amino acid sequence for each variant is described in the table. European individuals from phase 3 of the 1000 Genomes Project were used to calculated the MAF. Family ID followed by a letter (A or B) is used to denote the individuals with or without variants. For instance, 196A and 196B are siblings of family 196.

A
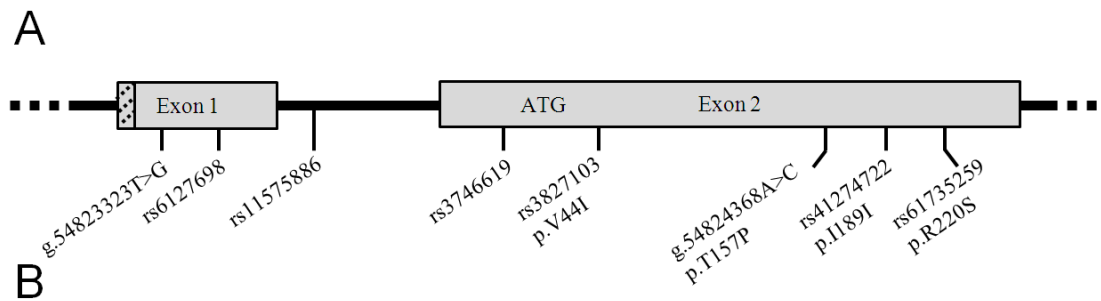
B

Variants identified in *MC3R* through Sanger sequencing of 151 sibling pairs with CF								
hg19 bp	54823323	54823416	54823550	54823805	54824029	54824368	54824466	54824557
rsID	none	rs6127698	rs11575886	rs3746619	rs3827103	none	rs41274722	rs61735259
Type of variant	5' UTR	5' UTR	intron	5' UTR	missense	missense	synonymous	missense
Nucleotide change	T → G	G → T	A → G	C → A	GTT → ATT	ACC → CCC	ATT → ATA	CGC → AGC
Amino acid change	N/A	N/A	N/A	N/A	V44I	T157P	I189I	R220S
Calculated MAF	0.34%	48.80%	12.20%	9.20%	9.20%	0.17%	0.34%	0.34%
1000 Genomes MAF	N/A	45.00%	11.20%	8.00%	8.00%	N/A	0.30%	0.20%
Siblings with rare variants (genotype)	196A (T/G) 196B (T/G)					419B (A/C)	480A (A/T) 480B (A/T)	209A (A/C) 560A (A/C)
Siblings without rare variants (genotype)						419A (A/A)		209B (C/C) 560B (C/C)

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To assess the association of the variants to lung disease severity, we tested the minor alleles using linear regression. Lung disease severity was quantified using SaKNorm, a survival-adjusted average of CF-specific percentiles for FEV_1 using three years of data (112). These analyses used the Z-score corresponding to a given percentile, related by normal transformation. Significant association with lung disease severity as measured by FEV_1 was observed for three rare variants located in the coding region: g.54824368A>C (p.T157P), rs41274722 (p.I189I), and rs61735259 (p.R220S) (Table 3.1) (Fig. 3.2). None of the common variants was associated with lung function.

To evaluate whether the *MC3R* alleles identified by Sanger sequencing could explain the former linkage signal on chr20q13.2, we assessed the LOD score contributions of the individuals carrying the rare variants that were significantly associated with lung function. One IBD=2 sibling pair carrying rs41274722 (p.I189I) contributed positively to the LOD score. However, the remaining three sibling pairs had negative LOD scores: one IBD=0 pair in which one sibling carries g.54824368A>C (p.T157P) and two IBD=0 pairs in which one sibling per pair carries rs61735259 (p.R220S). There is insufficient evidence that the rare variants in *MC3R* are responsible for the linkage signal on chr20q13.2.

We next performed haplotype analysis to assess whether a particular combination of common and rare variants was associated with lung function. Haplotype analysis revealed that *MC3R* belongs in one block exhibiting moderate linkage disequilibrium (LD) (Fig. 3.3A-B). SNPs rs3746619 and rs3827103 are in complete LD, but rs6127698 and rs11575886 show partial LD. The correlation values (r^2 and D') and haplotype frequencies derived for the CF siblings are concordant with those of the HapMap3 CEU

Table 3.1: Association of *MC3R* variants with lung disease severity.

	g.54823323T>G	rs6127698	rs11575886	rs3746619	rs3827103	g.54824368A>C	rs41274722	rs61735259
P>|t| for association with FEV_1	0.624	0.499	0.177	0.315	0.315	**0.043***	**0.024***	**0.007***
ΔZ-score	N/A	N/A	N/A	N/A	N/A	1.58	-1.25	1.49

* Significant findings are in bold.

Figure 3.2: Histogram of CF patients according to lung disease severity. Lung

disease severity was quantified by FEV_1 percentile, which was transformed to z-score.

The green arrows represent three unrelated individuals with rare variants that were

associated with higher lung function. The red arrows represent the sibling pair who carry

p.I189I variant, which was shown to be associated with poor lung function. The black

arrows represent the sibling pair who carry the g.54823323T>G variant that was not

associated with lung function.
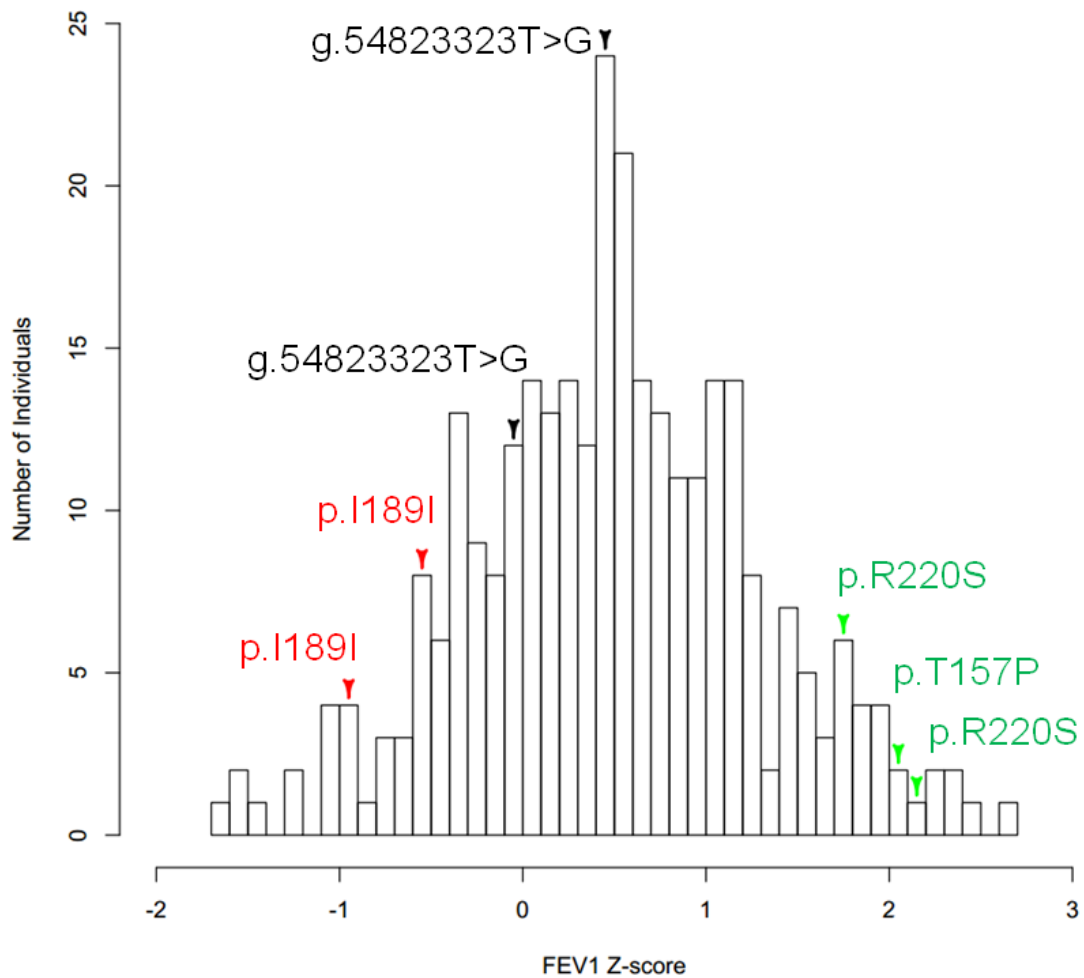
Figure 3.3: LD plots across the *MC3R* locus generated by Haploview. Based on the
criteria defined by Gabriel *et al.* (214), one haplotype block was identified for variants in
MC3R. (A) LD plot based on r^2. The physical position of each variant is presented in the
upper diagram. Each box provides estimated statistics of pairwise r^2 values, with darker
shades representing stronger LD. $r^2 = 1$ (black) means complete LD. (B) LD plot based
on D'. D' values are given in the cell intersecting each pair of SNPs. The LD color
scheme is stratified according to the LOD score and D': LOD <2 (white for D'<1 and blue
for D' = 1) or LOD >2 (shades of pink/red for D'<1 and bright red for D' = 1). (C) Four
major haplotypes A-D represent close to 99% of all haplotypes observed in this study.
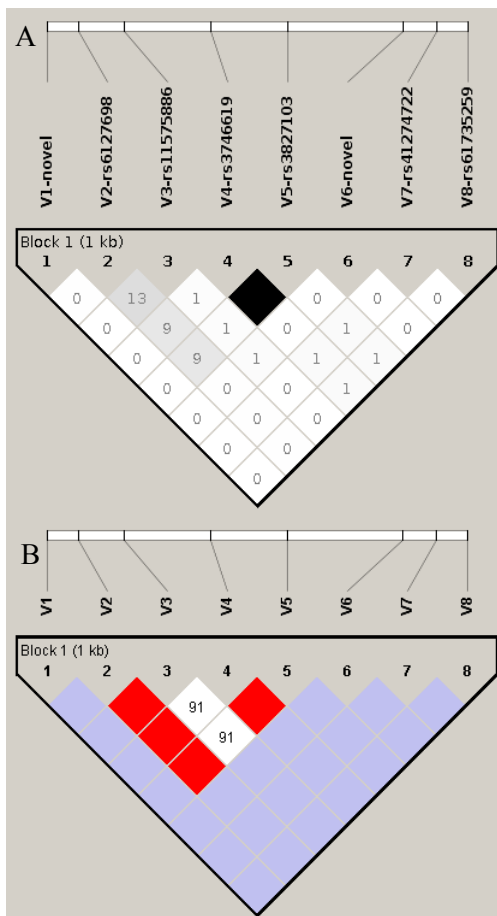
population. Of the eight haplotypes identified in *MC3R,* four major haplotypes (labeled A

