MIR-140-3P REGULATION OF TNF-α-INDUCED CD38 EXPRESSION IN HUMAN AIRWAY SMOOTH MUSCLE CELLS

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Abstract

CD38 is a membrane protein expressed in airway smooth muscle (ASM) cells and plays a role in cellular calcium dynamics and ASM contractility. In human ASM (HASM) cells, TNF-α induces CD38 expression through activation of MAPKs and NF-κB and AP-1, and its expression is differentially elevated in cells from asthmatics compared to the cells from non-asthmatics. The CD38 3’UTR has targets for miR-140-3p. We hypothesized that miR-140-3p regulates CD38 expression in HASM cells by altering CD38 mRNA stability. Basal and TNF-α-induced expression of miR-140-3p were determined in non-asthmatic ASM (NAASM) and asthmatic ASM (AASM) cells. NAASM and AASM cells were transfected with control or miR-140-3p mimic or miR-140-3p antagonirs and CD38 expression and CD38 mRNA stability were determined. Luciferase reporter assays were used to determine miR-140-3p binding to CD38 3’UTR. Activation of p38, ERK, JNK MAPKs, NF-κB and AP-1 were determined in miR-140-3p mimic-transfected NAASM. TNF-α attenuated miR-140-3p expression in NAASM and AASM cells, but to a larger magnitude in AASM cells. MiR-140-3p mimic attenuated CD38 mRNA expression in NAASM and AASM cells with comparable magnitude. Mutated miR-140-3p target on CD38 3’UTR reversed the inhibition of luciferase activity by miR-140-3p mimic. CD38 mRNA stability was unaltered by miR-140-3p mimic in NAASM or AASM cells following arrest of transcription. MiR-140-3p mimic attenuated TNF-α-induced activation of p38 MAPK and NF-κB. The findings indicate that miR-140-3p modulates CD38 expression in HASM cells through direct binding to CD38 3’UTR and indirect mechanisms involving activation of p38 MAPK and NF-κB. Furthermore, indirect mechanisms appear to play a major role in the regulation of CD38 expression.
Introduction

CD38 is a cell surface protein expressed in a variety of mammalian cells including airway smooth muscle (ASM) cells (30). CD38 possesses multiple enzymatic activities, with the ADP-ribosyl cyclase activity generating cyclic ADP-ribose (cADPR), a Ca\(^{2+}\) mobilizing agent (12, 18). Past studies in our laboratory have established that CD38/cADPR pathway plays an important role in cellular Ca\(^{2+}\) dynamics and ASM contractility (6, 7). In HASM cells, TNF-\(\alpha\) induces CD38 expression through activation of transcription factors NF-\(\kappa\)B and AP-1 and MAPK kinases (27). Among the MAP kinases, p38 and ERK MAP kinases mediate TNF-\(\alpha\)-induced CD38 expression through modulating the transcript stability (27). We recently reported that, in ASM cells obtained from subjects with history of asthma, TNF-\(\alpha\)-induced CD38 expression was differentially elevated, although the mechanistic basis of this differential elevation was not clearly understood (15).

MicroRNAs (miRNA) are non-coding small RNAs emerging as post-transcriptional regulators in various biological processes including inflammation. MicroRNAs can regulate expression of their target genes either by destabilizing the transcripts or through translational repression (2). Accumulating evidence has proven that miRNAs are associated with various pathological conditions in humans. Recent studies have shed light on the role of miRNAs in airway disorders like asthma, chronic obstructive pulmonary disorder (COPD) and idiopathic pulmonary fibrosis (reviewed in (22). Recent studies reported a role for miR-21 in determining the Th1/Th2 immune response to antigen, thus playing a role in the pathogenesis of allergic asthma (19, 20). Other studies have attempted to determine the role of specific miRNA in airway
inflammation and allergic airway hyperresponsiveness. The roles of let-7 and miR-155 in IL-13 signaling, and the potential role of miR-133 on Rho-A expression were some of the notable studies that highlight the roles of miRNA in the pathogenesis of airway inflammatory disorders (5, 19-21). In the present study, bioinformatic tools were used to determine potential miRNA response elements in the 3’UTR of human CD38 gene. Expression of miR-140-3p, which came as a top hit in one of the target prediction algorithms, and its functional role in CD38 expression were determined in asthmatic and non-asthmatic ASM cells. We hypothesized that miR-140-3p downregulates CD38 expression in HASM cells through post-transcriptional mechanisms.
Materials and Methods

