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Regulation of miR-155 biogenesis in cystic fibrosis lung epithelial cells: Antagonistic role of two mRNA-destabilizing proteins, KSRP and TTP

Sharmistha Bhattacharyya [a], Parameet Kumar [a,b], Motohiro Tsuchiya [c], Anirban Bhattacharyya [a], Roopa Biswas [a,*]

[a] Department of Anatomy, Physiology and Genetics, School of Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD, United States
[b] Johns Hopkins School of Medicine, Baltimore, MD, United States
[c] Molecular and Cell Biology Program, USUHS, Bethesda, MD, United States

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ABSTRACT

Cystic fibrosis (CF) is characterized by a massive pro-inflammatory phenotype in the lung arising from profound expression of inflammatory genes, including interleukin-8 (IL-8). We have previously reported that IL-8 mRNA is stabilized in CF lung epithelial cells, resulting in concomitant hyper-expression of IL-8 protein through elevated expression of miR-155. We therefore investigated what factors promote the enhanced aberrant expression of miR-155 in CF. Here we report for the first time, the role of mRNA-destabilizing inflammatory RNA-binding proteins, KSRP and TTP, in the regulation of miR-155 biogenesis in CF lung epithelial cells. We find that KSRP and TTP have an antagonistic role in miR-155 biogenesis. While KSRP promotes enhanced processing of miR-155 precursors to mature miR-155, over-expression of TTP in the CF lung epithelial cells suppresses expression of miR-155. We find that TTP induces the expression of miR-1, which appears to be a regulator of miR-155 biogenesis in CF lung epithelial cells. These data provide novel insights into the mechanisms that induce hyper-inflammatory phenotype of CF, and are potential candidates for anti-inflammatory therapeutics for CF.

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1. Introduction

Cystic fibrosis (CF), the most common autosomal recessive disease in the U.S. and Europe, is caused by mutations in the cystic fibrosis trans-membrane conductance regulator (CFTR) gene [1–4]. CFTR mutations, of which the frequent is [ΔF508]-CFTR, cause a massive pro-inflammatory phenotype in the lung, which manifests in the airway by high levels of IL-8 and other pro-inflammatory cytokines and chemokines [5–7]. IL-8 is the most potent known chemotactic agent for neutrophils [8], and is constitutively secreted from CF lung epithelial cells [9]. CF patients also constitutively secrete high levels of IL-8 [10]. However, how a mutation in CFTR induces an inflammatory phenotype by targeting mechanisms that regulate inflammation remains poorly understood.

microRNAs (miRNAs, miRs), a class of ~22 nucleotide long endogenous RNA molecules, have been shown to negatively regulate gene expression by promoting mRNA degradation and/or translational suppression [11–14]. Furthermore, miRNAs can occasionally also switch from a normally translational repressor mode to that of a translational activator [15,16]. Recently, specific miRNAs have been reported to be associated with diabetes [17,18], cancer [19–22], heart disease [23,24], cell cycle [25], and development [12]. Consistent with their multiple essential roles, miRNA expression and biogenesis is regulated at either transcriptional or post-transcriptional levels [26–28]. However, the mechanisms by which miRNA processing is controlled in specific cells and tissues remain largely unknown. In mammalian miRNA biogenesis, primary (pri-) miRs are cleaved into hairpin intermediates, precursor (pre-) miRs, by nuclear RNase III Drosha. Pre-miRs are further processed into mature miRs by cytosolic Dicer [29,30]. The function and expression of mature miRs is controlled by mechanisms that regulate the processing of pri-miRs and pre-miRs.

We have recently reported the aberrant expression of miRNAs in CF lung epithelial cells compared to control cells (both in culture and in primary epithelial cells isolated from CF patient bronchial brushings) and have demonstrated how elevated level of miR-155 induces hyper-expression of IL-8 [31]. However, what causes aberrant elevated expression of miR-155 in CF cells, resulting in the pro-inflammatory disease phenotype, is not known. Here we

Abbreviations: CF, Cystic fibrosis; miRNA, microRNA; IL-8, Interleukin-8; UTR, untranslated region; TTP, tristetraprolin; KSRP, KH-type splicing regulatory protein; RBP, RNA-binding protein; AU, adenine–uridine; AREs, AU-rich elements.

* Corresponding author. Address: Department of Anatomy, Physiology and Genetics, School of Medicine, Room B4024, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814, United States.
E-mail address: roopa.biswas@usuhs.edu (R. Biswas).

