Translational repression of SLC26A3 by miR-494 in intestinal epithelial cells

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SLC26A3 [downregulated in adenoma (DRA)] is a Cl–/HCO3– exchanger involved in electroneutral NaCl absorption in the mammalian intestine. Altered DRA expression by miRNA, five different in silico algorithms were used to predict the miRNAs that target DRA. Of these miRNAs, miR-494 was shown to have a highly conserved putative binding site in the DRA 3′-untranslated region (3′-UTR) compared with other DRA-targeting miRNAs in vertebrates. Transfection with pmirGLO dual luciferase vector containing DRA 3′-UTR (pmirGLO-3′-UTR DRA) resulted in a significant decrease in relative luciferase activity compared with empty vector. Cotransfection of the DRA 3′-UTR luciferase vector with a miR-494 mimic further decreased luciferase activity compared with cells transfected with negative control. The transfection of a miR-494 mimic into Caco-2 and T-84 cells significantly increased the expression of miR-494 and concomitantly decreased the DRA protein expression. Mutation of the seed sequences for miR-494 in 3′-UTR of DRA abrogated the effect of miR-494 on 3′-UTR. These data demonstrate a novel regulatory mechanism of DRA expression via miR-494 and indicate that targeting this miRNA may serve to be a potential therapeutic strategy for diarrheal diseases.

DRA; microRNA; 3′-UTR

The coupled operation of Na+/H+ and Cl–/HCO3– exchangers has been shown to be the predominant route for electroneutral NaCl absorption in the human ileum and colon (9, 13). Studies have shown that two members of SLC26 gene family, SLC26A3 or downregulated in adenoma (DRA) and SLC26A6 or putative anion transporter-1 (PAT-1), are the main candidate genes for luminal intestinal Cl–/HCO3– exchangers (38). However, DRA appears to be the major Cl–/HCO3– exchanger mediating vectorial chloride absorption in intestine based on its established role in congenital chloride diarrhea (6, 20, 25, 35, 37). Also, diarrheal phenotype due to loss of luminal membrane Cl–/base exchange activity is the predominant feature exhibited by DRA knockout mice but not by PAT-1 knockout mice (42). Furthermore, DRA expression is significantly reduced in animal models of inflammatory and infectious diarrhea and in inflammatory bowel disease patients (4, 49). Thus DRA has emerged as a potential novel therapeutic target for diarrheal diseases.

Earlier studies from our group have shown the transcriptional regulation of DRA gene by various agents such as proinflammatory cytokine interferon-γ (41), soluble factors secreted by probiotic Lactobacillus acidophilus (39) and lysophosphatidic acid (43). However, other mechanisms regulating DRA gene expression have yet to be investigated. Specifically, to date, there are no studies available pertaining to the potential role of microRNAs (miRNA) in the regulation of DRA expression.

miRNAs are evolutionarily conserved, single-stranded, 20–22 nucleotide noncoding RNA molecules that negatively regulate gene expression at the posttranscriptional level by either repressing translation or degrading mRNA (51). To date, >1,000 human miRNAs have been identified (see miRBase, www.mirbase.org). Each miRNA is predicted to have as many as 200 target miRNAs and, overall, miRNAs are predicted to regulate up to one third of all genes (24). Recent studies have demonstrated the role of miRNAs in various processes in intestinal epithelial cells such as cell differentiation, proliferation, and apoptosis (18).

Interestingly, specific miRNAs have also been implicated in modulating expression of genes involved in intestinal barrier and transport functions. For example, specific miRNAs have been shown to regulate expression of occludin, a tight junction protein (50); cystic fibrosis transmembrane regulator (CFTR), a chloride channel, Na+/K+/Cl– cotransporter (15); and pepT1, an oligopeptide transporter (8). In this regard, recent studies have shown global changes in miRNA expression in gut inflammation along with repression of DRA expression (3, 7, 47).

