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microRNA regulation of expression of the cystic fibrosis transmembrane conductance regulator gene

Austin E. Gillen, Nehal Gosalia, Shih-Hsing Leir, and Ann Harris¹

Human Molecular Genetics Program, Children's Memorial Research Center, Chicago, IL 60614, U.S.A., and Department of Pediatrics, Northwestern University Feinberg School of Medicine, Chicago, IL 60611, U.S.A.

Abstract

The *CFTR* (cystic fibrosis transmembrane conductance regulator) gene shows a complex temporal and spatial pattern of expression that is controlled by multiple *cis*-acting elements interacting with the basal promoter. Although significant progress has been made towards understanding these genomic elements, there have been no reports of post-transcriptional regulation of *CFTR* by miRNAs (microRNAs). In the present study, we identify two miRNAs, hsa-miR-145 and hsa-miR-494, which regulate *CFTR* expression by directly targeting discrete sites in the *CFTR* 3' UTR (untranslated region). We show that at least 12 miRNAs are capable of repressing endogenous *CFTR* mRNA expression in the Caco-2 cell line. Ten of these also inhibit expression of a reporter construct containing the *CFTR* 3' UTR in one or more cell lines, and five repress endogenous *CFTR* protein expression in Caco-2 cells. Moreover, at least three are expressed in primary human airway epithelial cells, where *CFTR* expression is maintained at low levels in comparison with intestinal cell lines. Three of the miRNAs that target *CFTR*, hsa-miR-384, hsa-miR-494 and hsa-miR-1246, also inhibit expression of a reporter carrying the Na⁺ – K⁺ – Cl[–] co-transporter *SLC12A2* [solute carrier family 12 (Na⁺ – K⁺ – Cl[–] transporters), member 2] 3' UTR, suggesting that these miRNAs may play a more general role in regulating chloride transport in epithelial cells.

Keywords

cystic fibrosis transmembrane conductance regulator (*CFTR*); microRNA (miRNA); solute carrier family 12 (Na⁺ – K⁺ – Cl[–] transporters); member 2 (*SLC12A2*); post-transcriptional regulation

INTRODUCTION

CF (cystic fibrosis), the most common lethal autosomal recessive disorder in Caucasians, results from mutation of the *CFTR* (CF transmembrane conductance regulator) gene [1]. The *CFTR* locus spans 189 kb of DNA and includes 27 exons, which encode a 6.2 kb mRNA transcript, including a 1.5 kb 3' UTR (untranslated region). The 1480-amino-acid *CFTR*

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¹To whom correspondence should be addressed (ann-harris@northwestern.edu).

AUTHOR CONTRIBUTION

Austin Gillen and Ann Harris designed the experiments and analysed the data. Austin Gillen, Nehal Gosalia and Shih-Hsing Leir performed the experiments. Austin Gillen and Ann Harris wrote the paper.

protein is a member of the ABC (ATP-binding cassette) family of transporters and functions primarily as a chloride channel. The gene is expressed at low levels in specialized epithelial cells of the airway, and at much higher levels in pancreatic duct, intestinal and male genital duct epithelium. The gene is also expressed in some non-epithelial cells, and may be involved in a wide range of cellular processes. *CFTR* expression is subject to temporal regulation during development of the airway, with high expression in fetal lung and low expression in adult airway, except in the serous portion of submucosal glands and some ciliated cells of the surface epithelium. Despite this complex regulation of expression (reviewed in [2]), the *CFTR* promoter lacks key cell-type-specific control elements, implicating elements elsewhere in temporal and tissue-specific regulation.

Indeed, several regulatory elements have been identified both flanking the locus and within introns (reviewed in [2,3]). Enhancer-blocking insulators were found both proximal and distal to the coding region of *CFTR* [4,5], and several cell-type-specific enhancers were characterized within introns that co-ordinate looping of the active locus [6–9]. Although these *cis*-acting genomic elements are likely to be the primary regulators of *CFTR* expression, post-transcriptional regulation of the *CFTR* mRNA may also be important. In addition to alternative splicing, which is known to influence *CFTR* expression levels (reviewed in [10]), targeting of the *CFTR* transcript by miRNAs (microRNAs) may have a post-transcriptional regulatory role. These short (~22 nt) endogenously expressed non-coding RNAs generally suppress gene expression in mammals by binding to the 3' UTR of transcripts and blocking translation or promoting degradation of the transcript by decapping or deadenylation (reviewed in [11]). In addition to suppression, miRNAs may also up-regulate expression, although this mechanism appears to involve binding in the 5' UTR rather than the 3' UTR of the transcript. miRNAs are predicted to regulate more than 50% of mammalian mRNAs post-transcriptionally [11], and there is evidence that 3' UTR length strongly correlates with miRNA regulation [12]. In this context, it may be relevant that *CFTR* has a long (1.5 kb) 3' UTR in comparison with the 740 bp average for human genes.

To date, several groups have examined the potential role of miRNAs in molecular pathways involved in the pathogenesis of CF, including lung inflammation [13,14]; however, despite its potential value as a therapeutic target, miRNA regulation of *CFTR* itself has not been investigated. An hypoxia-induced increase in expression of a group of miRNAs predicted to target *CFTR* correlates with hypoxia-induced down-regulation of *CFTR* expression, but no direct targeting of *CFTR* has been reported [15]. In the present paper, we describe the identification of several miRNAs which directly repress *CFTR* expression by binding to the 3' UTR of the transcript and are expressed in relevant primary human epithelial cells. Moreover, we demonstrate that three of these miRNAs also target the Na⁺–K⁺–Cl[–] co-transporter *SLC12A2* [solute carrier family 12 (Na⁺–K⁺–Cl[–] transporters), member 2], implying a more general role in co-ordinating chloride transport in epithelial cells.