– D) account for almost 99% of the variation (Fig. 3.3C). Two haplotypes (labeled D_1 and

D_2) carrying the rare variants, rs41274722 (p.I189I) or rs61735259 (p.R220S), are

derived from haplotype D. The third rare haplotype (labeled B_1) with g.54823323T>G

change rose from haplotype B. Due to missing parental haplotypes, we were not able to

distinguish between A or G at rs11575886 for the fourth rare haplotype (labeled B_2 or C_1)

with g.54824368A>C (p.T157P) variant. Haplotypes D_1, D_2 and B_1 were each observed

on two chromosomes. Haplotype B_2 (or C_1) was present on one chromosome. Omnibus

tests of the common (df = 3, *p = 0.357*) haplotypes did not reveal any association with

lung function though the presence of rare variants seem to drive the haplotypic effects

seen in haplotypes D_2 and B_2 (or C_1) (df = 7, *p = 0.027*) (Table 3.2). Independent effects

of rare SNPs on a major haplotype background were not possible as these variants were

underrepresented in our population (seen in a maximum of 2 pedigrees).

In silico and functional characterizations of coding variants in *MC3R* demonstrate

that g.54824368A>C (p.T157P) may be deleterious

The potential pathogenicity of the four coding variants was assessed using *in*

silico prediction tools, such as SIFT, PolyPhen2, Condel and GERP (Table 3.3). The

rs3827103 (p.V44I), a common variant, has been shown to have ligand binding and

signaling properties similar to wildtype *MC3R*, and was unsurprisingly predicted to be

benign (215). Whereas g.54824368A>C (p.T157P) was consistently found to be

deleterious and well conserved, rs61735259 (p.R220S) had varying results among the

Table 3.2: Association of *MC3R* haplotypes with lung disease severity.

	g.54823323T>G	rs6127698	rs11575886	rs3746619	rs3827103	g.54824368A>C	rs41274722	rs61735259	p-value*
Haplotype A		T	A	C	G				0.754
Haplotype B		G	A	C	G				0.926
Haplotype C		G	G	C	G				0.171
Haplotype D		G	A	A	A				0.223
F-statistic overall p-value									0.357
Haplotype A	T	T	A	C	G	A	T	C	0.754
Haplotype B	T	G	A	C	G	A	T	C	0.881
Haplotype C	T	G	G	C	G	A	T	C	0.265
Haplotype D	T	G	A	A	A	A	T	C	0.311
Haplotype D_1 (p.I189I)	T	G	A	A	A	A	A	C	**0.0245**
Haplotype D_2 (p.R220S)	T	G	A	A	A	A	T	A	0.101
Haplotype B_1 (g.54823323T>G)	G	G	A	C	G	A	T	C	0.637
Haplotype B_2 or C_1 (p.T157P)	T	G	?	C	G	C	T	C	**0.041**
F-statistic overall p-value									0.027

* for that haplotype against all others

Table 3.3: *In silico* predictions for pathogenicity of the coding variants in *MC3R*.

rsID or HGVS name	Substitution	SIFT[a]	PolyPhen2[b]	Condel[c]	GERP[d]
rs3827103	p.V44I	0.19 tolerated	0.098 benign	0.033 neutral	4.05
g.54824368A>C	p.T157P	0 damaging	0.989 probably damaging	0.831 deleterious	5.21
rs41274722	p.I189I	N/A	N/A	N/A	3.44
rs61735259	p.R220S	0.13 tolerated	0.92 probably damaging	0.503 deleterious	3.94

[a]SIFT: range from 0 to 1. The amino acid substitution is predicted to be damaging if the score is <= 0.05 and tolerated if the score is > 0.05.
[b]PolyPhen2: range from 0 to 1. The amino acid substitution is predicted to be probably damaging if the score is close to 1.
[c]Condel assesses the outcome of nonsynonymous variant by using a consensus deleteriousness score that combines SIFT, PolyPhen2 and MutationAssessor.
[d]GERP is a method for producing position-specific estimates of evolutionary constraint using maximum likelihood evolutionary rate estimation. GERP score ranges from -12.36 to 6.18. Positive scores represent a substitution deficit (i.e., fewer substitutions than the average neutral site) and thus indicate that a site may be under evolutionary constraint. Negative scores indicate that a site is probably evolving neutrally.

algorithms. Based on the positive GERP score, the synonymous variant rs41274722 (p.I189I) seems to be under evolutionary constraint.

To test the effect of the rare coding variants on *MC3R* mRNA expression, we measured the mRNA levels by qPCR (Fig. 3.4). RNA was extracted from human transformed lymphocytes of rare variant carriers, their siblings, and two additional individuals without any rare variants. Unlike the housekeeping genes TBP and PPIA, the level of *MC3R* mRNA was low in all samples. The presence of g.54824368A>C (p.T157P) variant in 419B decreased the level of *MC3R* mRNA compared to the sibling without the variant, 419A. Interestingly, the presence of a missense variant at rs61735259 (p.R220S) in 209A increased the level of *MC3R* mRNA compared to the sibling without the variant, 209B. The sibling pair 480A and 480B have the same *MC3R* genotype as they both carry a synonymous change at rs41274722 (p.I189I). However, we observed a large difference in the mRNA level of *MC3R*, suggesting that other factors may be involved in regulating the expression of this gene. Given that the sibling pair that is IBD=2 across *MC3R* has different levels of *MC3R* transcript, we are unable to interpret the result of IBD=0 sibling pairs. We will need to further investigate the effect of the rare variants on *MC3R* transcript levels using additional sibling pairs.

Modulation of MC3R alters NF-κB-mediated reporter gene expression

Previous investigations have demonstrated that melanocortin receptors, primarily MC1R and MC3R, are involved in the inflammatory pathways (116,121,144,216,217). Given that NF-κB is responsible for transcription of numerous cytokines that are involved in inflammation, we asked if modulation of MC3R can alter NF-κB-driven cytokine