In this study, an in silico analysis of five algorithms used to identify the putative miRNA binding sites showed that among the various miRNA candidates targeting DRA, miR-494 was highly conserved among various mammalian species and had a high context score. Our results further demonstrated that miR-494 was involved in regulating DRA expression in intestinal epithelial cells. Transient transfection of a miR-494 mimic into Caco-2 and T-84 cells significantly increased the expression of miR-494 and concomitantly decreased the DRA protein expression. These data demonstrate a novel regulatory pathway for regulating DRA expression and suggest that targeting miR-494 using anti-miR-494 may serve as a potential therapeutic tool for diarrheal diseases.

MATERIALS AND METHODS

Cell culture. Caco-2 and T-84 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and grown at 37°C

http://www.ajpgi.org
in a 5% CO₂-95% air environment in T-175 plastic flasks. Caco-2 and T-84 cells were grown in MEM and DMEM F-12 (1:1), respectively, supplemented with 50 U/ml penicillin and 50 μg/ml gentamicin. For Caco-2 and T-84 cells, 20 and 10% fetal bovine serum were used, respectively. For the present study, cells between passage 25 and 45 were used.

**Cloning.** A 388-bp fragment of the DRA mRNA-3'UTR was amplified by PCR and purified using Qiagen Gel extraction kit (Qiagen, Frederick, MD). Purified PCR product was cloned into multiple cloning site of pmirGLO Dual Luciferase miRNA target expression vector (Promega, Madison, WI) downstream of the firefly luciferase gene. The primer sequences flanked by Nhel and XhoI sites; used for PCR amplification are presented in Table 1. The resulting plasmid (pmirGLO-3'-UTR-DRA) was used to transform the competent JM-109 cells (Promega); single colonies were picked from ampicillin agar plates and plasmid DNA was prepared using a plasmid miniprep kit (Qiagen). The fidelity of the construct was confirmed by DNA sequencing.

**Bioinformatic analyses.** In silico identification of putative miRNAs targeting DRA-3'UTR was performed by employing common prediction algorithms TargetScan (http://www.targetscan.org/) (17); MicroCosm (http://www.ebi.ac.uk/enrightsrv/microcosm/htdocs/targets/v5/); miRDB (http://mirdb.org/miRDB/); miRGen (http://www.diana.pcbi.upenn.edu/cgi-bin/miRGen/v3/Targets.cgi); and miRanda (http://www.microrna.org/). The predicted miRNAs were ranked according to context scores and context score percentile (Table 3). Molecular Evolutionary Genetics Analysis 5 (MEGA-5) software was used for predicting the miRNA binding seed sequence conserved in 3'-UTR of several species.

**Table 2. miRNA target prediction: number of miRNAs predicted to target SLC26A3 by five different miRNA target prediction algorithms**

<table>
<thead>
<tr>
<th>Algorithms</th>
<th>miRNAs Predicted Targeting SLC26A3</th>
<th>Site</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TargetScan</td>
<td>65</td>
<td><a href="http://www.targetscan.org/">http://www.targetscan.org/</a></td>
<td>18</td>
</tr>
<tr>
<td>MicroCosm</td>
<td>62</td>
<td><a href="http://www.ebi.ac.uk/enrightsrv/microcosm/htdocs/targets/v5/">http://www.ebi.ac.uk/enrightsrv/microcosm/htdocs/targets/v5/</a></td>
<td>17</td>
</tr>
<tr>
<td>miRDB</td>
<td>14</td>
<td><a href="http://mirdb.org/miRDB/">http://mirdb.org/miRDB/</a></td>
<td>46-47</td>
</tr>
<tr>
<td>miRGen</td>
<td>46</td>
<td><a href="http://www.diana.pcbi.upenn.edu/cgi-bin/miRGen/v3/Targets.cgi">http://www.diana.pcbi.upenn.edu/cgi-bin/miRGen/v3/Targets.cgi</a></td>
<td>11, 22, 24</td>
</tr>
<tr>
<td>miRanda</td>
<td>44</td>
<td><a href="http://www.microrna.org/">http://www.microrna.org/</a> or <a href="http://www.ebi.ac.uk/enrightsrv/cgi-bin/miRanda">http://www.ebi.ac.uk/enrightsrv/cgi-bin/miRanda</a></td>
<td>11, 22, 24</td>
</tr>
</tbody>
</table>

