# miR-126 Is Downregulated in Cystic Fibrosis Airway Epithelial Cells and Regulates TOM1 Expression.

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**miR-126 is down-regulated in cystic fibrosis airway epithelial cells and regulates TOM1 expression**

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Running Title: miR-126 regulation of TOM1 in CF airway epithelium

**Abstract**

Cystic fibrosis (CF) is one of the commonest lethal genetic diseases in which the role of microRNAs (miRNAs) has yet to be explored. Predicted to be regulated by miR-126, TOM1 (target of Myb1) has been shown to interact with Tollip, forming a complex to regulate endosomal trafficking of ubiquitinated proteins. TOM1 has also been proposed as a negative regulator of IL-1β and TNF-α induced signaling pathways. miR-126 is highly expressed in the lung and we now show for the first time differential expression of miR-126 in CF versus non-CF airway epithelial cells both *in vitro* and *in vivo*. miR-126 down-regulation in CF bronchial epithelial cells correlated with a significant up-regulation of TOM1 mRNA, both *in vitro* and *in vivo* when compared to their non-CF counterparts. Introduction of synthetic pre-miR-126 inhibited luciferase activity in a reporter system containing the full length 3'-untranslated region (3'UTR) of TOM1 and resulted in decreased TOM1 protein production in CF bronchial epithelial cells. Following stimulation with LPS or IL-1β, over-expression of TOM1 was found to down-regulate NF-κB luciferase activity. Conversely, TOM1 knockdown resulted in a significant increase in NF-κB regulated IL-8 secretion. These data show that miR-126 is differentially regulated in CF versus non-CF airway epithelial cells and that TOM1 is a miR-126 target that may have an important role in regulating innate immune responses in the CF lung. To our knowledge this is the first report of a role for TOM1 in the TLR2/4 signaling pathways and the first to describe miRNA involvement in CF.

## Introduction

Cystic fibrosis (CF) is an inherited disorder characterised by chronic airway inflammation. Bronchial epithelial cells contribute significantly to the pulmonary inflammation evident in CF. LPS and IL-1β, which bind to Toll-Like Receptor 4 (TLR4) and the IL-1 Type-I receptor (IL-1RI) respectively, also play a pivotal role in this process. These agonists can activate the innate immune response culminating in pro-inflammatory gene expression leading to neutrophil-dominated airway inflammation and tissue damage in the CF lung. IL-1RI and TLRs are present on a variety of cell types, including both immune cells and epithelial cells within the lung, and in the context of CF airway epithelial cells have been shown to promote pro-inflammatory gene transcription following stimulation with their cognate agonists (1, 2). For example, in airway epithelial cells of non-CF and CF origin triacylated lipopeptide, LPS or unmethylated CpG DNA can induce IL-6, IL-8 and TNF-α production via TLRs 2, 4 and 9 (1). Similarly, IL-1β can up-regulate production of a plethora of pro-inflammatory cytokines (2). Thus and/or TLRs and their signaling intermediates represent potential therapeutic targets for CF. Despite significant advances in treatment regimes CF remains a condition for which there is no effective cure. Therefore investigating the expression and function of miRNAs in CF will shed light on previously unidentified regulatory mechanisms controlling changes in gene expression and direct the development of future therapeutic strategies for this debilitating and fatal disorder.

Expanding interest in miRNAs over the past decade has uncovered their importance in several biological processes and has identified disease states with altered miRNA expression patterns. miRNAs are approximately 20-25 nucleotides long and negatively regulate gene expression at a post-transcriptional level. Within

each miRNA there exists a 2-8 nucleotide "seed region" thought to be critical for target selection (3). Mature miRNAs use this seed region to bind selectively to microRNA recognition elements (MRE) within the 3'UTR of target mRNAs. Different target genes may have several MREs and therefore be regulated by numerous miRNAs. The number of and distance between MREs are considered important for the biological activity of miRNAs. Relatively few miRNAs have been studied in detail and hence the biological relevance of the majority remains to be uncovered. Expression levels vary greatly among tissues and it is believed that dysregulation of miRNA can contribute to disease pathology (4). Therefore we considered it plausible to investigate whether unique miRNA expression profiles exist in CF, particularly in CF bronchial epithelial cells and explore their effects on influencing signaling pathways.

We performed expression profiling comparing miRNA expression in CF and non-CF bronchial brushings. Based on these studies we selected miR-126 for further investigation given that its expression is known to be highest in vascularised tissues such as the lung, heart and kidney (5-7) and as it has been shown to be present in bronchial epithelium (8). miR-126 is 21 nucleotides in length, located on chromosome 9q34.3 and is contained within intron 5 of its host gene epidermal growth factor like-7 (EGFL-7) (6, 9). In recent studies miR-126 has been shown to have functional roles in angiogenesis (10, 11), to be down-regulated in a number of malignancies (8, 12) and to act as a tumor suppressor in breast cancer (13). *In silico* analysis of a number of miRNA target prediction databases shows that TOM1 is a potential target of miR-126. TOM1 is a member of a family of proteins containing an N-terminal VHS (Vps27p/Hrs/STAM) domain reported to be involved in intracellular trafficking (14). Previous studies have shown that TOM1 forms a complex with Tollip (Toll-

interacting protein) a negative regulator of TLR2, TLR4 and IL-1RI signaling. This complex regulates endosomal trafficking of ubiquitinated proteins (15). Moreover this complex has been shown to traffic IL-1RI to the endosome for degradation (16). TOM1 has also been proposed as a negative regulator of IL-1β and TNF-α induced signaling pathways, whereby its over-expression can suppress the activity of the transcription factors NF-κB and AP-1 (17). In this paper we explore the presence of miRNA in CF for the first time. We investigate the expression of miR-126 in CF and non-CF airway epithelial cells both *in vitro* and *in vivo* by quantitative real time PCR (qRT-PCR) and miRNA expression profiling and explore the potential mechanism responsible for altered miRNA expression in CF bronchial epithelium. We determine whether TOM1 is a valid target of miR-126, as predicted by *in silico* analysis, and further investigate the role of TOM1 in IL-1β, LPS and lipopeptide-mediated airway inflammation in the CF lung using over-expression and knockdown approaches.

## Material and Methods

***Cell culture and treatments*** All cell lines were maintained in a 37°C humidified $CO_2$ incubator in appropriate media. 16HBE14o$^-$ and 9HTEo$^-$ (human bronchial and tracheal epithelial cell lines respectively), CFBE41o$^-$ and CFTE29o$^-$ (human ΔF508 homozygote bronchial and tracheal epithelial cell lines respectively) were obtained as a gift from D. Gruenert (California Pacific Medical Center Research Institute, San Francisco, CA). HepG2 (human hepatocellular liver carcinoma cell line), HEK293293 (human embryonic kidney cell line), A549 (type II-like human lung epithelial cell line), THP-1 (human acute monocytic leukemia cell line), U937 (monocytic cell line) and U373 MG (human glioblastoma-astrocytoma, epithelial-like cell line) were obtained from the European Collection of Cell Cultures. Prior to agonist treatment, cells were washed with serum-free media and placed in media containing 1% FCS for *Pseudomonas aeruginosa* LPS (Sigma), IL-1β (R&D Systems), flagellin (Alexis Biochemicals), triacylated lipopeptide (palmitoyl-Cys((RS)-2,3-di((palmitoyloxy)-propyl)-Ala-Gly-OH, Pam3) (Bachem), unmethylated CpG DNA (uCpG 5'-TCGTCGTTTTGTCGTT-3') (MWG) and poly:IC (Sigma) stimulation experiments.

***Study populations and bronchial brush sampling*** Nineteen individuals in total were recruited into this study, six of which had CF (confirmed by sweat testing and/or genotyping) and thirteen which were non-CF controls with a mean age of 22.8$\pm$1.6 years and 51$\pm$4.2 years respectively. For expression profiling studies five individuals with CF (three male and two female) and five non-CF controls (four male and one female) were selected from the study group. All participants (CF and controls) were undergoing diagnostic and/or therapeutic fibre-optic flexible bronchoscopy as part of routine care. Full informed consent was obtained pre-procedure and appropriate

approval obtained from our institutional review board. Following completion of the bronchoscopy and prior to the withdrawal of the bronchoscope, an area 2 cm distal to the carina (medially located) in either the right or left main bronchus was selected and washed twice with 10 ml sterile 0.9% NaCl. Following this, a sterile 10 mm x 1.2 mm bronchial brush (Olympus Medical Systems Corp, Japan) was inserted through the appropriate port on the bronchoscope and the chosen area sampled with two consecutive brushes by scraping the area gently. The brush was withdrawn and immediately placed in 5ml MEM+Glutamax supplemented with 10% FCS and 1% penicillin-streptomycin (Gibco). Brushes were gently agitated to dislodge cells into the media which was centrifuged at 1,200rpm for 5 minutes and cell pellets re-suspended in 0.5 ml of Tri Reagent (Sigma) prior to RNA extraction.