EXPERIMENTAL

Cell culture

The human colon carcinoma cell line Caco-2 [16], bronchial epithelial cell line 16HBE14o- [17] and pancreatic adenocarcinoma cell line PANC-1 [18] were grown by standard

methods. Primary HTEs (human tracheal cells) and NHBEs (normal human bronchial and tracheal epithelial cells; Lonza) were grown as described previously [19].

qRT-PCR (quantitative reverse transcription PCR)

Total RNA for qRT-PCR was isolated using TRIzol[®] (Invitrogen). *CFTR* expression was assayed using a TaqMan probe and primer set spanning exons 5 and 6 (TAQEX5/6) [7]. *SLC12A2* expression was measured using a primer set spanning exons 13 and 14 [20]. miRNA expression was assayed using TaqMan miRNA assays (Applied Biosystems) according to the manufacturer's protocols. 18S rRNA (*CFTR/SLC12A2*) and U6 snRNA (small nuclear RNA) (miRNAs) were used as endogenous normalization controls.

Western blot analysis

CFTR expression was assayed by Western blot analysis using the anti-CFTR antibody 570 [21]. Cells were lysed in RIPA buffer with 1% Triton X-100, using previously published protocols [22]. Cell lysates were separated by SDS/PAGE (3%/6% gels) and transferred on to Immobilon membranes (Millipore). Western blot quantification was performed using ImageJ software (NIH; <http://rsb.info.nih.gov/ij/>).

Luciferase reporter constructs

The *CFTR* 3' UTR (chr7:117307180–117308839; hg19) was amplified by PCR from pCOF [24] and cloned into the MCS (multiple cloning site) of the 3' UTR luciferase reporter vector pMIR-REPORT luciferase (Ambion) to create pMIR-CFTR. Mutant variants of pMIR-CFTR were created using the QuikChange[®] Lightning Site-Directed Mutagenesis Kit or Lightning Multi Site-Directed Mutagenesis Kit (Agilent). The *SLC12A2* 3' UTR (chr5:127522279–127525419; hg19) was amplified from human genomic DNA using Pfu DNA polymerase (Agilent) and cloned into the MCS of pMIR-REPORT luciferase to create pMIR-SLC12A2.

Transient transfections

For evaluating the effects of miRNAs on endogenous *CFTR* mRNA or protein expression, cells were reverse-transfected with 20 nM (final concentration) of Pre-miR[™] miRNA mimics (Ambion) using Lipofectamine[™] RNAiMAX (Invitrogen). For luciferase reporter assays, cells were co-transfected 48 h after plating with 200 ng/ml luciferase reporter (e.g. pMIR-CFTR), 200 ng/ml pMIR-REPORT β -galactosidase (Ambion) and 20 nM (final concentration) Pre-miRs using Lipofectamine[™] 2000 (Invitrogen). Assays were performed 48 h post-transfection. All data were normalized to transfection with the Pre-miR[™] Negative Control #2 (Ambion).

Primer sequences

Primer sequences and genomic locations used for cloning and mutagenesis are shown in Supplementary Table S1 at <http://www.BiochemJ.org/bj/438/bj4380025add.htm>

RESULTS

The 3' UTR of the *CFTR* transcript is approximately 1.5 kb and so is a plausible candidate for regulation by miRNAs. We first used an *in silico* approach to search for predicted miRNA seed sites within the *CFTR* 3' UTR.

Multiple miRNAs are predicted to target the *CFTR* 3' UTR

TargetScan (version 5.0) [25] was used to predict miRNAs that bind to the *CFTR* 3' UTR since it is reported to be the most accurate algorithm currently available for *in silico* prediction of miRNAs that may bind to a gene, and does not rely exclusively on site conservation [26]. The 106 miRNAs predicted to target the *CFTR* 3' UTR were ranked according to context score (the TargetScan raw binding affinity score, a measure of the affinity of a miRNA for a specific DNA sequence), and context score percentile (a measure of the context score of a predicted miRNA target site in *CFTR* relative to that of other predicted targets). The top 11 candidate miRNAs by context score are shown in Table 1 and the location of the predicted binding sites in the *CFTR* 3' UTR for each are shown in Figure 1. Also shown are hsa-miR-101, the only highly conserved miRNA with a conserved target predicted by a previous version of TargetScan (version 4.0), and hsa-miR-939, for which sites are predicted in the 3' and 5' UTRs of *CFTR*, and thus may form a miRNA bridge [27].

Since *CFTR* shows a complex pattern of cell-type-specific expression, with high expression in intestinal, pancreatic duct and epididymis epithelial cells, and low expression in the airway epithelium, we next evaluated previously published human expression data for the 13 candidates [28–34] (Table 1). Two candidate miRNAs, hsa-miR-600 and hsa-miR-607, were identified in colorectal epithelial cells, either the Caco-2 colon carcinoma cell line (hsa-miR-607) or primary normal colonic epithelium (hsa-miR-600) [32], hsa-miR-1246 and hsa-miR-1290 were identified in human embryonic stem cells [28], and hsa-miR-1827 was identified in HeLa cervical carcinoma cells [29]. hsa-miR-101, hsa-miR-145, hsa-miR-331-3p, hsa-miR-376a/b, hsa-miR-377, hsa-miR-494 and hsa-miR-939 are expressed in a wide range of cell types, with hsa-miR-101 expression highest in lymphocytes and hsa-miR-145 expression highest in the prostate, uterus and epididymis [30,31,33]. No human expression data are available for hsa-miR-384. This is likely to be due to the exceptionally low melting temperature of this miRNA making detection difficult [35].

miRNAs predicted by TargetScan repress endogenous *CFTR* expression

To investigate whether the TargetScan-predicted miRNAs influence *CFTR* mRNA and/or *CFTR* protein expression, miRNA mimics [dsRNAs (double-stranded RNAs) which are processed like endogenous pre-miRNAs] were transfected into Caco-2 cells. After 48 h, total RNA and protein were isolated in separate experiments and the effect of the miRNAs on *CFTR* mRNA and protein levels were assayed by qRT-PCR (Figure 2A) and Western blot analysis with anti-*CFTR* antibody 570 (Figure 2B) respectively. Twelve out of the 13 predicted miRNAs repressed *CFTR* mRNA expression by at least 40% relative to a negative control miRNA. The sole exception was hsa-miR-101, which has the second weakest TargetScan context score among the miRNAs assayed. Only hsa-miR-939 has a lower context score, but this does not account for the predicted site in the 5' UTR, which may

make a functional contribution [27]. Consistent with the qRT-PCR data, hsa-miR-145, hsa-miR-331-3p, hsa-miR-384, hsa-miR-494 and hsa-miR-1290 individually decreased CFTR protein levels by 30–60%, whereas hsa-miR-101 had no effect (Figure 2B). A representative Western blot is shown in Supplementary Figure S1 at <http://www.BiochemJ.org/bj/438/bj4380025add.htm>. Transfection of multiple miRNAs did not enhance knockdown of either protein or mRNA in Caco-2 cells relative to the transfection of individual miRNAs (results not shown).