**Transient transfection and 3'-UTR activity.** Caco-2 and T-84 cells were transiently transfected with 1.5 μg of pmirGLO-3'-UTR-DRA alone or in combination with 20 nM of different miRNA mimics/negative control miRNA (Sigma, St. Louis, MO). For transfecting mimic or negative control miRNA alone in Caco-2 cells, we utilized Lipofectamine RNAiMax (Invitrogen Life Technologies, Carlsbad, CA; Ref. 15), which gave the best results. For this purpose, Caco-2 cells grown in T150 flask to 70% confluency were trypsinized and suspended in complete growth medium without antibiotic at a concentration of 40–100 cells/μl. Transfection materials (100 μl optiMEM, 1 μl of RNAiMax reagent, and 20 nM of mimic/negative control) prepared according to manufacturer’s instructions were added to the cell suspension and plated into 24-well plates. Forty-eight hours posttransfection, cells were lysed in passive lysis buffer (Promega). The luciferase activity was determined using the Dual Luciferase Assay Kit (Promega) and a GLOMAX 2020 Luminometer (Promega) equipped with double injectors. The effect of 3'-UTR activity on the reporter gene was calculated as a ratio of firefly luciferase to renilla luciferase and is expressed as percentage of control.

**RNA extraction and real-time PCR.** To quantitate the DRA mRNA, total RNA from Caco-2 and T-84 cells was extracted with Qiazol using miRNeasy mini kit (Qiagen) according to the manufacturer’s instruction. The quantity and total RNA were determined by Beckman DU640 spectrophotometer. Extracted RNA was amplified by Brilliant SYBR Green qRT-PCR Master Mix Kit (Agilent Technologies, Santa Clara, CA) utilizing a gene-specific primer for DRA and GAPDH (Table 1). The relative RNA levels of DRA were
expressed as percentage of control normalized to GAPDH used as internal control gene.

Quantification of mature miR expression. For quantitating the mature miRNA expression, 1 μg of total RNA was reverse transcribed to produce cDNA using the NCode miRNA first-strand cDNA synthesis kit (Invitrogen) in a 10-μl reaction according to the manufacturer’s instructions. Mature miRNA levels were quantified by Express SYBR Green qPCR supermix; universal reverse primer provided in the NCode miRNA first-strand cDNA synthesis kit (Invitrogen) and the specific miRNA forward primers for miR-21, miR-142–5p, miR-190, miR-382, miR-494, miR-568, and small RNA U6 (used as housekeeping gene) were obtained from (MWG operon Eurofins, Huntsville, AL) primer sequences listed in Table 1. Real-time PCR amplification and data capture were performed using the Stratagene MX3005P (Agilent Technologies).

Cell lysates and Western blotting. After 48 h of transient transfection with mimics, cells were washed with ice-cold 1× PBS and lysed in 1× cell lysis buffer (Cell Signaling, Danvers, MA) and 1× protease cocktail inhibitor mixture (Roche, Indianapolis, IN). The cells were lysed by sonication and the lysate was centrifuged at 7,000 rpm for 7 min at 4°C. Protein concentration was determined by the Bradford assay and the lysates were frozen at −80°C until use.

To examine the protein levels of DRA and GAPDH, 75–100 μg of cell lysates were loaded on 7.5% SDS-polyacrylamide gels and transblotted to nitrocellulose membranes. 1× PBS and 5% nonfat dry milk was used as a blocking buffer for 1 h. The membranes were then probed with human DRA (1:100 dilution) or GAPDH antibodies (1:3,000 dilution) in 1× PBS and 2.5% nonfat dry milk overnight at 4°C. The membranes were washed four times with the wash buffer containing 1× PBS and 0.1% Tween-20 for 5 min. Finally, the

Fig. 1. The downregulated in adenoma (DRA) 3′-untranslated region (UTR) sequence cloned into pmirGLO and the location of predicted microRNA (miRNA) seed sites. A: the DRA 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. Placental Mammal Basewise Conservation is based on sequence alignment of 33 mammalian genomes, including those appearing individually below. The figure is based on the output from http://genome.ucsc.edu (hg19 assembly) and http://www.targetscan.org (B) miR-494 binding site on DRA 3′-UTR in various mammalian species using MEGA 5 software.