***miRNA expression profiling in CF bronchial brushings*** miRNA expression profiling was performed in bronchial brushings (CF; n=5, non-CF; n=5) by a stem-loop real-time PCR-based miRNA expression profiling method using the Taqman MicroRNA Arrays v2.0 (released June 2009) from Applied Biosystems. The content is derived from the miRBase microRNA registry, providing comprehensive coverage of miRNAs from release 10.0 using the most up-to-date TaqMan MicroRNA Assays. Two array cards (A and B) for each sample were run on the Applied Biosystems 7900HT fast real time PCR system which measured expression levels of 667 different human miRNAs in each sample and three positive and one negative control per card. RNA (30 ng) from clinical samples was reverse transcribed with the Megaplex primer pool (Applied Biosystems), allowing simultaneous reverse transcription of 430 miRNAs and 36 endogenous controls in one RT pool (18). A Pre-amplification step was performed on the Megaplex RT product (5µl) using TaqMan PreAmp Master Mix

(2x) and PreAmp Primer Mix (5x) (Applied Biosystems). The PreAmp primer pool contained forward primers specific for each miRNA, and a universal reverse primer (Applied Biosystems). All miRNAs with Cycle threshold (Ct) values greater than 35 were considered 'non amplified' or 'not expressed' and excluded from analysis. Mean normalisation was carried out by subtracting the mean sample Ct from the individual miRNA Ct values (19). Relative quantification of gene expression was determined using the comparative cycle threshold method ($2^{(-\Delta\Delta Ct)}$) as previously described (20). In the case of the non-CF samples, the mean Ct value was calculated for each individual miRNA, and this was used to calculate the ΔCt for the calibrator sample. Array data have been deposited in NCBI's Gene Expression Omnibus (21) and are accessible through GEO Series accession number GSE19431(http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE19431)

***Quantitative assessment of miRNA levels by Real Time PCR*** miR-126 expression was measured using Taqman miRNA assays (Applied Biosystems) according to the manufacturer's instructions and qRT-PCR was performed on the Roche LC480 Lightcycler. The expression of miR-126 relative to miR-16 was determined using the $2^{(-\Delta\Delta Ct)}$ method. All qRT-PCR experiments were performed in triplicate, including no-template controls.

***miR-126 regulation*** 16HBE14o$^-$ and CFBE41o$^-$ cells ($1x10^5$ in triplicate) were left untreated or treated with LPS (10 μg/ml) or IL-1β (10 ng/ml) for 3, 6 and 24 h following which miR-126 expression was measured by qRT-PCR as described above. 16HBE14o$^-$ cells ($1x10^5$ in triplicate) were treated with Thapsigargin (1 μM) (a pharmacological ER stress inducer) or $CFTR_{inh}$-172 (5 μM and 10 μM) for 4 h and 24

h, (all reagents were purchased from Sigma). DMSO was the vehicle control. RNA was isolated and miR-126 expression assessed by qRT-PCR.

***miR-126 target predictions*** *In silico* analysis of a range of miRNA target prediction databases was performed namely, TargetScan 4.2, picTar, PITA, RNA Hybrid, RNA22 and miRanda. TOM1 was a predicted target of miR-126 in all databases but miRANDA. Tollip was listed as a predicted target of PITA and RNA 22 only.

***TOM1 and Tollip expression analysis*** Total RNA was extracted using Tri Reagent, equal quantities of which was reverse transcribed into cDNA using Quantitect Reverse Transcription Kit (Qiagen) following the manufacturer's protocol. Primers for TOM1, Tollip and β-actin were obtained from MWG Eurofins Genetics (TOM1-F 5'-ATTCTGTGGGCACTGACTCC-3' and TOM1-R 5'-CACTCACCATCTCCAGCTCA-3', β-actin-F 5'-GGACTTCGAGCAAGAGATGG-3' and β-actin-R 5'-AGGAAGGAAGGCTGGAAGAG-3', TOLLIP-F 5'-CAAGGTGGAGGACAAGTGGT-3', TOLLIP-R 5'-ACATGTCCTGGATGGCTTTC-3'). Expression of TOM1 or Tollip relative to β-actin was determined using the $2^{(-\Delta\Delta Ct)}$ method. All qRT-PCR experiments were performed in triplicate, including no-template controls.

***Luciferase reporter plasmid construction*** The full length 3′UTR of TOM1 was PCR amplified using a proof reading Phusion flash High-Fidelity PCR master mix (Finnzymes) with the following primers TOM1 3′UTR-F 5'-CTGCTCTCACACCCTTAGGC-3' and TOM1 3′UTR-R 5'-TGCTAGCAGGGTGGTTTTCT-3'. The amplified 740bp product was inserted into the *Hind*III and *Spe*I sites of the miRNA expression vector pMIR-REPORT (Applied Biosystems) immediately downstream of the luciferase gene and termed pMIR-

TOM1-3'UTR. Transformants were validated by restriction mapping and sequencing (MWG Eurofins Genetics).

***Transfection of pre-miR-126 and reporter plasmids*** HEK293293 cells ($1x10^5$ in triplicate) were transiently transfected with 250 ng of pMIR-TOM1-3'UTR and 100 ng of reference *Renilla* luciferase reporter plasmid pRLSV40. Cells were co-transfected with either 30 nM of synthetic pre-miR-126 or a scrambled control. Transfections were performed using Genejuice (Novagen) for plasmid DNA and Ribojuice (Novagen) for siRNA in OptiMEM reduced serum media (Gibco) as per the recommended conditions. Lysates were prepared at 24 h post-transfection and assayed for both firefly and *Renilla* luciferase using Luciferase assay system (Promega) and coelenterazine (MGT), respectively. Firefly luciferase activity was normalized to the *Renilla* luciferase activity.

***miR-126 over-expression*** CFBE41o$^-$ cells ($1x10^5$ in triplicate) were left untransfected (NT) or reverse transfected with either 30nM of a scrambled control or synthetic pre-miR-126 using NeoFX transfection reagent (Applied Biosystems). Twenty four hours post-transfection cells were washed with PBS, whole cell lysates prepared and separated by electrophoresis on a 10% SDS-polyacrylamide gel. Nitrocellulose membranes (Sigma-Aldrich) were probed using a mouse monoclonal antibody to TOM1 (Abcam) and a rabbit polyclonal β-actin antibody as a loading control (Abcam). Signals were detected using the appropriate horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technologies) and visualized by chemiluminescence (Pierce) on the Syngene G:Box chemi XL gel documentation system. Membranes were analyzed by densitometry using GeneTools software on the same system.

***TOM1 over-expression and NF-κB luciferase reporter activity*** CFBE41o$^-$ cells ($6x10^4$ in triplicate) were co-transfected for 48 h (Genejuice) with 250 ng of pCDNA3 (empty vector) or a TOM1 overexpression plasmid pTOM1-Myc (a gift from Dr. K. Nakayma, Kyoto University), and 100 ng of an inducible $(NF\text{-}\kappa B)_5$-promoter (firefly) luciferase reporter plasmid and 100 ng of the constitutive *Renilla* luciferase reporter plasmid pRLSV40. Post-transfection (42 h) cells were treated with LPS (1μg/ml) or IL-1β (10ng/ml) for a further 6 h. Lysates were prepared and assayed for both firefly and *Renilla* luciferase as described above.