miRNAs that repress *CFTR* expression target the 3' UTR

To determine whether miRNAs that repress endogenous *CFTR* expression act directly through the *CFTR* 3' UTR, a series of luciferase reporters were generated in pMIR REPORT (Figure 3A). pMIR-*CFTR* contains the complete wild-type 1.5 kb *CFTR* 3' UTR; pMIR-*CFTR*mut145 and pMIR-*CFTR*mut494 carry mutations in the predicted hsa-miR-145 and hsa-miR-494 target sites respectively within this 1.5 kb (Supplementary Table S1). pMIR-*CFTR*, pMIR-REPORT β -galactosidase [CMV (cytomegalovirus) β -galactosidase transfection control] and miRNA mimics were co-transfected into Caco-2 colon carcinoma cells and PANC-1 pancreatic adenocarcinoma cells. Several miRNAs acted directly on the *CFTR* 3' UTR in this assay (Figures 3B and 3C). hsa-miR-384 and hsa-miR-494 each repressed luciferase expression by >30% in both cell lines ($P < 0.001$). hsa-miR-145 and hsa-miR-331-3p also significantly inhibited reporter expression in both cell lines ($P < 0.001$), but more effectively in Caco-2 (>30% repression) than PANC-1 (10–20%) cells. hsa-miR-1246 produced significant knockdown in PANC-1 cells ($P < 0.001$), but was less effective in Caco-2 cells ($P < 0.05$). hsa-miR-1290- and hsa-miR-1827-dependent repression was significant in PANC-1 ($P < 0.001$), but not in Caco-2, cells, whereas hsa-miR-376b showed the opposite result. Five out of the 11 miRNAs that caused *CFTR* mRNA inhibition were also assayed in 16HBE14o- bronchial epithelial cells (Figure 3D) and hsa-miR-384 and hsa-miR-494 again caused significant knockdown ($P < 0.001$). hsa-miR-600 and hsa-miR-607 targeted *CFTR* in 16HBE14o- cells, but were ineffective in the other cell lines.

To confirm that several of these miRNAs were acting directly through their predicted seed sites in the *CFTR* 3' UTR, we next evaluated the effect of mutation of the TargetScan-predicted binding sites for hsa-miR-145 and hsa-miR-494. pMIR-*CFTR*mut145 and pMIR-*CFTR*mut494 were co-transfected with pMIR-REPORT β -galactosidase and miRNA mimics into Caco-2 and PANC-1 cells. Mutation of the miR-145 and miR-494 seed sites significantly reduced repression of reporter expression in comparison with the wild-type sequence in Caco-2 cells ($P = 0.001$) (Figure 3E), thus confirming the functional importance of these sites. Repression by another miRNA (hsa-miR-384) was unaffected in pMIR-*CFTR*mut145 and pMIR-*CFTR*mut494.

miRNA-145 and miRNA-331-3p are differentially expressed in Caco-2, 16HBE14o-, PANC-1 and primary human airway epithelial cells

To investigate the relative expression levels of each miRNA in the cell lines employed in the present study, we used TaqMan miRNA assays for hsa-miR-101, hsa-miR-145, hsa-miR-331-3p, hsa-miR-384 and hsa-miR-494. Expression profiles for Caco-2, 16HBE14o- and PANC-1 cells are shown in Figure 4(A), together with HT29, a human colorectal

carcinoma cell line included in the NCI-60 cancer cell panel, for comparison with published NCI-60 miRNA expression data [36,37]. There was no significant difference between Caco-2, PANC-1 and 16HBE14o- cells in the expression levels of hsa-miR-101 or hsa-miR-494, but expression of hsa-miR-101 was significantly higher in HT29 ($P < 0.001$) than in any other cell line. Levels of hsa-miR-145 were significantly higher in Caco-2 cells ($P < 0.001$) than in the other cell lines, whereas hsa-miR-331-3p levels were significantly higher in 16HBE14o- than in Caco-2 ($P < 0.01$) or PANC-1 ($P < 0.001$) cells. No hsa-miR-384 expression was detected in any of the cell lines tested (including K562, T47D, MCF7 and B549; results not shown), consistent with the lack of any published hsa-miR-384 expression data. We next measured expression of hsa-miR-101, hsa-miR-145, hsa-miR-331-3p and hsa-miR-494 in primary HTE cells and cultures of NHBEs in comparison with 16HBE14o- cells (Figure 4B). Three primary cultures of each cell type were used to account for individual variation.

hsa-miR-384, hsa-miR-494 and hsa-miR-1246 also target the SLC12A2 3' UTR

SLC12A2 (*NKCC1*) is among the group of ion channels, ion exchangers and transporters that interact directly with CFTR and influence its activity [38]. We examined the 3.1 kb 3' UTR of *SLC12A2* for predicted miRNA seed sites and identified sites for seven of the 12 miRNAs predicted to repress *CFTR* expression, together with a site for hsa-miR-101 (Figure 5A). To determine whether *SLC12A2* is also functionally targeted by these miRNAs, we transfected miRNA mimics into Caco-2 and PANC-1 cells and assayed *SLC12A2* expression by qRT-PCR (Figures 5B and 5C). All seven miRNAs predicted to target *SLC12A2* were assayed in Caco-2 cells (Figure 5B), and six of these (excluding hsa-miR-939, which has a context score of 0.0) were evaluated in PANC-1 cells (Figure 5C). In contrast with miRNA-mediated *CFTR* mRNA repression, only three miRNAs significantly repressed *SLC12A2* mRNA: hsa-miR-494 in Caco-2 cells ($P < 0.001$), and hsa-miR-377 and hsa-miR-1246 in PANC-1 cells ($P < 0.01$). To assay for direct targeting of the *SLC12A2* 3' UTR by these miRNAs, a pMIR-REPORT construct was generated that encompassed the full 3' UTR of the transcript. pMIR-*SLC12A2*, pMIR-REPORT β -galactosidase and miRNA mimics were co-transfected into PANC-1 cells (Figure 5D). Four miRNAs repressed reporter expression by >35% ($P < 0.01$), including three that also functionally target the *CFTR* 3' UTR, hsa-miR-384, hsa-miR-494 and hsa-miR-1246.

