POSTTRANSCRIPTIONAL REGULATION OF INFLAMMATORY MOLECULES IN CYSTIC FIBROSIS

by

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Abbreviation

Akt	serine / threonine-protein kinase Akt
ARE	adenosine-uridine rich element
ATRA	all-trans retinoic acid
BALF	bronchoalveolar lavage fluid or bronchioalvelar lavage
CCL2	C-C motif chemokine 2
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
Chk	serine / threonine-protein kinase Chk
COPD	chronic obstructive pulmonary disease
COX	cyclooxygenase
CSNK	casein kinase
CTGF	connective tissue growth factor
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELAV	embryonic lethal abnormal visual protein
ELAVL	ELAV-like protein
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
Ets1	Protein C-ets-1
FBLN	fibulin
FKBP12	FK506-binding protein 12
FOS	proto-oncogene c-fos
FXR1	fragile X mental retardation syndrome-related protein 1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony-stimulating factor
HCV	hepatitis C virus
hnRNP	heterogeneous nuclear ribonucleoprotein
Hu	human antigen
IFN	interferon
IL	interleukin
IP	immunoprecipitation
KSRP	far upstream element-binding protein 2, also known as KH type-splicing regulatory protein
LPS	lipopolysaccharide
LT	lymphotoxin
MAPK	mitogen-activated protein kinase
MDM2	E3 ubiquitin-protein ligase Mdm2
miR	microRNA
MK2	MAP kinase-activated protein kinase 2

MMP	matrix metalloprotease
mTOR	mammalian target of rapamycin
MYB	transcriptional activator Myb
MYC	Myc proto-oncogene protein
NF-κB	nuclear factor κB
NR2F2	nuclear receptor subfamily 2 group F member 2, also known as COUP transcription factor 2
nt	nucleotide(s)
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PI3K	posphoinositide-3-kinase
PKA	protein kinase A
RAR	retinoic acid receptor
Rheb	Ras homolog enriched in brain
RICTOR	RPTOR-independent companion of mTOR
RISC	RNA-induced silence complex
RNA	ribonucleic acid
RPTOR	regulatory-associated protein of mTOR
RT-PCR	real time PCR
RXR	retinoid X receptor
SHIP1	phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 1
siRNA	small interfering RNA
SIRT1	NAD-dependent protein deacetylase sirtuin-1
SIS	platelet-derived growth factor subunit B
SLC	solute carrier family
SMAD	mothers against decapentaplegic homolog
TGF	transforming growth factor
TLR	Toll-like receptor
TNF	tumor necrosis factor
TOM1	target of Myb1
TTP	tristetraprolin
uPA	urokinase-type plasminogen activator
UTR	untranslated region
VEGF	vascular endothelial growth factor

Dissertation Abstract

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in a chloride ion channel called cystic fibrosis transmembrane conductance regulator (CFTR). CF patients frequently develop chronic lung inflammation and refractory respiratory infection, most commonly by *Pseudomonas aeruginosa* (*P. aeruginosa*, PA). The CF lung disease is one of the major causes of the morbidity and mortality in CF patients, and development of anti-inflammatory therapeutics for CF is a major goal. Inflammatory molecules are regulated through posttranscriptional mechanisms by microRNAs (miRNAs) and mRNA-binding proteins. miRNAs are endogenous non-coding RNA molecules that have been found to regulate various cellular processes by destabilizing target mRNAs. Recent studies indicate that microRNAs are key molecules in disease development and therapy. Additionally, several RNA-binding proteins (RBP) have been shown to coordinate with miRNAs and regulate expression of inflammatory genes. A class of RBPs recognize and bind to adenosine-uridine rich regions (ARE regions) in the 3' UTRs of mRNAs and are known as ARE-binding proteins (AUBPs). However, the role of these factors in the regulation of the inflammation in CF has not been well studied. Therefore, my overall research theme is to determine posttranscriptional regulation of inflammatory molecules in CF. Based on previous findings from our laboratory, my thesis especially focuses on the mechanisms of posttranscriptional regulation of IL-8 expression in CF by miR-155 and HuR. Our findings indicated that inflammatory mediators and *Pseudomonas* infection modulate IL-8 and as well as miR-155 expression in CF lung epithelial cell. To further investigate mechanisms of posttranscriptional regulation of IL-8 mRNA, we subsequently searched for proteins and miRNAs which bind to the ARE region of IL-8 mRNA specifically in CF. Our data show that HuR and miR-16 bind to the ARE region, and regulate IL-8 mRNA stability. Additionally we examined how miRNA regulates CF disease development. We focused on miR-155, one of the significantly upregulated miRNAs in CF, and searched for novel direct targets of miR-155 which contributes to the lung inflammation in CF. We validated RPTOR as a miR-155 target, and demonstrated that

lower RPTOR expression might lead to higher CTGF expression in CF, which is considered to be associated with fibrosis in CF lungs.

Table of Contents

Chapter 1: Dissertation Introduction

Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in chloride ion channel called cystic fibrosis transmembrane conductance regulator (CFTR) (114). Intracellular domains of CFTR include a regulatory domain (R domain) and two ATP binding sites (NBF1 and NBF2) (21). When serine residues of the R domain are phosphorylated by PKA, CFTR channel is poised to bind ATP to NBF1 and NBF2 (21). CFTR channel opens by hydrolysis and separation of ATP. Since water follows ion movement, CFTR eventually affects water intake. For example, in case of CFTR inhibition, lumen is more viscous (CF lung), while CFTR overstimulation leads to water loss. Among the Caucasian population, CF is the most common autosomal recessive disease, affecting one patient in 3300 people and one carrier in 25 people (36). There are various types of mutations, but the most common pattern is a deletion of the phenylalanine residue at 508 (Phe508del) from CFTR (22). In a specific population, other traits are more common, such as W1282X in the Ashkenazi Jewish population (125). After birth, the natural history of cystic fibrosis begins with steatorrhea and failure of gaining weight during infancy, immediately followed by intermittent or chronic respiratory infection (25). Series of autopsy of steatorrhic children who died of respiratory infections revealed dilated (cystic) acini and ducts and fibrotic surrounding stroma in the pancreas. This was considered to lead to less excretion of digestive enzymes and subsequent malabsorption, especially lipid-soluble vitamins A and D (46). Although malabsorption can be treated with nutrient supplementation, respiratory infection is still a life-threatening factor for CF patients. Patients with CF tend to develop chronic infection and inflammation systemically, but especially in the respiratory tract. Chronic infection is most commonly caused by bacteria such as *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus*, and *Haemophilus influenzae* (82; 118), because of viscous airway

mucosa caused by defective chloride ion export from airway epithelial cells, which leads to more severe inflammation (41). In fact, the number of neutrophils is higher in bronchioalveolar lavage fluid (BALF) from CF, and inflammatory products from neutrophils, such as elastase and IL-8, were shown to increase in sputum (23) and BALF (54) from CF patients.

Definition of inflammation has not yet been established exclusively (122). Pathologically, inflammation generally refers to a series of responses of vascularized tissues to infections and damaged tissues that brings cells and molecules of host defense from the circulation to the sites where they are needed, in order to eliminate the offending agents (67). These offending stimuli include microbial pathogens, foreign bodies, toxins, hypoxia, burns, physical forces (trauma) and so on. When tissue is injured, local blood vessels become permeable and humorous components and leukocytes leak into the injured site. Depending on the stimulus, leukocytes kill or seal it, and finally the impaired tissue is repaired by fibroblasts. During this process of inflammation, many molecules are mediating each reaction among endothelial cells, leukocytes, damaged epithelial cells, and pathogens. These molecules are called inflammatory mediators, including cytokines, chemokines, arachidonic acid derivatives, growth factors, complements, extracellular matrices, and reactive oxygen species.

Whether cystic fibrosis cells are inherently inflammatory or not has been controversial. According to the explanation above, it follows that cystic fibrosis does not develop inflammation if a patient is not infected, because there are no definitive external stimuli. Also, the association between an ion channel mutation and inflammation is not promptly inferable. Although in most cases, histopathological findings of cystic fibrosis lung specimen include acute and chronic inflammation as well as dilation of bronchioles and hyperplasia of mucus glands (139), the airways are usually histologically normal during the first few months of life (58), indicating that the inflammation can be because of infection that occurs later but not necessarily because of the nature of CF. To determine whether it is a possibility, several studies were done using cultured CF cell lines under sterile environment. Both samples of BAL fluid and bronchial epithelial cells

from uninfected CF patients showed higher IL-8 mRNA expression compared with normal uninfected control (90). CF cell line with Phe508del mutation showed increased sensitivity to bradykinin which activated phospholipase A2, leading to more production of arachidonic acid (9). Human bronchial goblet cells isolated from CF and non-CF patients were cultured and IL-8 was found to be increased (131), and also the other lab showed that CF bronchial epithelial cell line, IB3-1 (*CFTR*$^{p.Phe508del/W1282X}$), has increased IL-8 mRNA expression (5). However, another study with respiratory epithelium from CF patients without infection did not show significant difference in IL-8 mRNA compared with non-CF controls (51). Whether CF is inflammatory in nature or not, at least it is commonly accepted that CF epithelial cells may have abnormal regulation of inflammatory cytokines independent of infection.

Function of microRNAs

MicroRNAs (miRNAs, miRs) are approximately 22 nucleotide-long non-coding RNAs which regulate translation or stability of target mRNAs by binding to specific mRNA sequences through complementarity. Phenomenon of RNA-mediated gene silencing had been known since before the discovery of microRNA. In 1984, mouse L-cells were transfected with a plasmid containing the antisense sequence of thymidine kinase (TK) gene of herpes simplex virus (HSV) (45). When TK-negative L-cells were co-transfected with one plasmid with wild type TK gene and another plasmid with flipped TK sequence, TK expression level significantly decreased (45). Later, miRNA was first described in *Caenorhabditis elegans* (70). Previously it had been known that LIN-14 protein expression was reduced by *lin-4* gene product, and that the 3'UTR of *lin-14* mRNA was necessary for this inhibition. In 1993, Lee *et al* discovered that there were two small transcripts from the *lin-4* gene (61 nt-long *lin-4*L and 22 nt-long *lin-4*S) which are not protein-coding (70). While *lin-4*L was predicted to have characteristic hairpin loop structure, these transcripts include a same sequence which is complementary to the 3'UTR of the *lin-14* mRNA,

and thus *lin-4*L were thought to be a precursor of *lin-4*S which was thought to bind to the 3'UTR of *lin-14* mRNA and inhibit the translation of lin-14 (70). Together with other microRNAs, such as *let-7*, these were initially called small temporal RNA (stRNA).

Although several mature miRNAs had been reported since then, the processing mechanism was not clear until another similar RNA, siRNA, was discovered. In 1998, Fire *et al* synthesized double-stranded RNA molecules of several genes and transfected them into *C. elegans*, which led to deficient phenotypes of corresponding genes (31). In later study, an *in vitro* silencing experiment showed that this is caused by mRNA degradation which requires nuclease activity and guiding RNA, and this complex was termed RNA-induced silencing complex (RISC) (39). Almost at the same time, it was also shown that dsRNAs were cleaved into approximately 22 nt-long small sequences and separated into single-stranded RNAs, which were thought to function as a guide to the target mRNA (160). However, the RISC did not have the ability to cut dsRNA into 22 nt-ssRNA. In 2001, *Drosophila* S2 cells were transfected with one of plasmids containing several different RNase III genes with T7 tag (10). Subsequent immunoprecipitation with T7 antibody revealed 22 nt-long RNA molecules in a sample with CG4792 plasmid (renamed as Dicer-1), and this RNA molecule migrated in gel down to the same position as RNA isolated from RISC (10).

Because of similar length of siRNA to stRNA, it was predicted that Dicer-1 was also involved in stRNA processing. Four months later, while examining the phenotype of *dcr-1* mutant homozygote of *C. elegans*, it was shown that this developmentally defected phenotype was similar to that of *let-7* mutant (37; 53). Indeed, *lin-4* and *let-7* expressions decreased and precursors accumulated, and therefore it was concluded that Dicer-1 was necessary for pre-stRNA processing to mature stRNA (37; 53). Although precursor stRNAs had been first thought to be a primary sequence, there seemed to be another longer and more primary product than pre-stRNA, based on the existence of a group of several miRNAs which are located in one cluster sharing a common upstream regulatory region. By using primers binding to a sequence outside of

pre-miR-30, a primary precursor was detected (371 nt-long pri-miR-30a), exclusively in the nucleus, and when this sequence was inserted in plasmid and expressed, this was processed to 65 nt-long pre-miR-30a, and finally to the mature miR-30a (72). Finally, an RNase III which cleaves pri-miRNAs was sought. Because of the nuclear localization and unestablished roles, Drosha was examined. Drosha was tagged with flag, collected by immunoprecipitation, and treated with pri-miRNA *in vitro* (71). pri-miRNA was successfully processed to pre-miRNA, and Drosha knockdown resulted in accumulation of pri-miRNA (71).

There are some proteins which bind to miRNAs and mRNAs and regulate their processing (63). For example, KH-type splicing regulatory protein (KSRP) and heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) were shown to bind to the terminal loop sequence of pri-miRNAs, and KSRP also binds to pre-miRNAs to facilitate maturation of its target miRNAs (89; 137). However, in case of let-7a processing, hnRNPA1 inhibits its processing at Drosha level by physically competing with KSRP (88).

So far, 30424 microRNAs have been reported in plants, insects and animals as of June 2013 (60), and it is estimated that up to 30% of protein expression is influenced by miRNA (30). MicroRNA genes have been identified in introns of protein-coding and non-coding genes, or in exons of protein non-coding transcription units (such as miR-155 in *BIC* transcript which does not code a protein) (56). As described above, miRNAs are transcribed by RNA polymerase II (63). Primary transcript (pri-miRNA) has stem loop structure(s) where a ribonuclease Drosha binds and processes pri-miRNA to shorter precursor (pre-miRNA). Canonically, pre-miRNA is exported into the cytoplasm through exportin 5. In the cytoplasm another ribonuclease Dicer binds and cleaves the loop, producing a duplex composed of miRNA/complementary miRNA-star sequence. One of the two strands of this duplex is determined to be incorporated into RNA-induced silencing complex (RISC) and guided to target mRNAs to degrade or reduce the stability of target mRNAs. A figure from a review article summarizing this mechanism is shown at the end (103). Some miRNAs stimulate translation or stability of its target mRNAs (141). Classically,

miRNA binds to 3'-untranslated region (3' UTR) of its target mRNAs, while some miRNAs were shown to bind to 5'UTR or coding region, or several sites together (69; 99).

Prediction of miRNA targets

Since the discovery of miRNA, it has been thought that a miRNA interacts with its target mRNA by complementary binding. When *lin-4S* was discovered as the first miRNA against *lin-14* mRNA, it was also found that there were two short blocks of *lin-4* sequence which are complementary to an element repeated seven times in the 3'-UTR of *lin-14* mRNA (70). The first block of complementary sequence was 9 nt-long at the very 5' end of *lin-4S*, and the second block, and the second block was at least 4 nt-long at the 3' end of *lin-4S*. The reasons why this potential antisense complementarity seemed critical was because the seven repeated elements in *lin-14* are conserved between *C. elegans* and *C. briggsae*; because screening for loss-of-function mutant revealed a mutation located within a block of *lin-14*-complementary sequence of *lin-4*; and because the seven *lin-4*-complementary elements occur in the *lin-14* region deleted in *lin-14* gain-of-function mutant (70). In a later study, a ^{32}P-labeled chemically synthesized 24-nucleotide *lin-4S* RNA was incubated with *in vitro* transcribed *lin-14* 3'-UTR RNA bearing the seven putative *lin-4* binding sites, and complex formation was confirmed by gel electrophoresis (38). When *lin-14* RNA had helix-disrupting mutations in each of the seven binding sites, the final product migrated down to the position where unbound RNA was detected (38). In this report, there was also a predicted binding site where miRNA-mRNA duplex forms a bulge (38). Although this bulged binding was not proven by electrophoresis, *in vivo* observation suggested some biological effects on larva development (38). Thus it was proven that microRNAs bind to their targets by base pairing.

Based on known miRNA sequences at that time, an algorithm was developed (MiR scan) to predict and find miRNAs in *C. elegans* and *C. briggsae* genes (76). Its prediction method was

largely based on the presence of hairpin structures, and conservation between these two species (76). They identified 88 *C. elegans* miRNA genes, and succeeded in grouping them into 48 families including one to eight genes per each, so that, within families, sequence identity either spanned the length of the miRNAs or was predominantly at their 5' terminus (76). Thus it was suggested that the 5' end sequence was particularly conserved and involved in base pairing with their target mRNAs (76). Later on, another algorithm was developed to predict the targets of vertebrate miRNAs, called TargetScan (74). This program combines thermodynamics-based modeling of RNA:RNA duplex interactions with comparative sequence analysis to predict miRNA targets conserved across multiple genomes (74). Given an miRNA that is conserved in multiple organisms and a set of orthologous 3'-UTR sequences from these organisms, TargetScan searches the UTRs for segments of perfect Watson-Crick complementarity to 2nd to 8th bases (or longer if they still match) from 5' end of the miRNA (74). This 7 nt segment of the miRNA was referred to as the "miRNA seed", and UTR heptamers with perfect Watson-Crick complementarity to seed as "seed matches" (74). TargetScan further extends to search miRNA to optimize base pairing of the remaining 3' portion of the miRNA to the 35 bases of the UTR (74). By using this program, it was shown that the ratio of the average number of predicted mRNAs per miRNAs to that of randomized miRNAs became significantly higher when conservative miRNAs were chosen from broader range of species (74). It was also shown that this ratio was particularly high when the seed sequence was within first to ninth bases from 5' end of miRNA (74).

miRNAs in Cystic Fibrosis

In relation to cystic fibrosis, miRNAs play roles in two aspects. One is for posttranscriptional regulation of CFTR mRNA and protein, and the other is for upregulation of inflammation. First of all, overall miRNA expression profiling studies in CF are mentioned. One study has shown that 24 miRNAs were significantly upregulated in the ilea of CF mice (6).

Another study has been carried out with bronchial brushing samples from five CF patients and five non-CF patients (96). In this study, among 667 miRNAs examined, 56 were significantly downregulated, and 36 were upregulated. Third study performed miRNA expression profiling in CF bronchial epithelial cell line (IB3-1) as well as in lung epithelial cells isolated from bronchial brushings of CF patients (12). Of 356 miRNAs examined, 4 were significantly downregulated and 18 were upregulated. Each of these studies focused on different aspects of regulation of the disease phenotype in CF.

There are several reports which showed regulation of CFTR mRNA and protein by miRNAs. miR-145 and miR-494 were shown to directly target 3'-UTR of CFTR mRNA in human bronchial cell lines by luciferase assay (35). Similarly, another study showed miR-101 and miR-494 targeting CFTR mRNA in HEK-293 cell line (85). Another study also demonstrated that miR-101 (and miR-144 which has the identical binding site) targets CFTR mRNA in HEK-293 cell line treated with cigarette smoke (40). Samples from bronchial brushings of CF patients showed increased miR-145, miR-223 and miR-494 expression, and these miRNAs were further shown to directly target CFTR mRNA in HEK-293 cell line (97). CFTR can also be regulated indirectly by miRNA. For example, miR-138 directly targets a histone deacetylase SIN3A mRNA, which binds and represses the promoter of CFTR gene (110).

miRNAs can also modulate the effects of inflammation and infection in CF. miR-145 was increased in nasal epithelial cells from CF patients, and luciferase assay showed that miR-145 targets SMAD3 mRNA in HEK-293 cells (84). Although SMAD3 is involved in TGF-β1 pathway to inhibit NF-κB signaling, a relationship between miR-145 and NF-κB signaling pathway was not demonstrated in this article (84). Another study was performed to compare miRNA expression in bronchial brushing samples between CF and non-CF patients (96). In this study, miR-126 was found to decrease in CF, and miR-126 was validated to target an mRNA of TOM1 which is involved in intracellular trafficking and an inhibitor of inflammatory signaling (96). In a CF cell line, decreased miR-126 caused increased TOM1 expression which finally led

to decreased cytokine production after LPS and IL-1 stimulation (96). Another study showed that, when a CF cell line was treated with *Pseudomonas aeruginosa*, miR-93 expression decreased (28). miR-93 was further shown to bind to the 3' UTR of IL-8 mRNA, and IL-8 increased after *Pseudomonas* infection because of decreased miR-93 (28). Our lab also previously showed that miR-155 was upregulated in CF cell line, which led to activation of PI3K/Akt pathway by directly targeting SHIP1, an inhibitor of the pathway (12). In CF, activated PI3K/Akt pathway resulted in increased transcription and stability of IL-8 mRNA (12).

RNA-binding proteins and inflammation

Eukaryotic mRNA has some distinct features. Nascent precursor mRNA (pre-mRNA) is transcribed by RNA polymerase II, and at almost the same time, mRNA goes through several events such as 5' capping, splicing and polyadenylation at the 3' end (8; 147; 161). These events are necessary for efficient translation and stability for mRNAs. For example, mRNA was shown to be circular so that poly(A) tail interacts with the 5' end through poly(A) binding protein and eukaryotic initiation factor 4, which is required for starting translation. It means, in turn, that poly(A) length is an important determinant for its protein expression and stability of mRNA. This has been known for long time, but a recent study examined poly(A) length of mRNAs at transcriptome-wide scale, and showed a positive correlation of poly(A) tail length with an ability to interact with poly(A) binding protein Pab1p and mRNA translation efficiency (ribosomal density) in *Saccharomyces cerevisiae* (7). However, this study did not show the correlation between poly(A) length and mRNA half-life (7).

On the other hand, another specific feature of 3' UTR was found to be an important determinant for mRNA stability regulation. Compared with proto-oncogene v-*fos*, its eukaryotic cognate c-*fos* had been known to be unstable to transform fibroblasts in vivo (87). Meijlink *et al* examined their major sequence difference in 3' UTR, and by deleting various segments of c-*fos* 3'

UTR, they found that deletion of a 67-nucleotide stretch containing adenosine and uridine bases was sufficient to activate its transforming potential (87). AT-rich sequence was also identified in genes of some inflammatory molecules such as TNF, LT, IL-1α and –β, IFN-α and –β, and GM-CSF, and it was recognized that there was a common 33-nt sequence in the 3' UTR that was conserved between mouse and human (16). This sequence was composed entirely of A and T residues and contains the repeating octamer TTATTTAT (16). More detailed features were revealed in granulocyte-monocyte colony stimulating factor (GM-CSF) mRNA, which had been known to express only transiently in T lymphocytes. GM-CSF mRNA also has ARE regions in its 3' UTR, and this sequence was found to be conserved in mouse and human (124). When this conserved ARE sequence was inserted into 3' UTR of stable β-globin mRNA, it was rapidly decayed after actinomycin D treatment (124). Thus ARE sequence was shown to regulate mRNA stability (124). In this study, it was further shown that ARE sequences were recognized and conserved in other mRNAs of several lymphokines and proto-oncogenes, such as α-IFN, β-IFN, γ -IFN, IL-1, IL-2, IL-3, TNF, cFOS, cSIS, cMYC, and cMYB (124). Analysis of these sequences also revealed that the largest sequence motif common to all of these genes is AUUUA (124). To determine if there is a cytoplasmic protein which interacts with the 3' UTR of unstable mRNA through the AUUUA element, *in vitro* transcribed, labeled synthetic RNA that contained four adjacent reiterations of the AUUUA motif was incubated with lymphocyte cytoplasmic extract (81). After treatment with RNase A, band-shift assay revealed a stable, RNase A-resistant complex, which was abolished by prior treatment with proteinase K (81). Thus there was shown to be a protein which binds to ARE sequence, and this protein was termed AU-binding factor (AUBF) (81). Since then several ARE-binding proteins had been reported, and among them was a group of RNA-binding proteins called Elav-like proteins, consisted of HuC, HuD, and Hel-N1. These proteins were ARE-binding proteins and expressed only in the brain. In addition to these three proteins, another protein of this family which especially expresses in non-neuronal cells was sought (78). RT-PCR analysis of mRNA from HeLa cell revealed a new sequence composed of

RNA recognition motif (RRM) III with adjacent 140 nt-long conserved region (78). This product was found to be highly related to, but distinct from, HuD, HuC and Hel-N1, and newly termed as HuR (78). The predicted protein had an identical domain arrangement as the other Elav-like family members, including RRMI and II for ARE-binding and RRMIII for poly(A) tail binding (78). Furthermore, it was also shown that HuR binds to ARE region, particularly AUUUA sequence of IL-3 mRNA (78).