***TOM1 knockdown*** CFBE41o$^-$ cells ($1x10^5$ in triplicate) were left untreated (NT) or transfected with either 30 nM of an siRNA to TOM1 (siRNA ID 137971 5' CCUCUUGGUUGAUCCUCUUtt 3' Sense, 5' AAGAGGAUCAACCAAGAGGtg 3' Antisense), GAPDH (positive control, *Silencer*® GAPDH siRNA (Human) Part Number AM4605) or a scrambled control (Scr, *Silencer*® Negative Control #1 siRNA Part Number AM4611) siRNA (Applied Biosystems) using Ribojuice according to the manufacturer's instructions. Twenty four hours post-transfection cells were stimulated with LPS (10 μg/ml), IL-1β (10 ng/ml), triacylated lipopeptide (palmitoyl-Cys((RS)-2,3-di((palmitoyloxy)-propyl)-Ala-Gly-OH, Pam3) (10 μg/ml), flagellin (1μg/ml) or poly:IC (100 μg/ml) for 6 h and uCpG DNA (100 μg/ml) for 24 h. Supernatants and cells were collected for ELISA and RNA extraction respectively. siRNA transfection was also performed ($3x10^5$ cells in triplicate) for protein analysis. Cells were lysed 24 h post-transfection and western blot analysis carried out as described above.

***IL-8 ELISA*** IL-8 protein concentrations in the cell supernatants were determined by sandwich ELISA (R&D Systems).

***Statistical analysis*** All analyses was performed using GraphPad PRISM 4.0 software package (San Diego CA). Results are expressed as the mean ± SEM and were compared by student *t*-test (non-parametric, one tailed) or ANOVA as appropriate. Differences were considered significant at $p \leq 0.05$

## Results

### *miRNA expression profiling and miR-126 expression levels* in vivo

Profiling to examine the expression of 667 different human miRNAs was performed on bronchial brushings (CF; n=5 and non-CF; n=5) using Taqman Low Density Arrays (TLDAs) v2.0. Of the 667 miRNA examined, no appreciable target detection (Ct >35) occurred for 263 miRNA across all samples. Ninety three miRNA were significantly differentially expressed ($1.5 \geq RQ \leq 0.7$) in at least 3 of 5 CF patients. Of these 57 were down regulated and 36 up regulated (Supplemental Table 1). miR-126 was found to be significantly decreased in four of the five CF samples compared to controls, ($p = 0.0143$) (Fig. 1*A*). miR-16 and miR-491-5p were not significantly decreased or increased in CF versus non-CF samples. We independently confirmed this observation by measuring miR-126 expression by qRT-PCR (CF; n=6 and non-CF; n=13) where miR-126 was again significantly decreased in CF compared to non-CF controls, ($p = 0.0037$) (Fig. 1*B*).

### *miR-126 expression levels in cells lines*

We next investigated whether miR-126 was differentially expressed in CF versus non-CF airway epithelial cell lines and again performed qRT-PCR on CF tracheal (Fig. 2*A*) and bronchial epithelial cell lines (Fig. 2*B*) and their non-CF counterparts. This revealed that miR-126 was down regulated in CF compared to non-CF cells and

significantly so in bronchial epithelial cells ($p = 0.05$) which we continued to use for the rest of the study. We also determined miR-126 levels in a variety of cell lines by qRT-PCR and, consistent with other reports, observed higher expression of miR-126 in lung airway epithelial versus non-lung cells (Fig. 2*C*).

***Regulation of miR-126***

We then attempted to address the mechanism responsible for reduced miR-126 levels in CF epithelial cells by inducing either endoplasmic reticulum (ER) stress or defective chloride ion channel secretion in 16HBE14o$^-$ cells using thapsigargin or $CFTR_{inh}$-172, respectively. Following 4 h treatments there were no significant changes in miR-126 expression (data not shown) however, 24 h post treatment miR-126 was significantly reduced ($p=0.0296$) by the ER stress inducing agent (Fig 3). There was no change observed in cells treated with the CFTR inhibitor. We also examined the effects of LPS and IL-1β on miR-126 expression in both 16HBE14o$^-$ and CFBE41o$^-$ and found no effect (data not shown).

***TOM1 is a functional target of miR-126***

In order to identify potential targets of miR-126 both for experimental validation and functional studies in airway inflammation we performed *in silico* analysis of a range miRNA target prediction databases. From the databases analyzed all presented TOM1 as a target of miR-126 with the exception of miRANDA. RNA Hybrid provided a schematic of miR-126 predicted binding within the TOM1 3'UTR showing a minimum free energy (mfe) of -21.5kcal/mol (Fig. 4*A*) and notably Targetscan 4.2 illustrated the predicted pairing region in the TOM1 3'UTR to be conserved across species (Fig. 4*B*). We initially confirmed TOM1 expression in CFTE29o$^-$,

16HBE14o$^-$, CFBE41o$^-$, 9HTEo$^-$ and human lung RNA by semi-quantitative RT-PCR (Fig. 4*C*). TOM1 expression relative to β-actin was then determined in CFBE41o$^-$ versus 16HBE14o$^-$ cell lines by qRT-PCR resulting in reciprocal levels ($p = 0.05$) compared to miR-126 in these cells (Fig. 1*C*), and again this observation was confirmed *in vivo* in four of the five bronchial brushings from CF versus non-CF individuals ($p = 0.0143$) (Fig. 4*D*). The same databases were interrogated to assess whether Tollip is also a potential miR-126 target. Both PITA and RNA 22 but none of the other databases listed Tollip as a target. Therefore we examined Tollip mRNA expression in CFBE41o$^-$ versus 16HBE14o$^-$ cell lines by qRT-PCR. Figure 4*E* demonstrates reduced expression of Tollip in CF compared to non-CF cells ($p = 0.0286$).

Next in order to determine whether TOM1 is a molecular target of miR-126, we constructed a luciferase reporter vector containing the full-length TOM1 3'-UTR (pMIR-TOM1-3'UTR). The sequenced transformant showed 100% base pair match to the TOM1 3'UTR and importantly maintained an intact miR-126 binding region. HEK293293 cells, which exhibit low levels of miR-126 expression, were used for transient transfections with pMIR-TOM1-3'UTR. Co-transfection with pre-miR-126 (a synthetic miR-126 mimic) resulted in a significant decrease in luciferase gene expression from the reporter vector containing the TOM1 3'-UTR when compared to a scrambled control (Fig. 5) demonstrating direct targeting by miR-126, ($p = 0.0011$)

***Over-expression of miR-126 decreases TOM1 protein***

We next assessed the effect of miR-126 over-expression on TOM1 protein. Transfection of pre-miR-126 into CFBE41o$^-$ cells resulted in a significant increase in

miR-126 expression compared to non-transfected or scrambled-transfected cells, as measured by qRT-PCR (data not shown). Subsequent western blot analysis of TOM1 in CFBE41o$^-$ cells showed that miR-126 over-expression caused a reduction in TOM1 protein production compared to non-transfected (NT) cells or cells transfected with a scrambled siRNA (Scr) (Fig. 6*A*). Representative densitometry for western blots is shown (Fig. 6*B*).

***pTOM1-Myc inhibits LPS - or IL-1β-induced NF-κB reporter gene expression in CFBE41o$^-$ cells***

In order to determine functional effects of TOM1 in the context of the CF lung we transfected CFBE41o$^-$ cells with a TOM1 over-expression plasmid, pTOM1-Myc, and assessed its effects on NF-κB activity in these cells in response to inflammatory stimuli common in the CF lung utilising an NF-κB reporter system. NF-κB reporter gene expression in CFBE41o$^-$ cells was measured in response to stimulation with LPS or IL-1β for 6 h. Each of the agonists significantly increased NF-κB reporter gene expression (* $p = 0.05$) compared with controls, whilst over-expression of TOM1 inhibited this effect (# $p = 0.05$) (Fig. 7).

***Knockdown of TOM1 increases IL-8 protein production in response to LPS, IL-1β or lipopeptide in CFBE41o$^-$ cells***

IL-8 is an NF-κB regulated gene and a key cytokine present in the CF lung. We assessed the effect of TOM1 knockdown on IL-8 protein production in CFBE41o$^-$ cells in response to a range of pro-inflammatory stimuli namely lipopeptide, poly:IC LPS, flagellin, uCpG DNA or IL-1β whose cognate receptors are TLR2, TLR3, TLR4, TLR5, TLR9 and IL-1RI, respectively. Cells transiently transfected with a

GAPDH or TOM1 siRNA resulted in 80% and 65% knockdown, respectively when compared to a scrambled control (Fig. 8*A*). Transfection efficiency was normalized to GAPDH gene expression. Knockdown of TOM1 resulted in a 20-50% decrease of TOM1 protein as determined by western blot, representative blot shown (Fig 8*B*). IL-8 protein production in CFBE41o$^-$ was measured in response to stimulation with LPS or IL-1β, lipopeptide, flagellin or poly:IC for 6 h and uCpG DNA for 24 h. LPS, IL-1β and lipopeptide significantly increased IL-8 protein production ($p < 0.05$) compared with untreated cells (data not shown) and also in the presence of GAPDH siRNA (Fig 8*C*). TOM1 knockdown in these cells potentiated the stimulatory effects of LPS, IL-1β and lipopeptide with IL-8 secretion significantly increased in these cells (Fig 8*C*). TOM1 knockdown did not enhance IL-8 secretion following treatment with flagellin, poly:IC or uCpG DNA (data not shown).