DISCUSSION

It is well known that miRNAs contribute to the pathogenesis of a number of important human diseases, including cancer. The *Bcl2* (B-cell lymphoma 2) oncogene, an anti-apoptotic protein that is down-regulated in several cancers, is a proven target of hsa-miR-15 and hsa-miR-16. Both of these miRNAs are able to down-regulate *Bcl2* expression and induce apoptosis when expressed in cell lines, and both are down-regulated in chronic lymphocytic leukaemias [39,40]. In contrast, the 'oncomiR' hsa-miR-21 is overexpressed in a variety of tumours, where it acts by repressing *PTEN* (phosphatase and tensin homologue) and *PDCD4* (programmed cell death 4) expression [41,42]. miRNAs have also been implicated in cardiac and neurodegenerative diseases, diabetes, inflammation and diseases of the immune system (reviewed in [43]). Hence they show great promise as therapeutic

targets, largely due to their potential for inhibition by artificial antisense miRNAs (antagomirs or anti-miRs). LNA (locked nucleic acid) anti-miRs are exceptionally stable, non-toxic and soluble in aqueous solution. Delivery of LNA anti-miRs directed against miR-122 in monkeys results in long-lasting and reversible knockdown of the targets of miR-122 with no evidence of toxicity [44]. This ease of *in vivo* inhibition highlights the importance of identifying miRNA-target interactions relevant to disease states.

In the present study we demonstrate that miRNAs post-transcriptionally regulate expression of the *CFTR* gene. Moreover, we show that at least three of these miRNAs (hsa-miR-145, hsa-miR-331-3p and hsa-miR-494) are expressed in primary human airway epithelial cells, which have very low levels of *CFTR* expression in comparison with intestinal epithelial cells. Of the 12 miRNAs that repress expression of *CFTR* mRNA in Caco-2 cells (Figure 2A), ten of these directly target the *CFTR* 3' UTR in Caco-2, PANC-1 or 16HBE14o- cells (Figures 3B–3D). For hsa-miR-145 and hsa-miR-494, this targeting occurs at least partially through the sites predicted by TargetScan. All of the top 11 TargetScan-predicted miRNAs are able to repress endogenous *CFTR* mRNA expression by >40% in Caco-2 cells, but only four repress 3' UTR reporter expression by >30% in the same cell line. This may be due to miRNA regulation of upstream (transcription) factors involved in *CFTR* expression, or these miRNAs binding to sites outside of the 3' UTR. The fact that the luciferase reporter is repressed by all but one of the top 11 miRNAs (hsa-miR-377) in Caco-2, PANC-1 or 16HBE14o- cells, but not necessarily in all of the cell lines, warrants further discussion. Although it is possible that target site utilization is cell-type-dependent, or that there are cell-type-specific cofactors required for repression with some miRNAs, these results may reflect significant differences in miRNA transfection efficiency between cell lines and reagents. Although Lipofectamine™ 2000 (used in luciferase experiments) was the most efficient reagent available for co-transfection of small dsRNA and plasmid DNA [measured by hsa-miR-1 knockdown of *TWFI* (twinfilin-1) mRNA; results not shown], it was significantly less efficient than Lipofectamine™ RNAiMAX (used in qRT-PCR experiments) for miRNA delivery in Caco-2 cells. This difference was less evident in PANC-1 cells, where both reagents were as efficient as Lipofectamine™ RNAiMAX in Caco-2 cells. Thus, in Caco-2 cells, the functional miRNA concentrations in experiments that measured direct effects on the *CFTR* 3' UTR were likely to be lower than in those with Lipofectamine™ RNAiMAX or in PANC-1 cells with either reagent.

It is of interest that the transient transfections using the pMIR-*CFTR*mut145 and pMIR-*CFTR*mut494 mutant reporters confirmed the functional relevance of the binding sites predicted by TargetScan. The fact that mutation of these sites fail to relieve repression completely suggests that there may be additional functional binding sites in the *CFTR* 3' UTR. Indeed, while TargetScan predicts only a single target site for hsa-miR-494, the *CFTR* 3' UTR contains two sequences complementary to the miR-494 seed site (GAAACA). The second site, located 292 bp downstream of the TargetScan-recognized site, is not predicted by this program because it has neither an 'A' opposite base 1 nor pairing at base 8 of the miRNA sequence. RNAHybrid, another tool for modelling RNA–RNA interactions, predicts slightly stronger pairing between the mRNA and miRNA at this 3' site, based on calculated minimum free energy of hybridization. There is only one sequence complementary to the

hsa-miR-145 seed site in the *CFTR* 3' UTR, suggesting a different mechanism for maximal hsa-miR-145-mediated repression.

The physiological levels of miRNAs that are required to regulate *CFTR* expression in normal cells *in vivo* are of interest, particularly given the large variation in endogenous expression of the miRNAs seen in the different cell lines utilized in these experiments; however, there are currently few studies that directly address this question in primary human epithelial cells. The results of the present study show that the relatively high level of hsa-miR-145 expression in Caco-2 cells is not sufficient to maximally repress *CFTR*, as the introduction of exogenous hsa-miR-145 further represses expression of endogenous *CFTR* and reporters carrying the *CFTR* 3' UTR. However, miRNA inhibition experiments would be necessary to definitively establish the effect of endogenous hsa-miR-145 on *CFTR* expression in Caco-2 cells.

Of the ten miRNAs that target the *CFTR* 3' UTR in the luciferase reporter assay, three also target the *SLC12A2* 3' UTR in PANC-1 cells: hsa-miR-384, hsa-miR-494 and hsa-miR-1246. Of these, only hsa-miR-494 has a validated target, *PTEN* [45], and none have yet been implicated in the regulation of epithelial chloride channels and transporters. However, the results of the present study show that they are able to functionally regulate two of the primary chloride transporters in epithelial cells, and thus they may have a wider role in coordinating epithelial ion transport.