These two structures (poly(A) and ARE region) described above were shown to be associated to each other. When mouse NIH3T3 cells were transfected with a plasmid of human c-*fos* gene with deleted ARE region, the poly(A) length from that mRNA was shown to be longer, and more stable than that with ARE region (151). HuR was also shown to stabilize ARE-containing mRNAs, such as fos and GM-CSF (29). But when RRMIII of HuR was deleted, these mRNAs were not stable (29). Since RRMIII has been assigned a role in associating with the poly(A) tail (78), and since deadenylation was shown to be the first step of degradation, it was suggested that interactions with both ARE and poly(A) were essential for effective mRNA stabilization by HuR and this serves initially to protect the mRNA from deadenylation (29).

Since then, HuR has been shown to bind to the ARE regions in 3' UTR of several mRNAs of cytokines and proliferative molecules, and also stabilize the target mRNAs. Early examples of HuR targets include mRNAs of VEGF (73), cyclin A and B1 (145), and TNF-α (32). In malignant glioma cell line, HuR was shown to bind to 3' UTR of IL-8 mRNA (92). It was further confirmed that HuR bound to ARE region of IL-8 mRNA by UV cross-linking of salivary protein lysate with IL-8 ARE, which led to stabilization of IL-8 mRNA (102). The expression and function of HuR were shown to be regulated by several stimuli and signaling pathways. Stimulation of MAPK pathway in mammary epithelial cells by docetaxel led to activation of MK2, which was shown to be required for stabilizing an HuR target, COX2 mRNA (129). In another study of HuR binding to the ARE region of uPA mRNA, HeLa cells were treated with constitutively active form of MK2, and HuR was shown to translocate to the cytoplasm and more

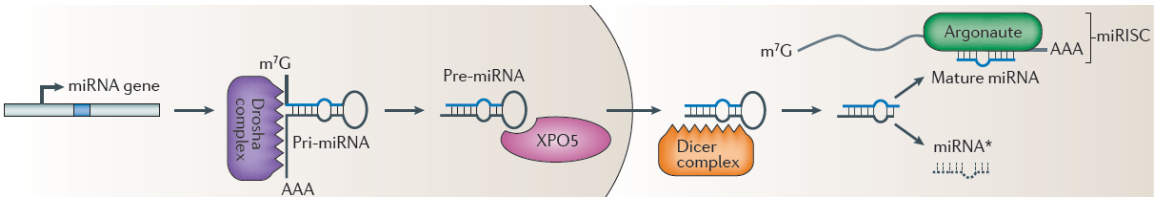
HuR bound to uPA ARE which led to stabilization of uPA mRNA (138). Another mechanism of HuR regulation is phosphorylation by Chk2 (1). When WI-38 cells were treated under oxidative stress, Chk2 was activated and the S100 residue of HuR was phosphorylated, and dissociated from its target, SIRT1 mRNA (1). In this study, more HuR translocated to the cytoplasm under oxidative stress, but HuR was dissociated and SIRT1 mRNA was degraded (1). These signaling pathways are provoked by inflammatory stimuli, and therefore, HuR is an important molecule in inflammatory signaling pathways which is controlled by upstream stimuli, and then regulates stability of its target cytokine mRNAs.

Conclusion and specific aims

Based on the literature review above, knowledge about posttranscriptional regulation mechanisms by miRNAs and ARE-binding proteins have grown recently. Especially, the discovery of the presence of ARE sequences in 3' UTR of many inflammatory genes progressed further research and revealed substantial contribution of ARE-binding proteins to mRNA stability of these inflammatory molecules in several diseases. However, little has been studied about the posttranscriptional regulation mechanisms of inflammatory genes in CF. Indeed, a recent review describes several studies of miRNA-mediated regulation of CFTR mRNA, but not as many of miRNA-mediated regulation of inflammation (126). Furthermore, the roles of ARE-binding proteins have not been well studied in CF. Therefore, my overall research theme is to describe posttranscriptional regulation of inflammatory molecules in CF. Based on previous findings from our laboratory, here we especially focussed on the mechanisms regulating IL-8 expression in CF by miR-155 and HuR for these aims. First, we analyzed how inflammation and infection affected the expression and of IL-8 mRNA and miR-155. Subsequently we analyzed how ARE-binding proteins and miRNAs which bind to the ARE sequences of IL-8 mRNA regulate its expression in CF lung epithelial cells. Finally we examined how miRNA contributes to CF disease

development, focusing on miR-155, one of significantly upregulated miRNAs in CF. We identified novel direct targets of miR-155 which contribute to the lung inflammation in CF.

Figure



(Cited from Pasquinelli AE. (103))

Figure Legend

Figure: microRNA biogenesis. In animals, microRNA (miRNA) genes are typically transcribed as primary miRNA (pri-miRNA) transcripts that undergo processing by Drosha-containing complexes. The resulting hairpin precursor miRNAs (pre-miRNAs) are transported to the cytoplasm by exportin 5 (XPO5). The Dicer complex removes the loop region from pre-miRNAs, and one strand of the resulting duplex is bound by Argonaute to form a miRNA-induced silencing complex (miRISC), which targets mRNAs for degradation and / or translational suppression. The other strand, which is often called the star strand (miRNA*), is degraded. The figure and legend is cited from Pasquinelli AE. (103)

Chapter 2: Specific aim 1

Differential regulation of inflammation by inflammatory mediators in cystic fibrosis lung epithelial cells

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Running head: Regulation of IL-8 expression in CF

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Abstract

Cystic Fibrosis (CF) is due to mutations in the *CFTR* gene, which cause a massively proinflammatory phenotype in the CF airway. The chemical basis of the inflammation is hyper-production of interleukin-8 (IL-8) by CF airway epithelial cells, based on both an intrinsic mutation-dependent mechanism, and by infection. In infection-free, cultured CF lung epithelial cells, high levels of the microRNA (miR), miR-155 is responsible for hyper-expression of IL-8. However, whether infection-induced IL-8 expression in CF cells is also mediated by miR-155 is not known. We have hypothesized that miR-155 might be a general mediator of enhanced IL-8 expression in CF cells, either in response to other cytokine/chemokine mediators of inflammation, or following exposure to infectious agents. Here we find that a reduction in miR-155 accompanies suppression of IL-8 by either the anti-inflammatory cytokine IL-10, or by inhibition of ambient IL-1β with a neutralizing antibody. However, attempts to elevate IL-8 levels with either intact bacteria (*viz.* a mucoid strain of *Pseudomonas aeruginosa (PA)*), or lipopolysacchride (LPS), were unable to elevate miR-155 above its intrinsically high level in the absence of these agents. Instead, in response to PA-infection, the CF cells modestly suppress the expression of miR-155, and express a novel set of miRs, including miR-215. We find that ex-vivo CF lung epithelial cells also express high levels of both miR-155 and miR-215. The predicted module of infection-induced mRNA targets focuses on activation of the NFκB signaling pathway, and on the pro-apoptotic p53 signaling pathway. We interpret these data to suggest that that CF lung epithelial cells respond to *P. aeruginosa*, or bacterial cell products with a novel microRNA program that may carry with it serious challenges to survival.

Key words: cytokines, chemokines, inflammation, microRNAs, mRNA, gene regulation

Introduction

Cystic fibrosis (CF) is the most common fatal autosomal recessive disease in the U.S. and Europe, and is due to mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (19; 33; 59; 107). CF is characterized by a massive pro-inflammatory phenotype in the lung, due to the secretion of high levels of IL-8 and other pro-inflammatory cytokines and chemokines (3; 13; 23; 113; 115). IL-8 is the most potent known chemotactic agent for neutrophils (115), and is secreted from CF lung epithelial cells (13).The enhanced secretion of IL-8 seems to be an intrinsic property of the CF epithelium, since fetal CF lung epithelium spontaneously secretes high IL-8 levels into the airway as early as the 15th week of gestation and in the absence of any detectable infection (54; 135). Furthermore, most infection-free, cultured CF lung epithelial cells also hyper-secrete IL-8 (26).

However, the airway of the CF patient is chronically infected, most often with mucoid strains of *Pseudomonas aeruginosa* (PA). This chronic infectious state therefore adds to the already high IL-8 expression level by added signaling through the toll-like receptor (TLR) and NFκB signaling pathways. Therefore a combination of defective intrinsic regulation of IL-8 expression, coupled with infection-mediated increase in IL-8 expression, may result in a catastrophic proinflammatory quagmire of chemokine activities, bacteria and bacterial products, neutrophils, and mucins in the CF airway. However, it remains unknown whether a common intrinsic defective mechanism is responsible for both the intrinsic defect in IL-8 expression, as well as the defective response to the extracellular presence of bacteria in the CF airway.

The expression of inflammatory genes, including the pro-inflammatory chemokine IL-8, is known to be regulated by diverse processes. We have previously demonstrated that CF lung epithelial cells in culture not only secrete large amounts of IL-8 protein, but also have high levels of very stable IL-8 mRNA (5). Recent studies have also shown that IL-8 expression in CF cells is regulated by the microRNA, miR-126 (95). More recently, we have specifically shown that miR-

155 is directly responsible for stabilizing IL-8 mRNA and up-regulating of IL-8 protein expression in CF lungs (12). Elevated levels of miR-155 are also found in primary cultures of airway epithelial cells from CF patients, as well as in neutrophils from peripheral blood of CF patients (12). However, whether this mechanism is also responsible for massively elevated levels of IL-8 in response to bacteria has not yet been determined.

We have therefore hypothesized that miR-155 might be a general mediator of enhanced IL-8 expression in CF cells, including in response to other cytokine/chemokine mediators of inflammation, or following exposure to infectious agents. To test this hypothesis, we have challenged CF lung epithelial cells with conditions which either raise or lower IL-8, and have measured changes in miR-155. Here we report as anticipated, that agents such as the anti-inflammatory cytokineIL-10, or neutralizing antibodies against IL-1β, lower both IL-8 and miR-155. However, while *PA* raises IL-8 levels, we find it fails to further elevate miR-155. Instead, *PA* modestly suppresses miR-155, and recruits an entirely unique set of miRNAs. This unique set of miRs includes elevated miR-215, whose focus with other *PA*-dependent miR changes, is to drive the CF cell into a p53-centric survival challenge. These data suggest that CF is associated with both an intrinsic proinflammatory defect, and an infection-elicited defect, and is therefore of importance in resolving this ongoing controversy.

Materials and Methods

Reagents

LHC-8 media, Trypsin-EDTA (0.05%) and Lipofectamine transfection reagent were purchased from Invitrogen (New York). Actinomycin D (ActD) was purchased from Sigma Chemical Co (St. Louis, MO). RNA aqueous and miRVana kit for isolation of RNA from CF cells was obtained from Life Technology (Grand Island, NY). Anti-IL-1β antibody was purchased from R&D systems (Minneapolis, MN). The human IL-10 was purchased from Cell Signaling Technology, Inc. (Danvers, MA).

Cell culture and transfection

IB3-1 CF lung epithelial cells were maintained in LHC-8 serum free medium in humidified 5% CO_2 as previously described (26). Transfections were performed using the Lipofectamine Transfection Reagent (Invitrogen). The *Pseudomonas aeruginosa* infection was performed according to published protocol (18). Briefly, 1.5 x 10^6 cells were treated with high (HA) or low (LA) algenate form of mucoid *P. aeruginosa* (MPA) for 24 h. RNA was isolated and analyzed by quantitative real time PCR. The dosage of bacterial treatment is non-toxic to the CF cells and was selected based on the published protocol of Chattaraj, et al. (18).

Measurement of mRNA and microRNA by qRT-PCR

RNA was isolated from the CF cells and mRNA expression levels were analyzed with qRT-PCR as described earlier (5).The primary bronchial epithelial cells were obtained from lung brush biopsies of CF patients under a USUHS Institutional Review Board approved protocol as described earlier (5). The cells were stored in RNA later or Trizol and total RNA was isolated using the miRVana kit. The microRNA expression profile was analyzed by Taqman Low Density Arrays (ABI) as described in Bhattacharyya et al. (12). Briefly, 350 ng of RNA was used for the

profiling. Each of the multiplex Reverse Transcription performed with TaqMan miRNA Reverse Transcription Kit (ABI) was diluted and mixed with TaqMan Gene expression Master Mix (2X). 100 µl of the RT reaction-specific PCR reaction mix was loaded into the corresponding fill ports of the TaqMan Low Density Human MicroRNA Panel v2.0 (Early Access). The individual miR-specific Taqman assays were performed with 20 ng of RNA with Taqman probes specific for mature miRs (ABI).

Statistical analysis

Statistical analysis was performed using Excel. Significance values ($p \leq 0.05$) were determined by student's t-test for the 3 hour time points. Error bars on graphs represent SEM.

Results

IL-10 treatment of CF cells suppresses IL-8 and miR-155 expression

CF epithelial cells are also functionally deficient in IL-10, an important anti-inflammatory, immune-modulatory cytokine (142). To test if IL-10 treatment could suppress IL-8 in CF lung epithelial cells, we treated IB3-1 CF cells with IL-10 (10ng/ml) for various intervals of time. We subsequently measured IL-8 mRNA levels. As depicted in **Fig. 1A**, we find that a 2 hour incubation of the CF cells with 10 ng/ml IL-10 promotes the most significant and efficient reduction in IL-8 mRNA. **Fig. 1B** shows that IL-10 treatment also suppresses miR-155 by ca. 20% ($p < 0.05$). There is precedence for this kind of observation, since IL-10 has been shown to suppress miR-155 expression in mouse macrophages (83).

We have previously shown that the miR-155 mechanism for IL-8 activation depends on suppression of the inositol 5-phosphatase SHIP1, resulting in activation of the PI3K/Akt/MAPK signaling pathway (12). Activation of MAPK by this mechanism results in stabilization of the IL-8 mRNA. Consistently, **Fig. 1C** shows that reduction of miR-155 through treatment with IL-10 also results in enhanced degradation of IL-8 mRNA. Thus the miR-155 mechanism contributes to IL-8 suppression by IL-10.

Anti- IL-1β suppresses IL-8 gene expression and miR-155 in CF lung epithelial cells

IL-1β has been shown to induce IL-8 expression in CF cells (91) and also to promote stabilization of IL-8 mRNA in various cell types (130). We therefore examined the role of IL-1β in the hyper-expression of IL-8, as well as in the up-regulation of miR-155 by treating CF cells with different doses of neutralizing anti-IL-1 β antibody for 24 hours**.** As shown in **Fig. 2A,** a dose of 50 ng/ml of the antibody (1:10,000 fold dilution), is effective in suppressing IL-8 expression. Treatment with this dose of anti-IL-1 β reduces IL-8 mRNA levels by ca. 60% and protein levels by ca. 75%. Subsequently, we examined the stability of IL-8 mRNA at this dose of

antibody treatment. As shown in **Fig. 2B**, neutralization of IL-1 β promotes enhanced degradation of IL-8 mRNA ($p<0.05$). There is rapid reduction of IL-8 mRNA within 1 hour, followed by decay of IL-8 message to *ca*. 65% of its original level. This rate and extent of IL-8 mRNA decay is comparable to that observed in CFTR-repaired IB3-1/S9 lung epithelial cells (5). These data have historical precedents in earlier studies of other cell types showing effects of anti- IL-1 β on both IL-8 mRNA transcription and mRNA stability (91; 130)**.**

Based on our earlier findings that IL-8 expression is regulated by miR-155 in CF cells, we further analyzed miR-155 expression levels in the anti-IL-1 β-treated IB3-1/CF cells. **Fig. 2C** shows that miR-155 expression is indeed suppressed by neutralizing IL-1 β. However, the level of miR-155 suppression is reduced by only *ca*. 20%. Nonetheless, the reduction is significant ($p < 0.05$), and there are precedents in the literature for small changes in miR expression to have significant impact on target genes (123). Conclusively, these data strongly support the concept that elevated IL-1β is one of the factors in CF cells that promotes hyper-expression of IL-8. These data are also consistent with the possibility that miR-155 expression helps to mediate this effect on IL-8 mRNA expression and mRNA stability in CF lung epithelial cells.

Pseudomonas aeruginosa **infection elevates IL-8 expression, and enhances IL-8 mRNA stability, but decreases miR-155**

High alginate (HA) strains of mucoid *Pseudomonas aeruginosa* (MPA) are often closely associated with CF lung disease. We therefore examined the effects of both low alginate (LA) as well as the high alginate (HA) forms of MPA on IL-8 expression in CF lung epithelial cells. As shown in **Fig. 3A**, the HA form of MPA induced the most significant increase in IL-8 mRNA levels in IB3-1 CF cells. Inasmuch as increased IL-8 expression in the infection-free state is, in part, due to enhanced stabilization of the IL-8 message (5), we proceeded to examine whether MPA infection had any effect on IL-8 mRNA stability.

As shown in **Fig. 3B**, IL-8 mRNA stability is further enhanced (by *ca*. ~3 fold increase in $t_{1/2}$) in MPA treated cells, compared to media-treated CF cells. The log/linear plot shows that even though IL-8 mRNA stability is the CF cells is quite high to begin with, IL-8 stability can be even further enhanced by the added presence of bacteria. However, as shown in **Fig. 3C**, we find that increased expression of IL-8 mRNA in the MPA-infected CF cells does not promote further increase in miR-155 expression. Rather it modestly, but significantly suppresses miR-155 expression. These data suggest that the miR-155-dependent mechanism for regulation of IL-8 expression in infection-free CF cells might be modified by the concomitant presence of infection.

Lipopolysaccharide (LPS) further stimulates IL-8 mRNA expression and IL-8 mRNA stability in CF cells without inducing miR-155

Dead bacterial cell walls, represented by purified lipopolysaccharide (LPS), can interact with specific Toll-like Receptors (TLRs) on the epithelial cell surface, and promote proinflammatory intracellular process that are similar to those evoked by living bacterial cells. We therefore treated CF lung epithelial cells to LPS, and analyzed the IL-8 mRNA response. **Fig. 4A** shows that IL-8 mRNA levels are significantly increased in IB3-1 cells over many hours. However, **Fig. 4B** shows that no significant increase is observed in the corresponding miR-155 expression levels over the same time period. Finally, **Fig. 4C** shows that LPS significantly stabilizes IL-8 mRNA compared to the already stabilized levels observed in media-treated CF cells. Thus the miR-155-independent effects of living mucoid PA on IL-8 mRNA levels, IL-8 expression, and IL-8 stabilization can be similarly observed in the presence of a purely chemical stimulus by LPS.

Differential expression of miRNAs in mucoid PA-infected CF lung epithelial cells

Based on the preceding data with intact bacteria and purified LPS, we considered the possibility that the added presence of infection modifies the miR-155 dependent, intrinsic

proinflammatory program in CF lung epithelial cells. To test this hypothesis further, we analyzed the miRNA expression profile in IB3-1 CF treated with mucoid *Pseudomonas aeruginosa* (MPA), and compared that to media-treated IB3-1 CF cells. As shown in **Table 1**, the difference is substantial and mechanistically informative. The data indicate that 75 miRNAs are significantly ($p < 0.05$) and differentially-expressed in CF IB3-1 cell treated with mucoid *Pseudomonas aeruginosa* bacteria for 24 hours. Of these significantly changed miRs, miR-215 is the only miRNA whose expression is elevated (*ca*.10-fold), while the other miRs are down-regulated compared to uninfected IB3-1 CF cells. According to the prevailing paradigm for understanding miR function, an elevated miR would suppress target mRNAs, while reduced miRs would release target mRNAs from tonic inhibition.

To determine the possible relevance of these miR data to clinical CF, we performed the same screen on *ex-vivo* CF lung epithelial cells. As shown in **Fig. 5A**, we found that miR-215 was also elevated by *ca*. 1000-fold in CF cells, compared to control cells. Inasmuch as most CF patients have a history of chronic *Pseudomonas aeruginosa* infection, the *in vivo* miR phenotype, at least with regard to miR-215, appears to parallel, in some fashion, infection-treated IB3-1 cells. MicroRNA-215 has been shown to be induced by the tumor suppressor gene p53 (106). Consistently, we find ~2-fold increase in p53 expression in the *Pseudomonas aeruginosa*-infected IB3-1 CF cells compared to uninfected control cells and ~3-fold increase in p53 in CFBB cells compared to control NHBE cells (**Fig. 5B**).

To further investigate the mechanistic significance of the entire 88 member miR pattern, including miR-215, we performed a set of network analyses using the Ingenuity Pathway Analyses (IPA) algorithm. As shown in **Fig. 5C,** IPA analysis identifies a network of miRNAs that target messenger RNAs for pro-inflammatory signaling, including MAPKs, NFκB and IL-8. Since, aside from miR-215, all the infection-induced miRs are color-coded in green (meaning reduced), the objective trajectory of the predicted, composite signaling ensemble is to drive inflammation. The IPA algorithm also identifies other signaling networks, albeit at lower levels

of significance. In the present case, **Fig. 5D** shows that the ensemble of infection-mediated miRNAs form a significant network that also focuses the tumor suppressor gene p53 (TP53). The p53 network includes 4 miRNAs (in addition to the 75) that were largely suppressed by PA infection and were therefore detected only in the uninfected IB3-1 CF cells (high-lighted in grey, **Table 1**). Consistently, deletions and mutations in the TP53 gene have also been shown to promote dysregulated inflammatory signaling (104; 127). In addition, p53 expression has been shown to be affected by certain pathogens including human papilloma virus (104). Moreover, p53 has been shown to induce microRNAs, including miR-215 (106). Additionally, p53 responsive miRs including miR-215 have been shown to regulate the cell cycle (15; 34). Thus the miR-155-dependent proinflammatory signaling pathway characterizes infection-free CF lung epithelial cells. However, when CF lung epithelial cells encounter infection, the response appears to be supplemented by a different, but still proinflammatory, miR-215-based signaling pathway.