***Discussion***

This study is the first to provide evidence for differential expression of a known miRNA in CF versus non-CF airway epithelial cells and our data supports the emerging body of evidence implicating miRNAs in innate immunity. Having established that expression of miR-126 is down-regulated in CF versus non-CF airway epithelial cells *in vivo* by miRNA expression profiling and qRT-PCR of bronchial brushings obtained from CF individuals and non-CF controls, we replicated this observation *in vitro* using CF airway epithelial cell lines. We also observed miR-126 to be markedly increased in lung versus non-lung cell lines consistent with other reports (6, 22). Using bioinformatic tools, targets of miR-126 were identified and TOM1 was selected for experimental validation given its known role in the innate immune response (17). As it is of general consensus that a target predicted by a combination of algorithms may have more functional relevance than those predicted using a single algorithm alone we analyzed a range of databases (23). TOM1 was listed as a predicted target in 5/6 of the target prediction databases interrogated. Here we have experimentally validated TOM1 as a target of miR-126 by showing that over-expression of pre-miR-126 results in a decrease of TOM1 protein production and reduced luciferase activity in a reporter system containing the full length TOM1 3'UTR, demonstrating direct targeting by miR-126. We also present a functional role for TOM1 in the signaling pathways induced in response to LPS and lipopeptide and link this observation with regulation of TOM1 by miR-126. Together these data show that miRNA can be differentially expressed in CF airway epithelium and may regulate pulmonary inflammation in CF.

Expression profiling studies have identified altered miRNA expression patterns in a variety of human diseases. A number of miRNAs are routinely under- or over-expressed in a variety of tumors e.g. miR-34a, miR-143, miR-145, miR-21 (24-26). Several miRNAs are also differentially expressed in specific types of cancers (26, 27). However there is also increasing evidence for important roles for miRNAs in regulating innate immunity (28-30). We hypothesized that unique miRNA expression profiles exist in CF versus non-CF bronchial epithelial cells and that these differential molecular miRNA signatures can regulate pro-inflammatory gene expression. This held true for miR-126 which was decreased in CF versus non-CF bronchial epithelial cells but also identified microRNAs that are not differentially expressed.

Expression of miR-126 was consistently and reproducibly decreased in CF versus non-CF airway epithelial cells and correlated with a reciprocal increase in expression of its predicted target TOM1 both *in vivo* and *in vitro*. Whilst we found miR-126 over-expression decreased production of luciferase from a reporter gene regulated by the 3'UTR of TOM1, this conflicts with another report that failed to demonstrate regulation of TOM1 by miR-126 (9). Notably, however, we cloned the entire TOM1 3'UTR, rather than smaller fragments, into our reporter system; an approach which Kuhn *et al* (31) have highlighted as being important for validation purposes. Since our initial *in silico* analysis which indicated that TOM1 was targeted by miR-126 alone, Targetscan 5.1 now lists an additional 58 miRNA families that may target TOM1 (including miR-126). Of these 23 were not analysed in our expression profiling, 19 were not detected in any sample and of the remaining 16 miR-126 was the only one to be significantly decreased in four of the five CF samples. Although the relatively small patient numbers used for the expression profiling may be considered a limitation of this study, we have subsequently

replicated our observations regarding miR-126 and TOM1 in additional patient samples.

Upon stimulation with IL-1β or LPS cells transiently transfected with a TOM1 over-expression plasmid exhibited a reduction in NF-κB luciferase activity. This is consistent with a previous report which proposed TOM1 as a negative regulator of signaling pathways induced by IL-1β and TNF-α whereby over-expression of TOM1 inhibited activity of the transcription factors NF-κB and AP-1 (17). Our work builds on this by showing that TOM1 can also negatively regulate signaling to NF-κB induced by LPS via TLR4. Our TOM1 knockdown studies corroborate this finding, showing a significant increase in secretion of the NF-κB regulated cytokine IL-8 in response to LPS or IL-1β and additionally lipopeptide, thus introducing a role for TOM1 in TLR2 signaling. Other pro-inflammatory stimuli tested which activate TLR3, 5 and 9 failed to elicit enhanced IL-8 secretion following TOM1 knockdown consistent with reports of Tollip's involvement solely in TLR 2 and 4 and IL-1RI signaling. To our knowledge this is the first report of involvement of TOM1 in the TLR2 and TLR4 signaling pathways. These findings extend our knowledge of TOM1 with respect to IL-1β and propose a new functional role for this protein in TLR2/4 signaling. Thus regulation of TOM1 has important implications not only for the pulmonary inflammatory manifestations of CF but for other inflammatory diseases too. We hypothesise that TOM1 may play an anti-inflammatory role in the CF lung and postulate that its increased expression may be an attempt to compensate for the high pro-inflammatory burden in this condition and support this hypothesis by demonstrating significantly higher expression of TOM1 *in vivo* in bronchial brushings from CF versus non-CF individuals. The CF lung is a highly pro-inflammatory milieu.

Bronchial epithelial cells are continuously exposed to multiple pro-inflammatory factors including neutrophil elastase, bacterial lipopeptides, LPS, flagellin and uCpG DNA, amongst others (1). Furthermore ER stress associated with accumulation of misfolded CFTR is also likely to activate additional pro-inflammatory pathways (32). Interestingly we have shown that ER stress actually contributes to down-regulation of miR-126 with concomitant up regulation of TOM1. Our studies show that in CF bronchial epithelial cells TOM1 provides a strong anti-inflammatory signal which we believe represents a possible compensatory mechanism of dealing with the chronic inflammation evident in CF. *In vivo* however TOM1's anti-inflammatory effect, whilst important may be overwhelmed due to the highly pro-inflammatory nature of the CF lung.

TOM1 is known to form a complex with Tollip and together they are responsible for the transport of ubiquitinated proteins to the endosome for degradation (33). IL-1RI is a substrate of the TOM1-Tollip complex and its passage to the endosome is facilitated by this complex (9). In a study by Hauber *et al* comparison of TLR4 levels in endobronchial biopsies from CF patients and healthy controls revealed a significant reduction of TLR4 and to a lesser extent TLR2 in the CF samples (34). In a more recent study CFBE41o$^-$ cells have also been shown to exhibit lower surface expression of TLR4 compared to a CFTR corrected counterpart (wild-type CFTR plasmid transfectant). The authors suggest this may contribute to the aberrant immune response evident in CF resulting in chronic bacterial infection of the CF airway due to decreased IL-8 secretion as a result of diminished TLR4 expression followed by delayed neutrophil chemotaxis (35). We show that miR-126 may be regulated by ER stress and in CF airway epithelial cells lower levels of miR-126 are concomitant with increased TOM1 expression. Thus, it may be that the TOM1-Tollip

complex, in addition to regulating IL-1RI expression also modulates cell surface expression of TLR2/4 by a similar mechanism adding to this impaired immune response.

Currently one of the burning questions in this field is 'what regulates miRNAs?' Expression of miR-146a, for example, is driven by NF-κB (36, 37). We evaluated the effect of LPS and IL-1β on induction of miR-126 in both CF and non-CF bronchial epithelial cell lines however no significant changes were observed with either stimuli. However, in human bronchial epithelial cells stimulated with the ER stress inducing agonist thapsigargin we saw a marked reduction in miR-126 expression after 24h. There was no effect on miR-126 expression levels at 4h and similarly $CFTR_{inh}$-172 did not have any effect on miR-126 in these cells suggesting that miR-126 may be regulated by chronic ER stress as seen in CF. Harris *et al* reported TNF-α had no effect on expression of miR-126 in a recent study investigating regulation of VCAM1 by miR-126 (6). However, similar to our study they proposed a regulatory role for miR-126 in inflammation, specifically in the vasculature. Saito *et al* have recently reported that down-regulation of miR-126 can be induced by inhibitors of DNA methylation and histone deacetylation (12).