The dominant mechanisms controlling *CFTR* expression levels *in vivo* are driven by *cis*-acting regulatory elements (reviewed in [2]). However, miRNAs could also play an important role in directing changes in the amounts of mature *CFTR* protein, which in turn could have a significant effect on cell physiology. Thus the identification of multiple miRNAs that target the *CFTR* gene may have a direct therapeutic application. This would be particularly relevant to certain classes of mutations in the *CFTR* gene that result in low and insufficient expression of a functional *CFTR* protein. Among others, these include mutations that reduce transcriptional activity, generate an unstable mRNA (e.g. stop mutations) and affect splicing (reviewed in [10]). In patients with these disease-causing mutations, inhibition (by antagomirs or anti-miRs) of miRNAs targeting *CFTR* in relevant tissues has the potential to elevate *CFTR* expression levels to within the normal range.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

Bcl2	B-cell lymphoma 2
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
dsRNA	double-stranded RNA
HTE	human tracheal epithelial cell
LNA	locked nucleic acid
MCS	multiple cloning site
miRNA	microRNA
NHBE	human bronchial and tracheal epithelial cell
PTEN	phosphatase and tensin homologue
qRT-PCR	quantitative reverse transcription PCR
SLC12A2	solute carrier family 12 (Na ⁺ –K ⁺ – Cl [–] transporters), member 2
UTR	untranslated region

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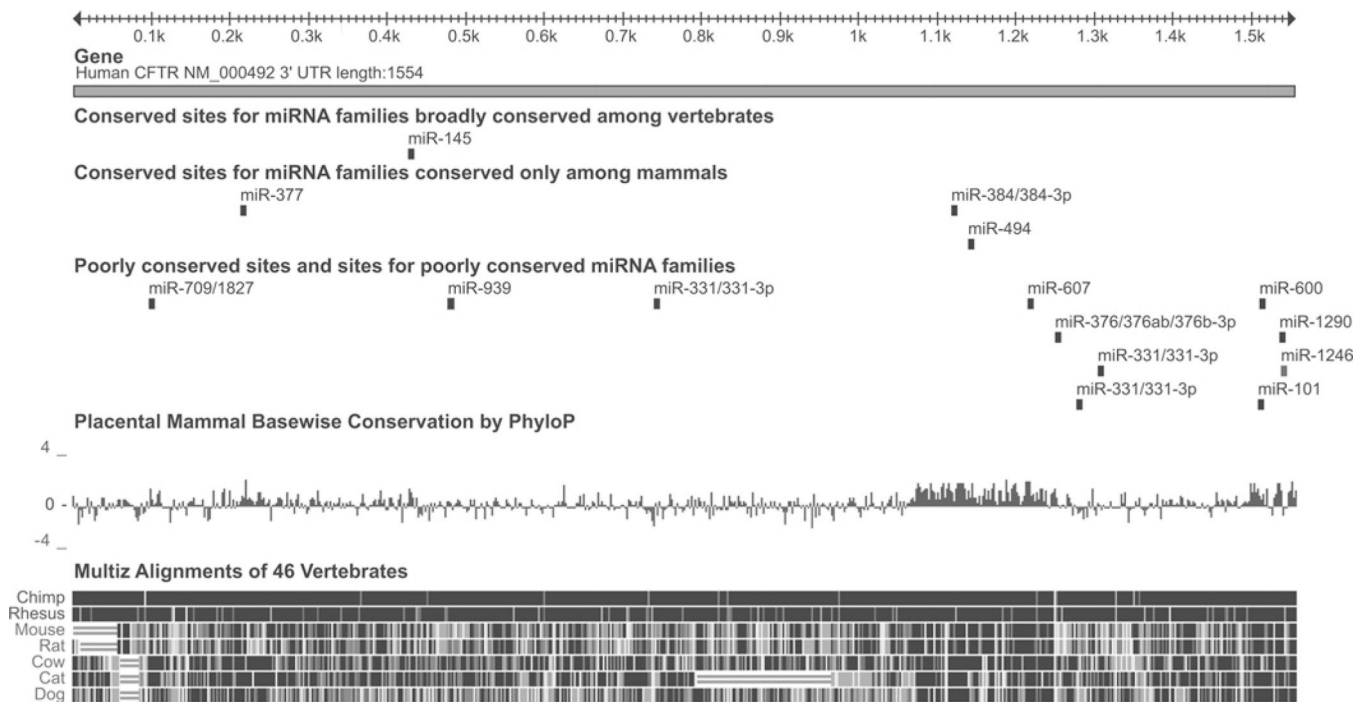


Figure 1. The CFTR 3' UTR and predicted miRNAs

The *CFTR* 3' UTR, and the location of predicted miRNA seed sites, mammalian conservation and sequence alignment with relevant mammals. 'Broadly conserved' miRNAs are conserved across most vertebrates; 'conserved' miRNAs are conserved across most mammals. 'Placental Mammal Basewise Conservation' is based on sequence alignment of 33 mammalian genomes, including those appearing individually below [46–48]. The Figure is based on the output from <http://genome.ucsc.edu> (hg19 assembly) [49] and <http://www.targetscan.org> [25].