Discussion

In this paper we have tested the hypothesis that miR-155 might be a general mediator of enhanced IL-8 expression in CF lung epithelial cells, either following exposure to cytokine/chemokine mediators of inflammation, or in response to infectious agents. We have previously reported that miR-155 stabilizes IL-8 mRNA, thereby enhancing IL-8 protein expression. Consistently, abnormally high levels of miR-155 have been shown in cultured CF lung epithelial IB3-1 cells, primary epithelial explants from CF lung, and acutely isolated, peripheral blood neutrophils from CF plasma (12). The data clearly show here that in the infection-free state, IL-10 mediates reduction in IL-8 mRNA expression, as well as miR-155 levels. The data further show that reduction in IL-1β with a neutralizing antibody against IL-1β has the same concurrent effects on suppression of both IL-8 and miR-155. However, exposure to either intact *Pseudomonas aeruginosa* (PA) or lipopolysaccharide (LPS), does raise IL-8 mRNA expression, but does not raise levels of miR-155 above the already high levels. In fact, miR-155 is somewhat suppressed in PA-infected CF cells. Instead, the miR-155 response appears to be supplemented, or replaced, by a different, but still proinflammatory, miR-215-based signaling pathway, which targets both NFκB and p53 signaling pathways. The increased targeting of NFκB is not so surprising, since the Toll-Like Receptors, which respond to intact bacteria and to LPS, feed downstream into the NFκB pathway through IKK (11). However, the additional focus on p53 is unexpected, and suggests that the new miR-based signaling pathways drive the CF cell into a p53-centric survival disadvantage when encountering infection. Importantly, the clinical relevance of these new observations is evident from the fact that a significant elevation in miR-215 (*ca*. 1000-fold) is also observed in ex-vivo primary lung epithelial cells.

Elevated miR-155 signaling in infection-free CF lung epithelial cells

The possibility of an intrinsic proinflammatory defect in CF lung epithelial cells has been the cause of ongoing controversy in the CF field for many years (26; 79; 112; 135). However, these data provide added support for the concept that the absence of infection, elevated miR-155, as previously reported (12), plays a direct role in the intrinsically elevated state of IL-8 expression in CF lung epithelial cells. The miR-155 encoding gene, BIC, was originally discovered as an oncogene for B cell lymphomas in chicken (20), and is also found to be elevated in human B cell lymphomas (27). By contrast, in non-cancer states, substantial elevation in miR-155 expression can be induced in cells of the innate immune system by many types of proinflammatory stimuli (93). Among others, sensitive proinflammatory systems include TLR3, a receptor for poly(I:C); TLR4, a receptor for LPS; TLR9, a receptor for hypo-methylated CpG; TLR2, a receptor for lipoprotein; IFN-β; TNFα-autocrine/paracrine signaling after stimulation by IFN-γ; and by activation of the JNK and NFκB signaling pathways. However, in the case of infection-free cultures of CF lung epithelial cells, the intrinsic elevation of miR-155 would appear to suggest that the system is responding as if *bona fide, yet unknown,* proinflammatory stress signals were active. But what that intrinsic signal might be, dependent as it is on the expression of mutant CFTR, is evidently not a response to inadvertent inclusion in the culture of bacteria such as *Pseudomonas aeruginosa*, or bacterial products such as LPS. These agents activate yet another, or an additional, set of microRNAs, including miR-215.

Elevated microRNA-215 signaling in infected CF lung epithelial cells

miR-215 was originally discovered as a p53-inducible miRNA, which mediates the p53 effect of cell cycle arrest at both G_1 and G_2M (34). From this perspective, miR-215 has been viewed as a tumor suppressor gene in its own right, and responsible for the extensively characterized transcriptional repression functions of p53. However, CF lung epithelial cells are not cancer cells. Consistently, levels of miR-215 in these cells are low. Yet, unexpectedly, in the presence of either high alginate PA, for 24 hours, the levels of miR-215 in the cultured cells

become elevated by *ca*. 10-fold. Just as unexpectedly, we find that miR-215 levels in *ex-vivo* CF lung epithelial cells are elevated by *ca.*1000-fold over control cells. Prospectively, we had chosen the 24 hour exposure of the cultured CF lung epithelial cells to model the chronic infectious state of the CF airway. It is therefore possible that the common elevation of miR-215 in the *in vivo* and *in vitro* conditions of CF lung epithelial cells is consistent with expectations. Thus, on the basis of pure discovery science, miR-155 would appear to be a biomarker for the absence of infection in CF cells, while miR-215 appears to be a biomarker for the presence of chronic infection.

However, at the level of mechanism, we may inquire how chronic/long-term infection of CF lung epithelial cells with PA, and/or exposure to LPS, leads to elevation of miR-215, strongly enhanced IL-8 expression, and a highly significant association with p53 signaling among many of the novel miRNAs co-induced with miR-215. One possibility rests in the fact that oxidative stress is among the principal drivers, including radiation and genotoxic drugs, for p53 expression (50; 98; 143). Uninfected cultures of CF lung epithelial cells express a chronic phenotype of low level oxidative stress termed "ER stress". This condition is thought to be based on the unfolded protein response (UPR) associated with the retention of the folding mutant [ΔF508]CFTR in the ER, and elevated levels of calcium (*viz*, $[Ca_i^{++}]$) in the ER. Under these conditions, we have demonstrated here that miR-155 is elevated and miR-215 is low. However, as has been previously reported by Ribeiro and colleagues (112), high levels of calcium are found in the ER of infected primary cultures of CF lung epithelial cells. This is a classic marker for ER stress, as emphasized by the authors (112); and, in the case of these cultures, is associated, in part, with TLR-driven NFκB signaling and high levels of IL-8. We consider this condition to parallel the condition of our acutely isolated *ex-vivo* CF lung epithelial cells, where we find high levels of miR-215. However, as the infection of the primary culture is gradually "cured" with antibiotics, Ribeiro and colleagues (112) find that ER stress and $[Ca_i^{++}]$ decline. Furthermore, secreted IL-8 drops to a much lower, but still significantly elevated level. It is possible that this "reduced-infection" state approaches that of an "infection-free" culture of IB3-1, or other of the common cultured CF lung

epithelial cell lines, where miR-215 is low, and the intrinsically elevated level of miR-155 is now evident. We conclude that the advantage of having a collection of microRNAs to study is that each miR may be targeting stability or translation of hundreds of mRNAs, allowing one to reduce the complexity of the system. However, because we need to fully understand the system, we anticipate that the next steps will include deep gene expression profiling of mRNAs as a function of exposure time and bacterial load.

MicroRNAs, inflammation and cystic fibrosis

The importance of the present paper rests in our delineation of miR-155 as a biomarker for uninfected, and of miR-215 as a biomarker for infected human CF lung epithelial cells. Importantly, this is a property shared by both the model CF lung epithelial IB3-1 cells and ex-vivo CF lung epithelial cells. These molecular biomarkers, representing hundreds of affected mRNAs, also indicate that these human CF cells carry with them an intrinsic proinflammatory defect in the uninfected state. The high level of miR-155 suppresses SHIP1, allowing the PI3K derived inositol tris- phosphate product to drive Akt, stimulate MAPKs, and stabilize the IL-8 mRNA. To this state, we find that infection induces an entirely different process, driven by miR-215, which is p53 centric, and may compromise CF lung epithelial cells when confronted with *P. aeruginosa*, or bacterial cell products. These data may also contribute to resolving the ongoing controversy over whether CF is associated with an intrinsic proinflammatory defect, or with an infection- elicited defect. In the case of human CF cells, the answer, which may satisfy both sides of this controversy, is "both", if infection is also present.

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Author Disclosure Statement

No competing financial interests exist.

GRANTS

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Figures and Tables

Figure 1

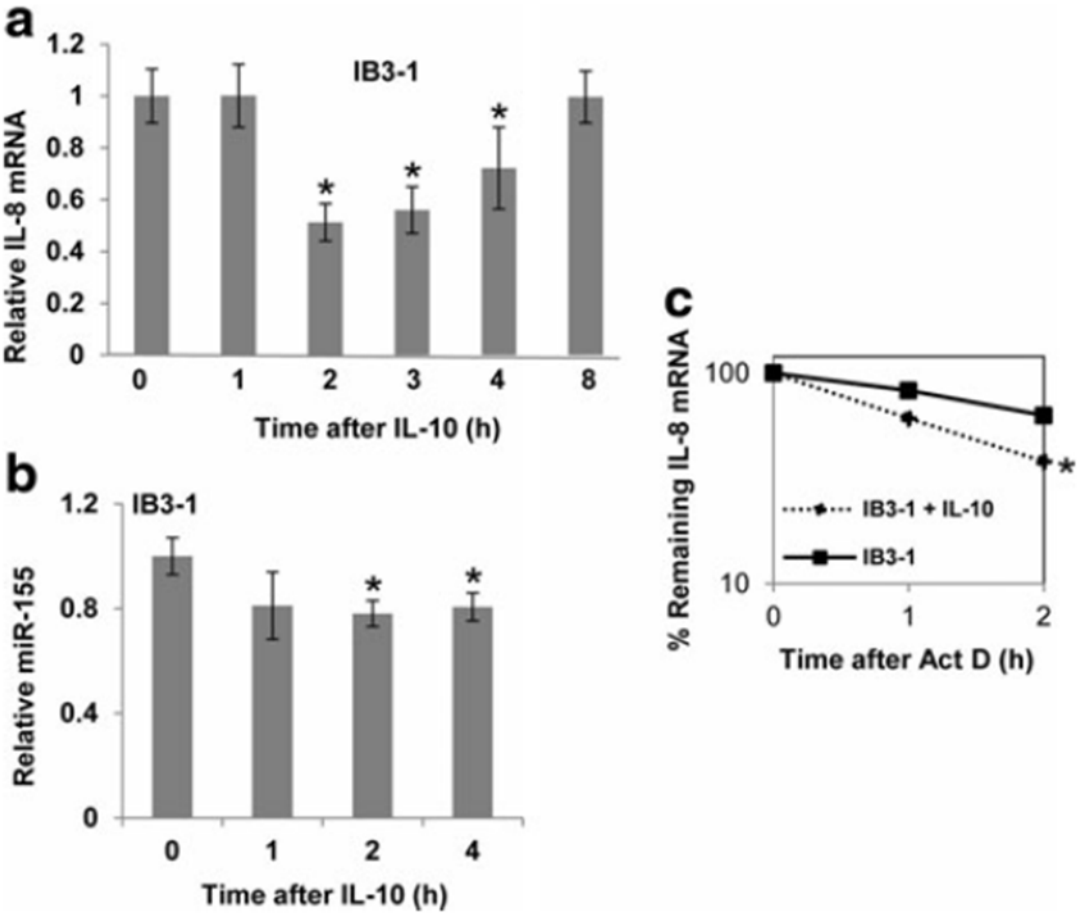


Figure 2

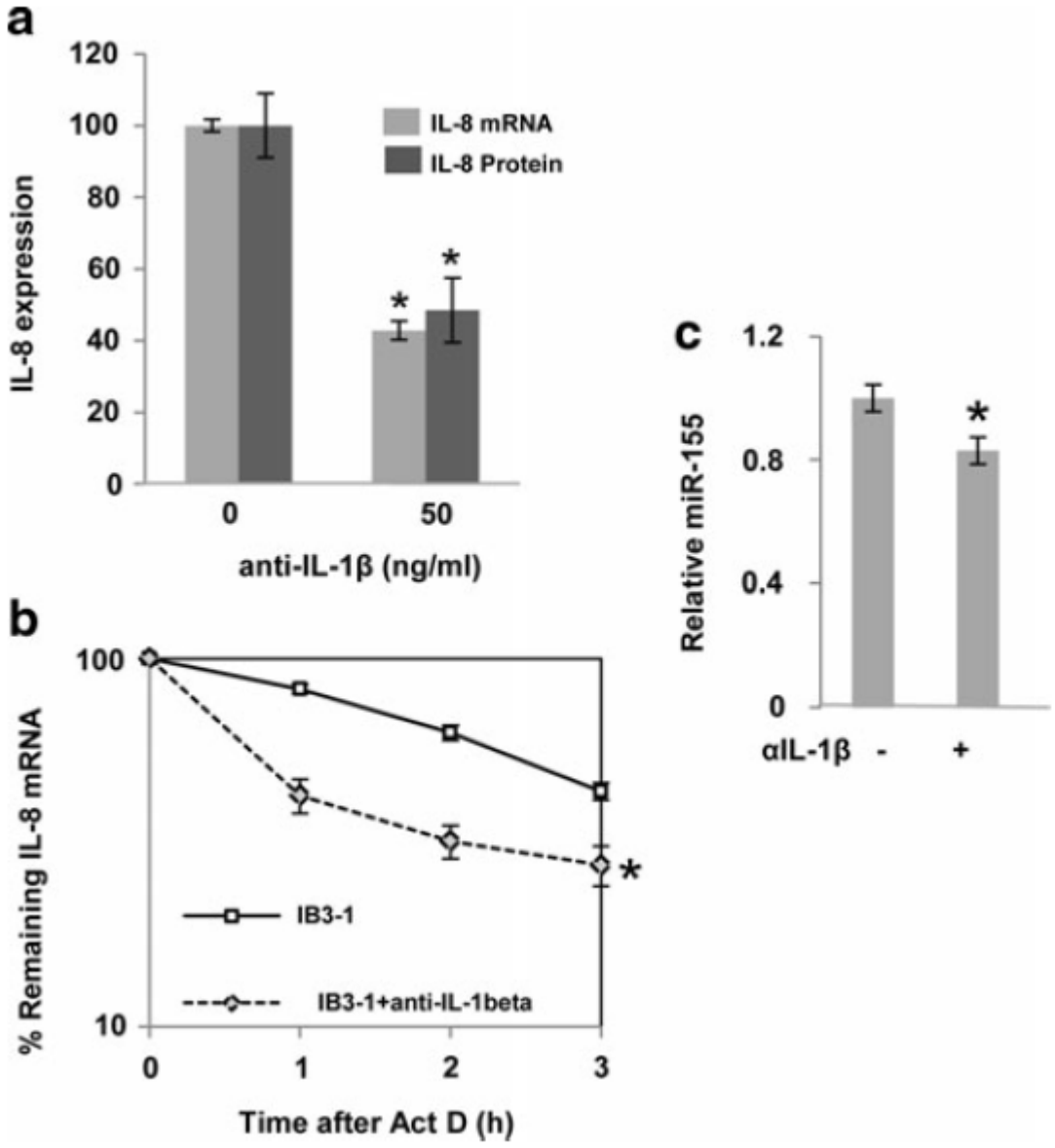
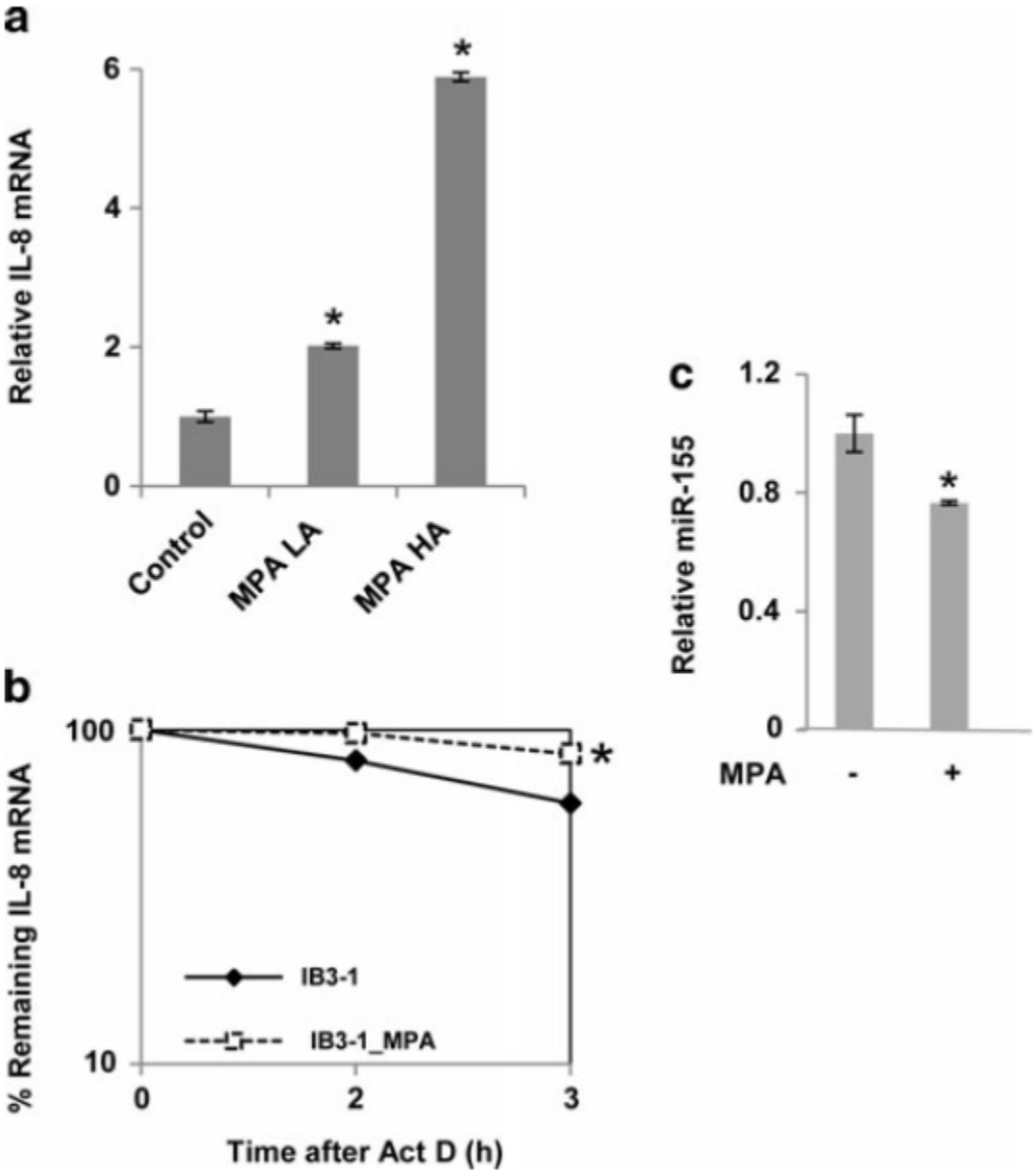

a
120
100
80
60
40
20
0
IL-8 expression
IL-8 mRNA
IL-8 Protein
*
*
0
50
anti-IL-1β (ng/ml)
b
100
10
% Remaining IL-8 mRNA
IB3-1
IB3-1+anti-IL-1beta
*
0
1
2
3
Time after Act D (h)
c
1.2
0.8
0.4
0
Relative miR-155
*
αIL-1β
-
+

Figure 3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Relative IL-8 mRNA
6
4
2
0
*
*
Control
MPA LA
MPA HA
b
% Remaining IL-8 mRNA
100
10
0
2
3
*
IB3-1
IB3-1_MPA
Time after Act D (h)
c
Relative miR-155
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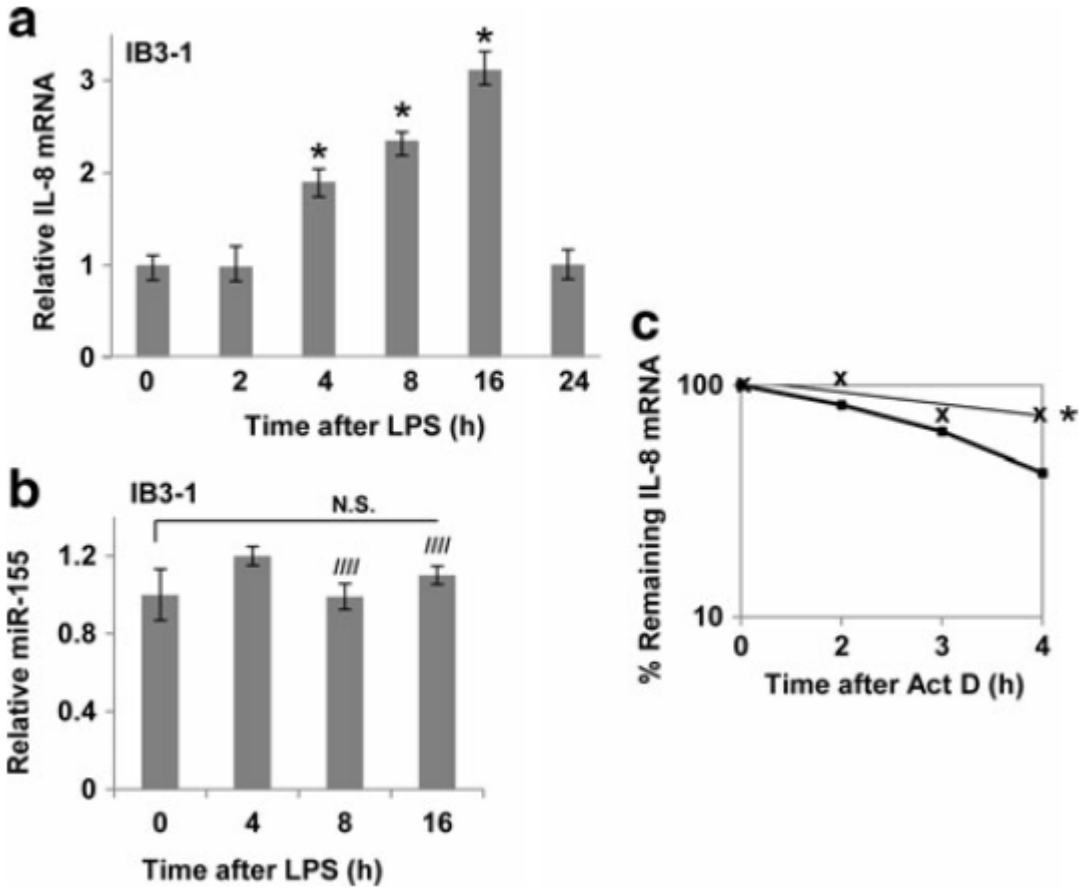
0.8
0.4
0
*
MPA
-
+

Figure 4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IB3-1
Relative IL-8 mRNA
0
1
2
3
0
2
4
8
16
24
Time after LPS (h)
b
IB3-1
N.S.
Relative miR-155
0
0.4
0.8
1.2
0
4
8
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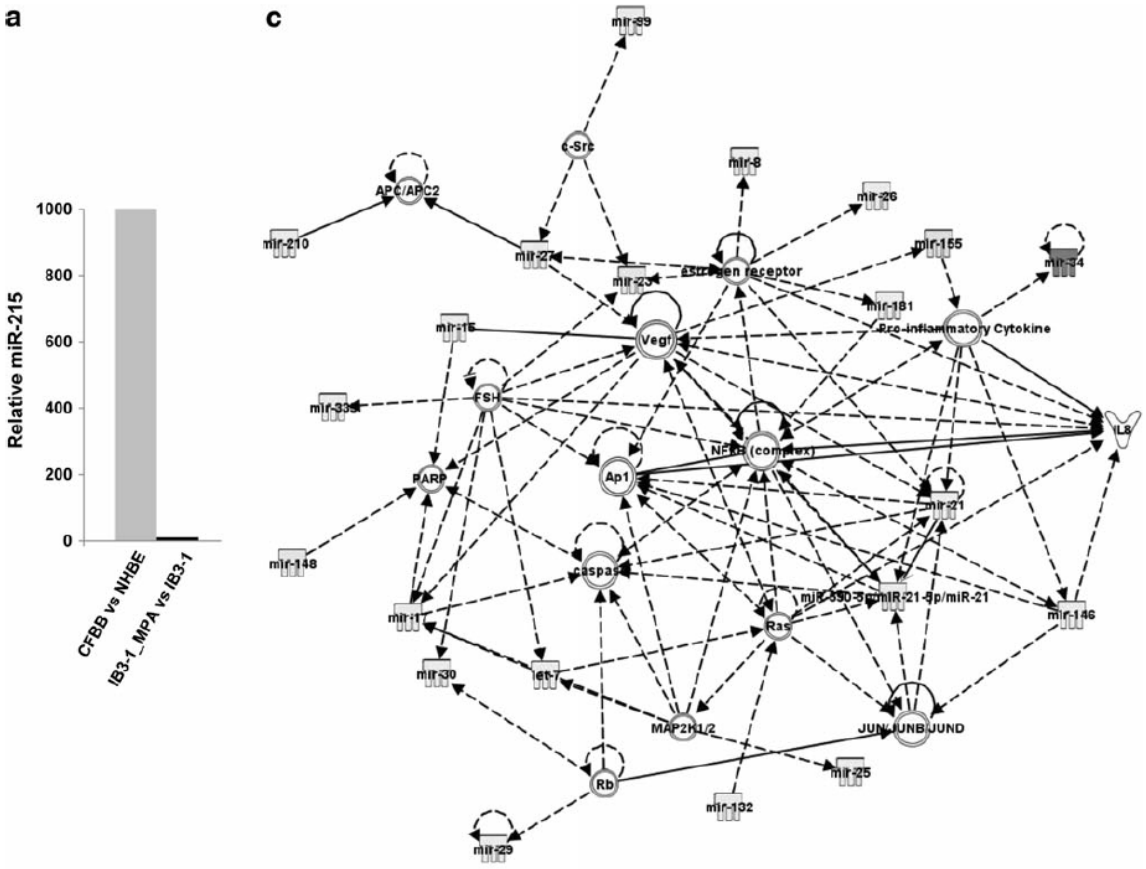
Time after LPS (h)
c
% Remaining IL-8 mRNA
100
10
0
2
3
4
Time after Act D (h)