Reagents
Tris base, glucose, HEPES, and other chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise noted. Human recombinant TNF-α (rhTNF-α) was purchased from R&D Systems (Minneapolis, MN). Hanks’ balanced salt solution (HBSS) and Dulbecco’s modified Eagle medium (DMEM) were purchased from Gibco-BRL (Grand Island, NY). Trizol, Superscript III reverse transcriptase, NCode miRNA first strand synthesis kit, Platinum SYBR Green qPCR mix and Lipofectamine RNAiMax were purchased from Invitrogen (Life Technologies, Carlsbad, CA). MiRVana RNA isolation kit was purchased from Ambion (Life Technologies, Carlsbad, CA). NE-PER nuclear/cytoplasmic extraction kit was purchased from Pierce (Rockford, IL) Antibodies for MAP kinases, NF-kB and Lamin A/C, Actin, α-tubulin, MKK3, MKP-1 and NRIP-1 were purchased from Cell Signaling Technology. Trans-AM ELISA kits (NF-κB and AP-1) were purchased from Active Motif (Carlsbad, CA). Quick Change Lightning multi site-directed mutagenesis kit was purchased from Agilent Technologies (Santa Clara, CA). Control oligonucleotide (C.ele-miR-67-), miR-140-3p mimic and antagonir oligonucleotides (hsa-miR-140-3p mature sequence 5’-UACCACAGGGUAGAACCACGG-3’) were purchased from Dharmacon (Lafayette, CO). The chemiluminescent substrate for HRP was purchased from Millipore (Billerica, MA).

Human Airway Smooth Muscle Cell cultures and treatments
The procedures for the isolation and culture of human airway smooth muscle (HASM) cells are described in earlier publications (6, 15). Briefly, HASM cells were obtained from Dr. Panettieri’s laboratory (University of Pennsylvania, Philadelphia, PA). The cells
were from de-identified healthy donors (non-asthmatic ASM cells-NAASM) and donors who have died due to severe asthma (asthmatic ASM cells-AASM). All experiments were conducted in HASM cells in their 4th or 5th passage. In all the experiments, prior to exposure to TNF-α, the cells were growth-arrested for 48 h in arresting medium without serum, but in the presence of transferrin and insulin. In experiments to determine the expression of CD38 and miR-140-3p, cells were exposed to vehicle (0.1% bovine serum albumin in PBS) or 10 ng/ml human recombinant TNF-α (rhTNF-α) for 0-24 hrs. To determine CD38 mRNA stability, cells were exposed to TNF-α for 12 hrs, and then washed to remove TNF-α before the addition of actinomycin D (5 μg/ml) to arrest transcription. Total RNA was collected at 0, 6, 12 and 24 hrs after transcriptional arrest. Data is reported for only 0 and 24 hr time points for brevity. In experiments to determine the activation of NF-κB or MAP Kinases, cells were exposed to rhTNF-α (10 ng/ml) for 1 hr or 15 min, respectively.

**Extraction of total RNA and cDNA synthesis**

Total RNA was extracted from HASM cells following manufacturer’s instructions (miRvana, Ambion). Briefly, HASM cells (50,000-200,000 cells) were collected in sterile PBS, centrifuged and the pellet was homogenized in a buffer provided in the kit. Column-eluted total RNA was quantified in Agilent Nano-drop bioanalyzer. To synthesize cDNA from small RNA, 200 ng of total RNA was poly-adenylated and cDNA was synthesized using NCode First strand synthesis kit according to manufacturer’s instructions. In parallel, 200 ng of total RNA was used to synthesize cDNA from larger RNA using Superscript II reverse transcription kit.

**Extraction of whole cell/nuclear lysates**
Whole cell lysates were obtained by sonicating HASM cells (200,000-500,000 cells) in lysis buffer (50 mM Tris, 100 mM NaCl, 50 mM NaF, 40 mM beta-glycerol phosphate, 2 mM EDTA, 0.2 mM Na3VO4, 1% Triton X-100, and protease inhibitor cocktail, pH 7.4). Nuclear extracts were collected from HASM cells (500,000-600,000 cells) using NE-PER cytoplasmic/nuclear extraction kit, according to the manufacturer’s protocols.

**Transient transfection of HASM cells**

HASM cells were plated into appropriate formats and cell number (200,000 cells/well in 6-well plate or 500,000 cells/plate in 100-mm culture plate) 24 hrs prior to transfection. Transfection was performed using Lipofectamine RNAiMax according to the manufacturer’s instructions. Briefly, control oligonucleotide (C.ele-miR-67) or miR-140-3p mimic oligonucleotides or miR-140-3p antagonirs were transfected in a range of concentrations (5-100 nM). Control oligonucleotide was transfected at 50 or 100 nM concentrations.