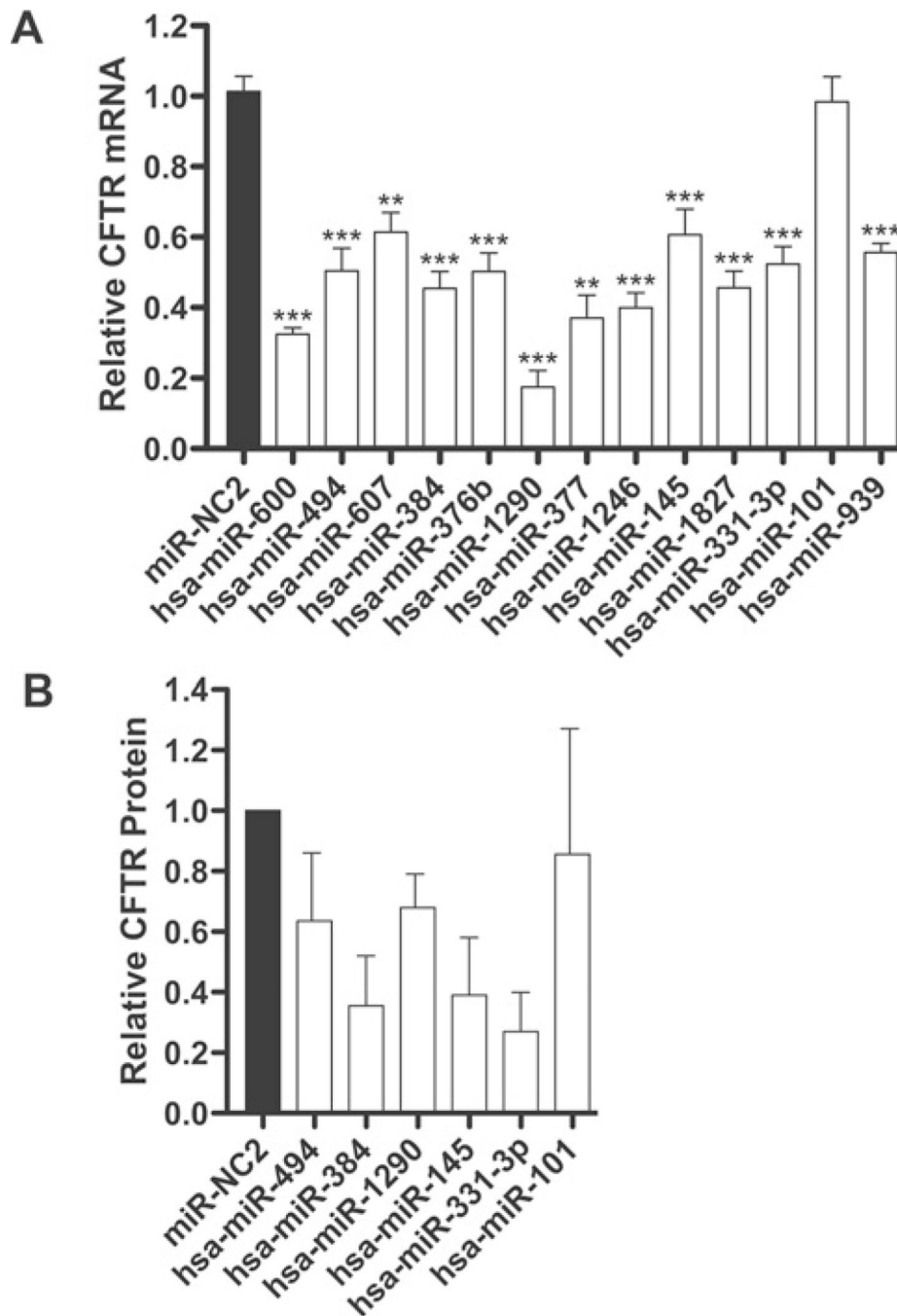


Figure 2. Repression of *CFTR* expression in Caco-2 cells by miRNAs predicted with TargetScan (A) Expression of *CFTR* mRNA in Caco-2 cells, measured by TaqMan qRT-PCR, after transient transfection of the indicated miRNA mimics. miRNAs are ordered by TargetScan context score, with the strongest predictions on the left-hand side. (B) ImageJ quantification of *CFTR* protein expression in Caco-2 cells, measured by Western blot analysis, after transient transfection of the indicated miRNA mimics (average of two biological replicates). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Values are means \pm S.E.M.