Figure 5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Relative miR-215
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800
600
400
200
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CFBB vs NHBE
IB3-1_MPA vs IB3-1
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APC/APC2
mir-8
mir-26
mir-210
mir-27
mir-23
Estrogen receptor
mir-155
mir-34
mir-181
mir-15
Vegf
Pro-inflammatory Cytokine
FSH
mir-335
IL8
NFkB (complex)
PARP
Ap1
mir-21
mir-148
caspase
mir-590-3p/miR-21-5p/miR-21
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Figure 5, continued

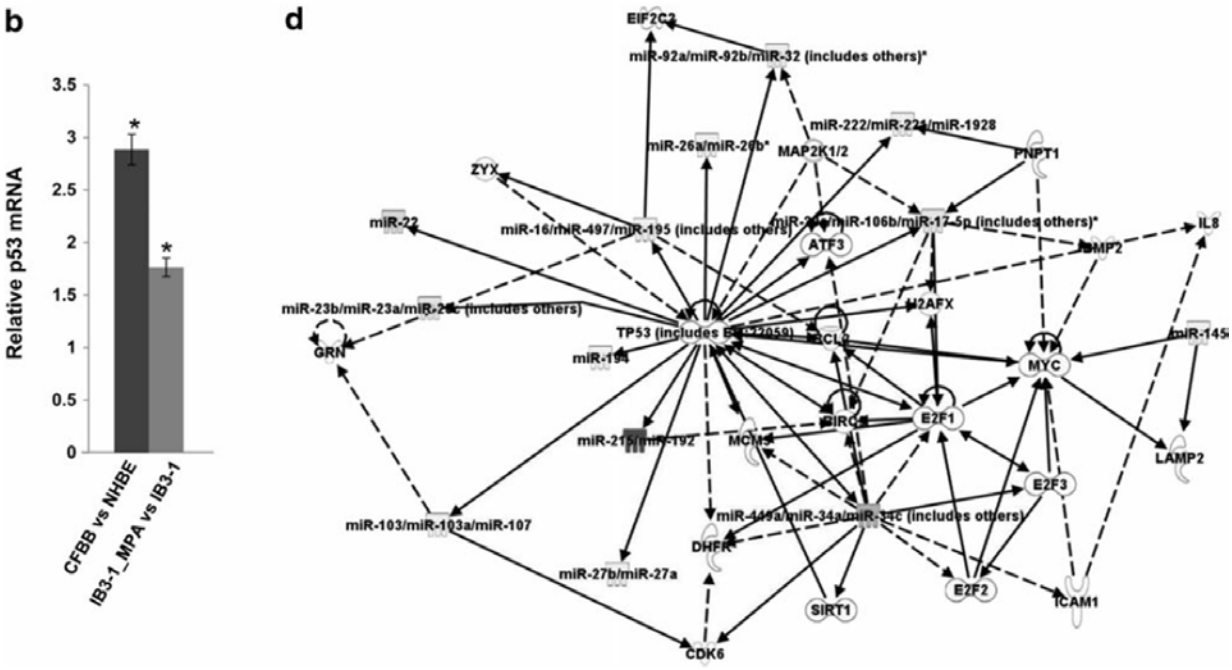


Table 1: Alteration in microRNA expression in *Pseudomonas aeruginosa*-infected IB3-1 CF cells compared to uninfected IB3-1 CF cells

Upregulated	*Fold change*↑		
hsa-miR-215	10.410		
Downregulated	*Fold change*↓	*Downregulated*	*Fold change*↓
hsa-miR-9	0.002393	hsa-miR-26a	0.037467
hsa-miR-27b	0.004225	hsa-miR-27a	0.040070
hsa-miR-494	0.004828	hsa-miR-30b	0.040088
hsa-miR-20b	0.005029	hsa-miR-365	0.045057
hsa-miR-671-3p	0.006352	hsa-miR-185	0.045307
hsa-miR-301b	0.007181	hsa-miR-30c	0.046848
hsa-miR-146a	0.008524	hsa-let-7e	0.048264
hsa-miR-28-5p	0.009504	hsa-miR-103	0.048460
hsa-let-7c	0.009630	hsa-miR-532-5p	0.048660
hsa-miR-92a	0.015382	hsa-miR-26b	0.049278
hsa-miR-500	0.015464	hsa-miR-100	0.053323
hsa-miR-98	0.016679	hsa-miR-340	0.056371
hsa-miR-194	0.016940	hsa-miR-19a	0.059764
hsa-miR-25	0.017312	hsa-miR-501-5p	0.059843
hsa-miR-32	0.017387	hsa-miR-324-3p	0.061743
hsa-miR-455-3p	0.017879	hsa-miR-598	0.062686
hsa-miR-15b	0.017940	hsa-miR-224	0.066118
hsa-miR-130a	0.018441	hsa-miR-140-5p	0.067280
hsa-let-7a	0.018751	hsa-miR-210	0.086570
hsa-miR-221	0.019009	hsa-miR-522	0.089965
hsa-miR-708	0.019436	hsa-miR-125b	0.091117
hsa-miR-652	0.019854	hsa-miR-93	0.091858
hsa-miR-19b	0.022858	hsa-miR-99a	0.093478
hsa-miR-301a	0.025830	hsa-miR-197	0.095958
hsa-miR-128	0.026975	hsa-miR-195	0.096128
hsa-miR-21	0.027510	hsa-miR-218	0.096480
hsa-miR-130b	0.029286	hsa-let-7g	0.108657
hsa-miR-20a	0.030067	hsa-miR-532-3p	0.112507
hsa-miR-423-5p	0.030313	hsa-miR-590-5p	0.125262
hsa-miR-744	0.030942	hsa-miR-212	0.133658
hsa-miR-181a	0.032339	hsa-miR-625	0.137320
hsa-miR-99b	0.032903	hsa-let-7b	0.138031
hsa-miR-18a	0.034687	hsa-miR-18b	0.151125
hsa-miR-328	0.036530	hsa-miR-17	0.155468
hsa-miR-886-3p	0.036622	hsa-miR-10a	0.158268
hsa-let-7f	0.036939	hsa-miR-31	0.183137
hsa-miR-29c	0.037180	hsa-miR-28-3p	0.189517
hsa-let-7d	0.037411	hsa-miR-34a	0.000495
		hsa-miR-22	0.006689
		hsa-miR-23a	0.016952
		hsa-miR-145	0.034436
		hsa-miR-335	0.008948
		hsa-miR-148	0.002627

Figure Legends

FIG. 1. Effect of IL-10 on IL-8 expression in CF cells.

(A) IB3-1 cells (1.5 x 106 cells) were treated with IL-10 (10 ng/ml) for the indicated period of time. RNA was subsequently isolated and analyzed by quantitative real time PCR. The data reflects averages of at least three independent experiments (* indicates $p<0.05$). (B)The expression of miR-155 was analyzed by Taqman qPCR assay(* indicates $p<0.05$). (C) RNA was isolated from IB3-1 cells pre-incubated with IL-10 (10 ng/ml) for 2h and then treated with actinomycin D (5μg/ml) for the indicated time intervals. The remaining mRNA was analyzed by quantitative real time PCR (* indicates $p<0.05$).

FIG. 2. Effect of inhibiting IL-1β on IL-8 mRNA stability and IL-8 protein in CF lung epithelial cells.

(A) IB3-1 (0.9 x 106 cells) cells were treated for 16h with a dose of 50 ng/ml of the neutralizing antibody against IL-1β. Both IL-8 protein (ELISA) and RNA (qPCR) were analyzed. The data reflects averages of at least three independent experiments (* indicates $p<0.05$). (B) RNA was isolated from IB3-1 cells pre-incubated with anti- IL-1β (50 ng/ml) for 16 h and then treated with actinomycin D (5μg/ml) for the indicated time intervals. The remaining mRNA was analyzed by quantitative real time PCR. The data reflect averages of at least three independent experiments (* indicates $p<0.05$).(C) The miR-155 expression in IB3-1 cells incubated anti-IL-1β (50 ng/ml) for 16 h was analyzed by Taqman assay (* indicates $p<0.05$).

FIG. 3. Mucoid *Pseudomonas aeruginosa* induces increased stability of IL-8 mRNA in CF cells.

(A) IB3-1 cells (1.5 x 106 cells) were treated with high (HA) or low (LA) algenate form of mucoid *P. aeruginosa* (MPA) for 24 h. RNA was isolated and analyzed by quantitative real time PCR. The data reflect averages of at least three independent experiments (* indicates $p<0.05$). (B)

RNA was isolated from IB3-1 pre-treated with MPA for 24h and then incubated with actinomycin D treatment for the indicated time intervals and the remaining mRNA was analyzed by quantitative real time PCR. The data reflect averages of at least three independent experiments (* indicates $p<0.05$). (C) The miR-155 expression in IB3-1 cells incubated with MPA for 24 h was analyzed by Taqman assay (* indicates $p<0.05$).

FIG. 4. LPS induces increased IL-8 expression in CF cells without affecting miR-155.

(A) IB3-1 cells were treated with *Pseudomonas* LPS (100 ng/ml/1.5 x 106 cells) for the indicated time intervals. RNA was isolated and was analyzed by quantitative real time PCR. The data reflect averages of at least three independent experiments (* indicates $p<0.05$). (B) The corresponding miR-155 expression was analyzed by Taqman assay (////// indicates $p>0.05$, N.S. not significant). (C) RNA was isolated from IB3-1 cells pre-incubated with LPS (100 ng/ml) for 2 h and then treated with actinomycin D (5µg/ml) for the indicated time intervals. The remaining mRNA was analyzed by quantitative real time PCR. The data reflect averages of at least three independent experiments (* indicates $p<0.05$).

FIG. 5. miRNA expression profile in PA-infected CF cells.

(A) Expression of miR-215 was analyzed in PA-infected IB3-1 compared to IB3-1 (~10 fold elevated) as well as in CF brush biopsies (CFBB) compared to normal human bronchial epithelial cells (NHBE) (~1000 fold elevated). (B) The expression of p53 was analyzed by sybr Green-based qRT-PCR in PA-infected CF cell lines and in CF brush biopsies compared to respective controls, uninfected CF cells and NHBE cells (* indicates $p<0.001$). IPA analyses indicate two major network foci (C) IL-8 (D) TP53.

TABLE 1. Alteration in miRNA expression in Pseudomonas aeruginosa-infected IB3-1 CF cells compared to uninfected IB3-1 CF cells.

miR-215 is up-regulated (↑) while all other miRNAs are suppressed (↓) in MPA-infected CF cells compared to uninfected IB3-1CF cells. Those miRNAs which were not detected in the infected CF cells (IB3-1_MPA), but only in uninfected IB3-1 CF cells, are shaded in grey.

Chapter 3: Specific aim 2

Introduction

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations of a chloride ion channel called cystic fibrosis transmembrane conductance regulator (CFTR) (114). Among several organs affected, CF lung disease is the major life-threatening factor for CF patients. Because of deficient export of chloride ion into lumen, the airway mucosa is viscous, which enables bacteria to cause intermittent respiratory infections, most frequently by *Pseudomonas aeruginosa* (82; 118). Furthermore, even without infections, CF lung has been shown to be inherently inflammatory, and CF patients eventually develop pulmonary fibrosis and airway remodeling and bronchiectasis (139). It is well known that CF lung epithelial cells produce pro-inflammatory cytokines and chemokines, such as IL-8, and that inflammatory signaling pathways are activated (24).

mRNAs of cytokines have adenosine-uridine rich region (AU-rich region, ARE) in their 3' UTR. ARE has been shown to be a regulatory sequence for stability and translation of its own mRNA (124). So far several proteins have been identified as ARE-binding proteins which regulate stability of mRNAs. For example, HuR has been shown to bind to the ARE regions in 3' UTR of several mRNAs of cytokines and proliferative molecules, and also stabilize the target mRNAs. Early examples of HuR targets include mRNAs of VEGF (73), cyclin A and B1 (145), and TNF-α (32). In malignant glioma cell line, HuR was shown to bind to 3' UTR of IL-8 mRNA (92). It was further confirmed that HuR bound to ARE region of IL-8 mRNA by UV cross-linking of salivary protein lysate with IL-8 ARE, which led to stabilization of IL-8 mRNA (102). Additionally, microRNAs have been identified and found to regulate various cellular processes by destabilizing target mRNAs. Recently comprehensive expression profiling data are obtained from microRNA and mRNA microarray analysis in several diseases, and indicate microRNAs to be important regulators of disease development and have therefore emerged as therapeutic targets.

In cystic fibrosis, one study showed that, when CF cells were treated with *Pseudomonas aeruginosa*, miR-93 expression decreased (28). miR-93 was further shown to bind to the 3' UTR of IL-8 mRNA and suppress IL-8 expression. Thus, *Pseudomonas* infection induced increased expression of IL-8 because of suppression of miR-93 (28). Previously our lab also showed that miR-155 was upregulated in CF patients and CF cells. miR-155 directly binds to SHIP1 mRNA, which is a phosphatase to inhibit PI3K/Akt signaling pathway (12). In CF, increased miR-155 therefore stimulated activated PI3K/Akt pathway, which eventually led to increased IL-8 production (12).

This study is targeted towards the identification of proteins and microRNAs which bind to the ARE region of IL-8 mRNA. We isolated IL8-mRNP complex including ARE region of IL-8 mRNA, and analyzed bound protein components by mass spectrometric analysis and miRNA by Taqman gene expression assays. We also demonstrated how these molecules affected IL-8 mRNA stability and translation, and proposed a potential mechanism of regulation of IL-8 mRNA in CF.

Materials and Methods

Reagents

LHC-8 media, Trypsin-EDTA (0.05%) and Lipofectamine transfection Reagent were purchased from Life Technologies. miRVana kit and RiboPure kit for RNA isolation, of total RNA from CF cells was obtained from Ambion Inc. (Austin, TX). Taqman qPCR reagents were purchased from Applied Biosystems.

Cell culture, RNA isolations and RNA transfection

IB3-1 CF lung epithelial cells and the control CFTR-repaired IB3-1/S9 cells were maintained in LHC-8 serum free medium (Life Technologies) in humidified 5% CO_2. The IB3-1-TTP cells were similarly maintained in LHC-8 medium containing puromycin (0.5µg/ml). Transfections with siRNA were done using siPORT NeoFX Transfection Reagent (Ambion).

RT-PCR and Western Blot

Total RNA was isolated from the IB3-1 and IB3-1/S9 cells using miRVana isolation kit (Ambion). Multiplex Reverse Transcription was performed with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). RNA was isolated from the cells and mRNA expression level were analyzed with qRT-PCR as described earlier (5). Following reverse transcription, each RT reaction was diluted and mixed with TaqMan Gene expression Master Mix (2X).

Statistical Analysis for qRT-PCR and Western blot

Ct values obtained from qRT-PCR for mRNA and miRNA (test genes) expression were analyzed by ΔΔCt method. The following calculation is based on the instruction described in Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR, which is supplied by Applied Biosystems. Each mRNA and miRNA from one sample was measured triplicated, and experiments were repeated at least three times under the same

conditions. ΔCt values were obtained by subtracting Ct values of control genes (β-actin for mRNA, RNU48 for miRNA) from those of test genes. ΔΔCt values were obtained by subtracting ΔCt of control condition from that of test condition, and finally, average and standard deviation of ΔΔCt were calculated (AvgΔΔCt and StdevΔΔCt). Relative expression is expressed by $2^{-\text{AvgΔΔCt}}$. The upper limit of error bars is the value of $2^{-\text{AvgΔΔCt+StdevΔΔCt}}$, and the lower limit of error bars is the values of $2^{-\text{AvgΔΔCt-StdevΔΔCt}}$. Therefore, positive and negative differences of error bars are not exactly equal value. Statistical difference of AvgΔΔCt between control and test conditions was evaluated by two-tailed *t*-test using StdevΔΔCt values, and considered as significant when p-value is less than 0.05.

Intensity of bands from Western blot films was quantified by ImageJ software. Intensity of bands of test proteins was divided by that of control protein (GAPDH for total cell lysate and cytoplasmic lysate, Lamin A/C for nuclear lysate). This divided value from control condition was normalized as 1, and relative intensity of test conditions were shown. Standard error of mean (SEM) was calculated from standard deviation of the normalized divided value. Since the divided value from control condition is always 1, SEM was not calculated for control condition. Significance of statistical difference was not evaluated because bands intensity is visibly different. Experiments were repeated at least three times, and representative blots are shown.

All experiments were repeated at least three times, unless otherwise indicated.

Plasmids and plasmid transfection

The pcDNA3.1 with Firefly luciferase ORF and S1 aptamer tag was given by Shobha Vasudevan (141). The plasmid was cut with XhoI and NotI, at the MCS located between the Firefly luciferase ORF and S1 aptamer tag, where a 60-nt of IL-8 3' UTR which includes four ATTTA sequences was amplified and inserted.

4 x 10^6 cells were plated on a 100 mm dish. Next day, 1.5 μg plasmid was incubated in 200 μl OPTIMEM (Life Technologies) with 12 μl Lipofectamine transfection Reagent (Life

Technologies), and incubated for 20 minutes, during which the cells were washed and incubated with 5 ml OPTIMEM. After 20 minutes, OPTIMEM was removed from the dish and transfection reagent mixture was added with 6 ml OPTIMEM, and incubated for 4 hours. After 4 hours, 9 ml of LHC-8 media was added and further incubated for 20 hours.

RNP cross linking and purification of mRNP

Cells transfected with the S1-tagged constructs and grown were washed with ice-cold PBS twice and incubated with 0.3% formaldehyde in PBS for 10 min at room temperature. Cells were lysed (150 mM KCl, 10 mM Hepes 7.4, 3 mM MgCl2, 2 mM DTT, 10% glycerol, and 0.5% Nonidet P-40), incubated for 10 min on ice, sonicated and centrifuged at 2000 g for 5 min.

The supernatant was filtered, and RNA-binding proteins were purified by FPLC with the Q column (GE Healthcare) (NaCl 150 – 1000 mM). The fraction with enriched RNA content was further incubated with streptavidin beads (Invitrogen) for 3 hours at 4 °C, and washed five times with wash buffer. The beads were kept in sample buffer for Western blot.

Results

Isolation of ARE-binding proteins

In order to identify proteins which bind to ARE regions of IL-8 mRNA, we first constructed a plasmid following the description by Vasudevan et al (141). The vector plasmid (control plasmid) is pcDNA3.1 with firefly luciferase and following S1 aptamer sequences which was given by Shobha Vasudevan. We made a 60 nt-long sequence from 3' UTR of IL-8 mRNA containing four ARE regions, and inserted it between luciferase and S1 aptamer (WT plasmid). We also made another sequence with mutations in ARE region, and inserted into control plasmid (mutant plasmid) (Figure 1A).

These plasmids were transfected into CF and control cell lines, and proteins which bound to the insert were identified after in-vivo crosslinking and subsequent affinity purification. In the purified complex with the insert, FXR1 binds to IL-8 ARE region more in control cells, while HuR binds more in CF cells (Figure 1B, C)).

HuR causes increased expression of IL-8 in CF lung epithelial cells

We next investigate the effects of these ARE-binding proteins. HuR has been studied well so far and was shown to regulate stability of ARE-containing mRNAs under various conditions such as infection, inflammation, and stress. Cystic fibrosis causes chronic inflammation and infection and shown to secrete high IL-8, therefore, we hypothesized that HuR might play roles in IL-8 mRNA regulation in CF cells. After silencing HuR expression by siRNA transfection (Figure 2A), total expression of IL-8 mRNA decreased (Figure 2B) and stability of IL-8 mRNA also decreased (Figure 2C). We further examined whether HuR also has any effects on translation of IL-8 mRNA, but polysomal fractionation analysis did not show significant differences in IL-8 mRNA content in ribosomes with or without HuR silencing. Altogether, these results suggest that HuR stabilizes IL-8 mRNA, but does not have any effects on IL-8 translation, in cystic fibrosis lung epithelial cells.

HuR expression is higher in the cytoplasmic fractions in CF epithelial cells

We next examined the difference in HuR expression between CF and control cells. HuR was shown to be a shuttling protein along the nucleus and the cytoplasm, and dominantly expresses in the nucleus. To make effects on target mRNAs, HuR has to be in the cytoplasm. In cystic fibrosis, more HuR was detected in the cytoplasm compared with control cells, although overall HuR expression in whole cell lysate was equal to each other (Figure 2D).

miR-16 binds to ARE region of IL-8 mRNA and regulates its translation

In addition to mRNA-binding proteins, microRNAs also bind to 3'UTR of their target mRNAs, and regulate the stability and translation. Therefore, we also measured one microRNA, miR-16, which was shown to bind to ARE region of TNF-α mRNA. As shown in Figure 3A, miR-16 was detected in IP samples from both CF and control cells. miR-16 binds more in CF cells to IL-8 mRNA compared to control cells. However, total miR-16 expression is almost equal between CF and control cells (Figure 3B), so, in order to emphasize the effect of miR-16 in CF cells, we overexpressed miR-16, and it was shown that miR-16 destabilizes IL-8 mRNA and also suppresses translation of IL-8 mRNA (Figure 3C, D).

Discussion

In the specific aim 2, we showed the effects of an RNA-binding protein and miRNA on IL-8 mRNA stability. More HuR was shown to bind to the ARE region of IL-8 mRNA in CF, and stabilized it. It was also shown that, although overall HuR expression was almost equal between CF and control cell line, more HuR was expressed in the cytoplasm in CF cell line. On the other hand, miR-16 also bound to the same region of IL-8 mRNA as HuR, and destabilized it. Additionally, miR-16 decreased translation of IL-8 mRNA.

As described in the introduction, HuR is an RNA-binding protein which binds to ARE region of some cytokine mRNAs, including IL-8 mRNA, and in most cases stabilizes them. Our results about HuR binding and subsequent effect of IL-8 increase are compatible with preceding reports. More abundant HuR expression in the cytoplasm of CF cell line also supports our speculation that HuR functions to stabilize IL-8 mRNA in CF.