Notwithstanding the current gaps in our knowledge it is clear that miRNAs have an important role in the regulation of innate immunity (28, 30, 38-41). For example miR-181a and miR-223 are implicated in establishing and maintaining the cell fate of immune cells (42), miR-146 is involved in innate immunity by regulating TLR signaling (37), Rodriguez *et al* highlighted the importance of miR-155 in maintenance of a normal immune response (43) whilst miR-181a has a role in regulation of T cell receptor signaling (44). Altered expression of miR-203 and miR-146 has been shown

in the chronic inflammatory skin condition psoriasis suggesting their involvement in immune-mediated diseases (45). In light of our findings it is appropriate to add miR-126 to the growing list of miRNAs with a role in fine-tuning of innate immune responses.

In summary, we describe for the first time miRNA involvement within CF. In particular we identify altered expression of miR-126, a negative regulator of TOM1, in bronchial epithelial cells. Whilst miR-126 is decreased in CF bronchial epithelium *in vivo* making it difficult to target, identifying miRNAs that are over-expressed in CF airway epithelium could lead to therapeutic targets which can be manipulated more easily. However, we believe these findings have important implications regarding regulation of innate immune responses in the CF lung which may impact on anti-inflammatory therapies currently under investigation for CF and help lead to better management of the disease. Future work on other differentially expressed miRNAs identified in our expression profiling studies will likely yield additional new therapeutic targets for CF.

***Acknowledgements***

We are grateful to Dr K. Nakayama (Kyoto University) for providing us with the pTOM1-Myc vector, Prof. D. Gruenert (University of Vermont) for the CFTE29o$^-$, CFBE41o$^-$, 16HBE14o$^-$ and cell lines and Dr. S. Smith (Trinity College Dublin) for help with the sequencing of pMIR-TOM1-3'UTR.

**Figure legends**

**FIGURE 1** miRNA expression profiling and miR-126 expression *in vivo*. ***A*** Expression levels of miR-126, miR-16 and mir-491-5p in bronchial brushings used in TLDAs (CF; n=5 and control; n=5). Data are represented as fold change compared to normalized controls. ***B*** miR-126 expression in bronchial brushings from individuals with and without CF (n=6 and n=13 respectively) measured by qRT-PCR. Relative expression levels determined by qRT-PCR were normalized to miR-16. Data are represented as mean ± SEM and were compared by *t* test. All qRT-PCR experiments were performed in triplicate and included no-template controls.

**FIGURE 2** miR-126 expression *in vitro*. ***A*** miR-126 expression levels were assessed in the CF tracheal airway epithelial cell line CFTE29o$^-$ compared to its non-CF counterpart 9HTEo$^-$, ***B*** in the CF bronchial epithelial cell line CFBE41o$^-$ compared to its non-CF counterpart 16HBE14o$^-$ and ***C*** in the bronchial airway epithelial cell line 16HBE14o$^-$ and non-lung cell lines THP-1, U937, HEK293, HepG2 and U373. The relative expression levels were determined by qRT-PCR using Taqman miRNA assays and normalized to miR-16. Data are represented as mean ± SEM and were compared by *t* test. All qRT-PCR experiments were performed in triplicate and included no-template controls.

**FIGURE 3** miR-126 regulation by ER stress. 16HBE14o$^-$ cells were treated with DMSO (vehicle control), thapsigargin (1µM) or $CFTR_{inh}$-172 (5µM and 10µM) for 24 h. Data is represented as mean ± SEM and was compared by *t* test; data is representative of three experiments.

**FIGURE 4** miR-126 targets the 3'UTR of TOM1. ***A*** *In silico* analysis of the miRNA target prediction database RNA Hybrid shows predicted pairing between TOM1 3'UTR and miR-126. The 7-mer seed region is shown between the black arrows. ***B*** Highly conserved predicted binding site for miR-126 within the 3'-UTR of TOM1 from interrogation of the Targetscan target prediction database (version 4.2) (Mm, *Mus musculus*; Rn, *Rattus norvicus*; Cf, *Canis familiaris*; Hs, *Homo sapiens*). ***C*** TOM1 mRNA expression in CFTE29o$^-$, 16HBE14o$^-$, CFBE41o$^-$ , 9HTEo$^-$, human lung RNA (Applied Biosystems) and no-template control (NTC) assessed by semi-quantitative RT-PCR. ***D*** TOM1 expression (both *in vitro* and *in vivo),* relative to β-actin in CFBE41o$^-$ versus 16HBE14o$^-$ cell lines measured by qRT-PCR. ***E*** Tollip expression relative to β-actin in CFBE41o$^-$ versus 16HBE14o$^-$ cell lines measured by qRT-PCR.

**FIGURE 5** miR-126 directly targets TOM1. Relative luciferase activity in HEK293293 cells ($1x10^5$ in triplicate) transiently transfected with pMIR-TOM1-3'UTR and pRLSV40 and co-transfected with a synthetic pre-miR-126. Firefly luciferase activity was normalized to the *Renilla* luciferase activity. Data are represented as mean ± SEM and were compared by *t* test; data is representative of three experiments. All qRT-PCR experiments were performed in triplicate and included no-template controls.

**FIGURE 6** Effects of pre-miR-126 over-expression. ***A*** TOM1 protein was analyzed by Western blot (n=3) using anti-TOM1 and anti β-actin antibodies in cell lysates from non-transfected control (NT), scrambled control (Scr) and pre-miR126

transfected CFBE41o$^-$ cells. ***B*** Representative densitometry of TOM1 relative to β-actin.

**FIGURE 7** Effects of TOM1 over-expression. CFBE41o$^-$ cells ($1x10^5$ in triplicate) were co-transfected with an empty vector (pCDNA3) or a TOM1 over-expression plasmid (pTOM1-Myc), an inducible NF-κB (firefly) luciferase reporter plasmid, and pRLSV40. Following incubation for 42 h, cells were stimulated with LPS (1μg/ml) or IL-1β (10ng/ml) for 6 h. Lysates were prepared using reporter lysis buffer (Promega). Luciferase production from both plasmids was quantified by luminometry using specific substrates. Relative NF-κB luciferase activity is shown. Data shown is representative of three experiments (*, $p < 0.05$ LPS/IL-1β vs. control (pCDNA3), #, $p < 0.05$ LPS and IL-1β vs. pTOM1-Myc).

**FIGURE 8** Effects of TOM1 knockdown. ***A*** Expression of GAPDH and TOM1 mRNA levels relative to scrambled control (Scr) following transfection of CFBE41o$^-$ cells with scrambled, GAPDH or TOM1 siRNAs. ***B*** TOM1 protein was analyzed by Western blot (n=3) using anti-TOM1 and anti β-actin antibodies following transfection of CFBE41o$^-$ cells with scrambled, GAPDH or TOM1 siRNAs. ***C*** Cells were stimulated with LPS (10μg/ml), IL-1β (10ng/ml) or lipopeptide (Pam3, 10μg/ml) 24 h post transfection for a further 6 h. IL-8 secretion in supernatants was measured by ELISA. Data are represented as mean ± SEM and were compared by *t*-test; data is representative of three experiments.