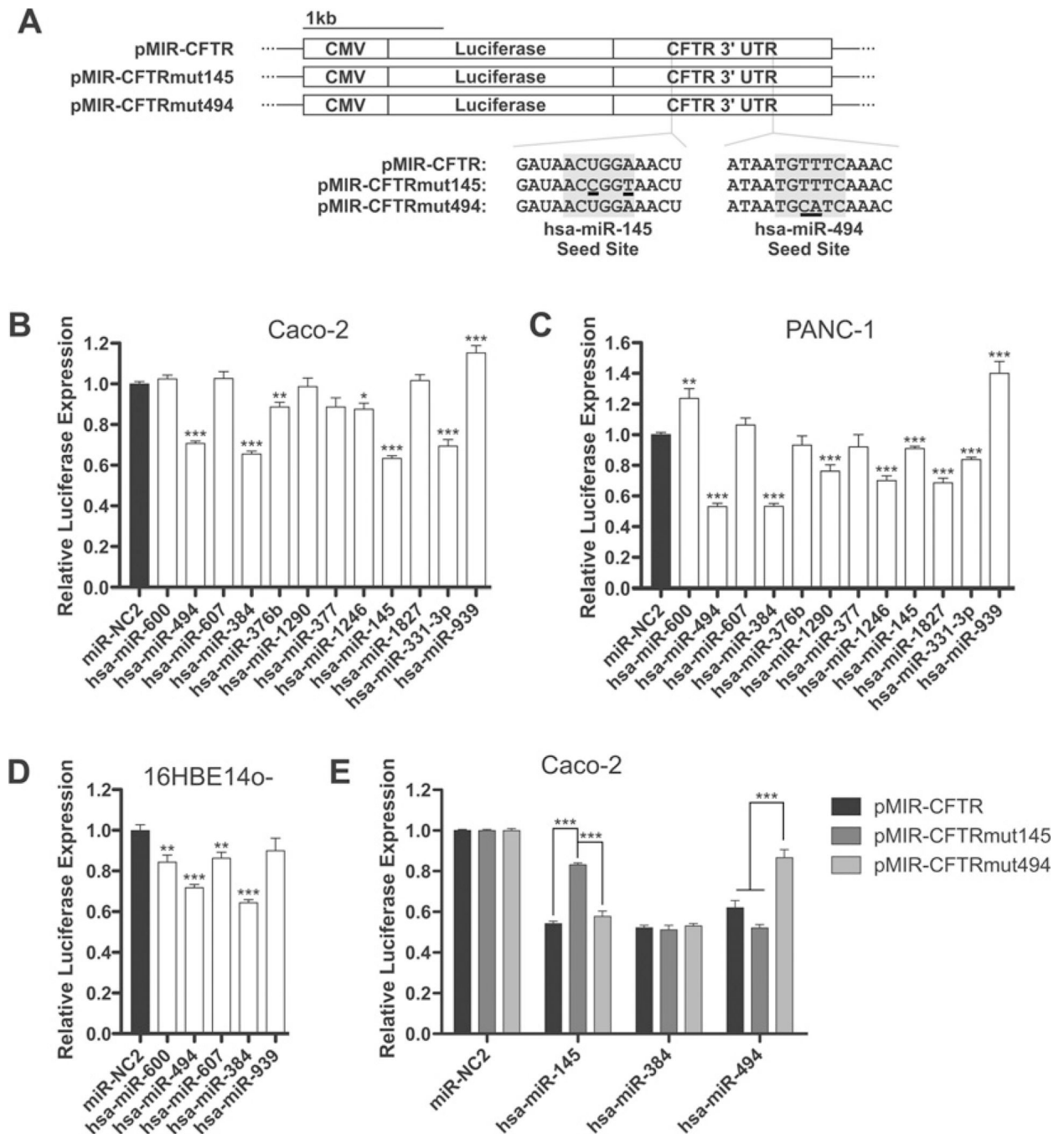


Figure 3. miRNA-mediated *CFTR* repression acts through the 3' UTR

(A) The pMIR-REPORT-based luciferase reporter constructs used in these assays. (B–D) Luciferase expression from pMIR-CFTR (full-length *CFTR* 3' UTR) after co-transfection with pMIR-REPORT β -galactosidase and the indicated miRNA mimics in Caco-2 (B), PANC-1 (C) and 16HBE14o- (D) cells. miRNAs are ordered by TargetScan context score, with the strongest predictions on the left-hand side. (E) Luciferase expression from pMIR-CFTR, pMIR-CFTRmut145 and pMIR-CFTRmut494 after co-transfection with pMIR-

REPORT β -galactosidase and the indicated miRNA mimics in Caco-2 cells. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Values are means \pm S.E.M. ($n = 9$).

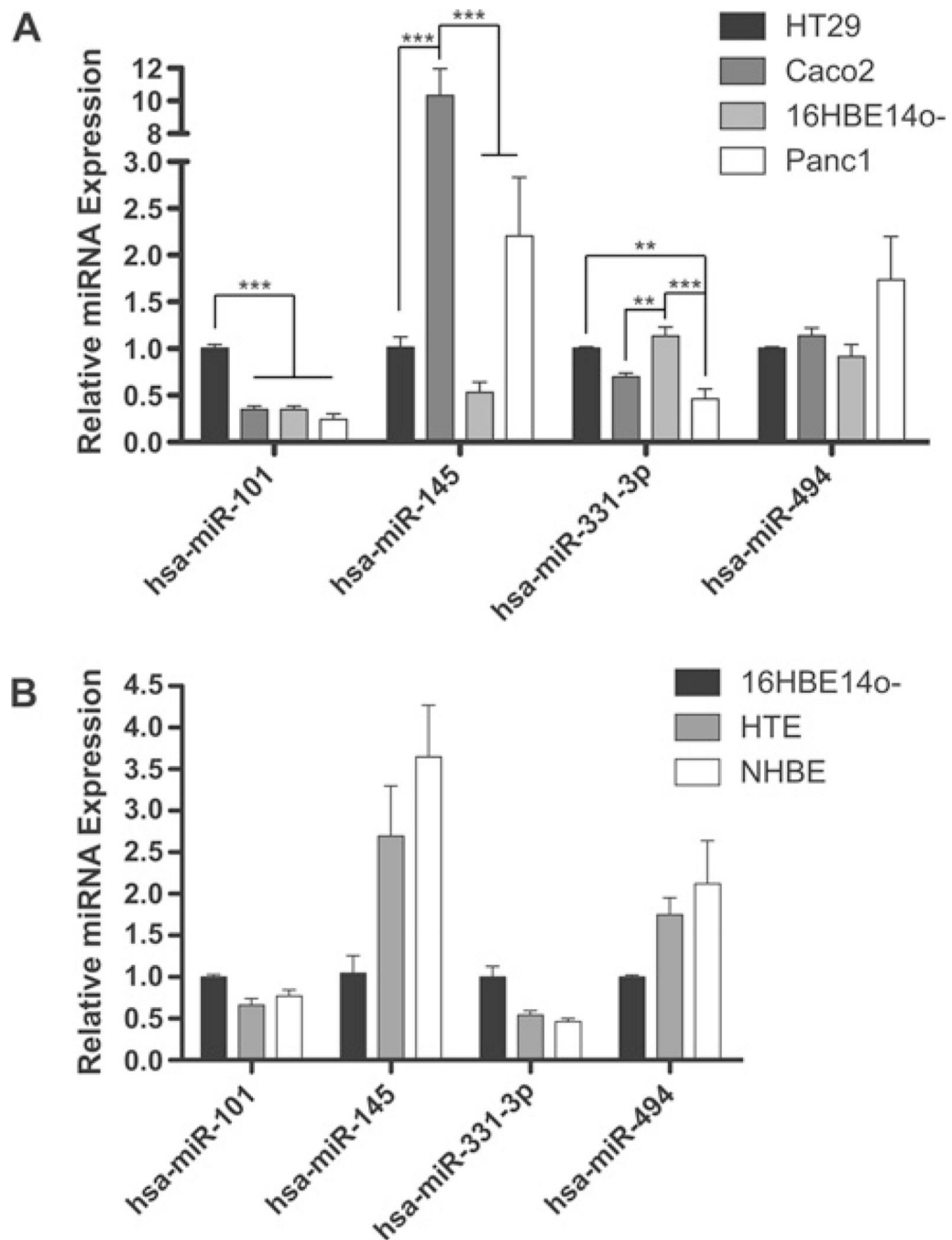


Figure 4. Expression of miRNA-101, miRNA-145, miRNA-331-3p and miRNA-494 in (A) cell lines and (B) primary human tracheal and bronchial cells (HTEs and NHBEs)
 Expression of hsa-miR-101, hsa-miR-145, hsa-miR-331-3p and hsa-miR-494, measured by qRT-PCR with TaqMan miRNA assays. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Values are means \pm S.E.M. Data from three cultures of HTEs and NHBEs are combined to account for variation between individuals.