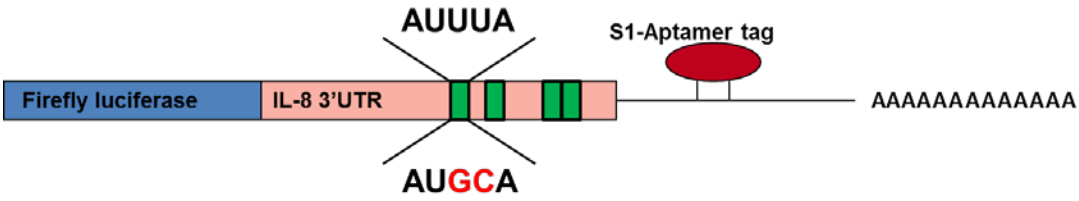
Findings about miR-16 are not simple for interpretation. First of all, miR-16 expression is not different between CF and control cell line, but our result showed more miR-16 binding to IL-8 ARE in CF. This experiments introduced exogenous plasmid with IL-8 ARE and this plasmid is estimated to be transcribed equally in CF and control cells, therefore, we speculate that, in CF, there is some driving force for miR-16 to particularly bind to IL-8 ARE. Next there are some reports which showed several RNA-binding proteins are associated when microRNAs bind to their targets. For example, tristetraprolin (TTP) was shown to be required when miR-16 to bind to its target region of TNF-α ARE (48). HuR was also shown to help let-7 to suppress translation of its direct target c-Myc mRNA (55). In this report authors proposed that HuR binds to facilitate let-7 binding to its target, so that let-7 gained higher affinity to its target site with higher concentration of HuR (55). Therefore, it is possible that miR-16 might be brought by HuR to IL-8 mRNA in CF. However, the effects of HuR on IL-8 mRNA is stabilization, so it is contradictory that HuR helps miR-16 to suppress translation of IL-8 mRNA. Also, there are several reports which showed HuR competed with miRNA for common binding sites, for example with miR-494

on nucleolin mRNA (136), and even with miR-16 on COX2 mRNA (158). Thus, more HuR binding with more miR-16 binding on IL-8 ARE is difficult to explain. Probably one possibility is that HuR might have other binding sites than the part of sequence we used for an insert. Our insert includes only four classical ARE regions but there are nine ARE regions in the whole 3'UTR of IL-8 mRNA. Even though miR-16 and HuR cooperate on the sequence we used, there might be additional opposite stabilizing effect on the other sites. The effect of miR-16 overexpression might have caused dominant occupation of IL-8 mRNA binding site over HuR, or less HuR expression by miR-16 directly targeting HuR mRNA (153), either of which can lead to less IL-8 translation.

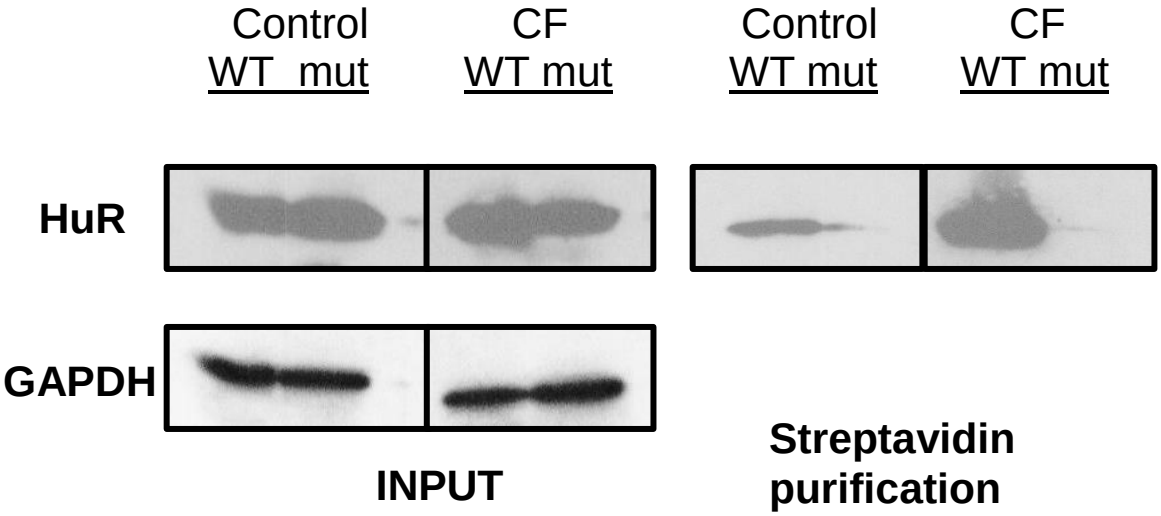
Figures

Figure 1

A



B



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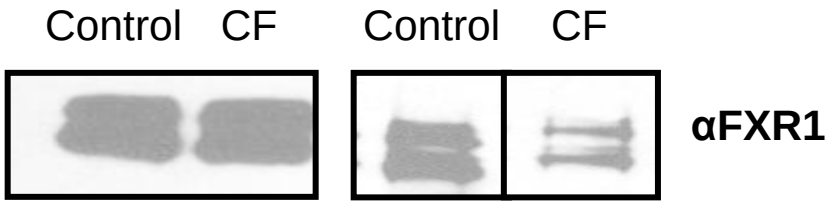
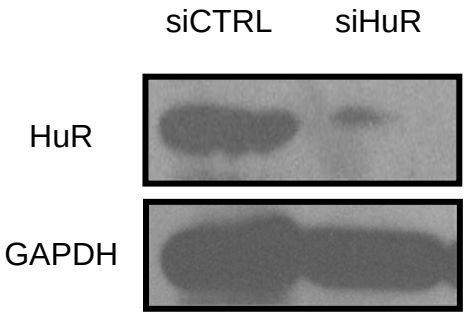
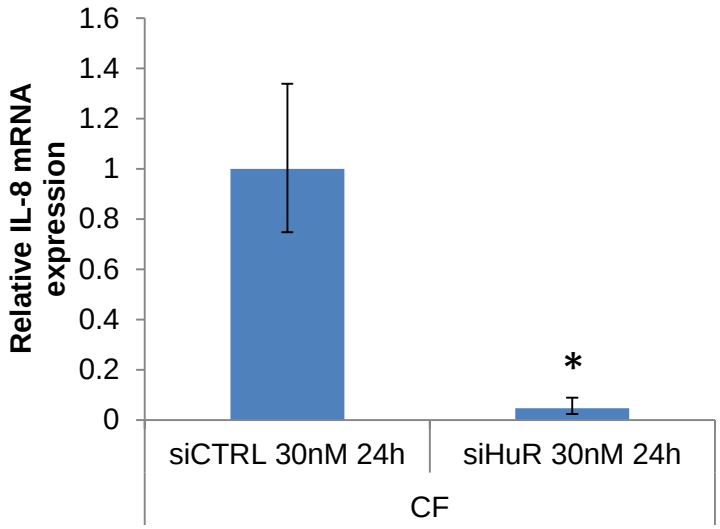


Figure 2

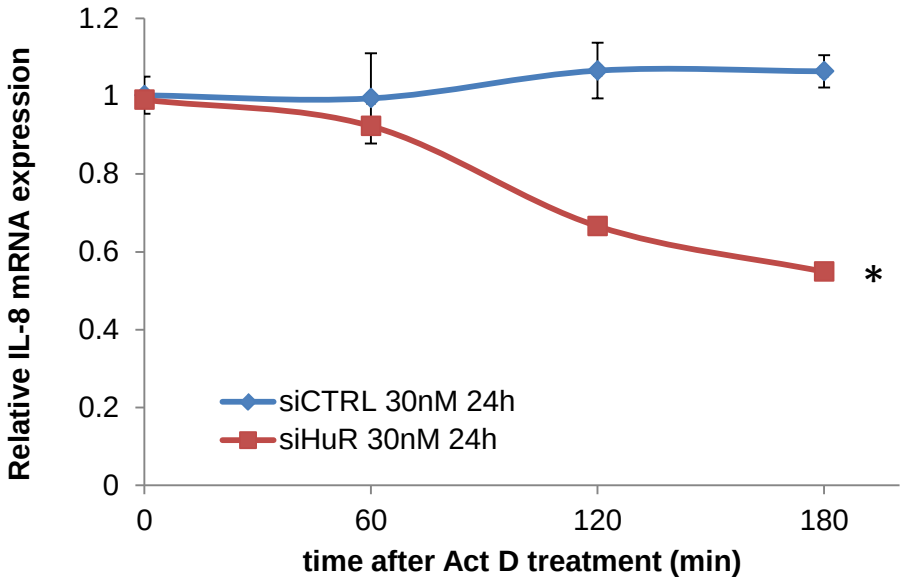
A


siCTRL
siHuR
HuR
GAPDH

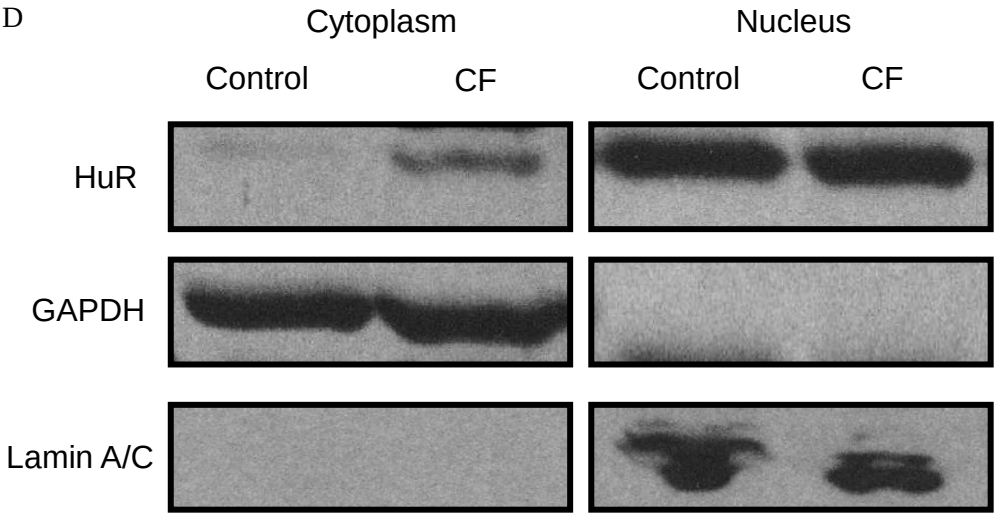
B


Relative IL-8 mRNA expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
siCTRL 30nM 24h
siHuR 30nM 24h
CF

C


Relative IL-8 mRNA expression
1.2
1
0.8
0.6
0.4
0.2
0
*
siCTRL 30nM 24h
siHuR 30nM 24h
0
60
120
180
time after Act D treatment (min)

D


Cytoplasm
Nucleus
Control
CF
Control
CF
HuR
GAPDH
Lamin A/C

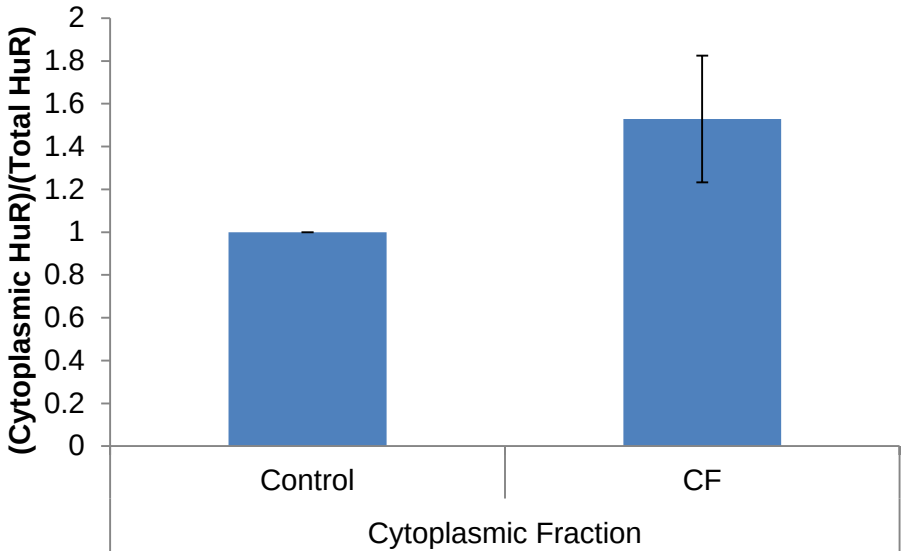
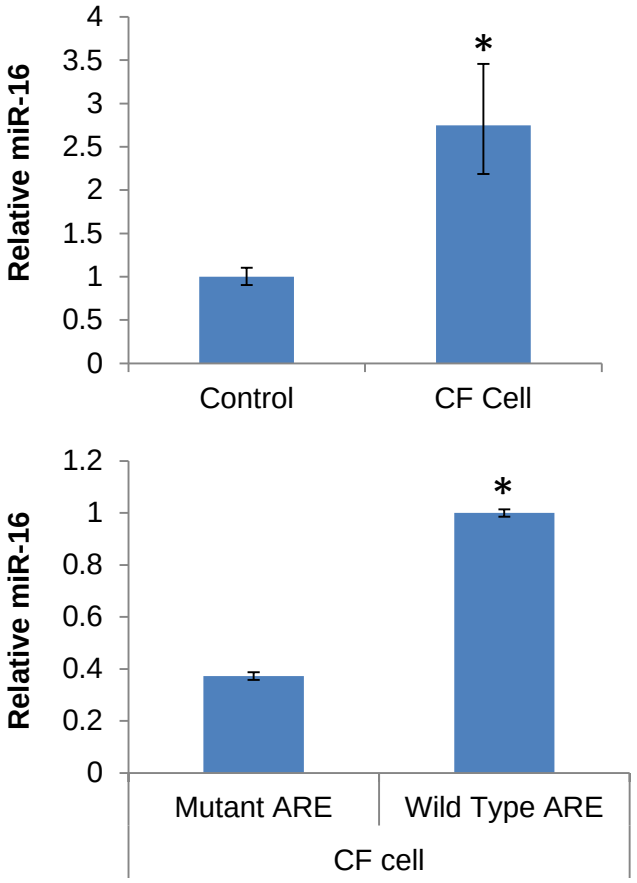
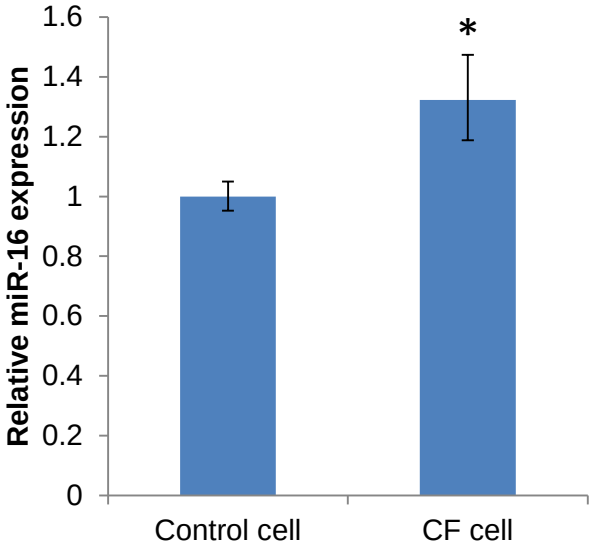

(Cytoplasmic HuR)/(Total HuR)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
CF
Cytoplasmic Fraction

Figure 3

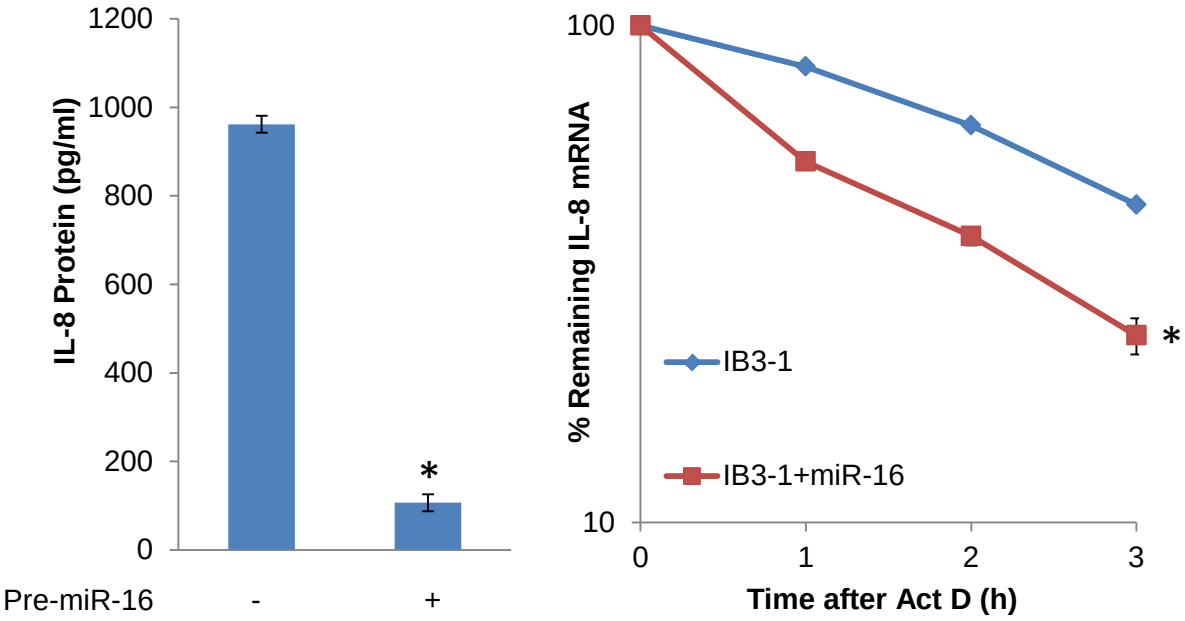
A


Relative miR-16
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Control
CF Cell
Relative miR-16
1.2
1
0.8
0.6
0.4
0.2
0
*
Mutant ARE
Wild Type ARE
CF cell

B


Relative miR-16 expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Control cell
CF cell

C (adapted from Kumar *et al*. (66))



D

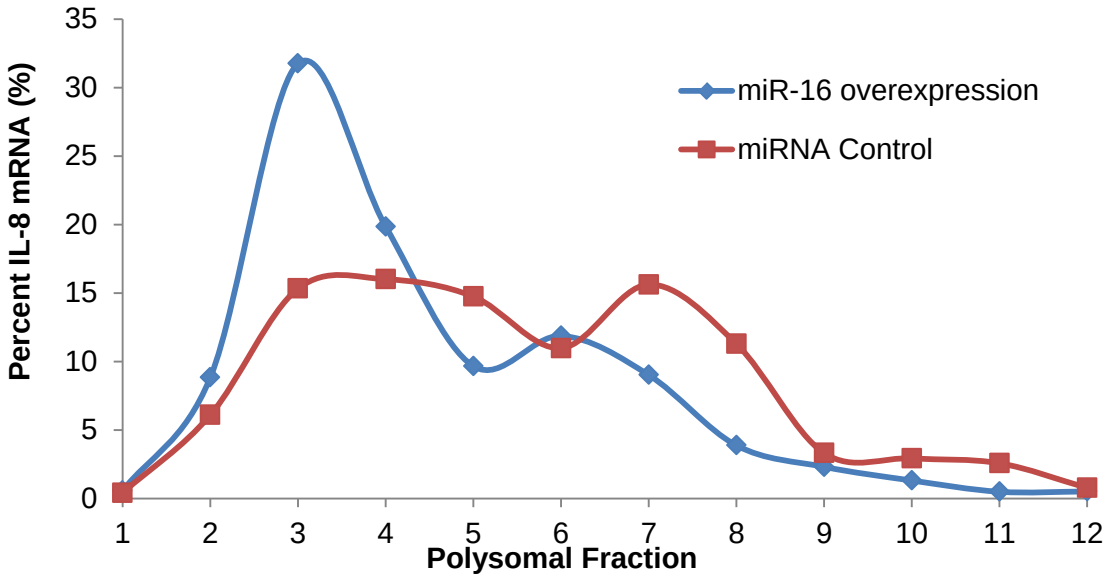


Figure Legend

Figure 1

HuR binding to the ARE regions of IL-8 mRNA. (A) The structure of the plasmid used, which contains wild type (WT) or mutant (mut) sequences of ARE. (B) HuR expression in total cell lysate (input) of IB3-1/S9 (control) and IB3-1 cells (CF) (left), and HuR detected in the streptavidin-purified samples (right). (C) FXR1 enrichment in the streptavidin-purified samples from IB3-1/S9 (control) and IB3-1 (CF) cells with wild type plasmid.

Figure 2

The effects of HuR on IL-8 mRNA expression. (A) HuR expression was knocked down with siRNA against HuR mRNA (siHuR) or control (siCTRL) (30 nM, 24 hours) in IB3-1 cell. (B) Relative IL-8 mRNA expression with siHuR or siCTRL in IB3-1 cell. *$p<0.05$. (C) IL-8 mRNA expression after indicated time points of actinomycin D (Act D) treatment in IB3-1 cell, with pretreatment with siHuR or siCTRL. Relative expression to mRNA level at $t = 0$. *$p<0.05$. (D) HuR expression in cytoplasmic and nuclear fractions from IB3-1/S9 and IB3-1 cells (top). Relative band intensity of cytoplasmic HuR expression to total HuR was quantified by ImageJ software, and normalized to that of IB3-1/S9 cell (bottom).

Figure 3

microRNA-16 binding to ARE regions of IL-8 mRNA. (A) Relative miR-16 enrichment in final purified samples from IB3-1/S9 (control) and IB3-1 (CF) cells. *$p<0.05$ (top). Relative miR-16 enrichment in final purified samples with wild type or mutant plasmid in IB3-1 (CF) cell. *$p<0.05$ (bottom). (B) Relative miR-16 expression in total cell lysates from IB3-1/S9 and IB3-1 cells. *$p<0.05$. (C) Relative IL-8 mRNA expression with siHuR or siCTRL in IB3-1 cell. *$p<0.05$. (C) Secreted IL-8 protein was measured using ELISA, after treatment with or without premiR-16 (25 nM, 48 hours). *$p<0.05$ (left). IL-8 mRNA expression after indicated time points of

actinomycin D (Act D) treatment in IB3-1 cell, with or without pretreatment with premiR-16 (25 nM, 48 hours). Relative expression to mRNA level at t = 0. *$p<0.05$. (right) The figure 3C was adapted from Kumar *et al* (66). (D) Polysomal fraction of IL-8 mRNA in IB3-1 cell with pre-miR-16 treatment (miR-16 overexpression) or premiR Negative control (miRNA CTRL) (25 nM, 72 hours). This experiment was performed only once.

Chapter 4: Specific aim 3

RPTOR, a novel target of miR-155, regulates CTGF in Cystic Fibrosis lung epithelial cells

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Short title: miR-155 targets RPTOR in CF

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Abstract

Cystic Fibrosis (CF) is caused by mutations in the CFTR gene, which prevents correct folding, trafficking and function of the mutant CFTR protein. The dysfunctional effect of CFTR mutations, principally the *F508del-CFTR* mutant, is accompanied by hypersecretion of the pro-inflammatory chemokines into the airway lumen, which contributes to inflammation and subsequent fibrosis causing airway destruction, leading to morbidity and mortality. We have previously reported that miR-155 is up-regulated in cystic fibrosis lung epithelial cells and induced hyper-expression of the pro-inflammatory interleukin-8 gene through activation of PI3K/Akt signaling. miR-155 has been implicated as a central regulator of the immune system. However, the molecular mechanism by which miR-155 regulates the manifestation of CF not fully clear. Here, we identified regulatory associated protein of mTOR, complex 1 (RPTOR) as a novel target gene of miR-155 in CF lung epithelial cells. We report that elevated levels of miR-155 resulted in decreased RPTOR expression in CF lung epithelial cells. This promoted increased expression of fibrosis inducing connective tissue growth factor (CTGF). Thus, we have demonstrated that miR-155 can regulate fibrosis of CF lungs by targeting RPTOR. We interpret these findings to suggest that these cellular pathways may constitute novel targets for CF therapy.