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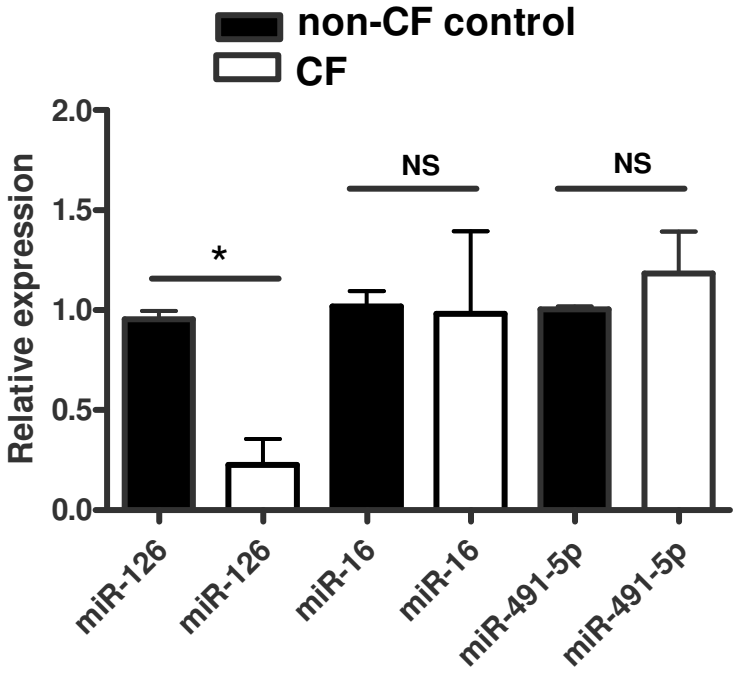
**Table 1.** miRNA differentially expressed in at least 3 of 5 CF vs. non CF bronchial brushing samples

| Down-regulated | Up-regulated |
|---|---|
| hsa-let-7b | hsa-miR-26b |
| hsa-let-7c | hsa-miR-27a* |
| hsa-miR-7 | hsa-miR-93* |
| hsa-miR-15a* | hsa-miR-101 |
| hsa-miR-16-1* | hsa-miR-127-3p |
| hsa-miR-17 | hsa-miR-138 |
| hsa-miR-20b | hsa-miR-140-5p |
| hsa-miR-222 | hsa-miR-142-3p |
| hsa-miR-23b | hsa-miR-142-5p |
| hsa-miR-29c | hsa-miR-143 |
| hsa-miR-29c* | hsa-miR-145 |
| hsa-miR-30a | hsa-miR-193a-5p |
| hsa-miR-30a* | hsa-miR-199a-3p |
| hsa-miR-30d | hsa-miR-212 |
| hsa-miR-30d | hsa-miR-221 |
| hsa-miR-31 | hsa-miR-223 |
| hsa-miR-34a | hsa-miR-223* |
| hsa-miR-34a* | hsa-miR-340 |
| hsa-miR-95 | hsa-miR-340* |
| hsa-miR-106a | hsa-miR-365 |
| hsa-miR-125a-5p | hsa-miR-374a |
| hsa-miR-126 | hsa-miR-374b |
| hsa-miR-126* | hsa-miR-425* |
| hsa-miR-130a | hsa-miR-450a |
| hsa-miR-135b | hsa-miR-483-5p |
| hsa-miR-140-3p | hsa-miR-494 |
| hsa-miR-141 | hsa-miR-526b* |
| hsa-miR-144* | hsa-miR-597 |
| hsa-miR-146a | hsa-miR-601 |
| hsa-miR-150 | hsa-miR-610 |
| hsa-miR-151-3p | hsa-miR-629* |
| hsa-miR-152 | hsa-miR-632 |
| hsa-miR-190 | hsa-miR-801 |
| hsa-miR-193b | hsa-miR-877 |
| hsa-miR-200a* | hsa-miR-886-3p |
| hsa-miR-200b | hsa-miR-886-5p |
| hsa-miR-200b* | |
| hsa-miR-200c | |
| hsa-miR-203 | |
| hsa-miR-205 | |
| hsa-miR-320 | |
| hsa-miR-324-3p | |
| hsa-miR-361-5p | |
| hsa-miR-362-5p | |
| hsa-miR-378 | |
| hsa-miR-429 | |
| hsa-miR-451 | |
| hsa-miR-452 | |
| hsa-miR-502-3p | |
| hsa-miR-532-3p | |
| hsa-miR-532-5p | |
| hsa-miR-565 | |
| hsa-miR-574-3p | |
| hsa-miR-598 | |
| hsa-miR-671-3p | |
| hsa-miR-708 | |
| hsa-miR-769-5p | |

FIGURE 1

A


non-CF control
CF
Relative expression
2.0
1.5
1.0
0.5
0.0
*
NS
NS
miR-126
miR-126
miR-16
miR-16
miR-491-5p
miR-491-5p

B

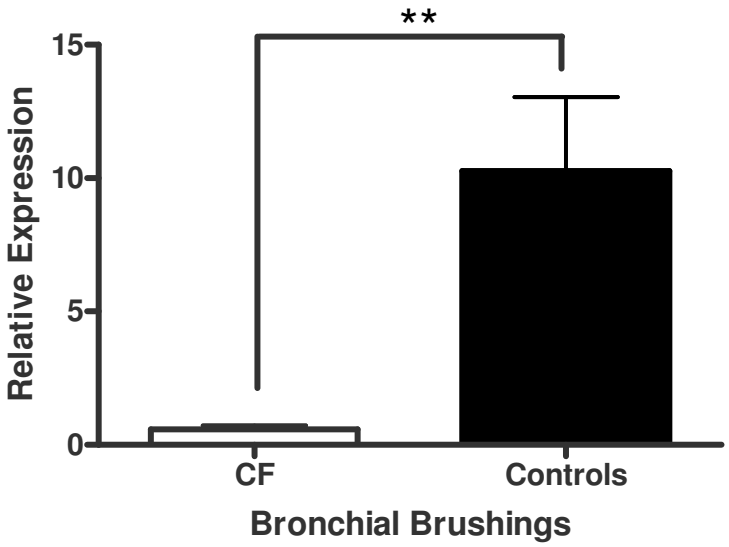
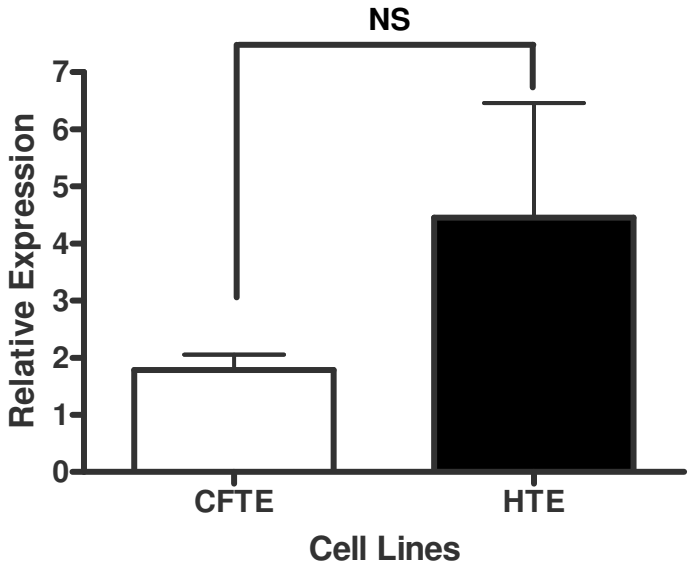
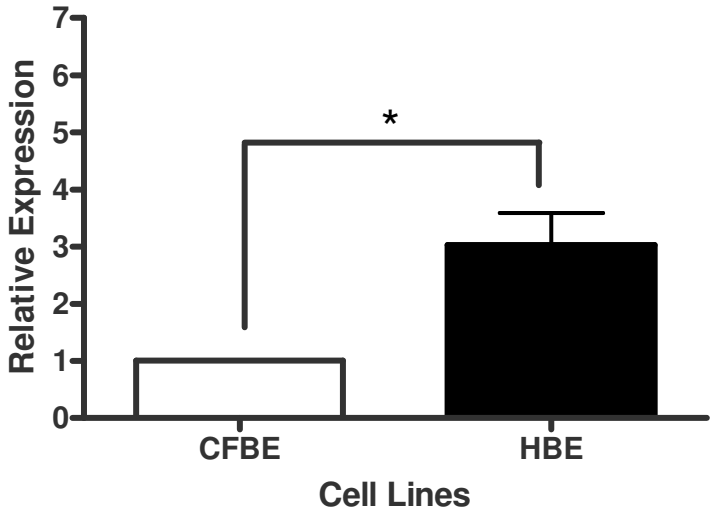