Figure 3.4: Differential mRNA expression of *MC3R* in human transformed lymphocytes. *MC3R* mRNA expression was normalized to TBP and PPIA. Error bars represent s.e.m. calculated across four replicates from three to four independent experiments.
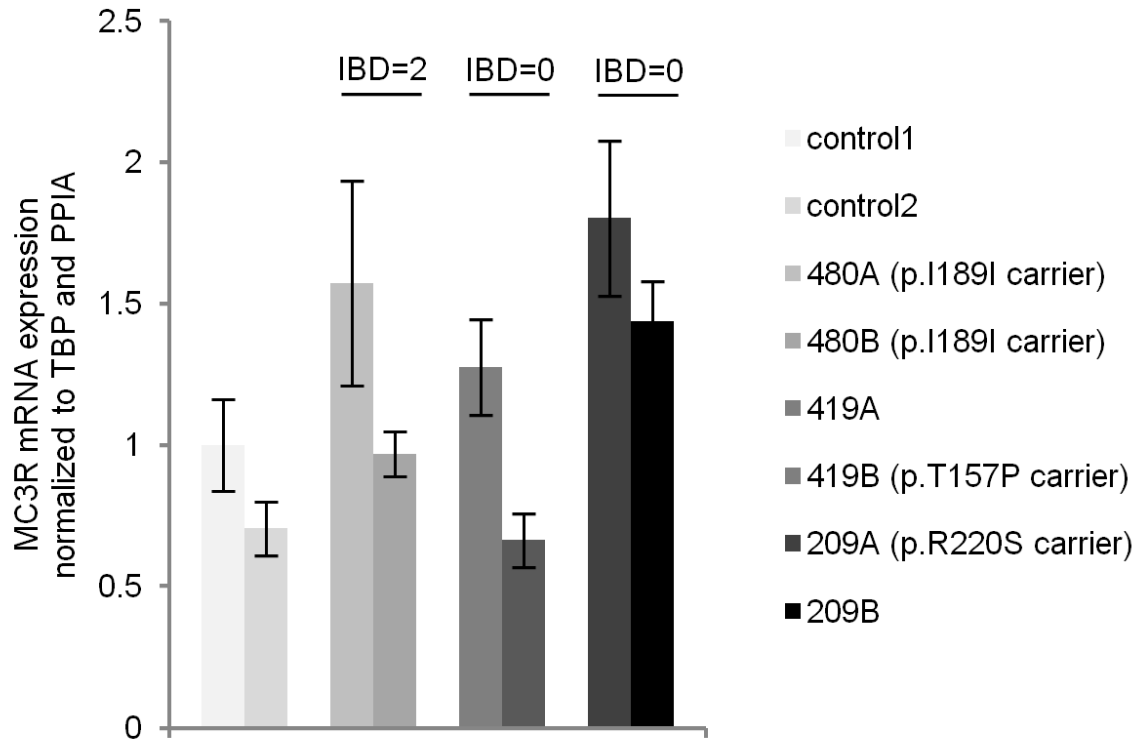
expression. To determine the dose and incubation length of siRNAs for effective

reduction of MC3R in K562 cells, we tested a range of *MC3R* siRNAs (0-100 pmol) and

collected the lysates 2-4 days post transfection. Four days following transfection, *MC3R*

siRNAs were successful in reducing the level of MC3R by 79% (Fig. 3.5A).

It has been reported that melanocortin receptors suppress NF-κB (121). Since the

CMV promoter has four binding sites for NF-κB (218,219), we hypothesized that

reduction of MC3R would prevent inhibition of NF-κB, which would allow the

transcription factors to bind and activate the CMV promoter. In this study, we used CFTR

as a reporter to measure NF-κB-activated CMV promoter function (Fig. 3.5B). K562

cells were initially transfected with siRNAs on Day 0, subsequently transfected with

various CFTR plasmids driven by the CMV promoter on Day 2, and lysed on Day 4.

When MC3R expression was significantly decreased by *MC3R* siRNAs (Fig. 3.5C), we

observed a 3-5 fold increase in the level of GFP-WT-, GFP-F508del- and F508del-CFTR

(Fig. 3.5D). These results suggest that reduction in MC3R protein increases NF-κB

activity in K562 cells consistent with findings in airway epithelial cells (116).

Modulation of MC3R alters NF-κB-driven cytokine expression

NF-κB drives expression of pro-inflammatory cytokines, such as interleukin (IL)-

8, that are known to be elevated in CF (220-222). Thus, we wanted to determine if

modulating NF-κB by reducing MC3R expression would alter IL-8 levels. We

determined the proper dose and incubation length of phorbol 12-myristate 13-acetate

(PMA) that stimulates IL-8 expression in K562 cells. Various concentrations of PMA

(0.2 – 5 ng/ml) were used to treat the cells, and serum IL-8 levels were measured by

Figure 3.5: Reduction in MC3R expression using siRNAs increases NF-κB-mediated reporter gene expression in K562 cells. (A) K562 cells were transfected with 0, 50 or 100 pmol of *MC3R* siRNAs. Cell lysates were collected 2, 3 or 4 days post transfection. The level of MC3R expression was normalized to GAPDH. After 4 days, 100 pmol of *MC3R* siRNAs was effective in reducing the expression of MC3R by 79%. Error bars represent s.e.m. calculated from four independent experiments. *$P < 0.05$ relative to control. ** $P < 0.005$ relative to control. (B) The CMV promoter contains four binding sites for NF-κB transcription factors. CFTR is used as a reporter to quantify NF-κB-activated CMV promoter function. (C) K562 cells were transfected with negative (no siRNA, negative control siRNA #1 or #2) or *MC3R* siRNAs. Two days later, the cells were transfected with three types of CFTR plasmids driven by the CMV promoter: GFP-WT-CFTR (dark grey bar), GFP-F508del-CFTR (grey bar), or F508del-CFTR (light grey bar) plasmid. Cells were lysed 4 days after initial transfection. When K562 cells were transfected with *MC3R* siRNAs, there was a 40-79% decrease in MC3R expression by 4 days post transfection. (D) When the level of MC3R has been significantly reduced in K562 cells, there is a 3 to 5-fold increase in the level of CFTR protein: GFP-WT-CFTR (298%, p<0.0001), GFP-F508del-CFTR (479%, p<0.03) and F508del-CFTR (336%, p<0.04). Error bars represent s.e.m. calculated from three independent experiments.* $P < 0.05$, ** $P < 0.005$

A

MC3R

B

pCMV | NFκB binding sites | Reporter (CFTR)

C

MC3R

siRNA

D

CFTR
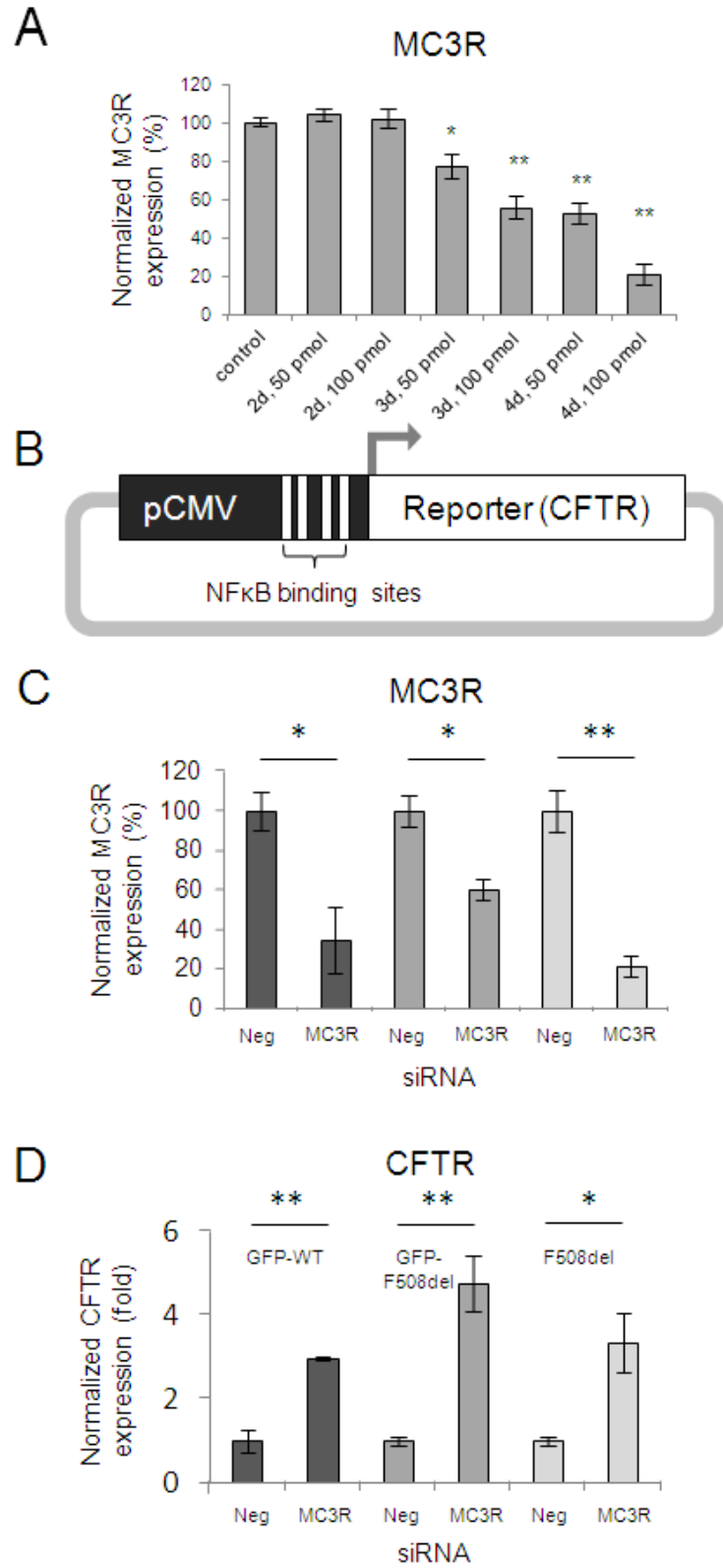
siRNA

ELISA at different timepoints (0 – 6 hours). PMA evoked a dose- and time-dependent secretion of IL-8 in K562 cells (Fig. 3.6A). PMA-induced IL-8 production was significantly increased in K562 cells in which MC3R expression has been reduced by siRNAs (Fig. 3.6B). Combining this result with prior observations, we concluded that a decrease in MC3R protein results in an increase in NF-κB, which then induces the expression of IL-8 upon PMA stimulation. We next asked if activation of MC3R via agonist binding would decrease IL-8 expression upon PMA stimulation. Administration of a selective MC3R agonist, [D-Trp^8]-γ-MSH, inhibited IL-8 expression in a dose-dependent manner in K562 cells (Fig. 3.6C). Taken together, altering MC3R expression by RNAi or agonist binding changes IL-8 levels following stimulation with PMA.