Key words: Cystic fibrosis, *F508del*-CFTR, microRNA, miR-155, RPTOR

Introduction

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations of a chloride ion channel called cystic fibrosis transmembrane conductance regulator (CFTR) (114). Among several organs affected, CF lung disease is the major life-threatening factor for CF patients. Because of deficient export of chloride ion into lumen, the airway mucosa is viscous, which enables bacteria to cause intermittent respiratory infections, most frequently by *Pseudomonas aeruginosa* (82; 118). Furthermore, even without infections, CF lung has been shown to be inherently inflammatory, and CF patients eventually develop pulmonary fibrosis and airway remodeling and bronchiectasis (139). CF lungs are characterized by increased expression of pro-inflammatory cytokines and chemokines (24).

MicroRNAs have been identified and found to regulate various cellular processes by destabilizing target mRNAs. Recent comprehensive transcriptomic analyses in several diseases indicate that microRNAs are key molecules in disease development therapy effectiveness. In cystic fibrosis, miRNA expression profiling analyses have been reported by several studies (6; 12; 96). Especially, miR-101, miR-145, and miR-494 were shown to bind to the 3' UTR of CFTR mRNA and destabilize it (40; 85). However, little is known about regulation of inflammation and fibrosis by miRNAs. So far, miR-126 was found to decrease in CF, and was it was further validated that TOM1 mRNA was a direct target of miR-126 (96). TOM1 is involved in intracellular trafficking and an inhibition of inflammatory signaling, and in a CF cell line, increased TOM1 expression led to decreased responses to LPS and IL-1 stimulation (96). Another study showed that, when a CF cell line was treated with *Pseudomonas aeruginosa*, miR-93 expression decreased (28). This miR-93 was further shown to bind to the 3' UTR of IL-8 mRNA, and IL-8 increased after *Pseudomonas* infection because of decreased miR-93 (28). Previously we have shown that miR-155 is upregulated in CF lung epithelial cells, both cell line and primary cells isolated from lung explants of CF patients. We demonstrated that the elevated expression.

miR-155 induced increased IL-8 expression by suppressing SHIP1 and subsequent activation of PI3K/Akt signaling pathway (12).

In this study we aimed to elucidate further involvement of miR-155 in CF disease processes. Here we have identified regulatory associated protein of mTOR, complex 1 (RPTOR) as a novel target gene of miR-155 in CF lung epithelial cells. We report that elevated levels of miR-155 resulted in decreased RPTOR expression in CF lung epithelial cells. This promoted increased expression of fibrosis inducing connective tissue growth factor (CTGF). We also identified COUP transcriptional factor 2 (NR2F2) as an indirect target of miR-155. Thus, we have demonstrated that miR-155 can regulate the disease development of CF lungs. We interpret these findings to suggest that these cellular pathways may constitute novel targets for CF therapy.

Materials and Methods

Biotinylated microRNA-155 transfection

IB3-1 CF lung epithelial cells and the control CFTR-repaired IB3-1/S9 cells were maintained in LHC-8 serum-free medium (Invitrogen, 12678-017) in humidified 5% CO_2. 3.0 x 10^6 IB3-1/S9 cells were plated on one 100 mm dish and incubated in LHC-8 media for 24 hours. 24 hours later, 10 nM of 3'-biotinylated microRNA-155 (Dharmacon) or control 5'-biotinylated cel-miR-67 (gift from Ashish Lal, National Cancer Institute) were transfected into cells with siPORT-NeoFX (Invitrogen, AM4511), and incubated for 24 hours. Cells were washed twice with PBS and lysed with 700 µl lysis buffer (20 mM Tris pH 7.5, 5 mM $MgCl_2$, 100mM KCl, 0.3% NP40), including 50 U RNaseOUT (Invitrogen, 10777-019) and proteinase inhibitor (Sigma-Aldrich, S8820). Cells were scraped and incubated on ice for 5 minutes, and centrifuged under 10000g at 4 °C. Supernatant was collected.

Streptavidin purification

Streptavidin-coated magnetic beads (Invitrogen, 11205D) were incubated for 2 hours at 4 °C with blocking buffer (20 mM Tris pH 7.5, 5 mM $MgCl_2$, 100mM KCl, 0.3% NP40, 1 mg/ml yeast tRNA, 1 mg/ml BSA). After blocking, cell lysate was added to magnetic beads, and incubated for 4 hours at 4 °C. After 4 hours, beads were washed five times with lysis buffer. RNA was isolated from the beads by the method described previously (140).

MicroRNA-mimic transfection

2.5 x 10^5 IB3-1/S9 cells were plated on a 6-well plate, and incubated in LHC-8 media for 24 hours. 24 hours later, 10 nM of microRNA-155 mimic or control-mimic (gift from Dr. Ashish Lal) was transfected into cells with siPORT-NeoFX and incubated for 48 hours. RNA was isolated from cells by the method described previously (140).

mRNA expression profiling

mRNA expression profiling for biotinylated microRNA transfection and microRNA-155 mimic transfection were done with HumanHT-12 v4 Expression BeadChip Kit (Illumina, BD-103-0204). The raw signals were normalized by Z-transformation and differences of Z-scores of test samples and controls samples were calculated for all mRNA signals. mRNAs which were detected more in biotinylated miR-155 treatment and less after miR-155 mimic transfection were selected for further investigation. The heat map was made by Microsoft Excel based on the Z-scores from the analyses, ranging red (low) to green (high). The places where data are not available, or false discovery rate (FDR) is more than 0.3 are marked in gray.

mRNA, miRNA and protein expression

Total RNA was isolated from the IB3-1 and IB3-1/S9 cells using the miRVana isolation kit (Life Technologies, AM1560). Real-time quantifications of individual mRNA and miRNAs were performed with specific TaqMan gene expression or microRNA assay (Life Technologies). Real-time PCR data were analyzed. The data were normalized to the endogenous control β-actin (for mRNA expression) or RNU48 (for miRNA expression) and filtered for Ct values >35. To determine the protein expression, 2.5 x 10^5 cells were plated in a six well plate. After 24 hours, (70-80% confluent) IB3-1/S9 cells were transfected with 50 nM of pre-miRs (Ambion) or pre-miR negative control #1 (Ambion) with siPORT-NeoFX and incubated for 48 hours. Western blotting was done and membranes were probed with primary antibody for RPTOR (Millipore, 09217, 1:1000), NR2F2 (Abcam, ab41859, 1:1000) and corresponding secondary antibody (1:5000). GAPDH was used as a loading control. Quantification of protein expression was performed with ImageJ software.

Statistical Analysis for qRT-PCR and Western blot

Ct values obtained from qRT-PCR for mRNA and miRNA (test genes) expression were analyzed by ΔΔCt method. The following calculation is based on the instruction described in Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR, which is supplied by Applied Biosystems. Each mRNA and miRNA from one sample was measured triplicated, and experiments were repeated at least three times under the same conditions. ΔCt values were obtained by subtracting Ct values of control genes (β-actin for mRNA, RNU48 for miRNA) from those of test genes. ΔΔCt values were obtained by subtracting ΔCt of control condition from that of test condition, and finally, average and standard deviation of ΔΔCt were calculated (AvgΔΔCt and StdevΔΔCt). Relative expression is expressed by $2^{-\text{AvgΔΔCt}}$. The upper limit of error bars is the value of $2^{-\text{AvgΔΔCt+StdevΔΔCt}}$, and the lower limit of error bars is the values of $2^{-\text{AvgΔΔCt-StdevΔΔCt}}$. Therefore, positive and negative differences of error bars are not exactly equal value. Statistical difference of AvgΔΔCt between control and test conditions was evaluated by two-tailed *t*-test using StdevΔΔCt values, and considered as significant when p-value is less than 0.05.

Intensity of bands from Western blot films was quantified by ImageJ software. Intensity of bands of test proteins was divided by that of control protein (GAPDH for total cell lysate and cytoplasmic lysate, Lamin A/C for nuclear lysate). This divided value from control condition was normalized as 1, and relative intensity of test conditions were shown. Standard error of mean (SEM) was calculated from standard deviation of the normalized divided value. Since the divided value from control condition is always 1, SEM was not calculated for control condition. Significance of statistical difference was not evaluated because bands intensity is visibly different. Experiments were repeated at least three times, and representative blots are shown.

Plasmids for Luciferase assay

Plasmid for luciferase assay was obtained from pMIR-REPORT™ miRNA Expression Reporter Vector System (ThermoFisher Scientific, AM5795).

Around 60 to 80 nt-long parts of predicted sites of WT and mutant sequences of 3' UTR of RPTOR and NR2F2 were inserted between *Spe*I and *Hind*III restriction sites of pMIR-Reporter vector. Exact insert sequences are shown in the Table 1. The vector without any inserts was used as positive control as well (designated as Luciferase only).

Luciferase assay

Luciferase assay was performed with pMIR-REPORT™ miRNA Expression Reporter Vector System (ThermoFisher Scientific, AM5795). IB3-1/S9 cells (2.5×10^5) were plated in 6 well plates 24 hours prior to transfection. The cells were transfected with 250 ng/ml of pMIR-Reporter, pMIR-Reporter-WT 3'-UTR, or pMIR-Reporter-mutant 3'-UTR. To control for transfection efficiency, pMIR-Reporter-β-galactosidase control vector was co-transfected. 50 nM of pre-miR-155 or pre-miR negative control was also transfected simultaneously. Transfection was performed using siPORT-NeoFX (Invitrogen). After incubating for 24 hours cells were lysed with Tropix lysis solution and luciferase activity in the cell lysates were assayed using Dual-Light Luciferase and β-Galactosidase Reporter Gene Assay System (Life Technologies, T1003). Luminescence was measured with BioTek™ Synergy™ H1 Hybrid Multi-Mode Monochromator Fluorescence Microplate Readers (BioTek Instruments, Inc). The relative luciferase activity was normalized.

Treatment with rapamycin

IB3-1/S9 cells (2.5×10^5) were treated with 50 nM rapamycin (LC Laboratories, R-5000,) or equal volume of DMSO and incubated for 24 hours. Cell lysate was collected with RIPA buffer containing protease inhibitor (Sigma-Aldrich) and phosphatase inhibitor cocktail

(Sigma-Aldrich). The effect of rapamycin was confirmed by measuring total and phosphorylated mTOR protein expression by western blot as described previously (mTOR (7C10) Rabbit mAb #2983, phospho-mTOR (Ser2448) (D9C2) XP® Rabbit mAb #5536, Cell Signaling, 1:1000). CTGF protein expression was measured by western blot (CTGF, Santa Cruz biotechnology, sc-14939, 1:2000). CTGF mRNA expressions was measured by real-time quantitative PCR described above, by using TaqMan gene expression assay (#Hs01026927_g1, #Hs00998133_m1, Life Technologies).

All-*trans* retinoic acid (ATRA) (R2625, Sigma) was gifted from Dr. Mikiei Tanaka (Uniformed Services University of the Health Sciences). 2.5 x 10^5 IB3-1 cells were plated on a six-well plate, and incubated for 24 hours. After 24 hours, 10 nM or 100 nM ATRA or equal volume of DMSO (0.05 vol%) was added and further incubated for 48 hours.

Results

Identifying novel candidates direct targets of miR-155 in CF

We previously reported that miR-155 was upregulated in bronchial brushings of CF patients as well as CF bronchial epithelial cell line (12). Therefore in the current study, we sought novel direct targets of miR-155 which are involved in disease development of cystic fibrosis. To identify miR-155 targets, we conducted mRNA expression profiling analysis under three different conditions. Under the first condition, miR-155 expression was suppressed in CF cells with antagomiR-155, and compared with CF cells without miR-155 suppression. The second condition employed synthetic miR-155 with its 3' end biotinylated. This biotinylated miR-155 was transfected into control cell line (10 nM) for 24 hours, and cell lysate was purified with streptavidin so that only mRNAs bound to biotinylated miR-155 were enriched. This sample was compared with that of biotinylated cel-miR-67 treatment as a control. Under the third condition, control cells were treated with miR-155 mimic (10 nM) for 48 hours, and mRNA expression was compared with that with control mimic treatment.

We expected target mRNAs to be up-regulated in the first two categories, and down-regulated in the third category. Figure 1 depicts several genes obtained from mRNA expression profiling using ILLUMINA bead arrays (higher detection in green, lower detection in red). We analyzed mRNAs which satisfied at least two of the criteria above, and which are also predicted targets of miR-155 (TargetScan algorithm). Among these genes, based on any known involvement in inflammatory processes, we initially selected CSNK1G2, FBLN1, NR2F2, RPTOR and SLC44A2, and individually measured mRNA expression between CF and control cells (data not shown, except RPTOR and NR2F2 in figures 2A and 4A). RPTOR mRNA decreased in CF cells (Figure 2A), but NR2F2 mRNA did not decrease (Figure 4A). Although NR2F2 mRNA did not show significant decrease in CF cells, we still selected NR2F2, because there are two predicted miR-15-binding sites in NR2F2 3’ UTR, and mRNAs with more than two

predicted sites are more probable to be an actual direct target (86). Thus we finally selected RPTOR and NR2F2 for further analyses (shown in red letters).

Validation of RPTOR as a direct target of miR-155

To validate RPTOR as direct miR-155 target, we first measured mRNA and protein expressions of RPTOR in CF cells and CFTR-repaired control cells. As depicted in Figure 2A, RPTOR mRNA is significantly reduced in CF cells compared to control cells. We next determined whether RPTOR protein expression was also affected. As shown in Figure 2B, overexpression of miR-155 in IB3-1/S9 control cells decreased RPTOR protein expression.

We next performed luciferase assays to determine whether RPTOR is a direct target of miR-155. As shown in Figure 2C, luciferase activity with wild type RPTOR 3'-UTR sequence decreased after miR-155 overexpression in control cells, while it increased after miR-155 inhibition in CF cells. This indeed validates RPTOR as a direct target of miR-155

Roles of RPTOR and NR2F2 in CF pathogenesis

We next investigated whether RPTOR and NR2F2 have any effects on disease development of cystic fibrosis. First, to examine the roles of RPTOR, we used rapamycin, which was known to bind to FKBP12 and thus prevents RPTOR from binding to mTOR to form mTORC1 (100). The effect of rapamycin was confirmed by reduced phosphorylation of mTOR protein (Figure 3A). Since mTORC1 pathway was shown to suppress cytokine production after LPS treatment (121; 146), we first treated control cells with rapamycin for 48 hours, and then treated with LPS for up to 16 hours. Although we expected to detect increased expressions of cytokines such as IL-1β, IL-6, and IL-8 and decreased IL-10, we did not observe these changes (data not shown). We next tried to measure molecules involved in fibrogenesis. Connective tissue growth factor (CTGF) is induced by physical or chemical or biological external stimuli (such as mechanical stretch, hypoxia or inflammation), and activates fibroblasts for more collagen

synthesis which leads to fibrosis (52; 65). CTGF expression is high in several respiratory diseases such as asthma and emphysema (149; 162), but it has not been examined in CF. Also it was shown that CTGF expression was stimulated by rapamycin through TGF-β signaling pathway (101). Therefore we first compared CTGF expression in CF cells to control cells, and higher expression was observed in CF cells (Figure 3B). Also, CTGF expression increased after miR-155 overexpression in control cells (Figure 3C). Therefore, to determine whether higher CTGF expression is because of the effect of RPTOR, we treated control cells with rapamycin (50 nM for 48 hours) and measured these molecules, and found that CTGF expression increased (Figure 3D).

Next, we examined the role of NR2F2 in CF disease development. NR2F2 is an orphan nuclear receptor whose unique ligand has not been identified. However, it was shown that NR2F2 binds to retinoic acid receptors and regulates their functions as a transcriptional factor. Although its functional character is not exactly established and still controversial, there is one report which showed suppressive effects on cytokine production in the uterus (75). Therefore, with expectation to modulate the downstream effects of NR2F2, we treated CF cells with all-*trans* retinoic acid (ATRA), and measured the expression of inflammatory cytokines. As shown in Figure 4D, ATRA treatment reduced the IL-8 mRNA expression in CF.

Discussion

In the specific aim 3, we targeted towards determining other inflammatory target genes of microRNA-155 in CF, other than IL-8 (12). For this aim, we sought novel direct targets of miR-155, and identified RPTOR mRNA as a target of miR-155 in CF. Although NR2F2 is not a direct target, its expression was also suppressed by miR-155 overexpression. We further examined the effects of RPTOR and NR2F2 on pathogenesis of CF.

Our methods to identify microRNA targets are following an orthodox procedure described in a review (133). Also, we further employed a relatively new method developed by Lal *et al*, which enables us to identify mRNAs which physically bind to a specific miRNA (68). Although conventional methods have compared mRNA expression with or without miRNA overexpression, it remains elusive whether these mRNAs are truly direct targets of the miRNA, and also these methods cannot detect direct targets where the translation is directly regulated by the miRNA. Indeed, although not dominant, 6-26% of miRNA targets are estimated to be translationally repressed (49). Another conventional way is to immunoprecipitate protein-miRNA-mRNA complex by using antibody against Ago2, but this method cannot distinguish between miRNAs from which the mRNAs in the final samples come. Lal's method overcomes these shortcomings by using biotin-labelled microRNA and the microRNA-mRNA complexes are isolated by streptavidin (68). Thus the final analyses of mRNA expression profile can, theoretically, yield only and all candidate direct targets of this miRNA (68). In addition to the method above, we performed several conventional mRNA expression profiling with miR-155 suppression in CF cells and miR-155 overexpression in control cells. Based on these data and predicted targets of miR-155 (TargetScan) (74), we identified RPTOR mRNA and NR2F2 mRNA as potential direct targets of miR-155.

RPTOR mRNA was shown to be a direct target by miR-155, and also our results show altered RPTOR expression is partly attributing to airway remodeling in CF. This finding is not compatible with a past report which examined the effects of miR-155 on mTORC1 and mTORC2

pathways (144). In this study, miR-155 suppressed Rheb, mTOR and RICTOR expressions and confirmed that miR-155 directly targeted Rheb and RICTOR mRNAs by luciferase assay, but RPTOR expression did not change after miR-155 overexpression (144). There are no reports about RPTOR expression in cystic fibrosis, but mTORC1 pathway was shown to be upregulated in airway neutrophils compared with peripheral neutrophils in CF patients (80). mTORC1 is also involved in innate immunity response, but, although rapamycin has been used for anti-inflammatory therapy, this is not simply pro-inflammatory or anti-inflammatory. One study treated human monocytes with rapamycin, and cells were subsequently stimulated by LPS. After LPS stimulation, cells with rapamycin treatment increased cytokine production such as TNF-α, IL-6, IL-12p40, while IL-10 production decreased (146). In another study, rapamycin treatment decreased LPS-induced production of TNF-α, but increased IL-1β (121). We did not find change in these cytokine expressions after rapamycin and LPS treatment in CF cells. Instead, we found that CTGF and TGF-β1 increased after rapamycin treatment, and subsequently confirmed upregulated expression in CF cells and in control cells with miR-155 overexpression. Expression of CTGF has not been described in CF so far, but it was shown in other lung inflammatory diseases such as COPD and asthma, that CTGF was upregulated in these diseases, and contributed to increased fibrogenesis and remodeling of the airway tract (149; 162). However, there are contradictory reports about the effect of rapamycin. In a human mesangial cell line, mTORC1 pathway induced type 1 collagen synthesis and rapamycin treatment decreased type 1 collagen synthesis, while TGF-β activates mTORC1 and increased collagen synthesis (119). But on the other hand, it was also shown that rapamycin increased CTGF expression via TGF-β signaling pathway in lung epithelial cells and lung fibroblasts (101; 155). Our results are compatible with these latter findings, and suggest that CTGF may play a role in fibrotic change of CF airway tract possibly because of miR-155 overexpression leading to RPTOR suppression. To validate this hypothesis, we still need to confirm that TGF-β-SMAD pathway is the one which induced CTGF in cystic fibrosis. In order to examine the activation of this pathway, it is

necessary to show the phosphorylation of SMAD2 and SMAD3. TGF-β also stimulates other signaling pathways. For example, it was shown that rapamycin treatment increased CTGF through TGF-β, but independent of SMAD (155). In this report, PI3K/Akt pathway is the one which induced CTGF after TGF-β (155). Also, CF cells will be treated with inhibitors of the signaling pathway to validate the involvement of each pathway. Another gap to be elucidated is which downstream pathways of mTORC1 signaling are responsible for TGF-β or CTGF induction. It is also intriguing to examine CTGF expression in mouse or human CF lung tissue. Immunohistochemistry with anti-CTGF antibody is likely to show higher expression in CF compared to control tissues.