**qRT-PCR**

To determine the miR-140-3p expression, a forward primer specific to the miR-140-3p and a universal primer targeting the poly-T of the cDNA were used according to the manufacturer’s instructions. Mammalian small nuclear RNA U6 was used for normalization of miR-140-3p expression. To determine CD38 expression, qRT-PCR was performed using Brilliant SYBR Green master mix as described in a previous publication (27). Cyclophilin was amplified as the house-keeping control.

**Site-directed mutagenesis**
Site-directed mutagenesis was performed in luciferase-CD38 3’UTR reporter plasmids. CD38-3’UTR (481 bases long; UGC genome browser) has a predicted miR-140-3p target site at 8 bases after the stop codon in the CD38 mRNA. The first miR-140-3p target had a 7-base complementarity to the miR-140-3p. A second target site with a 6-base complementarity to miR-140-3p was found 21 bases away from the first target site. First target site (mutated 1) or both target sites (mutated 1+2) were mutated to determine the selectivity of miR140-3p binding to these sites. QuikChange Lightning multi site-directed mutagenesis kit was used to mutate four bases (taGaGa) at first target site (CTGTGGT) or three bases (aGaGa) at the second target site (TGTGGT) (Figure 2A, Upper panel). Twenty bases flanking the target site with mutation was designed as primers for mutation. The primers for each mutagenesis were as follows: First target site mutation primer: TCTGAGATCTGAGCCAGTCGtaGaGaGaTGTTTTAGCTCCTTGACTCC. Second target site mutation primer: TTTAGCTCCTTGACTCCTaGaGGaTTATGTATCATCATACTGACTCAGC. Wild type and mutant plasmids were expanded in E.coli and sequenced to confirm mutations.

**SDS-PAGE and Western Blot**

Ten micrograms of total protein was resolved in a 4-20% Tris-HCl SDS gel and electrophoretically transferred onto PVDF membrane. The blot was blocked in 5% skim milk solution in phosphate buffered saline containing 0.05% Tween 20 for minimum 4 hrs. The blot was probed with relevant primary antibodies followed by incubation with horse-radish peroxidase-conjugated secondary antibodies. After washes in PBS
containing 0.05% tween 20, the blots were treated with the chemiluminescent substrate for HRP and exposed to an X-ray film to visualize bands.

**Enzyme-Linked Immunosorbent Assay (ELISA)**

ELISA was performed to determine the NF-κB or AP-1 activation according to the manufacturer’s instructions (Activ Motif) and as previously described (15). Briefly, 3 μg of nuclear extracts from HASM cells were incubated in multi-well plate coated with oligonucleotides carrying consensus NF-κB or AP-1 sequences. Twenty pmol (20X excess) of competitor oligonucleotide was added to some of the reactions to determine the specificity of the binding.

**ADP-ribosyl cyclase assay**

The ADP-ribosyl cyclase activity of HASM cell lysates was quantified by measuring the reverse cyclase activity of CD38. HASM whole cell lysates containing 5 μg of total protein were incubated for 1 hr at 37°C with or without 10 mM nicotinamide in the presence of 0.45 mM cyclic-ADP ribose. The reverse cyclase reaction was stopped by adding 25 μl of 1M HCl, vacuum filtered through a protein-binding membrane (Immobilon, 0.45 μm, Millipore), neutralized with 15 μl of 2 M Tris-base. The filtrate was incubated with reagent mixture containing 2 μM rezasurin, 0.76% v/v ethanol, 4 μM Flavin Mononucleotide (FMN), 40 μg/ml alcohol dehydrogenase and 0.04 U/ml diaphorase in NaH₂PO₄/Na₂HPO₄ buffer, pH 6.8 at room temperature. The fluorescence was quantified (excitation at 544 nm and emission at 590 nm) in a fluorometer (FLUO star Galaxy, BMG biotechnologies) and the rate of fluorescence emission was calculated. The quantity of NAD generated in the reaction was calculated from a standard curve generated from known NAD.
Data analysis

Each experiment was performed 3-6 times (NAASM or AASM cells obtained from 3-6 donors were used). The data from mRNA fold change, enzymatic activity and densitometry ratio were expressed as mean±SEM and statistically analyzed by student t-test or one-way ANOVA (depending on the number of experimental groups analyzed) using GraphPad Prism software. The differences were considered significant when the p value was ≤ 0.05.
Results

MicroRNA-140-3p expression in HASM cells

Human airway smooth muscle cells were exposed to vehicle (0.1% BSA in sterile PBS) or TNF-α (10 ng/ml) for 24 hrs and the expression of miR-140-3p and CD38 mRNA were determined. In TNF-α-treated NAASM cells, miR-140-3p expression was marginally reduced compared to the vehicle-treated cells (Figure 1A). In TNF-α-treated AASM cells, miR-140-3p expression was significantly reduced compared to the vehicle-treated cells (Figure 1B). The basal miR-140-3p expression levels were comparable in NAASM and AASM cells (Figure 1C). Exposure to TNF-α for 24 hrs resulted in down regulation of miR-140-3p expression in both groups of cells, but to a higher magnitude in AASM cells (Figure 1C). Exposure to IL-13, a Th2 cytokine with critical role in asthma pathogenesis, did not alter miR-140-3p expression in either group of HASM cells (data not shown).