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report the mechanism of regulation of miR-155 expression in CF lung epithelial cells.

We have previously shown 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 [32]. The changes in mRNA turnover are mediated by specific trans-acting proteins that recognize certain motifs, including adenine–uridine rich elements (AREs). Two such ARE RNA-binding proteins (RBPs) known to destabilize ARE-mRNAs, including IL-8, are (i) KH-type splicing regulatory protein (KSRP), the KH domain containing splicing factor [33]; and (ii) tristetraprolin (TTP), a zinc finger protein also known as ZFP36 [34,35]. We have previously reported that one of the factors contributing to the profound inflammatory gene expression in CF is the presence of low endogenous levels of the anti-inflammatory protein, TTP [32].

Here we report the antagonistic role of the two inflammatory RBPs, KSRP and TTP, in the regulation of miR-155 biogenesis in CF lung epithelial cells. Our data indicate that suppression of KSRP in CF cells leads to inhibition of miR-155 maturation. Moreover, we find that the anti-inflammatory RBP, TTP, suppresses the pro-inflammatory miR-155 processing and expression in CF lung epithelial cells through the induction of another microRNA, miR-1. These data suggest that mechanisms regulating miRNA biogenesis may serve as potential novel miR-based anti-inflammatory therapeutic targets for CF.

2. Materials and methods

2.1. Reagents

LHC-8. media, Trypsin–EDTA (0.05%) and Lipofectamine transfection Reagent were purchased from Invitrogen (Life Technologies). miRVana kit and RNA-aqueous-micro kit for RNA isolation, Taqman Low Density V2 arrays, miRNA primer pools, and Taqman qPCR reagents were purchased from Applied Biosystems (Life Technologies).

2.2. Cell culture and siRNA, pre-miR and anti-miR transfection

IB3-1 CF lung epithelial cells and the control CFTR-repaired IB3-1/S9 cells were maintained in LHC-8 serum free medium in humidified 5% CO_2 as previously described [36]. The IB3-1-TTP cells were similarly maintained in LHC-8 medium containing puromycin (0.5 μg/ml). Bronchial brush biopsies were collected under an Institutional Review Board approved protocol as previously described [32]. Transfections with siRNA, pre-miR-1 and anti-miR-1 were done using siPORT NeoFX Transfection Reagent (ABI). The siRNA against KSRP was obtained from ABI (siRNA id # s16322). The pre-miR-1 is the miR-1 precursor mimic (ABI id# PM10617) and anti-miR-1 is the siRNA against miR-1 (ABI id# AM10617).

2.3. RT-PCR, and assays for mature and precursor miRNAs

Total RNA was isolated from the IB3-1 and IB3-1/S9 cells using miRVana isolation kit (ABI). Multiplex Reverse Transcription was performed with TaqMan MicroRNA Reverse Transcription Kit (ABI). RNA was isolated from the cells and mRNA expression level was analyzed with qRT-PCR as described earlier [32]. miRNA expression profile was analyzed by TaqMan Low Density Human MicroRNA Panel v2 as described in Bhattacharyya et al. [31]. The KSRP mRNA was analyzed by Taqman assay (ABI). The mature miR-155 was assayed using Taqman miR-155 specific assay (ABI, Assay ID: 002623). The pri-miR-155 was analyzed with ABI Taqman assay (Assay ID: Hs03303349_pri) as well by SYBR green-based qPCR with the primers: GTGTATGATGCCTGTTACTAGCA (forward) and GCCTGAAGTCTAAGTTTATCCAGC (reverse). The pre-miR-155 was analyzed by SYBR green qPCR reactions with the primers: TGCTAATCGTGATAGGGGTTTT (forward) and TGCTAATATGTAGGAGTCAGTTGGA (reverse). GAPDH was used as endogenous controls. For analyses of GAPDH, the cDNA was diluted 1:5 and amplified with the primers (5 μM): GCTCACTGGCATGGCCTTCCGTGT (forward) and TGGAGGAGTGGGTGTCGCTGTTGA (reverse).