On the other hand, western blot showed decreased expression or NR2F2 in CF cells and in control cells after miR-155 overexpression. NR2F2 had been known as an orphan nuclear receptor, but it was shown that 9-cis retinoic acid and all-trans retinoic acid bound and activated NR2F2 function (64). NR2F2 function was studied in development, and also in hormone-sensitive cancers, such as prostate cancer, ovarian cancer, breast cancer, and endometrial cancer, but it has not been reported in cystic fibrosis or in other inflammatory diseases (154). There is one study suggesting association between NR2F2 and cytokine production. When human endometrial stromal cells were isolated from healthy biopsy samples, and incubated with or without siRNA against NR2F2 mRNA, mRNAs of IL-6, IL-8, TNF, CCL2 as well as other inflammatory molecules increased (75). ELISA further confirmed protein production increase in IL-6 and IL-8 (75). This suggests inhibitory effects of NR2F2 on cytokine production, and our result showing decrease of NR2F2 in CF is compatible with this finding. It would also be interesting to understand how miR-155 regulates NR2F2 expression. Since NR2F2 mRNA has long 3' UTR with two predicted miR-155 binding sites far apart, we could not examine the effects of both sites together. Although NR2F2 needs to be further examined to be a direct target of miR-155, protein expression decreased after miR-155 overexpression, which suggests that there might be an indirect association. As there are no reports so far which suggested connection between NR2F2

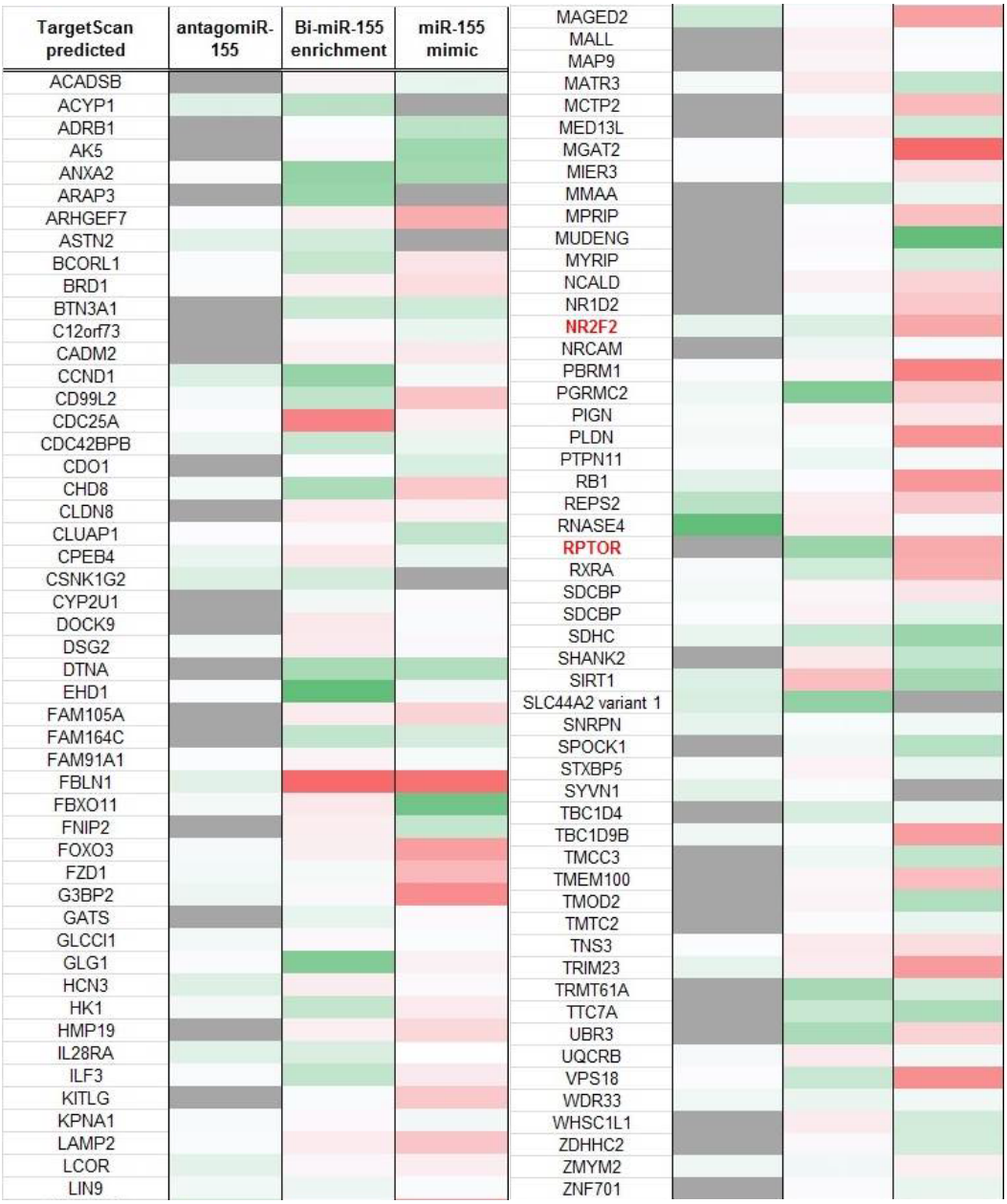
and miR-155, this warrants further study. There is a possibility that miR-155 regulation of NR2F2 is through Ets1 transcription factor. Ets1 was shown to be a direct target of miR-155 (116), and Ets1 facilitates transcription of NR2F2 mRNA (105). Thus when miR-155 is overexpressed, Ets1 decreases, and accordingly NR2F2 might decrease. NR2F2 mRNA was shown to be a direct target of miR-194 (47) and miR-302 (117). In addition, our study also showed possible relevance between NR2F2 function and inflammation in CF. Although further study is necessary to clarify the effects of NR2F2 on retinoid receptors (RARs and RXRs) in CF, the fact that all-*trans* retinoic acid treatment decreased inflammatory cytokine production may lead ATRA as potential therapeutic agents against CF.

Figures and Tables

Table 1

Insert	Sequence
RPTOR WT	5’-actagtccACCTCACTTTATTTCCATGTAATCAGAGCATTAGCTGC caagctt-3’
RPTOR mutant	5’- actagtccACCTCACTTTATTTCCATGTAATCAGACGTAAAGCTGC caagctt -3’

Figure 1


TargetScan predicted
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Bi-miR-155 enrichment
miR-155 mimic
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ACYP1
ADRB1
AK5
ANXA2
ARAP3
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ASTN2
BCORL1
BRD1
BTN3A1
C12orf73
CADM2
CCND1
CD99L2
CDC25A
CDC42BPB
CDO1
CHD8
CLDN8
CLUAP1
CPEB4
CSNK1G2
CYP2U1
DOCK9
DSG2
DTNA
EHD1
FAM105A
FAM164C
FAM91A1
FBLN1
FBXO11
FNIP2
FOXO3
FZD1
G3BP2
GATS
GLCCI1
GLG1
HCN3
HK1
HMP19
IL28RA
ILF3
KITLG
KPNA1
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MAP9
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MIER3
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NR1D2
NR2F2
NRCAM
PBRM1
PGRMC2
PIGN
PLDN
PTPN11
RB1
REPS2
RNASE4
RPTOR
RXRA
SDCBP
SDCBP
SDHC
SHANK2
SIRT1
SLC44A2 variant 1
SNRPN
SPOCK1
STXBP5
SYVN1
TBC1D4
TBC1D9B
TMCC3
TMEM100
TMOD2
TMTC2
TNS3
TRIM23
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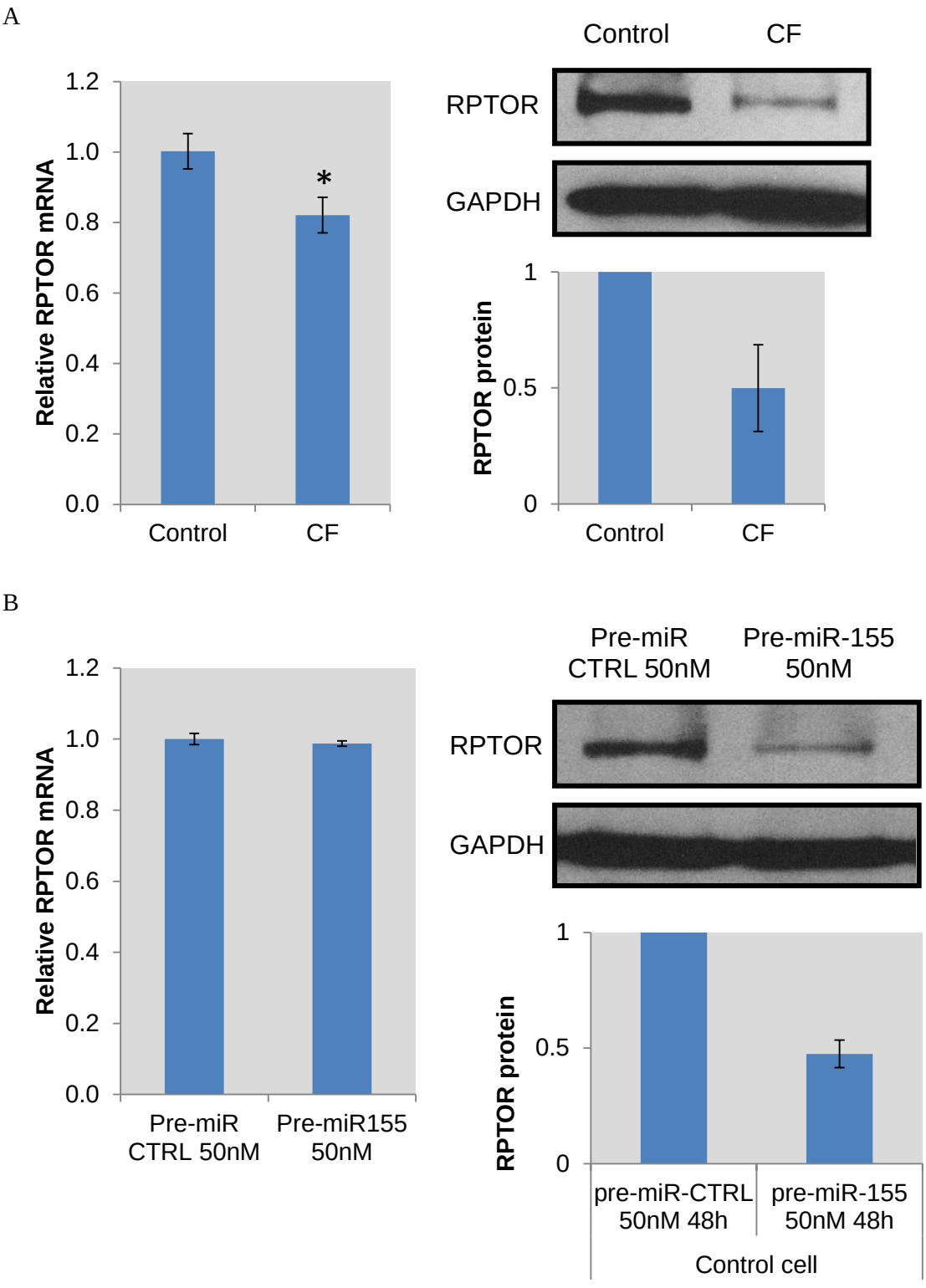
ZMYM2
ZNF701

Figure 2


A
Control
CF
RPTOR
GAPDH
Relative RPTOR mRNA
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
CF
RPTOR protein
1
0.5
0
Control
CF
B
Pre-miR CTRL 50nM
Pre-miR-155 50nM
RPTOR
GAPDH
Relative RPTOR mRNA
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pre-miR CTRL 50nM
Pre-miR155 50nM
RPTOR protein
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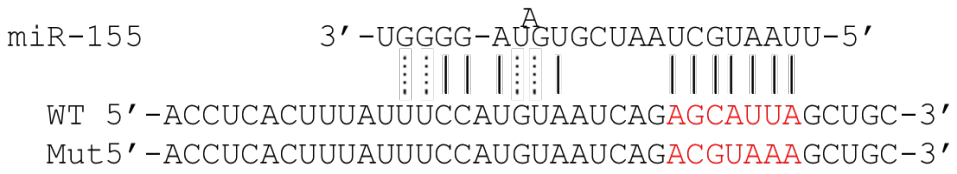
0.5
0
pre-miR-CTRL 50nM 48h
pre-miR-155 50nM 48h
Control cell

igure 2, continued

C



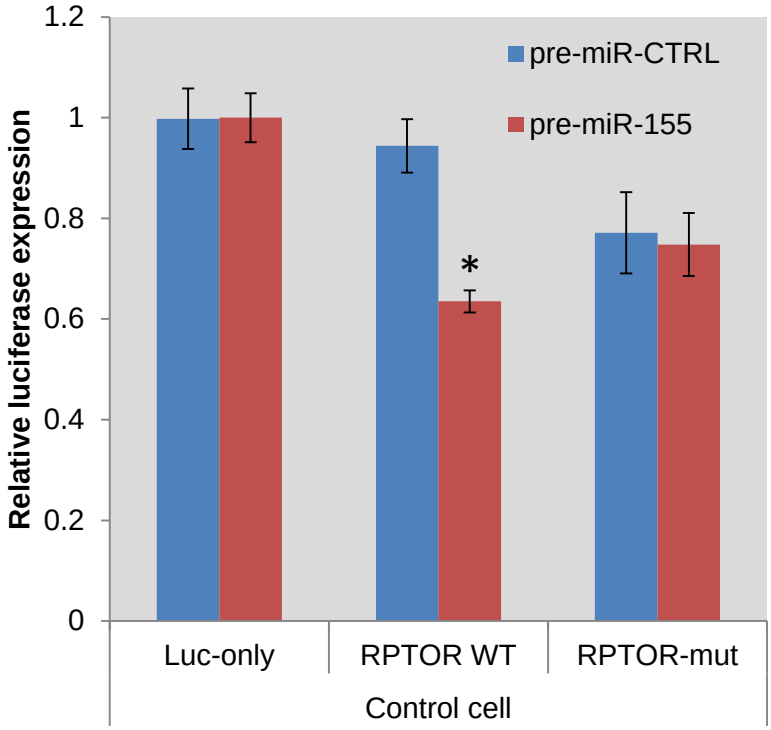
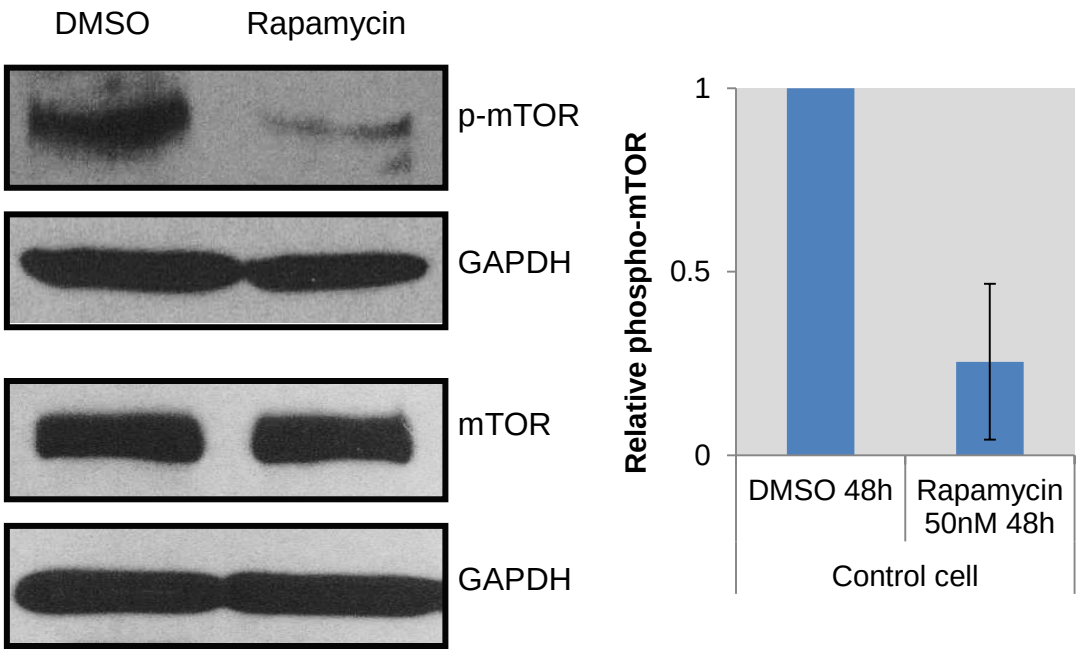


Figure 3

A


DMSO
Rapamycin
p-mTOR
GAPDH
mTOR
GAPDH
Relative phospho-mTOR
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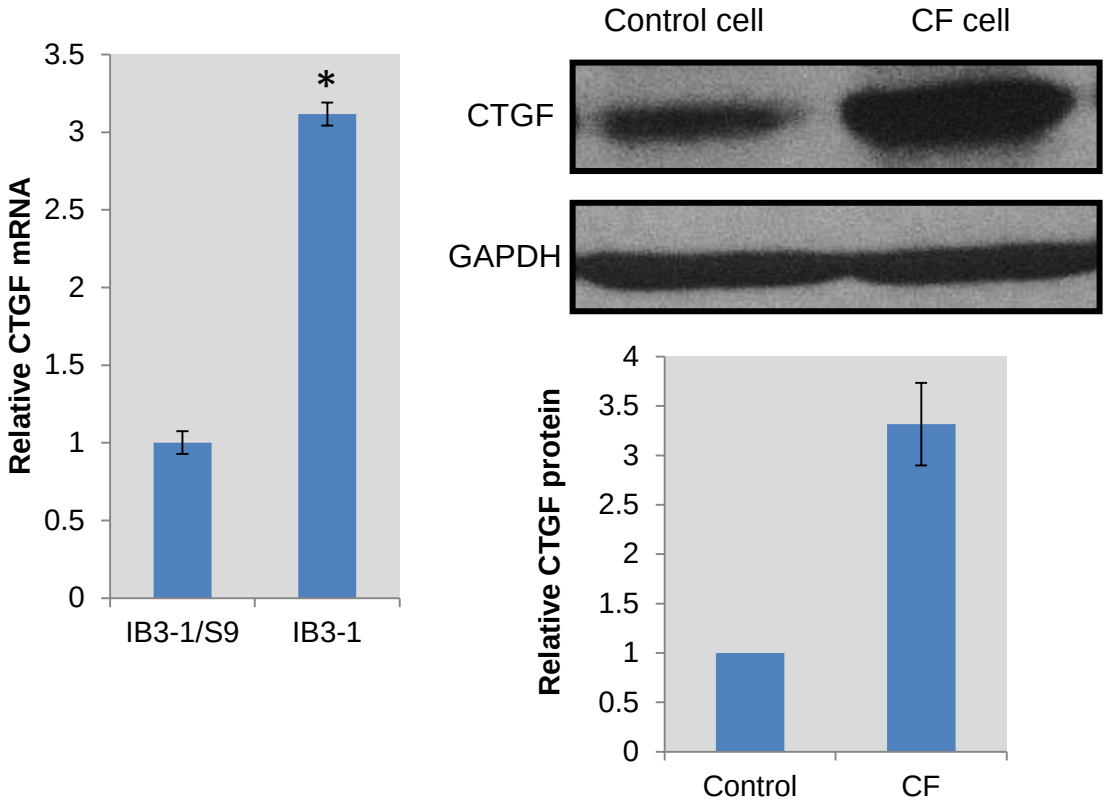
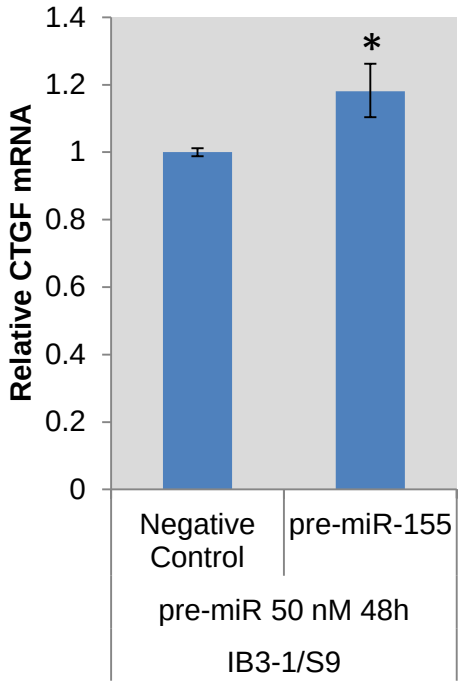
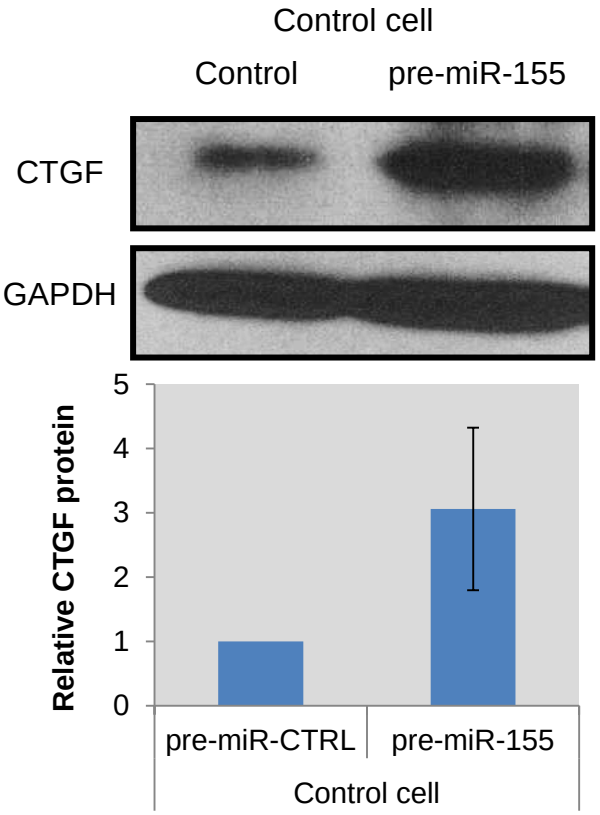
0.5
0
DMSO 48h
Rapamycin 50nM 48h
Control cell

B

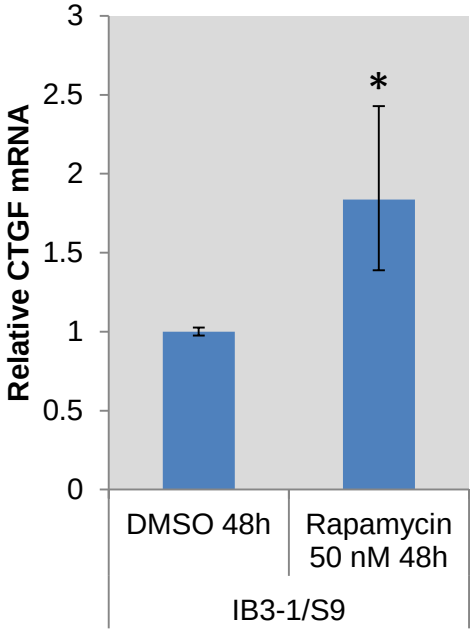

Relative CTGF mRNA
3.5
3
2.5
2
1.5
1
0.5
0
*
IB3-1/S9
IB3-1
Control cell
CF cell
CTGF
GAPDH
Relative CTGF protein
4
3.5
3
2.5
2
1.5
1
0.5
0
Control
CF

C


1.4
1.2
1
0.8
0.6
0.4
0.2
0
Relative CTGF mRNA
*
Negative Control
pre-miR-155
pre-miR 50 nM 48h
IB3-1/S9


Control cell
Control
pre-miR-155
CTGF
GAPDH
5
4
3
2
1
0
Relative CTGF protein
pre-miR-CTRL
pre-miR-155
Control cell

D


3
2.5
2
1.5
1
0.5
0
Relative CTGF mRNA
*
DMSO 48h
Rapamycin 50 nM 48h
IB3-1/S9

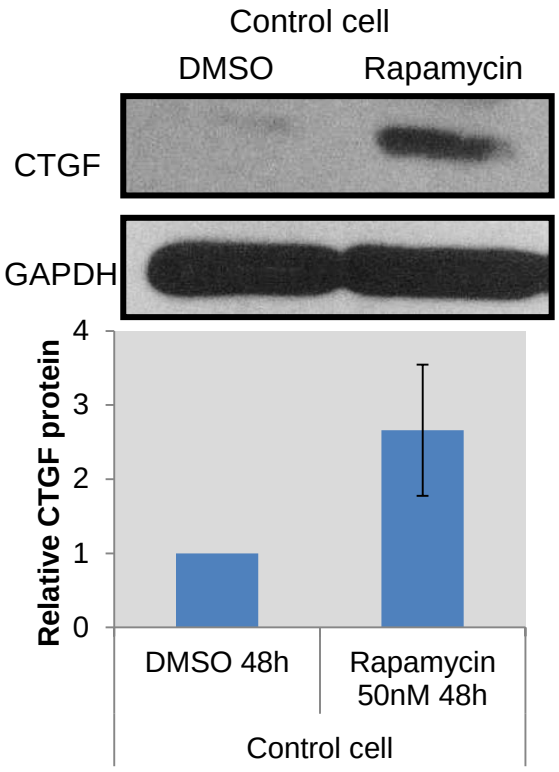
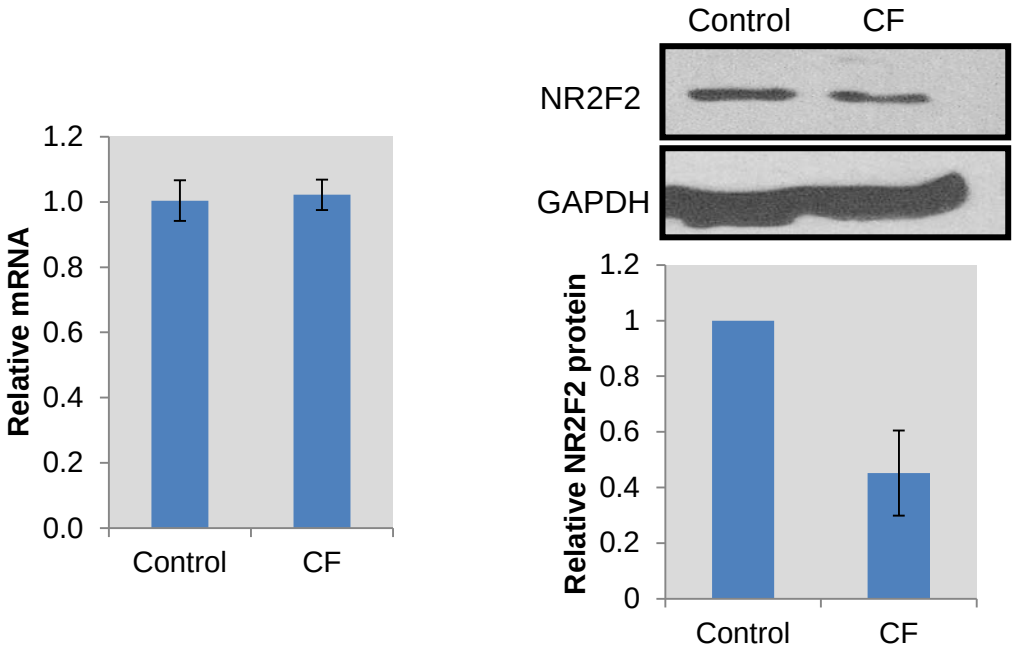