In another set of experiment, miR-140-3p expression was determined at different times following exposure to TNF-α. Significant attenuation of miR-140-3p expression was noted at 24 hrs of TNF-α exposure in AASM cells compared to expression in NAASM cells (Figure 1D). TNF-α induced CD38 mRNA expression in time-dependent manner in both NAASM and AASM cells, with larger magnitudes of CD38 expression in AASM cells, confirming our earlier findings (15) (Figure 1E).

MiR-140-3p mimic inhibits TNF-α-induced CD38 up regulation in HASM cells

To determine the functional role of miR-140-3p in CD38 expression in HASM cells, we transfected NAASM and AASM cells with mimic miR-140-3p oligonucleotides or
control oligonucleotides and determined the effects on CD38 mRNA expression and
ADP-riboysl cyclase activity. Transfection of NAASM cells with a range of miR-140-3p
mimic oligonucleotides resulted in a concentration-dependent increase in miR-140-3p
expression (Figure 2A). In cells transfected with miR-140-3p mimics, up regulation of
TNF-α-induced CD38 mRNA was significantly attenuated in a concentration-dependent
manner, although without a linear relationship to the mimic concentration (Figure 2B). At
higher concentrations of miR-140-3p mimic (20 and 50 nM), TNF-α-induced ADP-
ribosyl cyclase activity was significantly reduced compared to the cells transfected with
50 nM of control oligonucleotide (Figure 2C). At the optimal concentration (50 nM),
mir-140-3p mimic transfection attenuated TNF-α-induced CD38 mRNA expression in
NAASM and AASM cells to similar magnitude (Figure 2D). Transfection of NAASM
and AASM cells with miR-140-3p mimic oligonucleotides also resulted in comparable
inhibitory effects on the TNF-α-induced ADP-ribosyl cyclase activity (Figure 2E).
Transfection of antagomirs for miR-140-3p did not alter basal or TNF-α-induced ADP-
ribosyl cyclase activity in either group of HASM cells (Figure 2E).

**MiR-140-3p targets CD38 3’UTR**

To determine whether miR-140-3p brings about its effects through directly binding to the
3’UTR of CD38, dual luciferase reporter assays were performed in HEK293 or NIH3T3
cells. Co-transfection of cells with miR-140-3p mimic oligonucleotides and the wild type
Luc-CD38 3’UTR, resulted in a marginal 10-20% inhibition of luciferase activity in
HEK293 or NIH3T3 cell lines. Site-directed mutation of the first miR-140-3p target on
the CD38 3’UTR partially reversed the inhibition by miR-140-3p mimic oligonucleotide
in HEK 293 cells (Figure 3A). When the luciferase reporter assay studies were repeated
in NIH-3T3 cells, site-directed mutation of the first miR-140-3p target completely reversed the luciferase inhibition by miR-140-3p mimic (Figure 3B). Mutating both miR-140-3p targets on the CD38 3’UTR resulted in elevated luciferase activity in the presence of miR-140-3p mimic oligonucleotides (Figure 3B). To determine the effect of miR-140-3p mimic transfection on CD38 mRNA stability, NAASM and AASM cells were transfected with 20 nM of control oligo or mimic 140. At 12 hrs following TNF-α exposure (0 hr), mimic-transfected NAASM and AASM cells showed attenuated CD38 mRNA levels compared to control oligo-transfected cells (Figure 3C). There were no further reductions in CD38 mRNA levels in mimic-transfected cells at 6, 12 and 24 hrs in either group of HASM cells (Figure 3C, 6 and 12 hrs data not shown).