MC3R is expressed in the apical region of airway epithelial cells of normal and CF lung tissues.

If MC3R modifies lung function in CF, we hypothesized that MC3R may be expressed in the lung. We elected to analyze the expression of MC3R in normal and CF lung tissues by immunohistochemistry. Lung tissues were stained with two antibodies that recognize different epitopes on MC3R. Immunohistochemical staining of the normal and CF lungs revealed the presence of MC3R in the airway epithelial cells (Fig. 3.7C-F). The enlarged images in the inset showed intensity at the apical border in both normal and CF lungs. The same staining pattern was detected by two different MC3R antibodies, further validating its expression in airway lungs. The Abcam anti-MC3R antibody that had been neutralized with an antigenic peptide was used alongside the antibody in order

Figure 3.6: Modulation in MC3R expression followed by PMA stimulation alters NF-κB-driven cytokine expression in K562 cells. (A) K562 cells were incubated with different concentrations of PMA for 1, 3 or 6 hours. IL-8 levels were measured by ELISA. PMA induced IL-8 expression in a dose- and time-dependent manner. (B) K562 cells were transfected with no siRNA, negative control siRNA #1 or #2, or MC3R siRNAs. Four days after transfection, the cells were incubated with 0.5 ng/ml or 1 ng/ml of PMA for 6 hours. PMA-induced IL-8 production in supernatants of K562 cells was measured by ELISA. Error bars represent s.e.m. calculated from two independent experiments of 0.5 ng/ml PMA treatment or four independent experiments of 1 ng/ml PMA treatment. (C) K562 cells were incubated with increasing concentration of [D-Trp^8]-γ-MSH for 12 hours and then with PMA for 6 hours. PMA-induced IL-8 levels were measured by ELISA. Error bars represent s.e.m. calculated from five independent experiments.* $P < 0.05$, ** $P < 0.005$

A
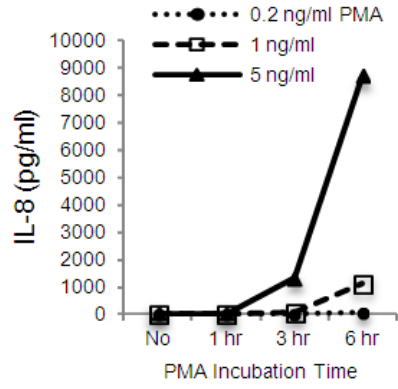
B
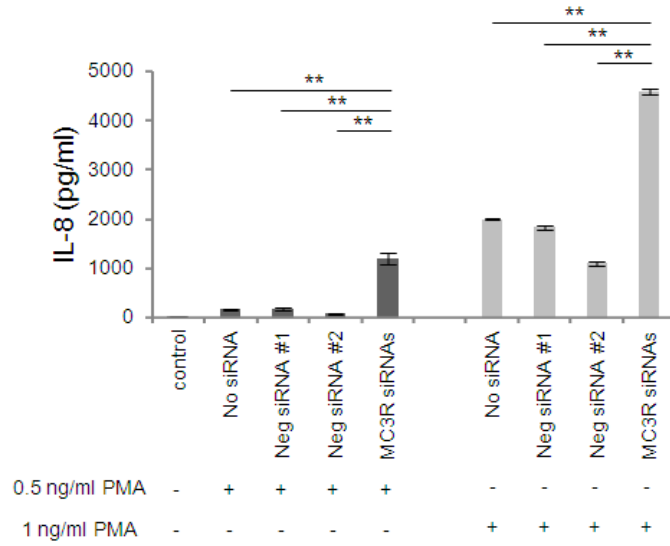
C
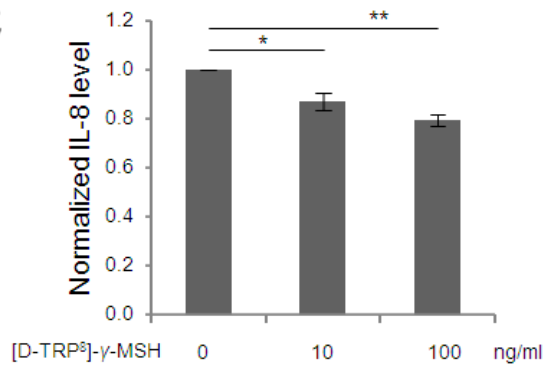
to determine that staining is specific (Fig. 3.7G-H). Elimination of the signal with the

blocked antibody indicated that staining is specific to MC3R.

Given that CFTR is known to be expressed in surface airway epithelial cells of the

lungs (223-225), we asked if MC3R is expressed in the same cell type as CFTR. Western

blot demonstrated that MC3R protein is present in primary airway epithelial cells

obtained directly from lung transplants from 5 individuals (Fig. 3.7I). Interestingly,

variability in the level of MC3R expression did not correlate with the *CFTR* genotype

(Fig. 3.7J).

Figure 3.7: Western blot and immunohistochemical staining reveal that MC3R is expressed in the airway epithelial cells of normal and CF lung tissues. The formalin-fixed, paraffin-embedded lung sections were detected by Dako EnVision™+ System-HRP, stained with hematoxylin, and visualized with a Nikon Eclipse 50i microscope at indicated magnification. Inset: higher magnification showing representative area. (Normal; n=2) (CF; n=2)

(A, B) A universal negative control for rabbit primary antibody, which contains immunoglobulin fraction of serum from non-immunized rabbits, was used to ascertain nonspecific binding and help interpret specific staining at the antigenic site. In both normal and CF lung tissues, there was an absence of non-specific binding with the negative control. (C, D) Positive staining was observed on the apical region of the airway epithelial cells of normal and CF lung tissues with anti-MC3R antibody from LS Bio. (E, F) Similarly, positive staining was observed in the brush border of the airway epithelial cells with anti-MC3R antibody from Abcam. (G, H) Neutralizing the Abcam antibody with the blocking peptide abolished the signal in both normal and CF lung tissues. (I) Western blot of cell lysates from the temporal lobe of human brain and the primary airway epithelial cells. The temporal lobe was used as a positive control for Western blot analysis of MC3R expression. Their *CFTR* genotypes are indicated above each lane. (J) A variable expression of MC3R was observed in the primary airway epithelial cells derived from five different individuals.

	Negative Control	MC3R antibody (LS Bio)	MC3R antibody (Abcam)	MC3R antibody (Abcam) blocked w/ peptide
Normal Lung	A 100x	C 400x	E 400x	G 400x
CF Lung	B 100x	D 400x	F 400x	H 400x

I

Primary airway epithelial cells

Brain 1. Wildtype 2. Wildtype 3. Wildtype 4. F508del/F508del 5. F508del/F508del

37 —

IB: MC3R (Abcam)

37 —

IB: β-actin

J

MC3R

Normalized MC3R Expression (%)

Primary airway epithelial cells

Discussion

Our search for a modifier of lung function in CF has focused upon a 1.3 Mb region on chr20q13.2. This region contains five genes among which *MC3R* is a promising candidate gene for modifying lung function in CF due to its known role in inflammation (115,116) and its effect on energy homeostasis (123-125). We hypothesized that *MC3R* may be responsible for regulating lung inflammation and ultimately affecting lung function in CF. Identification of three rare variants in *MC3R* that displayed significant association with lung function in individuals with CF provided evidence that variation in *MC3R* contibutes to CF lung disease severity. *In silico* prediction tools as well as qPCR analysis suggest that two of the three rare variants, g.54824368A>C (p.T157P) and rs61735259 (p.R220S), may affect MC3R function. Based on prior studies demonstrating that melanocortin receptors modulate NF-κB activity, we showed that reduction in MC3R increases the level of CMV promoter-driven CFTR. The 3- to 8-fold increase in PMA-induced IL-8 level after decreasing the expression of MC3R supports our hypothesis that MC3R alters pro-inflammatory signaling through NF-κB. The presence of MC3R in primary airway epithelial cells of the lungs provides additional evidence that MC3R could modify lung function. Toether, these results suggest MC3R as a potential mediator of the inflammatory response and a possible therapeutic target in the treatment of CF.

In CF, airway inflammation is responsible for the chronic infections and leads to progressive loss of lung function and respiratory failure (102). Thus, reduction of the inflammatory burden in the airways can prevent irretrievable lung damage and therefore be beneficial to all CF patients, regardless of their *CFTR* genotypes. Because NF-κB plays a key role in regulating the expression of a large number of cytokines involved in

inflammation, the NF-κB signaling pathway is an ideal target for pharmacological intervention (226-229). Our initial analysis using CFTR as a reporter to measure NF-κB activity indicates that reduction of MC3R expression increases the level of CFTR by 3- to 5-fold. This result highlights the potential of MC3R to regulate NF-κB activity.