Table 3. Key miRNAs predicted to target SLC26A3 3′-UTR based on their context score and context score percentile (TargetScan 5.1)

<table>
<thead>
<tr>
<th>Context Score</th>
<th>Context Score Percentile</th>
<th>Conserved Branch Length</th>
<th>Binding Position in SLC26A3 3′UTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-494</td>
<td>−0.48</td>
<td>98</td>
<td>1.433</td>
</tr>
<tr>
<td>hsa-miR-142-5p</td>
<td>−0.43</td>
<td>96</td>
<td>0.758</td>
</tr>
<tr>
<td>hsa-miR-382</td>
<td>−0.32</td>
<td>91</td>
<td>0.789</td>
</tr>
<tr>
<td>hsa-miR-190</td>
<td>−0.31</td>
<td>83</td>
<td>1.067</td>
</tr>
<tr>
<td>hsa-miR-190b</td>
<td>−0.31</td>
<td>82</td>
<td>1.067</td>
</tr>
<tr>
<td>hsa-miR-568</td>
<td>−0.29</td>
<td>78</td>
<td>0.444</td>
</tr>
</tbody>
</table>

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MIR-494 REPRESSES SLC26A3 EXPRESSION IN INTESTINE

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membranes were probed with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:2,000 dilution) for 1 h and the bands were visualized with Enhanced Chemiluminescence detection reagents.

**Site-directed mutagenesis.** Site-directed mutations were carried out in the miR-494 binding seed sequence of DRA 3'-UTR using the Quick change site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. Desired mutations were confirmed by sequencing. The primer sets used for site-directed mutagenesis are listed in Table 1.

**Statistical analyses.** All data were analyzed by Prism (Prism Graph Pad Software). Results are expressed as means ± SE and represent the data from three to six independent experiments. Student’s t-test or one-way ANOVA with Tukey’s multiple comparison test was used for statistical analysis. P < 0.05 was considered as statistically significant.

**RESULTS**

**Prediction of miRNAs targeting the DRA 3'-UTR.** miRDB, microcosm, miRanda, miRGen, and TargetScan algorithms were utilized to predict miRNAs with putative binding sites within the DRA 3'-UTR. As shown in Table 2, miRDB predicted 14, miRanda predicted 44, miRGen predicted 46, microcosm predicted 62, and Targetscan predicted 65 miRNAs targeting the DRA 3'-UTR. Of note, miR-494 was predicted by all five algorithms to possess a binding site in the DRA 3'-UTR. Further analysis of miR-494 demonstrated a high context score (−4.8), high context percentile (98) with a conserved branch length of 1.433 (Table 3). The potential binding site of several miRNAs along the length of DRA 3'-UTR (388 bp) and conserved sites for miRNA families among vertebrates and mammals are shown in Fig. 1A. Additionally, as shown in Fig. 1B MEGA 5 software details that binding region of miR-494, which is highly conserved in DRA 3'-UTR of various mammalian species. We selected miR-494 as well as several other miRNAs with high context scores (e.g., miR-142–5p, -190, -382, -568, and -21) for their ability to regulate DRA expression. In this regard, earlier studies have shown that miR-494 plays an important role in regulating CFTR expression (15). Since miR-494 was also identified as a highly conserved (among species) candidate for binding to DRA 3'-UTR, we were particularly interested in evaluating its role in regulation of DRA expression.

**Transient transfection with DRA 3'-UTR decreases luciferase reporter activity in intestinal epithelial cells.** To investigate the potential role of the DRA 3'-UTR in regulating gene expression, a 388-bp fragment of the DRA 3'-UTR was amplified and cloned in to a pmirGLO dual luciferase miRNA target expression vector. Caco-2 and T-84 cells were transiently transfected with pmirGLO empty vector or the pmirGLO-3'-UTR-DRA construct. As shown in Fig. 2, cells transfected with pmirGLO-3'-UTR-DRA demonstrated a significant decrease in luciferase activity in Caco-2 (56%) and T-84 (38%) cells compared with the empty vector. This decreased relative luciferase activity indicates that binding of certain miRNAs/RNA binding proteins to the DRA 3'-UTR may be responsible for the repression of the reporter luciferase activity.