**MiR-140-3p mimic attenuates activation of p38 MAP Kinase in HASM cells**

Results of the 3’UTR luciferase reporter assay indicated that direct binding of miR-140-3p mimic on the 3’UTR of CD38 only partially accounted for the inhibition of CD38 expression. We previously reported that the MAP kinases mediate TNF-α-induced CD38 expression in HASM cells (27). Therefore, to determine whether changes in MAP kinase activation were involved in miR-140-3p effect on CD38 expression, the activation of p38, ERK and JNK MAP kinases were determined in HASM cells following transfection with miR-140-3p mimic oligonucleotides. Transfection with miR-140-3p mimic reduced the TNF-α-induced p38 phosphorylation, with no significant changes in the expression of total p38 (Figure 4A and B). TNF-α-induced activation of ERK or JNK activation was not altered by miR-140-3p mimic transfection (Figure 4C and D). Expression of MAP kinase kinase 3 (MKK3), a Ser/Thr protein kinase upstream of p38 MAP kinase, was not
altered in the presence of miR-140-3p mimic oligonucleotides (Figure 4E, Upper and Lower Panels). We also determined the expression of dual-specificity phosphatase-1 (MKP-1), a phosphatase known to inactivate p38, ERK, and JNK MAP kinases. Transfection of HASM cells with miR-140-3p antagomir or mimic oligonucleotides did not alter the MKP-1 expression (Figure 4F).

**MiR-140-3p mimic attenuates activation of NF-κB in HASM cells**

We previously reported that transcription factors NF-κB and AP-1 mediate TNF-α-induced CD38 expression in HASM cells (16, 27). Transient transfection of HASM cells with miR-140-3p mimic oligonucleotides marginally attenuated the activation of transcription factor NF-κB (Figure 5A and B). MiR-140-3p transfection did not have a significant effect on TNF-α-induced AP-1 activation (Figure 5C). Expression of nuclear interacting protein (RIP) was not altered by mimic 140 transfection in HASM cells (Figure 5D).
Discussion

This is the first report of the regulatory role of miR-140-3p on CD38 expression in HASM cells. Our findings show that miR-140-3p expression is lower in HASM cells obtained from donors with history of severe asthma, in the presence of TNF-α. Our findings also indicate that the effects of miR-140-3p on CD38 expression are mediated through both direct binding of the miRNA to CD38 transcript and indirect mechanisms involving activation of p38 MAP kinase and NF-κB. The net result of the modest inhibition of activation of p38 and NF-κB by miR-140-3p transfection is the robust attenuation of CD38 expression in HASM cells.

Previous studies conducted in our laboratory have established that TNF-α-induced CD38 expression in HASM cells is mediated both at the transcriptional and post-transcriptional levels (16, 27). The MAP Kinases ERK1/2 and p38 play a role in the post-transcriptional regulation, whereas p38 and JNK MAP Kinases have a role in transcriptional regulation of CD38 gene (27). One of the objectives of the current study is to determine the role of miR-140-3p in the regulation of CD38 expression. Studies by other investigators have revealed that specific miRNAs, such as miR-21 and miR-133a, contribute to the pathogenesis of airway inflammatory disorders (5, 20). Chiba et al showed that IL-13, a Th2 cytokine with prominent role in allergic asthma, downregulates the expression of miR-133a (5). It is suggested that the downregulation of miR-133a leads to elevated RhoA, a pro-contractile protein in the ASM cells. CD38 contributes to the development of AHR in mouse models of asthma (10, 11). CD38 null mouse developed significantly lower levels of airway responsiveness compared to the wild type mouse, in response to contractile agonist methacholine (7). CD38 null mouse also
developed reduced AHR compared to that of wild type mouse, following brief exposure to TNF-α or IL-13 (10, 11). Therefore, investigating the miRNAs that target CD38 gene expression in HASM cells may lead to an understanding of the signaling pathways involved in the pathogenesis of AHR and asthma.

Multiple web-based target prediction algorithms (Target Scan-www.targetscan.org, miRWalk-www.ma.uni-heidelberg.de and miRbase-www.mirbase.org) were used to determine potential miRNA targets in the CD38 3’UTR. From the list of predicted miRNAs, miR-140-3p was chosen for further investigation because it has been reported as one of the highly expressed miRNAs in HASM cells (32). Furthermore, in HASM cells exposed to a mixture of inflammatory cytokines including TNF-α, there is significant downregulation of several miRNAs, including miR-140 and some miRNAs involved in the regulation of smooth muscle phenotype (17). In earlier publications by other investigators, miR-140 has been reported as a cartilage-specific miRNA in mouse and Zebrafish (29, 31). However, these reports were largely referring to miR-140-5p, one of the two mature miRNAs originating from the precursor miR-140. In the present study, we focused on miR-140-3p and its role in regulation of CD38 expression.