The NF-κB pathway in airway epithelial cells is believed to be critical in controlling lung inflammation through the release of several inflammatory mediators, such as IL-1β, IL-6, and IL-8 (230-232). There have been several studies that report the use of melanocortin peptides to inhibit the activation of NF-κB and repress the inflammatory signaling in various cell types, including inflammatory cells as well as airway epithelial cells (116,121,137-140). Given that MC3R has been previously implicated in inflammatory response, we sought to determine whether or not modulation of MC3R alters IL-8 levels in K562 cells. The cells were treated with PMA, a small molecule that activates protein kincase C (PKC), in order to stimulate an immune response. It is known that PKC activates IκB kinase, which degrades IκB and releases NF-κB to enter the nucleus in order to activate the expression of specific inflammatory genes, such as IL-8 (233-235). We found that upon reducing the expression of MC3R with siRNAs, IL-8 production was increased by 3- to 8-fold after PMA stimulation. On the other hand, activating MC3R with [D-Trp^8]-γ-MSH led to a subtle decrease in IL-8 level following stimulation with PMA. Our study confirms that increasing and decreasing signaling via MC3R alters the expression of pro-inflammatory genes, like IL-8. The evidence that MC3R may regulate the inflammatory response provides rationale for further investigation of the mechanisms underlying lung disease severity in hopes of designing synthetic analogs to treat inflammation.

MC3R expression is poorly characterized in human tissues. Here, we have demonstrated that MC3R is expressed in the airway epithelial cells of the lungs, which corroborates its potential role as a modifier of lung function. Several rationales have been considered to explain the differences seen in the level of MC3R in primary airway cells. The variable expression of MC3R may be attributed to *MC3R* genotype or heterogeneity of cell types obtained from lung transplants. The correlation between *MC3R* genotype and its protein expression warrants further investigation.

There is increasing evidence that support the role of rare variants in both monogenic and complex diseases (236-239). Analysis of 151 sibling pairs with CF identified three rare variants in *MC3R* that associate with lung disease severity. The rare variants, however, did not explain the linkage signal on chr20q13.2. In fact, just one out of four sibling pairs carrying a rare variant contributed positively to the LOD score. This may be attributed to an insufficient sample size and the extreme rarity of individual variants. It has been reported that rare variants, despite potentially large effect size, can only be detected in large samples (240,241). Given that only 151 out of 484 sibling pairs in TSS cohort were sequenced, it is possible that additional rare variants that contribute to the previous linkage signal have not been identified.

Significant association between three rare variants and lung function in the TSS cohort prompted us to search for protective and at-risk haplotypes. Though no haplotypes were associated with lung function, the small number of individuals harboring the rare haplotypes likely prevented potential significant associations to be uncovered. Furthermore, there have been other linkage studies in which the contribution of the at-risk haplotype to the linkage signal was not detected because non-carriers of the rare at-risk

haplotypes were the ones who were actually responsible for the majority of the linkage signal (15,242). Sequencing more sibling pairs may lead to the discovery of novel variants and haplotypes across *MC3R*, which can increase the power of association to detect the factors responsible for the linkage signal at chr20q13.2.

To assess the deleterious effect of coding variants in *MC3R*, we applied three publicly available programs (SIFT, PolyPhen2, and Condel) to predict whether or not a nonsynonymous variant is likely to affect protein function. GERP was used in conjunction to determine the degree of evolutionary constraint on specific nucleotides through quantification of substitution deficits. All four coding variants had positive GERP scores, indicating that all sites were under evolutionary constraint. The *in silico* prediction algorithms concordantly reported the common variant, rs3827103 (p.V44I), as benign and the rare variant, g.54824368A>C (p.T157P), as damaging. Interestingly, the rare variant, rs61735259 (p.R220S), was scored as tolerated by SIFT, probably damaging by PolyPhen2, and deleterious by Condel, which demonstrates that the classification of mutations by these prediction tools is not always accurate. A possible explanation as to why various tools differ in their predictions on the functional effects of nonsynonymous variants could be that PolyPhen2, unlike SIFT, incorporates structural information in addition to sequence information. A benign prediction may need to be further investigated to verify that a given variant is truly tolerated (243).

For this reason, we analyzed the *MC3R* transcript levels of three rare coding variants. *MC3R* mRNA level was lower in the individual carrying g.54824368A>C (p.T157P) variant compared to the sibling without the variant, which suggests that p.T157P is most likely deleterious. This conclusion is strongly supported by the

consistent prediction scores from three algorithms. On the other hand, *MC3R* mRNA level was higher in the individual with rs61735259 (p.R220S) variant compared to the sibling without the variant. Based on this result in combination with varying prediction scores of three algorithms, the pathogenic effect of p.R220S seems less likely. rs41274722 (p.I189I) is a synonymous change present in both siblings from one family. We expected the levels of *MC3R* mRNA to be similar in the siblings, but a large difference in *MC3R* mRNA implied that there may be variants in the promoter or trans-acting factors that regulate the expression of this gene. At this time, there is not enough conclusive evidence that validates whether or not the rare variants in *MC3R* affect its function. Further studies are necessary to elucidate the functional impact at the protein level, if any, of these rare nonsynonymous variants.

Despite considerable progress in CF therapy over recent decades, patients continue to experience longitudinal decline in lung function. The development of new therapies for CF requires identification of genetic loci that modify pulmonary disease severity as well as understanding of the mechanism at such loci. In this study, we evaluated the role of *MC3R* at chr20q13.2 as a potential modifier of lung function. Taken together, our study suggests that MC3R may modify CF lung disease severity by affecting IL-8 production through NF-κB signaling pathway. The development of a drug that selectively alters the expression of MC3R will be of great interest in modulating pro-inflammatory cytokines and subsequent airway inflammation in CF.

Materials & Methods

Genomic DNA Extraction and Sanger Sequencing

Peripheral blood was obtained from consented individuals and genomic DNA was

extracted by combination of phenol/chloroform method. The gDNA was amplified using

Taq DNA Polymerase (Invitrogen) and the following primers:

Primers	Direction	Sequence (5'– 3')
MC3R-all	F	CTT AAT CTC CCT GCA GAA TCC TC
	R	CAA ACG ACA AGT ACA ATC ATG G

Following a 6-min denaturation at 95°C, 30 cycles of 95°C for 45 s, 58°C for 45 s and

72°C for 2 min were performed, followed by a 10-min extension at 72°C. PCR products

were visualized by agarose gel electrophoresis with ethidium bromide staining. The

products were purified, Sanger sequenced and analyzed using Sequencher 5.1 from Gene

Codes Corporation.

Statistical analysis

Lung disease severity was quantified using SaKNorm, a survival-adjusted average of CF-

specific percentiles for FEV_1 using three years of FEV_1 data and then transformed to the

corresponding Z-score by normal transformation (112). Association of lung function with

the number of SNP minor alleles was tested using linear regression. Nominal statistical

significance was defined as *P < 0.05*. Study-wide significance was defined using

Bonferroni correction for 7 tests (8 variants in total but 2 variants are in complete LD)

(0.05/7 = 0.0071). Regression analyses were performed using Stata 11 (StataCorp,

College Station, TX).

Haplotype analysis

The variants discovered via Sanger sequencing were converted into .ped and .map formats. The final .ped file contained 298 founders and 7 non-founders (related individuals) each with corresponding FEV_1 coded as a quantitative trait. These files were used as input into both Haploview (244) and PLINK (245) software packages for both SNP and haplotype based association analysis. An omnibus test of haplotypic association was conducted for all haplotypes present in the population (8 haplotypes; 7 degrees of freedom) via the PLINK --chap command (additional: --maf 0, --each-vs-others). A SNP based association analysis was also conducted for all loci (8 variants) using the PLINK --assoc command. Comparable SNP and haplotype based association tests were performed using the Haploview GUI. Additionally, Haploview was used to define blocks of linkage disequilibrium in both the present dataset and the HapMap project phase 3 dataset (CEU and TSI populations included).

Pathogenicity prediction analysis

SIFT uses the degree of conservation of amino acid resides in sequence alignments derived from closely related sequences in order to predict whether an amino acid substitution affects protein function (246). The amino acid substitution is predicted damaging is the score is <= 0.05, and tolerated if the score is > 0.05. PolyPhen2 predicts the possible impact of an amino acid substitution on the structure and function of a human protein by relying on sequence homology as well as structural information characterizing the substitution (247). A mutation is reported as benign, possibly damaging, probably damaging or unknown based on false positive rate thresholds.

Condel computes a weighted average of the deleteriousness scores from SIFT, PolyPhen2 and MutationAssessor to assess the impact of non-synonymous variants on protein function (248). Results are given as neutral or deleterious. GERP identifies regions that exhibit nucleotide substitution deficits that reflect the intensity of past purifying selection and characterize evolutionary constraint (249). GERP scores range from -12.3 (least conserved) to 6.17 (most conserved).