**miR-494 targets the 3'-UTR of DRA.** To identify miRNAs interacting with DRA 3'-UTR, we cotransfected selected miRNA mimics with pmirGLO-3'-UTR-DRA in Caco-2 and T-84 cells. When the pmirGLO-3'-UTR-DRA and miRNA mimics were transiently cotransfected into Caco-2 cells, only the miR-494 mimic demonstrated a significant decrease in the relative luciferase activity compared with the negative control and the pmirGLO-3'-UTR-DRA transfected cells. (Fig. 3A), whereas the mimics for other selected miRNAs (e.g., miRs-142–5p, -190, -382, -568, and -21) showed no effect on DRA 3'-UTR (data not shown). Specifically, Caco-2 and T-84 cells transfected with the pmirGLO-3'-UTR-DRA and negative control mimic demonstrated a 46 and 49% reduction in luciferase activity compared with the empty vector, respectively (Fig. 3, A and B). The miR-494 mimic cotransfection into Caco-2 and T-84 cells resulted in a further 30 and 29% inhibition of luciferase activity compared with negative control cotransfected pmirGLO-3'-UTR-DRA, indicating a functional role of miR-494 in regulating DRA expression via the DRA 3'-UTR.

To further validate the hypothesis that miR-494 exerts its effects on DRA expression via direct interaction with specific putative target sequence in the DRA 3'-UTR, the seed sequence of the putative miR-494 binding site in DRA 3'-UTR was mutated (substitution mutation), using site-directed mutagenesis. The pmirGLO-DRA mut-494 was transfected alone and in combination with the miR-494 mimic in Caco-2 and T-84 cells. As shown in Fig. 4, the pmirGLO-DRA mut-494 construct, when transfected into Caco-2 and T-84 cells, resulted in an increase in basal luciferase activity, respectively, compared with the wild-type pmirGLO-3'-UTR-DRA cotransfected with mimic-494. Furthermore, mutating the putative miR-494 binding site in the pmirGLO-3'-UTR-DRA construct blocked the miR-494 mimic effect on luciferase activity.

**miR-494 does not alter endogenous DRA mRNA levels but decreases DRA protein levels.** Because miRNA binding to the 3'-UTR of mRNA may lead to either mRNA degradation or inhibition of protein translation, we investigated the specific influence of miR-494 on DRA mRNA and protein expression.

To study the effect of miR-494 on DRA mRNA expression, a miR-494 mimic was transiently transfected into Caco-2 and
After 48 h, total RNA was isolated and effect of the miR-494 mimic on DRA mRNA was assessed by realtime PCR. As shown in Fig. 5, in both Caco-2 and T-84 cells, there was no change in DRA mRNA levels in response to overexpression of miR-494. The results indicate that miR-494 does not destabilize the DRA transcript.

We next evaluated the effect of miR-494 overexpression on DRA protein levels in both the cell lines. As shown in Fig. 6, A and B, transfection with a miR-494 mimic resulted in a significant reduction in DRA protein expression both in Caco-2 (50.5%) and T-84 (58%) cell lysates compared with those from scrambled miRNA transfected cells, respectively. The data clearly indicate that miR-494 interaction with 3′-UTR of DRA results in translational repression of DRA protein expression defining a novel mechanism for the regulation of DRA expression in the intestine. While miRNAAs are known to regulate intestinal epithelial development T-84 cells. After 48 h, total RNA was isolated and effect of the miR-494 mimic on DRA mRNA was assessed by realtime PCR. As shown in Fig. 5, in both Caco-2 and T-84 cells, there was no change in DRA mRNA levels in response to overexpression of miR-494. The results indicate that miR-494 does not destabilize the DRA transcript.

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miR-31* does not alter endogenous DRA mRNA or protein levels. While we were preparing this article, a recent report suggested that miR-31* could play a role in modulating DRA expression (36). To investigate a possible direct involvement of miR-31*, we cotransfected mimic-31* with 3′-UTR of DRA in Caco-2 cells. There was no change observed in the relative luciferase activity compared with negative control (Fig. 7A).