**
Relative Expression
15
10
5
0
CF
Controls
Bronchial Brushings

FIGURE 2

A


NS
Relative Expression
0
1
2
3
4
5
6
7
CFTE
HTE
Cell Lines

B


*
Relative Expression
0
1
2
3
4
5
6
7
CFBE
HBE
Cell Lines

C

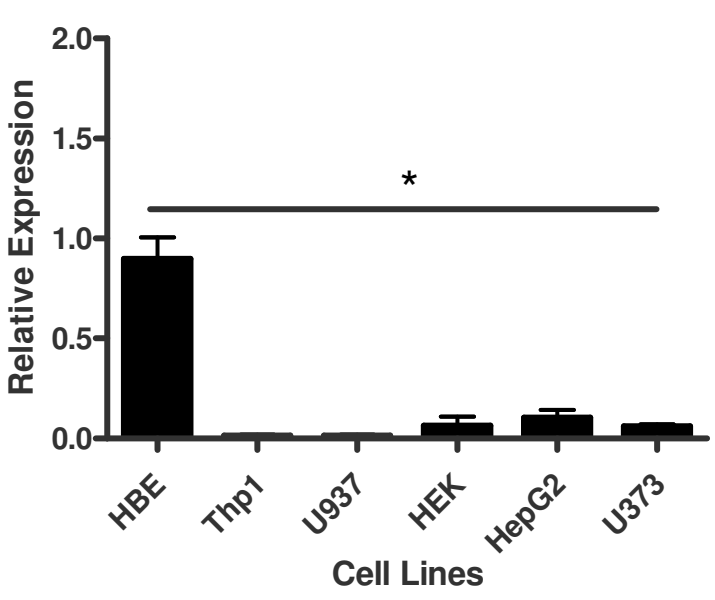
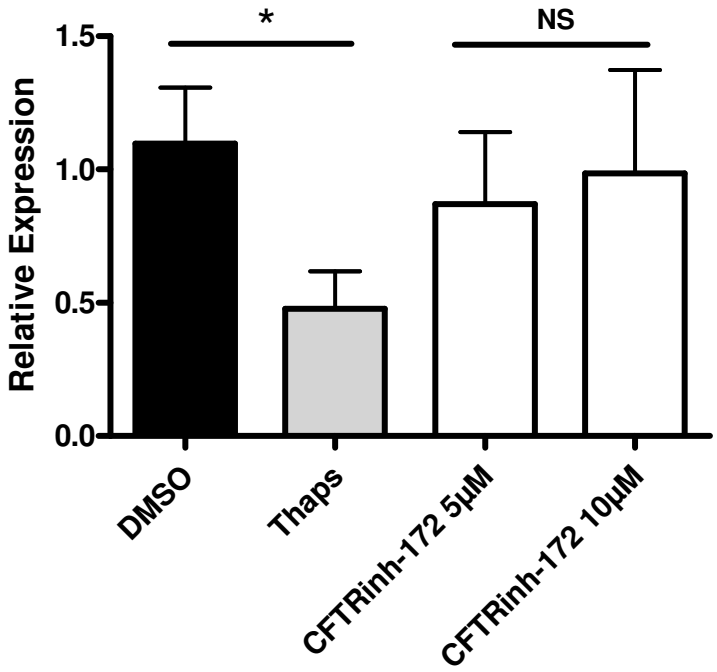
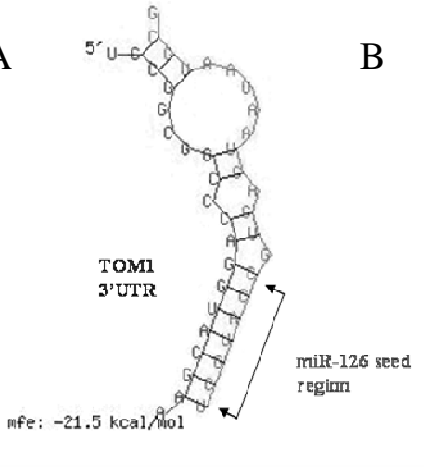

*
Relative Expression
0.0
0.5
1.0
1.5
2.0
HBE
Thp1
U937
HEK
HepG2
U373
Cell Lines

FIGURE 3


*
NS
1.5
1.0
0.5
0.0
Relative Expression
DMSO
Thaps
CFTRinh-172 5μM
CFTRinh-172 10μM

A




B

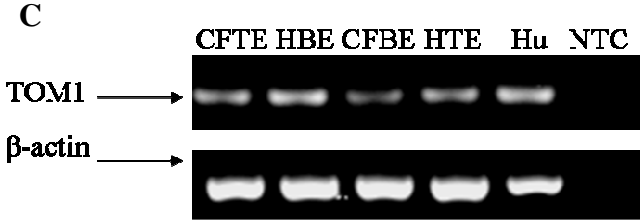
TOM1 3′ UTR

5′ ……530……539…… 3′

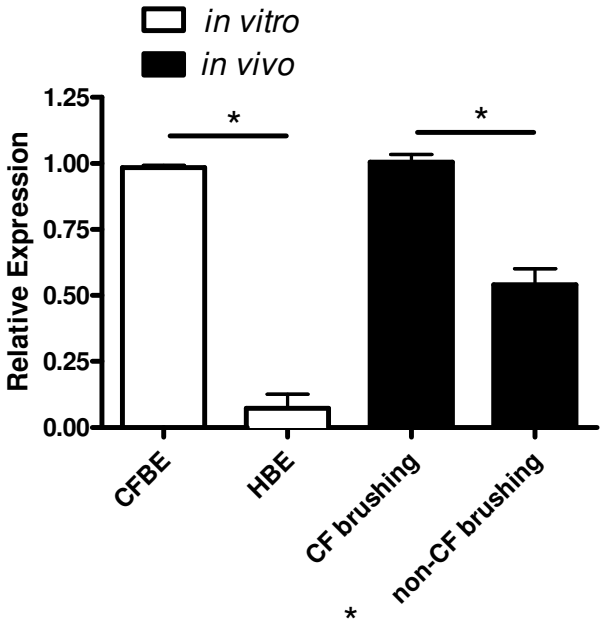
Mm ...GCUGCCCACUGUCCU**GGUACGA**AACU...
Rn ...GCUGCCCACUGUCCA**GGUACGA**AGCU...
Cf ...GCUGCCCGCUCUCCA**GGUACGA**AACU...
Hs ...AGCUGCGGCGGCCCA**GGUACGA**A...
hsa-miR-126 CGUAAUAAUGAGUG**CCAUGCU**

3′ 5′

C




D




E

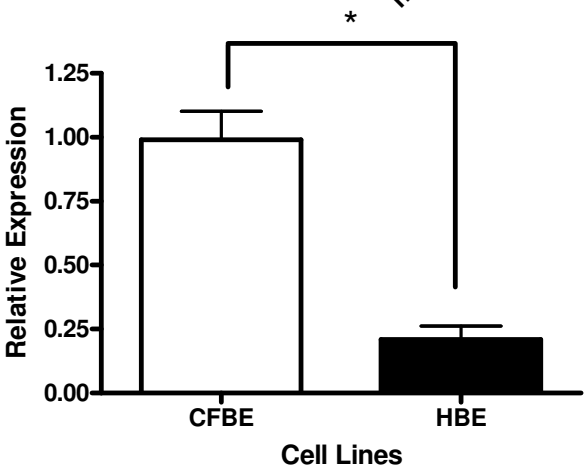


FIGURE 5

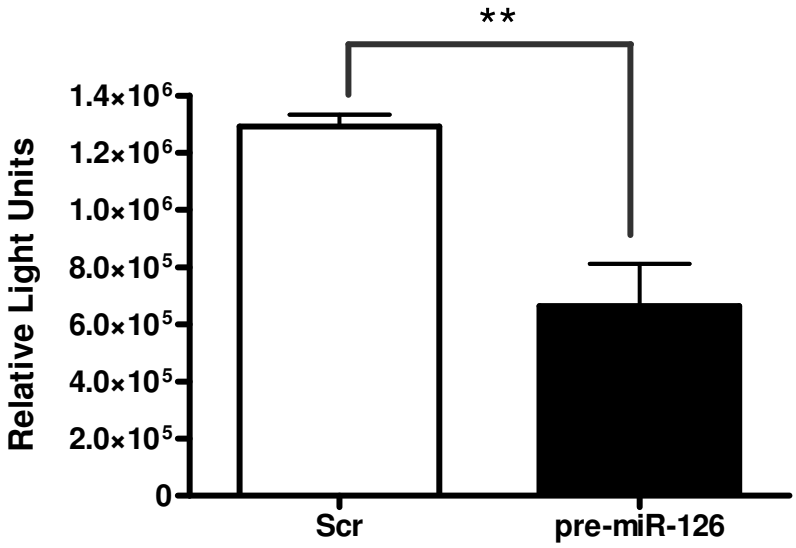
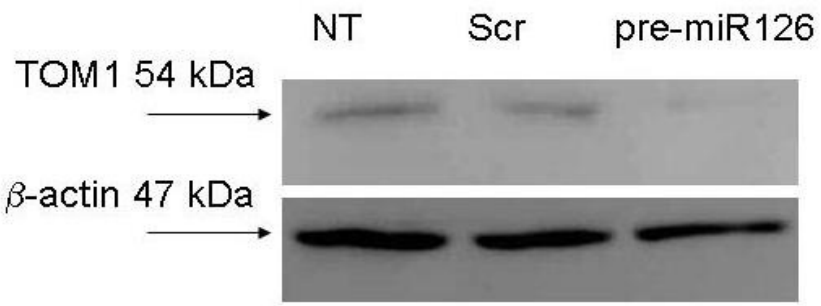