Cell Culture

K562 cells and human transformed lymphocytes were maintained in RPMI 1640 Medium (Gibco) at 37°C in an atmosphere of 5% CO_2. K562 cells were supplemented with 10% FBS (Cellgro) and 1% penicillin-streptomycin (Gibco). Human transformed lymphocytes were supplemented with 10% FBS (Cellgro). The lysates from primary human bronchial epithelial (HBE) cells were generous gifts from Dr. Joseph Pilewski (University of Pittsburgh).

RNA isolation and reverse transcription PCR (RT-PCR)

Total RNA was isolated from human transformed lymphocytes by using RNeasy Mini Kit (Qiagen) as described by manufacturer's protocol. It was treated with DNA-*free* DNase (Ambion) to remove contaminating DNA as described by manufacturer's protocol. The concentration of DNase-treated RNA was measured by NanoDrop™ 1000 Spectrophotometer (Thermo Scientific). One microgram (1 μg) of DNase-treated RNA was reverse transcribed using iScript™ cDNA Synthesis Kit (Bio-Rad). All cDNA was diluted 1:10 in water prior to qPCR.

Quantitative-PCR (qPCR)

qPCR was performed with SsoAdvanced™ SYBR Green Supermix (Bio-Rad) using

CFX96 Real-Time PCR Detection System (Bio-Rad). All samples were amplified in a

minimum of triplicates. Amplifications were performed with a 3-min denaturation at

95°C, followed by 40 cycles of 95°C for 10 s, 57°C for 10 s and 72°C for 15. Melt curves

for each pair of primers were inspected to assess amplification specificity. The C_q values

were normalized to two housekeeping genes: PPIA and TBP. Gene expression fold

changes were calculated using the $\Delta\Delta C_q$ method. The following primer pairs were used to

detect mRNA expression of *MC3R, PPIA* and *TBP*:

Primers	Direction	Sequence (5'– 3')
MC3R	F	ACA GAA GGA AGA CAG CTG AGG A
	R	TGA TGA AGA CCT GCT CAC A
PPIA	F	AGG TCC CAA AGA CAG CAG AA
	R	GAA GTC ACC ACC CTG ACA CA
TBP	F	AGA GTT CTG GGA TTG TAC CG
	R	TTC GTG GCT CTC TTA TCC TC

Western blot

Cells were lysed in buffer containing 1 M Tris (pH 7.4), 4 M NaCl, 0.5 M EDTA, 1%

NP-40, and protease inhibitor cocktail. The brain tissue was homogenized and sonicated

in RIPA buffer with protease inhibitor cocktail. Samples were denatured in Laemmli

sample buffer (Bio-Rad) at 42°C for 20 min for CFTR proteins or in NuPAGE LDS

sample buffer (Invitrogen) at 100°C for 5 min for all other proteins. Proteins were

subjected to electrophoresis in 7.5% SDS-PAGE gels (Bio-Rad) and transferred to PVDF

membranes (Bio-Rad). The membranes were blocked with 5% NFDM for an hour and

incubated overnight at 4°C with primary antibodies diluted appropriately in 0.1% PBST:

MC3R (Abcam ab93368) at 1:5,000; β-actin (Sigma) at 1:15,000; GAPDH (Sigma) at

1:15,000; β-tubulin (Sigma) at 1:15,000; CFTR (mAb596 and mAb570 generated by Dr. Riordan's group at UNC Chapel Hill) at 1:15,000. Then they were washed several times with 0.1% PBST for an hour and incubated for 1 h at room temperature with secondary antibodies diluted 1:50,000. The signal was detected by enhanced chemiluminescence Prime Western Blotting Detection Reagents (GE Healthcare). ImageJ software was used to quantify the bands.

Immunohistochemistry

After the tissues were fixed in 4% formalin for 24 hours and embedded in paraffin, 4 μm sections were cut and mounted on plus glass slides. The sections were deparaffinized in xylene and then hydrated through exposure with graded alcohols (100%, 95%, 70%, 50%, water, PBS). The slides were immersed in either antigen retrieval solution (Dako, citrate buffer, pH 6.0) and heated for 20 min or Proteinase K solution (Fisher) at 20 μg/ml in 37°C humidified chamber for 20 min. After cooling to room temperature, the slides were incubated with 0.03% hydrogen peroxide for 5 min to quench endogenous peroxidase activity (Dako). Subsequently, they were incubated with blocking solution (Dako) for 30 min to block non-specific binding. They were incubated with appropriately diluted primary antibodies (Dako; Abcam ab93368 at 1:1000; LS Bio LS-A452 at 1:100-1:500) overnight at 4°C. As negative controls, the primary antibodies were replaced with immunoglobulin fraction of serum from non-immunized rabbit (Dako) or with antibodies that have been incubated with the immunizing peptide at 5-fold excess (Abcam). After washing the primary antibodies with TBST, peroxidase labeled polymer conjugated to goat anti-rabbit immunoglobulins were added for 30 min. For signal detection, the slides

were incubated with AEC-chromogen solution (Dako) for 15 min, counterstained with hematoxylin (Dako) and mounted in mounting media (Dako). Photomicrographs were taken with Nikon Eclipse 50i.

Transfection

K562 cells were transfected with siRNAs using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. In certain experiments, K562 cells were subsequently transfected with 1.5 µg of CFTR plasmids using Lipofectamine 2000 (Invitrogen) 2 days after the initial siRNA transfection. Cells were harvest 4 days after the initial transfection. Two negative control siRNAs that have no homology to any mammalian gene were used as controls: AllStars Negative Control siRNA (Qiagen; "Neg. siRNA #1") and Silencer Select Negative Control siRNA (Ambion; "Neg. siRNA #2"). ON-TARGETplus MC3R siRNA SMARTpool, a mixture of 4 siRNAs provided as a single reagent, was used to target MC3R (Thermo Scientific). The sequences of the MC3R siRNAs are described below:

ON-TARGETplus SMARTpool siRNA J-005659-06, MC3R CCGACAUGCUGGUAAGUGU
ON-TARGETplus SMARTpool siRNA J-005659-07, MC3R GUGAGCAGGUCUUCAUCAA
ON-TARGETplus SMARTpool siRNA J-005659-08, MC3R GGAGGGAGAUUUUGUCUUU
ON-TARGETplus SMARTpool siRNA J-005659-09, MC3R GAAUUGCGCAACACCUUUA

ELISA

To examine the expression of IL-8, K562 cells were stimulated with indicated concentration of PMA for 6 hours. In some experiments, K562 cells were incubated with indicated concentration of [D-Trp^8]-γ-MSH for 12 hours prior to PMA stimulation. IL-8 secretion was measured using a human IL-8 ELISA kit (Invitrogen) according to the manufacturer's protocol. Briefly, the supernatants were harvested and added to IL-8

antibody-coated 96-well plates. After the addition of biotinylated antibody and

streptavidin-peroxidase, tetramethylbenzidin was used as a stabilized chromogen. The

absorbance of each well was read at 450 nm using a microplate reader. IL-8

concentrations were determined from a standard curve of recombinant human IL-8.

Chapter 4:

Conclusion and Discussion

CF is a Mendelian autosomal recessive genetic disorder caused by loss-of-function mutations in *CFTR*. Airway inflammation and chronic infection lead to progressive destruction of the lungs, making lung disease the major cause of morbidity and mortality in CF. Since the discovery of *CFTR* as the disease-causing gene for CF, considerable progress has been made in improving nutrition, adequate growth and lung function among patients with CF. Nearly half of all people with CF are now ages 18 and older. The steady increase in the median predicted age of survival in the last 25 years signifies the significant advances that have been made in the development of new drugs and therapies.

Despite its Mendelian pattern of inheritance, CF demonstrates considerable variability in disease severity and clinical presentation, independent of *CFTR* genotype. Therefore, it is critical to determine the magnitudes of non-*CFTR* genetic variation and environmental effects that contribute to the variability of the phenotypes. A high correlation for genetic modifier contribution to CF disease severity using twin-based assessment suggests that genes other than *CFTR* contribute to the disease manifestation. The heritability estimate of lung function based on FEV_1 ranged from 0.54 to 1, further confirming that genetic modifiers play an integral role in lung disease severity. The lack of a cure for this life-limiting disease fuels the search for modifier genes, which will serve as new targets for therapeutic intervention.

Kalydeco^{TM}, the first mutation-targeted drug to treat the underlying cause of CF, potentiates the open-channel probability of the defective CFTR gate. It was initially approved for those who have the G551D mutation. On Feb. 21, 2014, FDA approved expanded use of this drug for eight additional mutations: G1244E, G1349D, G178R,

G551S, S1251N, S1255P, S549N, or S549R. Substantial improvements in lung function and nutritional status have been observed in patients on Kalydeco[TM] (68). Nonetheless, Kalydeco[TM] has not shown to reduce or prevent inflammation that frequently results in chronic infection. The lack of change in inflammatory status may indicate that improvement of CFTR function does not reverse the imbalance in inflammation triggered by the consequence of CFTR dysfunction. Steroids have been used in the past to counteract the inflammatory process, but adverse effects have limited the use of such drugs (250,251). Identifying modifiers of the inflammatory process could provide new therapeutic targets to complement CFTR-directed therapy.