These data suggest that miR-31* has no direct effects on DRA 3′-UTR in Caco-2 cells.

**DISCUSSION**

The studies described here demonstrate for the first time that the expression of DRA, the mammalian intestinal Cl−/HCO3− exchanger, is posttranscriptionally regulated by miR-494. Further, we showed that ectopic expression of miR-494 in model human intestinal Caco-2 or T-84 cells did not alter DRA mRNA stability but caused translational repression of DRA protein expression defining a novel mechanism for the regulation of DRA expression in the intestine. While miRNAAs are known to regulate intestinal epithelial development
and function (33) and are also involved in various pathologies associated with aberrant crypt cell proliferation (29), our studies contribute to the extremely limited knowledge of miRNAs regulating intestinal ion transport processes (8, 15).

DRA plays an essential role in the intestinal luminal Cl⁻/HCO₃⁻ exchange activity and chloride absorption. Its altered expression and dysfunction have been associated with the pathophysiology of inflammatory and infectious diarrhea (14, 32). Since miRNAs are known to contribute to the pathogenesis of intestinal inflammatory diseases including inflammatory bowel disease (7), which exhibit remarkably low DRA repression (48, 49), we hypothesized that miRNAs are involved in modulation of DRA. We initiated our studies by examining the effects of transient expression of pmirGLO-3'-UTR-DRA in intestinal epithelial cells on pmirGLO reporter activity. Upon transient transfection of pmirGLO-3'-UTR in Caco-2 and T-84 cells, the relative luciferase activity declined compared with pmirGLO empty vector transfection, indicating that the DRA 3'-UTR indeed harbors binding sites for specific miRNAs or RNA binding proteins. Current studies were mainly focused on potential role of miRNAs in regulating DRA.

Next, using five different bioinformatic approaches, we identified potential miRNA-DRA 3'-UTR target pairs. These miRNA target prediction algorithms search for perfect or

Fig. 6. miR-494 mimic transfection decreases DRA protein expression. Cell lysates of Caco-2 (A) or T-84 (B) cells transiently transfected with control negative and mimic-494 were subjected to 7.5% SDS-PAGE followed by transfer to nitrocellulose membrane. The blot was probed with anti-DRA or anti-GAPDH antibody. Bottom: densitometric analysis of the relative band intensities using ImageJ. Results represent means ± SE of 5 different experiments. ***Significant differences between control negative vs. mimic-494-transfected groups (P < 0.001).

Fig. 7. miR-31* does not alter DRA expression in Caco-2 cells. Effect of cotransfection of mimic-31* or negative control with DRA 3'-UTR on relative luciferase activity (A), DRA mRNA expression (B), and DRA protein expression (C) as measured by Western blot (top) and densitometric analysis of relative band intensities (bottom).
nearly perfect pairing to the 3′-UTR sequence (the seed sequence match), evaluate the thermodynamic stability of miRNA-mRNA hybrids and perform comparative sequence analysis to identify evolutionary conservation (34). The candidate miRNAs targeting the DRA 3′-UTR predicted by Targetscan in common with other algorithms are short listed in Table 3. Predictions by Targetscan were given priority as it is reported to be the most accurate algorithm currently available and takes into account the complementarity in the seed region, which is the most important requirement for miRNA-mRNA interactions (11) together with other factors such as free energy calculation and conservation in orthologous UTRs (1, 12, 28). Of the six miRNA candidates selected (miRs-142–5p, -190, -382, -494, -568, and 21), only miR-494 showed a significant decrease in the relative luciferase activity compared with negative control and pmirGO-3′-UTR-DRA cotransfected cells. Additionally, it showed the best possibility of positive interaction based on high context score (−0.48, Table 3), which accounts for contribution from site-type, 3′-pairing, local AU effect, and position in the 3′-UTR (12, 17, 28), thus indicating a higher probability of in vivo functionality. Furthermore, the results obtained from analysis by MEGA-5 software showed that miR-494 binding site was conserved among various mammalian species (Fig. 1B). Hence miR-494 was chosen for further analysis.