Control cell
DMSO
Rapamycin
CTGF
GAPDH
4
3
2
1
0
Relative CTGF protein
DMSO 48h
Rapamycin 50nM 48h
Control cell

Figure 4

A


Control
CF
NR2F2
GAPDH
Relative mRNA
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
CF
Relative NR2F2 protein
1.2
1
0.8
0.6
0.4
0.2
0
Control
CF

B

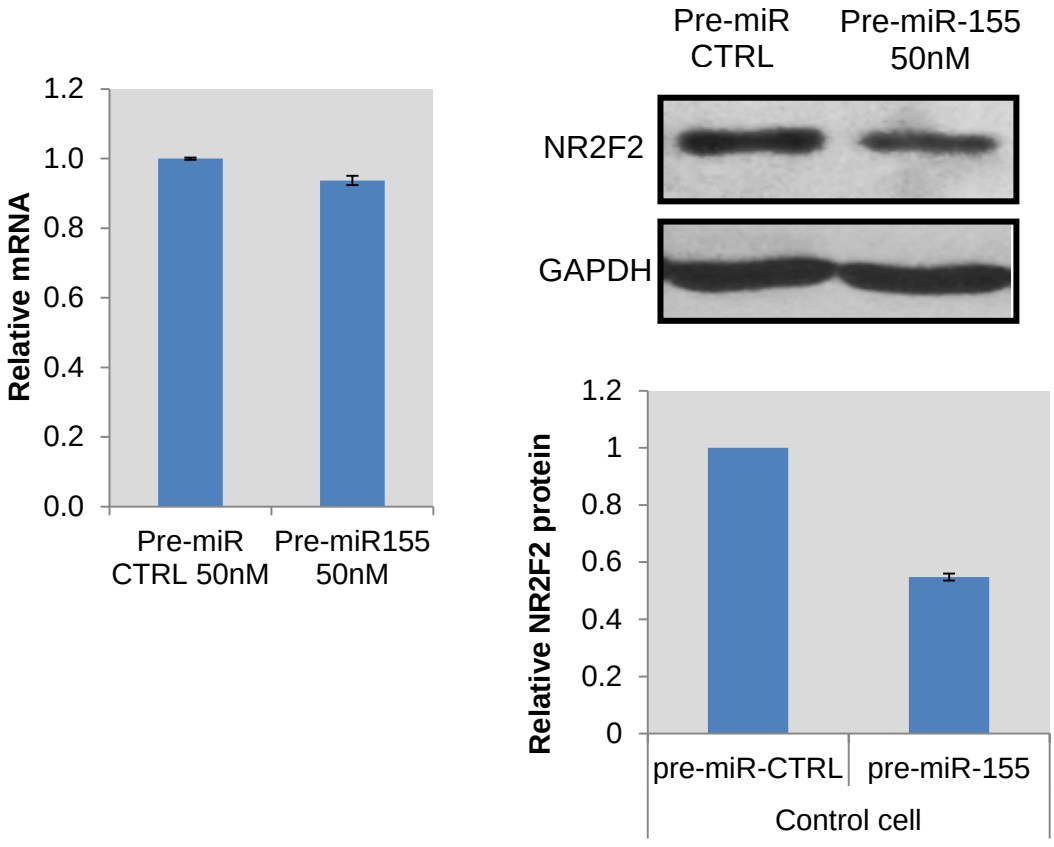

Pre-miR CTRL
Pre-miR-155 50nM
NR2F2
GAPDH
Relative mRNA
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pre-miR CTRL 50nM
Pre-miR155 50nM
Relative NR2F2 protein
1.2
1
0.8
0.6
0.4
0.2
0
pre-miR-CTRL
pre-miR-155
Control cell

Figure 4, continued

C

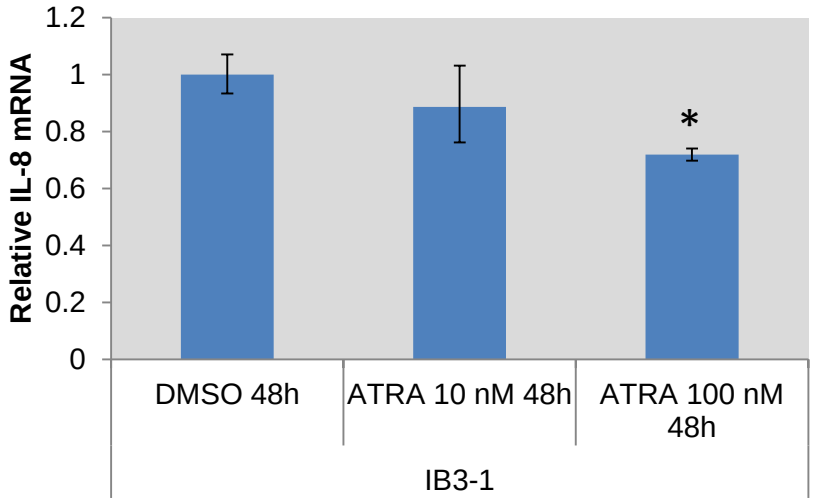


Figure Legends

Table 1. Insert sequences used to construct plasmids for luciferase assay. Small letters indicate restriction sites (*Spe*I and *Hind*III)

Figure 1. Expression and enrichment of genes predicted as miR-155 targets by TargetScan. Greener color indicates higher expression, and redder color indicates lower expression. The gray boxes indicate unavailable data, or the data with false discovery rate (FDR) more than 0.3. Genes with red letters are finally selected genes for further experiments.

Figure 2. Effects of miR-155 on RPTOR expression. (A) RPTOR expression in IB3-1/S9 (control) and IB3-1 (CF) cells. mRNA level (left) and protein level (right top) and band intensity quantification (right bottom) are shown. *$p<0.05$. (B) RPTOR expression in IB3-1/S9 cell (control) treated with negative control (pre-miR-CTRL) or pre-miR-155 (50 nM, 48 hours). mRNA level (left) and protein level (right top) and band intensity quantification (right bottom) are shown. *$p<0.05$. (C) Luciferase assay for predicted a target site in the 3' UTR of RPTOR mRNA. Predicted interaction between miR-155 and RPTOR mRNA. Red letters indicate predicted target site of miR-155 in RPTOR 3' UTR. WT: wild type, mut: mutant. (top). Luciferase assay in IB3-1/S9 cell with Negative control (blue bars) and pre-miR-155 (red bars) (50 nM, 24 hours). Luc-only: control plasmid without RPTOR sequence. *$p<0.05$.

Figure 3. Effects of miR-155 on CTGF expression. (A) Validation of rapamycin dose as decreased phosphorylation of RPTOR substrate mTOR. IB3-1/S9 cell (control) was treated with rapamycin (50 nM, 48 hours) or the equal volume of DMSO (0.005 vol%). (B) CTGF expression in IB3-1/S9 (control) and IB3-1 (CF) cells. mRNA level (left) and protein level (right top) and band intensity quantification (right bottom) are shown. *$p<0.05$. (C) CTGF expression in IB3-1/S9 cell (control) treated with pre-miR-155 or negative control (50 nM, 48 hours). mRNA level

(left) and protein level (right top) and band intensity quantification (right bottom) are shown. *p<0.05. (D) CTGF expression in IB3-1/S9 (control) cell treated with rapamycin (50 nM, 48 hours) or the equal volume of DMSO (0.005 vol%). mRNA level (left) and protein level (right top) and band intensity quantification (right bottom) are shown. *p<0.05.

Figure 4. Effects of miR-155 on NR2F2 expression. (A) NR2F2 expression in IB3-1/S9 (control) and IB3-1 (CF) cells. mRNA level (left) and protein level (right top) and band intensity quantification (right bottom) are shown. *p<0.05. (B) NR2F2 expression in IB3-1/S9 cell treated with pre-miR-155 or negative control (pre-miR CTRL) (50 nM, 48 hours). mRNA level (left) and protein level (right top) and band intensity quantification (right bottom) are shown. *p<0.05. (C) IL-8 mRNA expression after treatment of IB3-1 (CF) cell with all-trans retinoic acid (ATRA) (10 nM or 100 nM for 48 hours) or the equal volume of DMSO (0.05 vol%). *p<0.05

Chapter 5: Dissertation Discussion

Overall findings of the present studies showed the effects of miRNAs and RNA-binding proteins on posttranscriptional regulation of inflammatory molecules in cystic fibrosis. Specifically, a new target of miR-155 and its suggested involvement in disease development in cystic fibrosis is a truly new insight. A figure summarizing these three aims is shown at the end. In this chapter, updated discussion about the aim 1 based on more recent findings is first described, and subsequently, how the three aims can be integrated as a whole is discussed.

In the specific aim 1, we showed the effects of inflammatory molecules on IL-8 mRNA stability through posttranscriptional regulation, and further involvement of microRNAs in CF bronchial epithelial cell line, in the absence or presence of infectious agents. IL-1β increased steady state as well as stability of IL-8 mRNA. On the contrary IL-10, an anti-inflammatory molecule, decreased stability of IL-8 mRNA. We next examined the effects of *Pseudomonas aeruginosa* infection on IL-8 production in CF lung epithelial cells. Treatment of CF cell line with *P. aeruginosa* or with its endotoxin, LPS, increased the amount and stability of IL-8 mRNA. Unexpectedly, *P. aeruginosa* treatment decreased miR-155 expression, but LPS did not have significant effect on miR-155 expression. Consequently further analysis of microRNA expression profile identified miR-215 increase after *P. aeruginosa* treatment in CF cells. Subsequent analysis indicated the involvement of p53, which increased in CF cells with *Pseudomonas* infection.

The findings on effects of inflammatory molecules are compatible with conventional reports so far. Although there are some reports which showed that IL-1β production was not different between lung biopsy samples from normal and CF patients, and also between WT (S9) and CF (IB3-1) bronchial epithelial cell line (131; 132), stimulation by IL-1β led to more IL-8 generation in IB3-1 cell line (128). This was further examined in another report which showed that more IL-8 production under IL-1β stimulation in IB3-1 cell line was through increased activation of ERK1/2, p38, and NF-κB pathways (91). Activation of p38 after IL-1 stimulation was also reported for increased IL-8 mRNA stability (152), with which our result is also

consistent. It is also within expectation that IL-1β induces miR-155 because transcription of miR-155 was shown to increase by NF-κB, which is activated by inflammatory stimuli. Indeed there are studies which showed that IL-1β stimulated miR-155 production in mouse dendritic cells (77) and also in human melanoma cell lines (4). But there are some reports which showed that miR-155 decreased IL-1β production in dendritic cells (17) and bovine oviduct epithelial cells (43). This suggests some two-way regulations between miR-155 and IL-1β. But in particular in CF, our data suggest that IL-1β has dominant control to miR-155, given that IL-1β production is not different in CF while miR-155 increased in CF. The reason why miR-155 is low in control cells is probably because of effect of IL-10 whose expression is higher in control. This possibility is justified by the next data which showed lower miR-155 expression after treatment with IL-10, and also by another study as well (108).

The findings on effects of *Pseudomonas* infection and LPS treatment do not agree with published reports in the literature. LPS is an endotoxin expressed on the cell wall of gram-negative bacteria, including *P. aeruginosa*. Upon bacterial infection, LPS engages TLR4 on the cell surface and activates innate immune response. miR-155 was first recognized to play a role in normal and leukemic development of T and B cells, but was later established to mediate innate immunity in myeloid lineage cells. In most studies, miR-155 elevated after treatment with LPS, partly because of transcriptional upregulation by NF-κB and Ets2 (108) or because of activated KSRP function to facilitate miR-155 maturation (120; 137). But the final effects on inflammation vary. For example, miR-155 elevated after treatment of mouse macrophage with LPS, and inhibited the downstream NF-κB pathway, but at the same time miR-155 increased translation of TNF-α (134). Our results indicate that miR-155 decreases or unaltered after *P. aeruginosa* or LPS treatment of CF cells respectively, and this differs from conventional findings. There are, however, some reports which agree with our results. When bovine oviduct epithelial cell line was treated with LPS, miR-155 expression decreased (43). Also, when CF cell line (IB3-1) was treated with *P. aeruginosa*, the microRNA profiling analysis revealed slight decrease in miR-155

expression (28). We speculate that this discrepancy in miR-155 expression after LPS or infection is possibly because of cell type used, which means that, while most studies use macrophage or dendritic cells, the latter two studies and ours used epithelial cells. Taken together, our data suggest that a pro-inflammatory microRNA, miR-155 has a CF-specific regulatory role.

The findings on miR-215 increase after *Pseudomonas* infection is a new insight. Mechanism of how miR-215 affects infection or inflammation has not been shown so far. Earlier studies analyzed the regulation of miR-215 expression in infectious diseases. For example, when mice were orally infected with *Listeria monocytogenes*, ileal tissue showed decrease in miR-215, but co-infection with *Lactobacillus casei* to protect Listeria invasion led to recovery (increase) of miR-215 expression (2). Other studies showed, decreased miR-215 in blood after subcutaneous infection of *Staphylococcus aureus* and *Escherichia coli* to mice (111), decreased miR-215 in bovine oviduct epithelial cells after LPS treatment (43), and increased miR-215 in hepatoma cell line after HCV infection (44). These findings, together with our data, do not suggest any associations between miR-215 expression and general bacteriological features (Gram-positivity, biofilm production, aerobicity, and toxin production). On the other hand there are some reports about p53 involvement in inflammation. p53 was shown to inhibit the transcriptional activity of NF-κB after LPS treatment, and reduced subsequent cytokine production (57). And conversely, LPS treatment also suppressed p53 stability by enhanced binding of MDM2 to p53 through PI3K pathway (94). There is also another bacterial protein called azurin which is expressed in *P. aeruginosa* and renders blue color. Azurin from *P. aeruginosa* formed a complex with p53 and stabilized p53, leading to enhanced apoptosis in macrophage cell line (156). Through p53, azurin also increased expression of p21, another cell cycle regulator (157). Based on these earlier studies, we speculate that p53 increase after *P. aeruginosa* treatment was possibly because of the effect of azurin, but not because of LPS, and subsequently p53 induced miR-215. Furthermore, our results showing decrease in miR-155 expression after *P. aeruginosa* treatment, also supports the phenomenon of p53 increase, because miR-155 was shown to inhibit p53 activation (14).

There are still some experiments necessary to achieve the proposed aim 1. Previously our lab showed that IL-8 mRNA was posttranscriptionally regulated by miRNA-155, which is upregulated in CF (12). Also, since CF is characterized by increased expression of inflammatory molecules and frequent *Pseudomonas* infection, the aim 1 tried to target towards understanding posttranscriptional regulation of IL-8 expression and miR-155 in the absence or presence of *Pseudomonas* infection. As described above, we showed that IL-8 mRNA stability and miR-155 expression were regulated by inflammatory mediators, and miR-215 was induced after *Pseudomonas* infection. However, these experiments are not sufficient to conclude that this expression pattern is unique in CF. At least we needed to do same experiments with control cells and show any different patterns from CF cells. Also, our speculation that azurin might increase p53 after *Pseudomonas* infection, will be confirmed by using other bacteria. For example, *Staphylococcus aureus* and *Haemophilus influenzae* are also common infection in CF patients but they do not express azurin. Therefore, measuring p53 and miR-215 expression after these infections may render different expression pattern, and if so, we can characterize unique mechanism of *Pseudomonas* infection in CF.

The overall aim of this thesis is to describe posttranscriptional regulation of inflammatory molecules in cystic fibrosis. In this regard, to connect all three specific aims, HuR appears to play a central role in cystic fibrosis disease development. Indeed, in addition to the aim 2, involvement of HuR can be inferred for *Pseudomonas* infection (aim 1) and CTGF-mediated regulation (aim 3). In the aim 1, we examined the effects of IL-1β and IL-10 and *Pseudomonas* infection. Although direct association between IL-1β and HuR has not been reported, there are some reports which showed that IL-10 repressed HuR activation and expression (62). Another study showed a possible mechanism that IL-10 inhibited p38 MAPK pathway, which led to HuR decrease (109). Furthermore, it is interesting that HuR was also shown to increase p53 mRNA after LPS treatment in mouse cardiomyocytes (61). Although we could not describe any mechanisms about

p53 increase under *Pseudomonas* infection, this report suggests possible involvement of HuR for this phenomenon.

In the aim 3, we found miR-155 directly targets RPTOR which led to more CTGF expression. A previous report showed HuR to regulate CTGF expression. CTGF mRNA has ARE region in its 3' UTR, and HuR was shown to bind to this region and stabilize CTGF mRNA (42). Since CTGF stimulates fibroblasts to produce more MMP9, another report also supports the possibility of HuR regulation of CTGF, which showed MMP9 decreased after HuR knockdown in breast cancer cell line (159). Besides HuR, it is interesting that CTGF was also shown to be involved in *Pseudomonas* infection. When HeLa cells were infected with *P. aeruginosa*, CTGF expression increased (150). Another study showed that repeated *P. aeruginosa* infection on rats increased CTGF expression, and those rats manifested phenotypes similar to those observed in COPD (148).

There are, however, still some gaps in the three aims. In the aim 1 we showed that IL-8 mRNA was stabilized by inflammatory molecules and *Pseudomonas* infection, and this phenomenon should have been further investigated in the aim 2. For example, we could introduce the same construct with the IL-8 ARE region into CF cells treated with anti-IL-1β or IL-10 or *P. aeruginosa*, and analyze how HuR binding to IL-8 mRNA ARE region is affected. Specifically, effect of HuR on IL-8 mRNA stability could be examined after *P. aeruginosa* infection. This may address the contradiction of our previous report that miR-155 upregulates IL-8 mRNA (12) and the current study that *P. aeruginosa* decreased miR-155, yet increased IL-8 mRNA.

In summary, this dissertation aimed to identify inflammatory molecules in CF, and found miR-155, HuR and CTGF are key regulators of inflammation and fibrosis in CF.

Figure

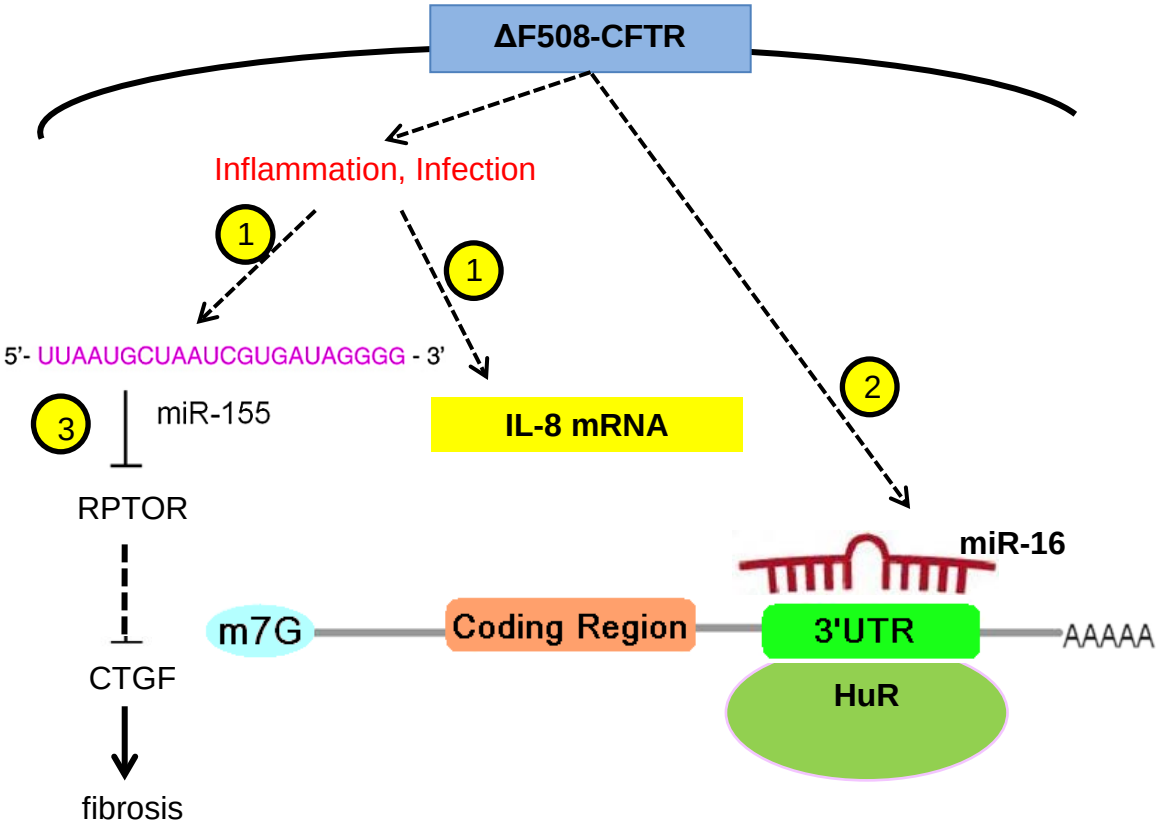

ΔF508-CFTR
Inflammation, Infection
1
1
2
5'- UUAAUGCUAAUCGUGAUAGGGG - 3'
3
miR-155
IL-8 mRNA
RPTOR
CTGF
fibrosis
miR-16
m7G
Coding Region
3'UTR
AAAAA
HuR

Figure Legend

The proposed model of posttranscriptional regulation of IL-8 mRNA, and CTGF induction by miR-155. Under infection and inflammation in CF, miR-155 and IL-8 mRNA increased. HuR and miR-16 regulate IL-8 mRNA stability. High miR-155 in CF suppresses RPTOR expression in CF which leads to high CTGF expression. This might be causes for fibrogenesis and airway destruction in CF. The numbers indicate corresponding specific aims.

Appendix: Correction of statistical analysis of Figure 3a of the specific aim 1

The purpose of the figure 3a is to choose the best condition which increases IL-8 mRNA expression, among three groups. For this purpose, repeating *t*-test analysis comparing control and each condition is not suitable. Here, we re-evaluate the statistical significance by employing Bonferroni correction. The significant p-values of three comparisons between each two groups are set as lower than 0.05/3, and performed *t*-test for each comparison. As shown below, all these p-values are confirmed to be lower than this preset value. Thus, we can still conclude that MPA-HA is significantly increasing IL-8 mRNA expression, and choose this condition.

Comparisons	p-value
Media Control and MPA LA	0.000326
Media Control and MPA HA	1.61E-05
MPA LA and MPA HA	3.68E-05

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