The work presented in this thesis has contributed to the field of molecular biology. In Chapter 2, we report molecular and cellular characterization of human *MC3R* gene. Prior to our work, *MC3R* was predicted to be a single-exon gene with uncertain translation start site. Our study was the first to unveil that *MC3R* is a two-exon gene that requires its 5' UTR to begin translation at the second in-frame ATG and apically localize within polarized cells. Our identification of the transcription start site of *MC3R* defined the location of the promoter and other regulatory element binding sites that control the gene expression. Those who are interested in understanding the mechanism behind *MC3R* gene regulation can now search for variants and haplotypes in the correct promoter region and design their assays accordingly. The understanding of the mechanism that governs *MC3R* translation initiation enables accurate interpretation of the functional consequences of DNA variants that confer risk for obesity and other phenotypes. In addition, we provide evidence for likely interaction between MC3R and MRAP2. Given that our data indicates that MRAP2 may have unknown roles besides being an accessory

factor for MC2R, determining if and how MRAP2 modulates the function of MC3R warrants further investigation. Taken together, our study demonstrates that a previously unannotated 5' exon directs translation of MC3R that localizes to the apical membranes of polarized cells and possibly interacts with (or is assisted by) MRAP2.

Moreover, this thesis makes several important contributions to the field of genetic modifiers in CF. In Chapter 3, we describe common and rare variants in *MC3R* identified through Sanger sequencing. We present evidence that three of the rare *MC3R* variants are associated with lung disease severity in CF. The benefit of sequencing is that this approach does not rely on strong LD with genotyped markers to determine causative alleles. In fact, array-based genotyping technologies as well as imputation methods cannot be used to detect rare variants that associate with many diseases. Therefore, our study design, while laborious, is well-suited to detect rare variants that may contribute to a trait. The identification of common and rare variants allowed us to construct the haplotypes across the *MC3R* locus. While we were unable to associate any of the haplotypes with lung function, our work on the derivation of the rare haplotypes from the major haplotypes provides the foundation for future haplotype analysis. Furthermore, our research reveals the role of MC3R in the regulation of the pro-inflammatory signaling. Since ameliorating airway inflammation can improve overall lung function in all patients with CF, it will be crucial to understand how MC3R alters the inflammatory response in order to design appropriate small molecules that target MC3R.

Rare variants play an important role in the etiology of complex traits and account for missing heritability unexplained by common variants (239,252). The coding variants are especially useful in understanding the function of a gene that is linked to a specific

phenotype. While rare variants tend to have large effect size, natural selection prevents them from becoming common if their impact is deleterious. Hence, analysis of individual rare variants requires very large samples (253), which makes Sanger sequencing an impractical choice. Besides Sanger sequencing, there are other ways to identify rare variants: deep- or low-coverage whole genome sequencing and targeted sequencing. Due to cost, deep-coverage whole genome sequencing can only be applied to limited numbers of samples. The benefit of this approach is that it provides the most complete catalog of variation. On the other hand, low-coverage whole genome sequencing can be performed on more samples, but the limitation of this approach is that not all rare variations will be ascertained with confidence. While targeted sequencing serves as a compromise between deep- vs. low-coverage whole genome sequencing, it requires a priori knowledge of what region of the genome to target. One of the our future studies includes targeted sequencing of MC3R as well as other modifier genes in additional samples in order to detect rare variants and establish rare haplotypes that can explain the linkage signal at chr20q13.2.

As an alternative to testing rare variants individually, efforts have been devoted to developing statistical methods to test associations between combinations of rare variants and complex traits. Because rare variant tests independently do not have enough power to detect its effects, they have been collapsed to test cumulative effects of rare variants in a so-called burden test. Aggregating rare variants substantially increases power, which depends on frequency and effect size. There are different examples of the burden tests: cohort allelic sum test (254), combined multivariate and collapsing method (255), and non-parametric weighted sum test (256). Each test makes different assumptions about the rare variant effects and has its own limitations. Once more rare variants are detected

through targeted sequencing of additional CF patients, we are interested in performing an appropriate burden test to find the association between rare variants in a region (i.e. *MC3R*) and lung disease severity.

The research presented in this thesis has advanced our understanding of the mechanism by which MC3R may regulate pro-inflammatory signaling pathway in CF. More work remains to be done to determine how rare variants affect MC3R function and alter the inflammatory response. Such *in vitro* functional studies will require the utilization of transformed lymphocytes or primary cells from the patients themselves or a creation of an appropriate cell line that contains the rare variants. Once we determine the most suitable system for studying the proposed mechanism, we hope to gain novel insights in the role of MC3R in inflammation.

Collectively, our study has uncovered a novel target, *MC3R*, that may be amenable to therapeutic interventions in patients with CF. Although the contribution of each genetic modifier to the overall variability in disease presentation is most likely small, identification of modifiers promotes alternative or additional ways to treat CF. If patients can increase the functional activity of their mutant CFTR protein through a drug like Kalydeco^{TM} while managing their inflammation with a different drug that targets a modifier gene, they may experience an improved quality of life and increased longevity. If a modifier of CF lung disease is implicated in another disease in which therapies already exist, CF patients may promptly benefit from existing treatments. Because genetic modifiers operate in the context of the genome in response to the environmental exposure, it may be difficult to tease out the complex relationship. Nonetheless, identification of true modifiers and their respective pathways signify potential new

targets for therapies against CF lung disease. Since airway inflammation impairs all CF patients' lung function regardless of *CFTR* genotype, development of a drug that can reduce or prevent inflammation will be beneficial to all CF patients.

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EDUCATION

Ph.D. in Human Genetics, **Johns Hopkins School of Medicine**   **Expected May 2014**

M. Ed., Teacher Education Program, **University of California, Los Angeles (UCLA)**   **2008**
Preliminary Single Subject (Biology) Credential with Cross-cultural, Language and Academic Development Emphasis

B. S., Molecular, Cell, and Developmental Biology, **UCLA**   **2007**
Minor in Asian Languages and Cultures (Korean)
Phi Beta Kappa, magna cum laude with highest departmental honors

RESEARCH

Institute of Genetic Medicine, Johns Hopkins School of Medicine   **2010-current**
Advisor: Dr. Garry Cutting, Department of Pediatrics
Molecular characterization of *MC3R* and evaluation of its potential role as a modifier of lung function in cystic fibrosis

Department of Human Genetics, UCLA   **Summer 2008**
Advisor: Dr. Karen Reue, Department of Human Genetics
Examination of the transcriptional regulation of *Diet1* gene, which is responsible for abnormalities in lipid and glucose metabolism in mice, and aimed to isolate the mutation that confers resistance to atherosclerosis

School of Education and Information Studies, UCLA   **2007-2008**
Advisor: Dr. Imelda Nava-Landeros, Teacher Education Program
Investigation of ways to build a college-bound classroom culture and to transform my classroom into the space of empowerment where students could develop a sense of responsibility for their success

Department of Molecular, Cell & Dev. Biology, UCLA   **2005-2007**
Advisor: Dr. L. Jeanne Perry, Director of UCLA Protein Expression Technology Center
Analysis of the structures and functions of *M. tuberculosis* proteins, Rv3878 and Rv3879c, for new drug therapy developments that would target the emergence of new drug-resistant tuberculosis strains

TEACHING

Department of Biology, Johns Hopkins University (Baltimore, MD)   **Winter 2014**
Instructor – Ethical Issues in Human Genetics (26 undergraduate students)
- Successfully proposed a new undergraduate course called *Ethical Issues in Human Genetics* for Johns Hopkins Winter Intersession Programs

- Created a curriculum that explored various ethical issues in human genetics, such as pre-implantation genetic diagnosis, newborn screening, rare disease research, genetic discrimination, and gene therapy

Department of Biological Sciences, Towson University (Towson, MD)   **2012-2013**
Adjunct Faculty
- Fall 2013: Biology 190, Introductory Biology for the Health Professions (48 undergraduate students)
- Fall 2012: Biology 120, Principles of Biology (44 students); Biology 120 Lab (24 students)
- Spring 2012: Biology 115, Biology - The Science of Life (42 students)

Molecular Biology and Genetics, Johns Hopkins School of Medicine (Baltimore, MD)
Teaching Assistant – *Fundamental of Genetics* (80 graduate students)   **Fall 2013**
- Worked with Dr. Jeremy Nathans to design a 100-point problem set that covered human genetics and population genetics portion of the course

Institute of Genetic Medicine, Johns Hopkins School of Medicine (Baltimore, MD)
Teaching Assistant – *Evolution of the Concept of a Gene* (12 graduate students)   **Fall 2011**
- Organized meetings with the first-year graduate students prior to their presentations
- Maintained a Blackboard course website

Los Altos High School (Hacienda Heights, CA)   **2008-2009**
Biology Teacher
- Taught 3 regular and 2 special education biology classes
- Created a class website on Blackboard to facilitate communication with students and parents
- Served as an advisor for Science Olympiad in the "Picture This" category (placed 2nd in state)