Altered miRNA expression profile has been reported in T cells obtained from severe asthmatics, indicating that miRNAs are one of the mechanisms involved in the cellular phenotypic changes observed in asthma (28). Attenuation of miR-140-3p expression in AASM cells by TNF-α suggests that this miRNA may have a role in the asthmatic phenotype in ASM cells. The mechanisms involved in miR-140-3p down regulation by TNF-α in HASM cells are yet to be determined. Recent studies in human
chondrocytes showed that the proximal upstream region of pri-miR-140 has functional
response elements for chondrogenic transcription factors Sox5/Sox6/Sox9, indicating
transcriptional regulation of both miR-140-3p and miR-140-5p expressions (33).
Different transcriptional regulators and epigenetic mechanisms such as DNA methylation
may be involved in the altered miR-140-3p expression in asthmatic ASM cells. The
finding that the miR-140-3p expression is attenuated to a larger magnitude in AASM
cells in the presence of TNF-α suggests an anti-inflammatory role for this miRNA. The
potential anti-inflammatory role for miR-140-3p is supported by findings of a previous
study that reported downregulated miR-140 expression in whole lung lysate from rats
exposed to cigarette smoke extract (14). We also found that IL-13, a Th2 cytokine with
major role in asthma, did not alter the expression levels of miR-140-3p in NAASM or
AASM cells (data not shown). These observations suggest that miR-140-3p may have a
functional role selective to TNF-α signaling. TNF-α is a cytokine with major role in the
pathogenesis of asthma (1, 26). TNF-α expression is elevated in the airways of asthmatic
patients and some recent therapeutics for asthma target TNF-α and its receptors in lungs
(3, 4, 13, 34). Therefore, defining the role of microRNAs involved in the regulation of
TNF-α-induced genes in airway smooth muscle may have a therapeutic potential.
Although TNF-α attenuates miR-140-3p expression to a greater extent in ASM cells from
asthmatics, it doesn’t appear to solely contribute to the differential induction of CD38
expression by TNF-α that we reported in a recent study (15).
Although the inhibitory effect of miR-140-3p mimic on Luciferase activity was
modest, mutating both miR-140-3p target sites reversed the inhibition, indicating
binding of the miRNA to CD38 3’UTR. Since the 3’UTR of CD38 transcript possesses
targets for other miRNAs as well, multiple miRNAs may be required to have a significant
effect on stability or translatability of the CD38 transcript. However, the significant
inhibition of CD38 mRNA and protein expression by miR-140-3p mimic
oligonucleotides suggests that additional mechanisms are involved in miR-140-3p
regulation of CD38 expression in HASM cells. In this context, we found that miR-140-3p
mimic attenuated p38 MAP kinase activation with no apparent effects on ERK or JNK
MAP kinases, demonstrating the selectivity of miR-140-3p for specific targets on cell
signaling pathways in HASM cells. The marginal inhibitory effect of miR-140-3p mimic
on p38 MAP kinase activation does not appear to be related to the upstream kinase
MKK3 or the MAP kinase phosphatase DUSP-1. However, it remains to be determined
whether other MAPK phosphatases with higher substrate specificity to p38 MAP kinase
mediate the miR-140-3p-mediated attenuation of p38 activation.

The significant attenuation of TNF-α-induced CD38 mRNA expression by miR-
140-3p mimic suggested that the regulation by miRNA may be through transcription of
CD38 gene in HASM cells. Transcription factors NF-κB and AP-1 mediate TNF-α-
induced CD38 expression in HASM cells (16). The role of NF-κB signaling in airway
inflammatory disorders has been demonstrated through in vitro studies and animal
models (8, 24). For this reason and due to the availability of numerous synthetic
inhibitors of NF-κB signaling, this pathway remains an attractive therapeutic target in
airway inflammatory disorders like asthma and COPD (reviewed in 9). Although the
DNA binding activity of NF-κB was only marginally reduced by miR-140-3p mimic
transfection, the reduction was a consistent finding in 5 independent experiments. Further,
a recent study reported attenuation of NF-κB activation by miR-140-3p in hepatocytes,
primarily by targeting nuclear receptor interacting protein 1 (NRIP-1), a co-activator of NF-κB (23). This is an unlikely mechanism in HASM cells since NRIP-1 expression was unaltered in cells transfected with miR-140-3p. The results of CD38 mRNA stability also showed that following transcription arrest, CD38 mRNA levels were maintained for up to 24 hrs. It should be noted that the expression levels of miR-140-3p remain high for up to 72 hrs following transfection. If the effects of miR-140-3p on CD38 transcript levels were due to binding to 3’UTR target sites, we would have seen a significant decline in transcript levels following transcription arrest. A lack of correlation between miR-140-3p and CD38 expression following TNF-α treatment strengthens the argument that miR-140-3p effects are largely indirect and time-dependent. Furthermore, overexpression of miR-140-3p causes a significant decline in CD38 transcript levels before arrest of transcription, with no further decline in transcript levels for up to 24 hrs, favoring a mechanism that involves indirect transcriptional regulation of CD38 expression in HASM cells.