2.4. Immuno-precipitation

Cells were lysed using RIPA buffer (50 mM Tris HCl pH 8150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing a protease inhibitor cocktail (Sigma) and DTT. Lysates were cleared by centrifugation and an aliquot was examined for protein concentration (BioRad). Lysates (1 mg Protein) were incubated with 25 μg anti-KSRP antibody (generous gift from Dr. Ching-Yi Chen, Department of Biochemistry and Molecular Genetics, University of Alabama, Birmingham) for 4 h at 4 °C. Subsequently 100 μg A/G coupled Dynabeads were added to the cell lysate and incubated at 4 °C for 1 h. The beads were incubated with 100 μl elution buffer (0.5% NP40, 1 μl of 10% SDS and 1 μl Proteinase K (20 mg/ml)) at 55 °C for 30 min. RNA was extracted using the RNAqueous – Micro kit (ABI).

2.5. Statistical data analyses

Statistical analysis was performed using Excel. Significance values ($p \leqslant 0.05$) were determined by student's *t*-test. Error bars on graphs represent SEM.

3. Results

3.1. miR-155 biogenesis is mis-regulated in CF lung epithelial cells

Aberrant expression in mature miRNAs is often observed in various human diseases, and can be due to the impairment of miRNA-processing steps. Our earlier data demonstrated that miR-155 expression is elevated in ΔF508-CF lung epithelial cells and that pharmacological inhibition of WT-CFTR activity also induces increased expression of miR-155 [31]. We, therefore, examined the expression of miR-155 precursors, both pri-miR-155 and pre-miR-155, in IB3-1 CF cells and CFTR-repaired IB3-1/S9 control cells. As depicted in Fig. 1, though level of the mature miR-155 is elevated (~5.7-fold) in CF cells compared to control cells, the expression of pri-miR-155 is lower (~40%). Moreover, we find that pre-miR-155 expression is relatively enhanced (~1.3-fold) in CF cells compared to controls. Thus, our data indicate that up-regulation of miR-155 in pro-inflammatory CF cells is not due to increased transcription of the precursor but rather enhanced processing of pri- as well as pre-miR-155.

3.2. KSRP induces enhanced processing of pri- and pre-miR-155 in CF lung epithelial cells

Several RNA-binding proteins, including KSRP, have been shown to bind to stems and terminal loops (TL) of miRNA precursors and regulate miRNA processing. Studies from Gherzi and colleagues have shown that the extracellular inflammatory stimulus, bacterial lipopolysaccharide (LPS), induces KSRP-mediated enhanced processing of miR-155 and promotes up-regulation of mature miR-155 in LPS-treated mouse macrophages [37]. Therefore, we analyzed the role of KSRP in the processing of miR-155 in CF lung epithelial cells and performed siRNA-mediated depletion of KSRP in CF cells. As depicted in Fig. 2A and B, a 30 pM dose of siRNA specific for KSRP can effectively suppress KSRP expression (~50%)

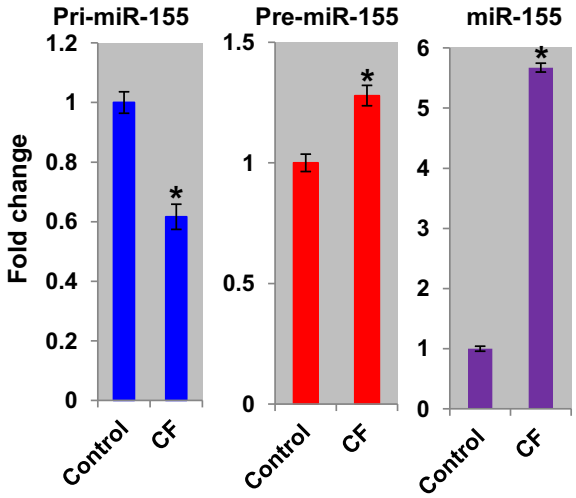

Fig. 1. miR-155 processing is enhanced in CF cells. Analyses of the relative expression of the precursors of miR-155, both pri- and pre-miR-155 by qPCR demonstrates that though pri-miR-155 is reduced in IB3-1 CF cells compared to control IB3-1/S9 cells (left panel), the expression of pre-miR-155 is increased (middle panel). Further enhancement in processing of pre-miR-155 promotes significant up-regulation in mature miR-155 expression in the CF cells compared to control cells (right panel). The data ($^*p < 0.05$) reflects average of three or more independent experiments.

both at mRNA as well as protein. This in turn leads to suppression in mature miR-155 (~30%, Fig. 2C) and a corresponding significant increases in pri- and pre-miR-155 expressions (Fig. 2D). Consistently, both pri- and pre-miR-155 precursors are immuno-precipitated from pro-inflammatory CF cells with KSRP antibody and there is increased association of pre-miR-155 with KSRP in CF cells compared to control cells (Fig. 2E). While the expression of KSRP does not show a significant change in CF cells compared to control cells, the KSRP mRNA is slightly elevated (~2-fold) in primary CF cells compared to the controls (Supplementary Fig. 1S). Thus, our data indicate that KSRP enhances miR-155 biogenesis in CF lung epithelial cells.