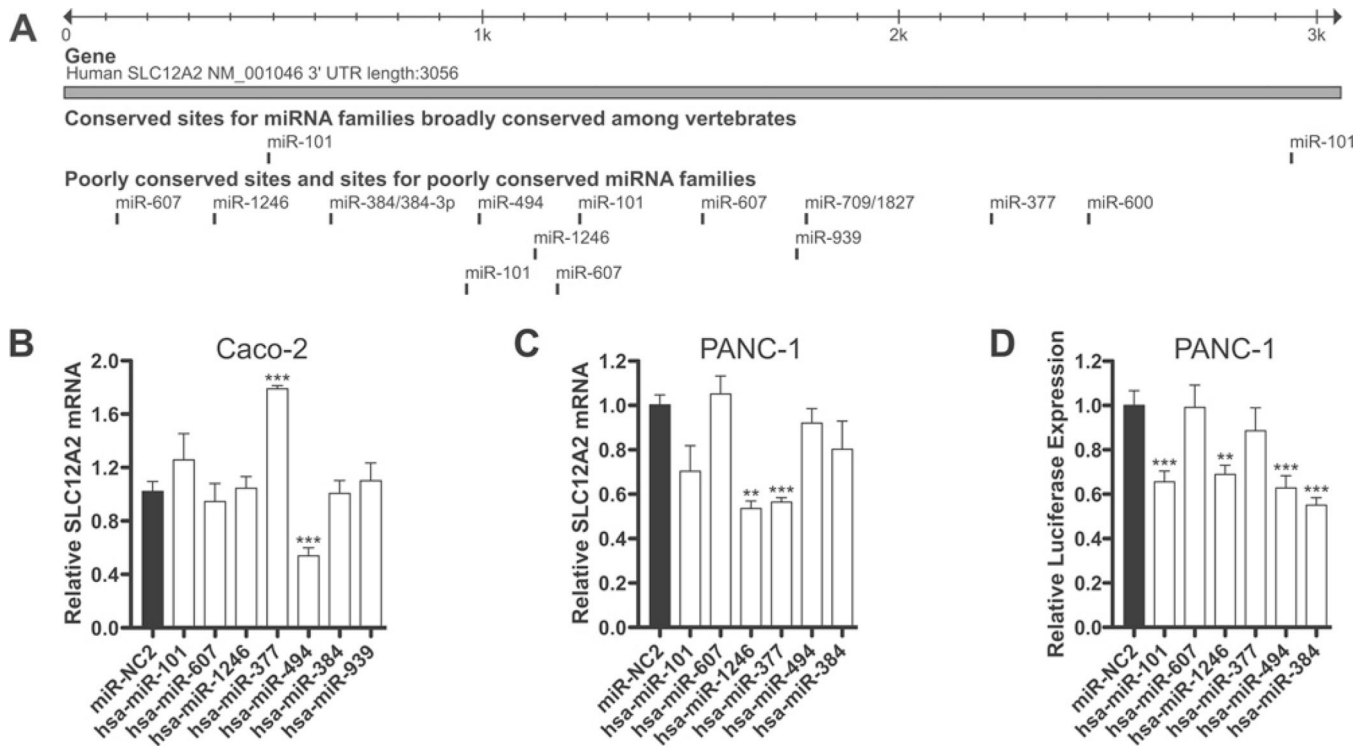


Figure 5. Repression of *SLC12A2* expression by miRNAs that also target *CFTR*
 (A) The *SLC12A2* 3' UTR, and the location of predicted miRNA seed sites. The Figure is based on the output from <http://genome.ucsc.edu> (hg19 assembly) [49] and <http://www.targetscan.org> [25]. (B–C) Expression of *SLC12A2* mRNA in Caco-2 (B) and PANC-1 (C) cells, measured by TaqMan qRT-PCR, after transient transfection of the indicated miRNA mimics. miRNAs are ordered by TargetScan context score, with the strongest predictions on the left-hand side. (D) Luciferase expression from pMIR-*SLC12A2* (full-length *SLC12A2* 3' UTR) after co-transfection with pMIR-REPORT β -galactosidase and the indicated miRNA mimics in PANC-1 cells. miRNAs are ordered by TargetScan context score, with the strongest predictions on the left-hand side. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Values are means \pm S.E.M. ($n = 9$).

Table 1

miRNAs predicted to target *CFTR* (TargetScan 5.1)

The top 11 miRNAs (sorted by context score) predicted to target the human *CFTR* 3' UTR are listed, along with published expression data [28–34]. Two additional miRNAs predicted to target the human *CFTR* 3' UTR are also included (see the text for details). CS, context score; N/A, not applicable.

miRNA	CS	CS rank	CS percentile	Number of sites	Expression
hsa-miR-600	-0.57	1	99	1	Normal colonic epithelium
hsa-miR-607	-0.53	2	99	1	Caco-2 (colon carcinoma)
hsa-miR-494	-0.53	2	99	1	Multiple cell types
hsa-miR-384	-0.5	4	99	1	N/A
hsa-miR-376a/b	-0.49	5	97	1	Multiple cell types
hsa-miR-1290	-0.47	6	97	1	Human embryonic stem cells
hsa-miR-1246	-0.45	7	98	1	Human embryonic stem cells
hsa-miR-377	-0.45	7	97	1	Various cell types
hsa-miR-145	-0.43	9	95	1	Multiple cell types, highest in prostate, uterus and epididymis
hsa-miR-1827	-0.42	10	99	1	HeLa (cervical carcinoma)
hsa-miR-331-3p*	-0.39	11	39, 40, 93*	3	Multiple cell types
hsa-miR-101	-0.27	27	80	1	Multiple cell types, highest in lymphocytes
hsa-miR-939	-0.11	75	65	1	Multiple cell types

* hsa-miR-331-3p has three predicted target sites in the *CFTR* 3' UTR.