In summary, miR-140-3p regulates TNF-α-induced CD38 expression in HASM cells through direct interaction with 3’UTR of CD38 mRNA and indirect mechanisms involving activation of p38 MAP kinase and transcription factor NF-κB. However, we cannot rule out other indirect mechanisms, such as competing endogenous RNAs (25) in the regulation of CD38 expression in HASM cells. Understanding the signaling pathways involved in miR-140-3p regulation of CD38 expression in HASM cells may reveal novel therapeutic targets for AHR and asthma.
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**Figure Legends**

**Figure 1. MiR-140-3p expression in NAASM and AASM cells**

Expression of miR-140-3p was determined in vehicle-treated (C) or TNF-α-treated (T10) NAASM and AASM cells. A) In the presence of TNF-α, miR-140-3p expression was marginally attenuated in NAASM cells compared to the cells treated with vehicle (C) (n=5). B) In AASM cells, TNF-α-significantly attenuated the miR-140-3p expression (n=6, *p<0.05). C) The basal miR-140-3p expression levels were comparable between NAASM and AASM cells. In the presence of TNF-α, miR-140-3p expression was significantly attenuated in AASM cells compared to the NAASM cells (*p<0.05, the data showed in this figure and figures A and B are from same experiments). D) When NAASM and AASM cells were exposed to TNF-α for various durations, both groups of cells showed attenuated miR-140-3p expression at 24 hrs, although the reduction was statistically significant only in AASM cells (n=3, *p<0.05). E) TNF-α caused a time-dependent increase in CD38 mRNA expression in both NAASM and AASM cells. The magnitude of CD38 induction in AASM cells was higher in AASM cells, although the differential increase was not statistically significant due to larger SEM in AASM cells.

**Figure 2. MiR-140-3p mimic inhibits TNF-α-induced CD38 expression in HASM cells**

Mimics of miR-140-3p or antagomirs for the miRNA were transiently transfected into NAASM and AASM cells and CD38 mRNA expression and ADP-ribosyl cyclase...
activity were determined. A) Transient transfection of NAASM cells with miR-140-3p mimic oligonucleotide resulted in a concentration-dependent increase in miR-140-3p expression (n=3). B) Transfection of miR-140-3p mimics in NAASM cells resulted in a concentration-dependent attenuation of TNF-α-induced CD38 mRNA expression, although the relationship between the mimic concentration and CD38 expression was not linear (n=3, *p<0.05, all concentrations compared to the control oligo transfection). C) In NAASM cells transiently transfected with miR-140-3p mimic oligonucleotides, ADP-ribosyl cyclase activity was significantly attenuated at higher concentrations of the mimic (n=3, *p<0.05, 20 nM or 50 nM compared to the control oligo transfection). D) When NAASM and AASM cells were transiently transfected with 50 nM of control oligonucleotides or miR-140-3p mimic oligonucleotides, CD38 mRNA expression was comparably attenuated in both groups of cells (n=2). E) TNF-α-induced ADP-ribosyl cyclase activity was attenuated to a comparable magnitude in NAASM and AASM cells in the presence of miR-140-3p mimic oligonucleotides (n=3, *p<0.05). Further, basal and TNF-α-induced ADP-ribosyl cyclase activities were unaltered in NAASM or AASM cells transiently transfected with miR-140-3p antagomir oligonucleotides (n=3, *p<0.05, 140 mimic-T compared to control oligo-T).

**Figure 3. MiR-140-3p targets CD38 3’UTR**

Luciferase reporter assays were performed in Luc-CD38-3’UTR constructs containing either wild type target (WT) or mutated target (Mutated). A) Upper panel: Diagram showing the two miR-140-3p targets on CD38 3’UTR and the mutations on each target. Lower panel: In HEK293 cells co-transfected with WT Luc-CD38-3’UTR and miR-140-3p mimic oligonucleotides, the luciferase activity was marginally reduced compared to
the cells that were co-transfected with scrambled-sequence oligonucleotides. In HEK293 cells co-transfected with target-mutated Luc-CD38-3’UTR and miR-140-3p mimic oligonucleotides, inhibition of luciferase activity was partially reversed (n= 3). B) In NIH3T3 cells co-transfected with WT Luc-CD38-3’UTR and miR-140-3p mimic oligonucleotides, the luciferase activity was marginally reduced compared to the cells that were co-transfected with scrambled-sequence oligonucleotides. When the first miR-140-3p target on Luc-CD38-3’UTR was mutated (Mutated 1), inhibition of luciferase activity was completely reversed. When both miR-140-3p targets on Luc-CD38-3’UTR was mutated (Mutated 1+2), the luciferase activity was significantly elevated in the presence of miR-140-3p mimic oligonucleotides (n=4). C) In NAASM and AASM cells transfected with miR-140-3p mimic, CD38 mRNA levels were comparably attenuated at 0 hr (NA-0 and A-0; following TNF-α removal and transcriptional arrest), compared to the cells transfected with control oligonucleotides. There were no further reductions in CD38 mRNA levels at 6, 12 or 24 hrs (NA-24 and A-24) following transcriptional arrest in mimic-transfected cells of either group. (*p<0.05, compared to wild type or mutated vector co-transfected with scramble oligonucleotide; **p<0.05, compared to wild type vector co-transfected with 140 mimic oligonucleotide).