3.3. TTP suppresses the maturation of miR-155 in CF cells

We have shown that pro-inflammatory CF cells, both in culture and ex vivo primary CF cells, exhibit negligible endogenous expression of the ARE-mRNA destabilizing protein TTP compared to control cells [32]. This is one of the factors leading to a profound hyper-expression of the pro-inflammatory chemokine IL-8 [32]. Our earlier studies have also indicated that over-expression of TTP in these cells suppresses inflammation by destabilization of IL-8 mRNA [32]. We subsequently developed a stable CF lung epithelial cell line, IB3-1-TTP (CF-TTP), expressing high levels of TTP. Interestingly, we find that the CF-TTP cells also exhibit (~2-fold) reduction in mature miR-155 expression compared to that in the pro-inflammatory CF cells and significant increases in the expression of miR-155 precursors, both pri- and pre-miR-155 (Fig. 3A) ($p < 0.05$). Thus it appears that the anti-inflammatory protein TTP inhibits miR-155 biogenesis.

3.4. miR-1 regulates TTP-mediated suppression of miR-155 biogenesis in CF lung epithelial cells

Subsequent to our previous observation that over-expression of TTP in CF cells can suppress miR-155, we analyzed the miRNA expression profile (ABI Taqman miRNA arrays) in these cells. We

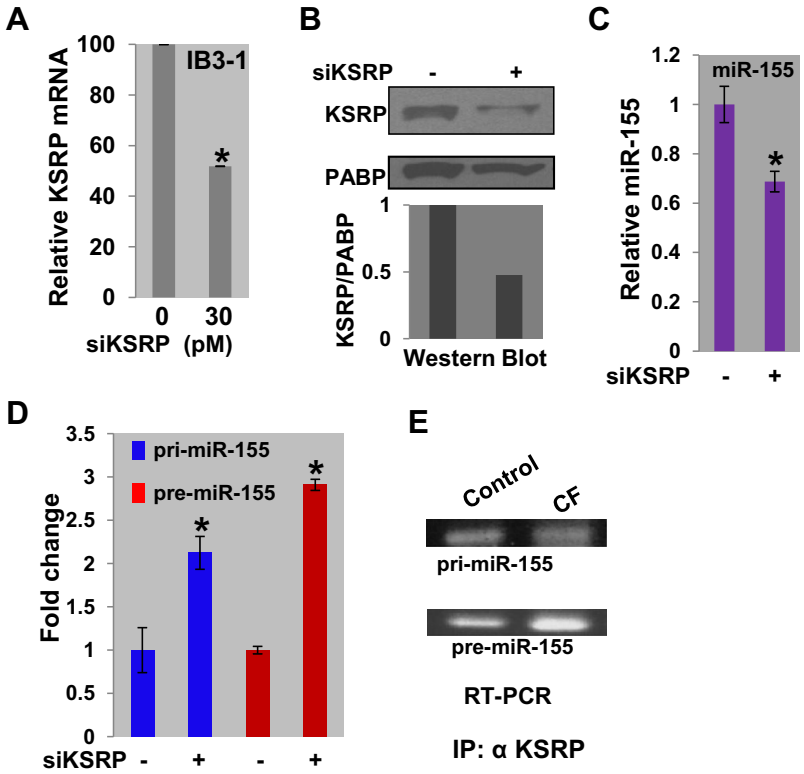

Fig. 2. KSRP enhances miR-155 processing in pro-inflammatory CF lung epithelial cells. (A) CF (IB3-1) cells were treated with siRNA specific for KSRP. A dose of 30 pM for 48 h effectively reduces KSRP mRNA to ~50%. (B) The corresponding KSRP protein also shows a ~50% reduction compared to PABP protein controls. The knockdown of KSRP promotes (C) suppression of mature miR-155 expression, while (D) there is a corresponding increase in the expression of pri- and pre-miR-155 (sybr green-based qRT-PCR). The data ($^*p < 0.05$) reflects average of three or more independent experiments. (E) IB3-1CF and IB3-1/S9 control cell extracts were immuno-precipitated (IP) with anti-KSRP antibody. The pri-miR-155 and pre-miR-155 expression in the IP were analyzed by RT-PCR.