**
Relative Light Units
1.4×10^6
1.2×10^6
1.0×10^6
8.0×10^5
6.0×10^5
4.0×10^5
2.0×10^5
0
Scr
pre-miR-126

FIGURE 6

A


NT
Scr
pre-miR126
TOM1 54 kDa
β-actin 47 kDa

B

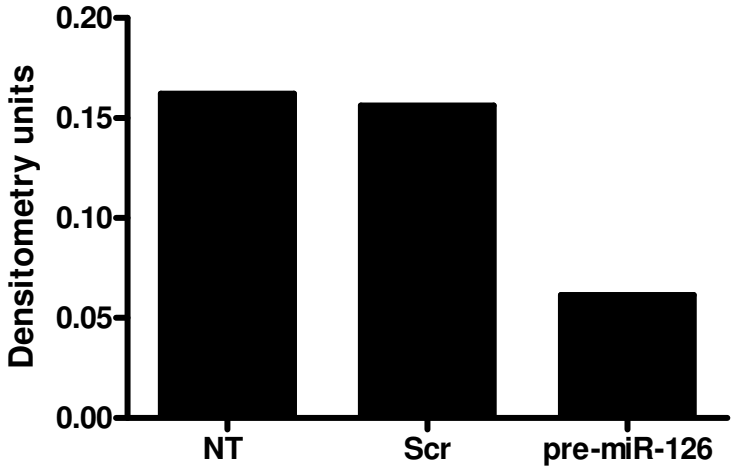

Densitometry units
0.20
0.15
0.10
0.05
0.00
NT
Scr
pre-miR-126

FIGURE 7

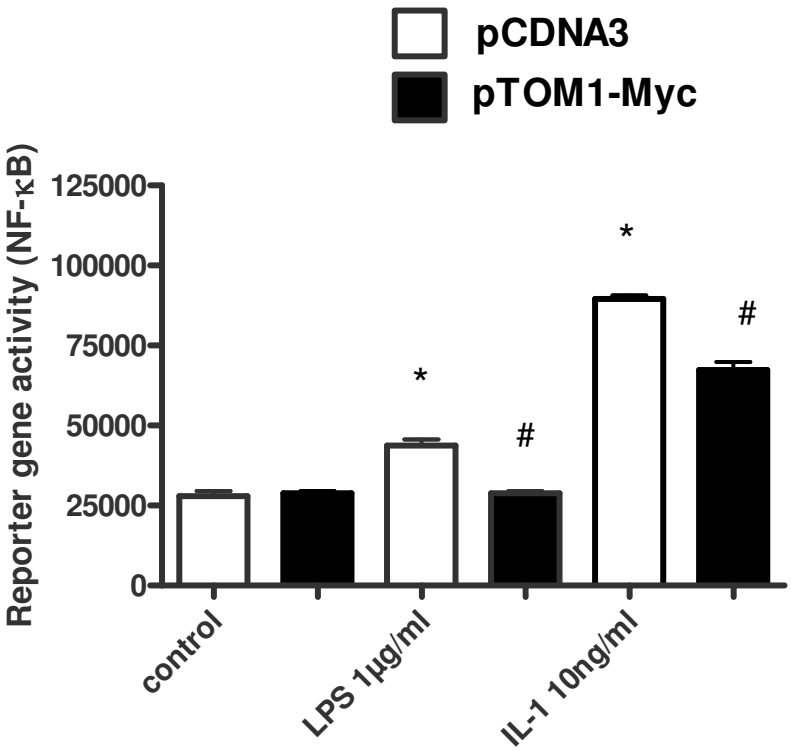
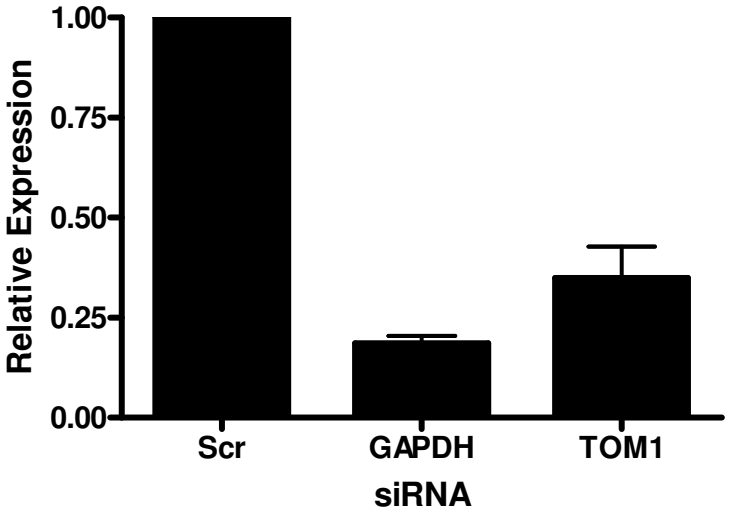
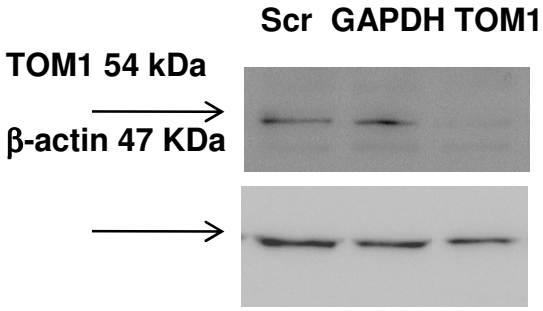

pCDNA3
pTOM1-Myc
Reporter gene activity (NF-κB)
125000
100000
75000
50000
25000
0
*
#
*
#
control
LPS 1µg/ml
IL-1 10ng/ml

FIGURE 8

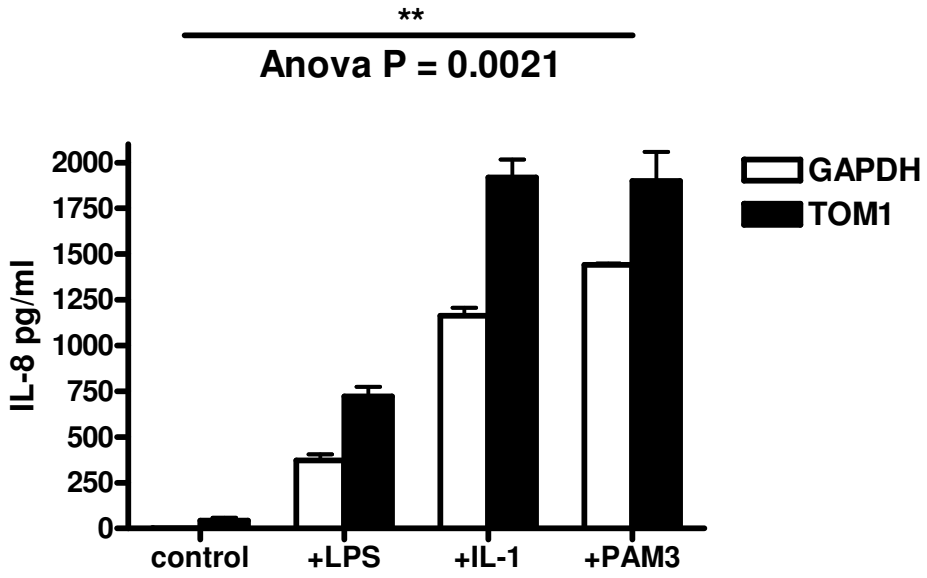
A


1.00
0.75
0.50
0.25
0.00
Relative Expression
Scr
GAPDH
TOM1
siRNA

B


Scr GAPDH TOM1
TOM1 54 kDa
β-actin 47 KDa

C


**
Anova P = 0.0021
2000
1750
1500
1250
1000
750
500
250
0
IL-8 pg/ml
control
+LPS
+IL-1
+PAM3
GAPDH
TOM1