Elite Educational Institute (Fullerton, CA)   **2008-2009**
SAT Math Instructor
- Prepared over 100 students for the math section of the SAT Reasoning Test

Locke High School (Los Angeles, CA)   **2007-2008**
Biology Teacher
- Taught 2 honors, 2 regular and 1 sheltered (for English Language Learners) biology classes in a Program Improvement school in the Watts community
- Organized and facilitated a professional development seminar for the biology department
- Provided outside educational opportunities for the students

University High School (Los Angeles, CA)   **2005-2007**
Advancement Via Individual Determination (AVID) Program Intern
- Taught biology, chemistry and geometry to 10th & 11th grade students in a Program Improvement school

MENTORING

Mentoring Undergraduates in Interdisciplinary Research (Baltimore, MD)   **2012-2013**
Mentor Scientist for Manpreet Kaur, a senior undergraduate from Notre Dame of Maryland University

- Designed an 1-year research project in which she characterized a potential modifier gene by identifying variants, determining its molecular mass, and confirming its expression in lung tissues
- Taught basic laboratory skills and molecular biology techniques, such as PCR, site-directed mutagenesis, gel electrophoresis, transformation, DNA extraction, Western blot, and Sanger sequencing
- Gave a lecture on Genomics and Its Impact on Science and Society: The Human Genome Project and Beyond to all undergraduate students participating in this program

Human Genetics Predoctoral Training Program (Baltimore, MD)   **Fall 2012**
Mentor Scientist for Kaitlin Victor, a first-year human genetics rotation student
- Supervised a graduate student who studied methylation pattern in the promoter region of *GNAS* gene in monozygotic twins with cystic fibrosis

Baltimore Excellence in STEM Teaching Project (Baltimore, MD)   **Summer 2011**
Mentor Scientist for Jon Rosenblum, a high school biology teacher
- Defined a 6-week research project in which he sequenced the exons of *CSE1L*, a gene that was discovered to be a negative regulator of CFTR-dependent fluid secretion
- Provided technical support and hands-on training for the teacher intern
- Helped develop an engaging and relevant genetics curriculum that contained lessons from the summer research

MANUSCRIPTS IN PREPARATION OR UNDER REVIEW

Sharma N, Sosnay P, Ramalho A, Douville C, Franca A, Gottschalk L, **Park J**, Siklosi K, Amaral M, Karchin R, Cutting GR. (2014) Accuracy of algorithms in predicting the consequences of splice site variants. [manuscript under review]

Park J, Sharma N, Cutting GR. (2014) Molecular and cellular characterization of the human melanocortin-3-receptor (*MC3R*). [manuscript under review]

Park J, Sharma N, Vecchio-Pagán B, Franca A, Lee M, Gottschalk L, Pilewski J, Belchis D, Blackman S, Cutting GR. (2014). Melanocortin-3-receptor (*MC3R*) modifies lung disease severity in individuals with cystic fibrosis. [manuscript in preparation]

PROFESSIONAL PRESENTATIONS

Park J, Rose A, Ingram M, Vieira N. *Beyond Mendel: Complexities of Simple Mendelian Disorders*. 64th Annual Meeting of the American Society of Human Genetics; 2014 Oct. 18-22 (expected); San Diego, CA. [invited session]

Park J, Pilewski J, Belchis D, Blackman S, Cutting GR. *MC3R modifies CF lung disease by increasing the level of CFTR*. 63rd Annual Meeting of the American Society of Human Genetics; 2013 Oct. 22-26; Boston, MA. [platform]

Park J, Vecchio-Pagán B, Cuppens H, Cutting GR. *SNP genotyping is a valuable tool for assessing the quality of next generation sequencing data.* 62nd Annual Meeting of the American Society of Human Genetics; 2012 Nov. 6-10; San Francisco, CA. [poster]

Park J, Sharma N, Cutting GR. *Evaluation of the melanocortin-3-receptor as a potential modifier of CF lung disease*. 26th Annual North American Cystic Fibrosis Conference; 2012 Oct. 11-13; Orlando, FL. [poster]

Kapa L, Sharma N, **Park J,** Cutting GR. *Creation of a CFBE41o- cell line with a single integration site for the expression of rare CFTR mutants*. 26th Annual North American Cystic Fibrosis Conference; 2012 Oct. 11-13; Orlando, FL. [poster]

Sharma N, Kapa L, **Park J**, Sosnay P, Cutting GR. *Overexpression of NHERF1 promotes recycling of a naturally occurring CFTR C terminus truncation mutant*. 25th Annual North American Cystic Fibrosis Conference; 2011 Nov. 3-5; Anaheim, CA. [poster]

Park J. *Building a college-bound classroom culture by encouraging students to develop responsibility for their success*. UCLA Teacher Education Program Secondary Resident Inquiry Poster Session; 2008 Jun. 4; Los Angeles, CA. [poster]

Park J, Eng A, Chiang J, Chan S, Pham M, Shin A, Elbogen J, Perry J. *Characterization of RD1 Proteins, Rv3878 and RV3879c, in Mycobacterium tuberculosis*. UCLA Annual Science Poster Day; 2006 May 24; Los Angeles, CA. [poster]

ACTIVITIES AND SERVICE

American Society of Human Genetics (Bethesda, MD)   **2014-current**
Information & Education Committee Member
- Carry out activities concerned with information relevant to human genetics as it relates to the education of students in general, the training of professionals in human genetics, and the awareness of human genetics by the general public

Johns Hopkins Diversity in Graduate Education Sub-committee (Baltimore, MD) 2012-2013
Graduate Student Member
- Wrote a proposal for a $200,000 grant over two years to increase and support diversity in graduate education at Johns Hopkins University School of Medicine
- Planned to create a bridge program for admitted graduate students to increase readiness for transitioning from a student to a professional, help them manage research time, provide networking opportunities, and enhance the feeling of community

Towson Teaching Fellowship Program (Towson, MD)   **2012**
Selection Committee Member
- Reviewed 21 applications for Teaching Fellows at Towson University

Towson Teaching Fellowship Program (Towson, MD)   **2011-2012**
Teaching Fellow
- Completed 3-semester teaching fellowship that is designed to prepare future university faculty to use research based pedagogical practices in college classrooms
- Attended pedagogy seminars focused on teaching a non-majors course and observed current instructors in the first semester of the program
- Was the instructor of record of an introductory biology course (Biology 115) and an adjunct faculty member of the Department of Biological Sciences at Towson University in the 2nd and 3rd semesters

Incentive Mentoring Program (Baltimore, MD)   2011-2012 / 2010-11 / 2009-10
Communications Director / Head of Household / Volunteer
- Diagnosed issues within the organization and provide a formal mechanism for addressing concerns
- Conducted organization-wide surveys and led a leadership training for new Heads of Household
- Managed a group of 10 volunteers to provide support for Kayla, an African American girl who had failed two years of school and lived with an unemployed father and a sick mother, by tutoring her twice a week, serving as a liaison to teachers, planning college visits, and providing transportation in times of need

UCLA Recreation (Los Angeles, CA)   2005-2008
Fitness Instructor
- Taught over 100 group exercise classes, such as Total Body Challenge, Super Step Circuit, Ultimate Upper Body, Extreme Step, Hip Hop Sets-N-Reps, etc.
- Earned Group Fitness Certification through American Council on Exercise (ACE)

AWARDS AND HONORS

Los Altos High School Teacher of the Month   October 2008

UCLA Graduate Opportunity Fellowship   2007
- Given to distinguished individuals from cultural, racial, linguistic, geographic and socioeconomic backgrounds that were underrepresented in graduate education
- $12500 one-year award that covers full tuition, stipend, and registration fees

UCLA Housing & Hospitality Services 'Hats Off To You!' Award   2007
- Recognized dedicated Apartment Coordinators who provided excellent service

UCLA Bruinlife Yearbook 'Senior of the Year' Award   2007
- Acknowledged seven most outstanding seniors that have excelled in academics, campus service and/or community service out of the graduating class of 7000 students

UCLA Chancellor's Service Award   2006
- Honored students who have donated their time and energy to making a positive difference at UCLA and in the community; recognized at the graduation ceremony

Phi Eta Sigma National Honor Society Scholarship   2006
- $5000 award given to those nominated by the local chapter for outstanding participation, evidence of creative ability and potential for success in chosen field

UCLA Undergraduate Research Scholarship   2006
- $2500 award given to students with a strong commitment to research and completion of an honors thesis

Freeman Award for Study in Asia   2004
- $5000 award presented to undergraduates who have been accepted to study abroad in Asia

UCLA Regents Scholarship   2003
- $5500 honorarium per year for four years awarded to the top 1.5% of the entering freshmen applicant pool based on academic excellence, leadership and exceptional promise

UCLA Alumni Scholarship   2003

- $1500 award per year for four years given to excellent incoming students who have committed to leadership and service through outstanding contributions

PROFESSIONAL AFFILIATIONS

American Society of Human Genetics
American Association for the Advancement of Science

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