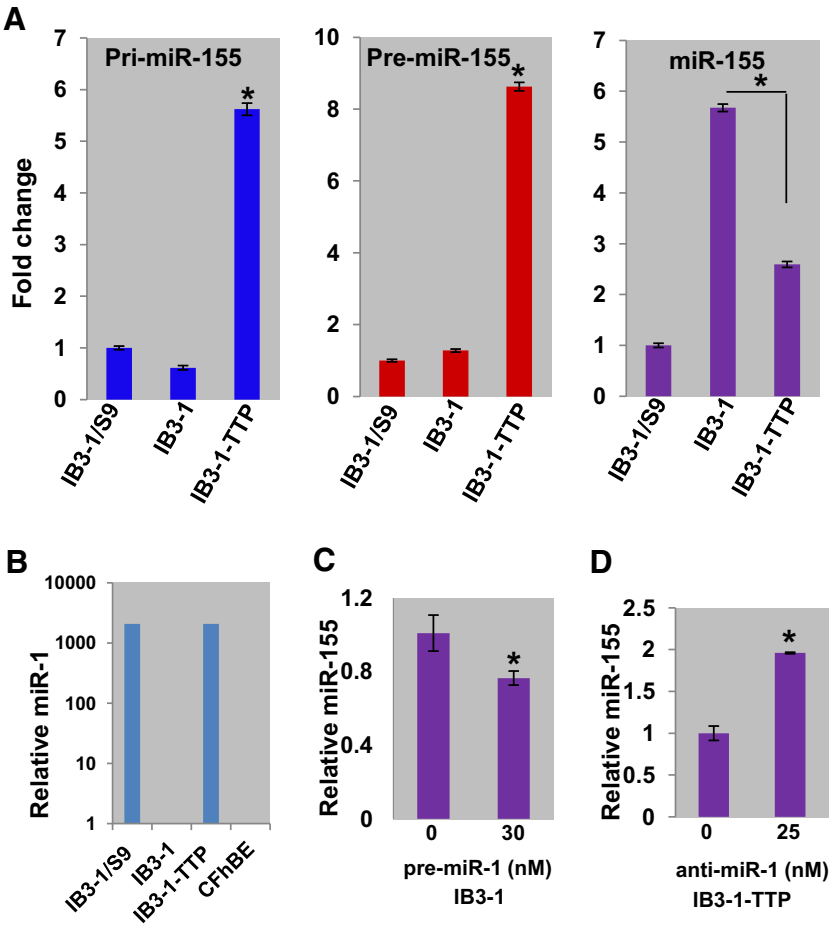

Fig. 3. TTP suppresses miR-155 expression in CF cells. The expression of pri- and pre-miR-155 was elevated in IB3-1-TTP cells compared to IB3-1 and IB3-1/S9 control cells (left and middle panel). The analyses of corresponding mature miR-155 expression (right panel), indicate suppressed expression in TTP over-expressing CF cells, IB3-1-TTP compared to the IB3-1 CF cells, which express low endogenous levels of TTP protein. (B) miR-1 expression was analyzed by Taqman assays in IB3-1/S9 control cells, IB3-1 CF cells, and in TTP over-expressing CF cells (IB3-1-TTP). IB3-1 CF cells and IB3-1-TTP CF-TTP cells were transfected with pre-miR-1 and anti-miR-1, respectively, for 24 h with the indicated doses. (C) Over-expression of miR-1 in IB3-1 CF cells with pre-miR-1 or (D) suppression of miR-1 in IB3-1-TTP cells with anti-miR-1 induces corresponding decreases and increases in mature miR-155, respectively. The data (*$p < 0.05$) reflects average of three or more independent experiments.

thus identified miR-1 as a possible regulator of miR-155 suppression in CF-TTP cells. As depicted in Fig. 3B, miR-1 was not detected in the IB3-1 CF cells, while both IB3-1/S9 control cells and IB3-1-TTP cells, which have increased expression of TTP, exhibit a remarkably significant up-regulation of miR-1. Consistently, we find that primary lung epithelial cells isolated from bronchial brushings of CF patients lack miR-1 expression (Fig. 3B). Moreover, we find that over-expression of miR-1 with pre-miR-1 (30 nM, 24 h) in IB3-1 CF cells significantly reduces expression of miR-155 (Fig. 3C) ($p < 0.05$). Additionally, knockdown of miR-1 with anti-miR-1 (25 nM, 24 h) in CF-TTP cells induces up-regulation of mature miR-155 (Fig. 3D) ($p < 0.05$). Thus, our data indicate miR-1 to be a potential regulator of miR-155 expression in CF cells.