Lately, miR-494 has been recognized as a target for gene therapy. Studies by Liu et al. (31) suggested that miR-494 functions as a micro-oncogene in carcinogenesis by downregulating the phosphatase and tensin homolog (PTEN) a tumor suppressor gene. Reports have also shown posttranscriptional regulation of the chloride channel CFTR (15, 34) by miR-494. DRA is functionally coupled to CFTR in the upper gastrointestinal tract (26), and its expression has been shown to be dependent on the presence of functional CFTR, whereas mutations or loss of CFTR has been shown to result in decreased DRA expression in tracheal epithelial cells (46). Therefore, it is of physiological relevance that miR-494 is a common regulator of both these transport proteins and may have clinical relevance due to the fact that a common miRNA could target both DRA and CFTR expression.

A significant reduction in luciferase activity on cotransfection of a miR-494 mimic and the DRA 3′-UTR construct in both Caco-2 and T-84 cells not only quantitatively detected direct interaction between the DRA 3′-UTR and miR-494 but also indicated that the effects are not cell line specific. Caco-2 cell line is a human colonic carcinoma cell line that manifests many anatomic and functional similarities to absorptive intestinal enterocytes upon differentiation, whereas T-84 cells represent secretary crypt cells derived from colon. Previous studies from our laboratory and others have clearly shown that both Caco-2 and T-84 express DRA (14, 27, 40). Based on the above observations with Caco-2 and T-84 UTRs, we proposed that if DRA 3′-UTR is the actual target of miR-494, manipulation in the endogenous levels of this miRNA should alter DRA mRNA or protein levels. Therefore, we expected that miR-494 overexpression would repress DRA expression. This was experimentally verified by transfecting Caco-2 and T-84 cells with the miR-494 mimic. Interestingly, miR-494 overexpression resulted in attenuation of DRA protein expression with no change in its mRNA levels, suggesting that miR-494 decreases DRA expression by inhibiting translation of the mRNA rather than via effects on mRNA stability. It is evident from published reports that miRNAs can regulate the expression of their targets in different ways. The choice of the mechanism may be dictated by the nature of promoter (23) or by the nature of the RNA-induced silencing complex (RISC), the functional protein complex involved in association between the miRNA and its target mRNA (19). In situations where level of mRNA is not changed but protein is downregulated, the base pairing in the mRNA 3′-UTR and the miRNA produces a translational inhibitory signal. As a result, the translation initiation complex protein is inhibited and the ribosomes run off from the mRNA translational machinery producing ribosome free mRNA that undergoes storage (30). Our results draw support from various similar studies where silencing by a miRNA is observed with either no change or significantly smaller change in mRNA levels compared with the protein levels (5, 30).

Our studies further demonstrated that mutations in the miR-494 binding region in 3′-UTR of DRA, which is complementary to the 5′-end of miR-494, abrogated the decrease in reporter activity caused by miR-494 overexpression. These data are suggestive of the fact that manipulations in the predicted target site can reduce the inhibitory function of miR-494. Thus it is likely that miR-494 directly interacts with DRA 3′-UTR and suppresses the corresponding protein product. While we were at a juncture to complete our miR-494 studies, a report in the literature (36) indicated that DRA suppression in metastatic colorectal cancer patients was via upregulation of miR-31*. This conclusion was based on their microarray results. However, when we performed detailed analysis of miR-31* using mimic-31* and DRA 3′-UTR as well as the levels of DRA mRNA and protein, our data showed that miR-31* has no direct effect on DRA expression.

The cytoplasmic life of an mRNA could be governed through both 5′- and 3′-ends. Previous studies from our laboratory have shown the transcriptional regulation of DRA gene (2, 14, 39, 41, 43). In the present study our data show for the first time a novel regulatory pathway of down regulation of DRA by miR-494 via mechanisms involving translational repression through its interactions with the DRA mRNA 3′-UTR. We speculate that targeting miR-494 using antagoniRs may have therapeutic potential for treating diarrheal conditions where DRA expression is decreased.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
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