Figure 4. MiR-140-3p mimic attenuates activation of p38 MAP Kinase in NAASM cells.

TNF-α-induced activation of p38, ERK and JNK MAP Kinases were determined in NAASM cells transiently transfected with 50 nM of control oligonucleotides or miR-140-3p mimic oligonucleotide. A) Representative western blot showing marginally reduced TNF-α-induced p38 MAP kinase activation in miR-140-3p mimic transfection. B)
Average relative densitometry measurement (n=5) of western blot, showing marginally reduced p38 MAP kinase activation in the miR-140-3p mimic-transfected NAASM cells. Basal or TNF-α-induced activation of C) ERK or D) JNK MAP kinases were not altered in the presence of miR-140-3p mimics (blots representative of 5 independent experiments). E) Upper and Lower Panels: Expression of MKK3, a kinase upstream of p38 MAP kinase, was not altered by transient transfection with miR-140-3p mimic oligonucleotides (blot representative of 3 independent experiments). F) A representative blot showing unaltered expression of MKP-1, a dual-specificity phosphatase (DUSP-1), in HASM cells transfected with miR-140-3p antagomir or miR-140-mimic oligonucleotides (n=3).

**Figure 5. MiR-140-3p mimic attenuates activation of transcription factor NF-κB in NAASM cells.**

TNF-α-induced activation of NF-κB was determined in NAASM cells transiently transfected with 50 nM of control oligonucleotide or miR-140-3p mimic oligonucleotides. A) Representative blot shows TNF-α-induced nuclear translocation of NF-κB (phosphorylated p65 subunit) was marginally attenuated in NAASM cells transfected with miR-140-3p mimic oligonucleotides. B) TNF-α-induced activation of NF-κB, measured as the binding of p65 subunit to a consensus DNA motif, was marginally reduced in NAASM cells transfected with miR-140-3p mimic oligonucleotides (n=5). C) TNF-α-induced activation of AP-1 (measured through binding of p-c-Jun to the consensus DNA sequence) was unaltered in the presence of miR-140-3p mimic oligonucleotides (n=4). D) Expression of nuclear receptor interacting protein (RIP), which is associated with NF-κB activation in other cell systems, was not altered in
HASM cells transfected with mimic 140 (representative blot and densitometry data average of 3 independent experiments, C-Vehicle; T-TNF-α).
REFERENCES


Figure 1. MiR-140-3p expression in NAASM and AASM cells
Figure 1. MiR-140-3p expression in NAASM and AASM cells (Contd.)

![Graph showing fold change in CD38 mRNA (relative to 0 hr)]
Figure 2. MiR-140-3p mimic inhibits TNF-α-induced CD38 expression in HASM cells.
Figure 3. MiR-140-3p targets CD38 3’UTR

A

End of ORF

5'-AGATCTGaGCGACTGCTGTGGTTTTTAGCTCCTTGACTCCTTTGTTTATGT-3'

1st target

2nd target

1st target mutation

2nd target mutation

B

Luciferase Activity (RLU)

Target

Wild type

Mutated 1

Oligo

Scramble

140 mimic

Target

Wild type/Mutated

Wild type

Mutated 1

Mutated 1+2

Oligo

Scramble

140 mimic

140 mimic

140 mimic
Figure 3. MiR-140-3p targets CD38 3’UTR (Contd.)

![Graph showing the change in CD38 mRNA fold change in mimic 140 transfection relative to control transfection over time (hrs after ActD).]
Figure 4. MiR-140-3p mimic attenuates activation of p38 MAP Kinase in NAASM cells.

A) Cont. oligo  |  Mimic 140

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B) p38 Activation (Density ratio)

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C) Cont. oligo  |  Mimic 140

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D) Phos-JNK  |  Total-JNK

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E) Cont. Oligo  |  140-mimic

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F) Cont. Oligo  |  140-antagomir  |  140-mimic

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Figure 5. MiR-140-3p mimic attenuates activation of transcription factor NF-κB in NAASM cells.