4. Discussion

We have previously reported aberrantly high levels of miR-155 in cultured CF lung epithelial IB3-1 cells, and in primary epithelial cells from lungs of CF patients [31]. Based on these findings, we analyzed the mechanism that regulates miR-155 expression in CF lung epithelial cells. Here we report, for the first time, that the aberrant up-regulation of miR-155 is due to the enhanced processing of the precursors in CF cells. As summarized in Fig. 4, the data clearly indicate the antagonistic role of the two inflammatory RNA-binding proteins, KSRP and TTP in the regulation of miR-155 biogenesis in CF cells. While KSRP induces increased processing of miR-155 precursors, over-expression of TTP appears to suppress this process and causes reduction in the mature miR-155 expression in CF lung epithelial cells. Moreover, the data also demonstrates that TTP induces the expression of another microRNA, miR-1, which appears to mediate the regulation of miR-155 processing in CF cells.

The mis-regulation of specific miRNAs leads to various human diseases including cancer, metabolic disorders, cardiovascular diseases, liver disease and immune dysfunction [17,18,20–22,24,25,38,39]. miR-155, a multifunctional miRNA, has been identified as an important regulator of the immune system and inflammation. It is therefore not unexpected that deregulation of miR-155 might be involved in CF pathophysiology.

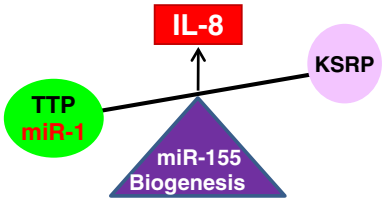

Fig. 4. Antagonistic regulation of miR-155 biogenesis by KSRP and TTP. The schematic summarizes our hypothesis that processing, expression and function of miR-155 is regulated by the inflammatory RBPs, TTP and KSRP, and that reciprocally miR-155 is also a potent inducer of pro-inflammatory genes including IL-8 in CF lung epithelial cells.

Recent studies indicate the role of RNA-binding proteins as regulators of miRNA processing [40,41]. Studies from Gherzi and colleagues have shown that the extracellular inflammatory stimulus, bacterial lipopolysaccharide (LPS), induces KSRP-mediated enhanced processing of miR-155 in mouse macrophages [37]. Analyses of miR-155 precursors, both pri- and pre-miR-155, in CF lung epithelial cells indicate that though mature miR-155 is up-regulated, the expression of pri-miR-155 is lower in CF cells compared to control cells. However, we observe increased expression of pre-miR-155 in CF cells, ultimately also leading to increased mature miR-155 expression. These data are consistent with previous reports that indicate enhanced processing of miR-155 induced enhanced expression of the mature miR-155. We, subsequently, analyzed the role of the inflammatory RBP, KSRP, in miR-155 biogenesis in CF cells. The data clearly indicate that KSRP promotes enhanced processing of the miR-155 precursors, pri- and pre-miR-155, in the pro-inflammatory CF lung epithelial cells.

We have previously reported that the deficiency in the anti-inflammatory AU-binding protein TTP is one of the major factors responsible for enhanced expression of IL-8 in the CF lungs [32]. Interestingly, we find that the anti-inflammatory TTP not only suppresses IL-8 expression but also significantly reduced the expression of the pro-inflammatory mature miR-155. Comprehensive miRNA expression profile analyses of the CF cell derivatives, constitutively expressing high levels of TTP (CF-TTP), indicated miR-1 as a potential regulator of miR-155. This is the first report on the regulation of the biogenesis of miR-155 by the anti-inflammatory RBP, TTP. Future goals are targeted towards further analyzing these mechanisms. These findings will lead to novel anti-inflammatory therapeutic targets for CF and related pulmonary diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.025.

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