



Downregulation of miR-140-3p Is a Cause of Upregulation of RhoA Protein in Bronchial Smooth Muscle of Murine Experimental Asthma

To the Editor:

Airway hyperresponsiveness (AHR) to a wide variety of stimuli is a common feature of allergic bronchial asthma. One of the factors that contribute to the exaggerated airway narrowing is an abnormality of the property of airway smooth muscle (ASM). Indeed, the ASMs from individuals with asthma exhibited increased agonist-induced contraction (1). Rapid relief from airway limitation in patients with asthma by β_2 -stimulant inhalation also suggests an involvement of augmented ASM contraction in the airway obstruction. It is thus important for development of asthma therapy to understand changes in the contractile signaling of ASM cells associated with the disease.

RhoA, a monomeric GTP-binding protein, is a key protein involved in the contraction of smooth muscles, including ASMs. Activation of RhoA and its downstream Rho-kinases (ROCKs) causes an inhibition of myosin light-chain phosphatase, resulting in the Ca^{2+} sensitization of contraction (i.e., an augmentation of contraction under a constant intracellular Ca^{2+} level) (2). Increasing evidence suggests that the augmented RhoA/ROCKs-mediated Ca^{2+} sensitization of ASM contraction is associated with the AHR in experimental asthma (3–5). Although the mechanism is unclear now, an upregulation of RhoA in the ASMs has also been demonstrated (3–5).

MicroRNAs (miRNAs) are small single-stranded noncoding RNAs that negatively modulate gene expression. There is increasing evidence that dysregulation of miRNAs is found in multiple human diseases, including chronic obstructive pulmonary disease and asthma (6–9). Evidence suggest that miR-140-3p is a candidate miRNA that contributes to dysfunction of ASM in asthma by targeting contractile proteins, such as CD38 (cluster of differentiation 38) (10). On the other hand, it has also been known that one miRNA can target multiple genes within one cell type. Here, we show that RhoA is one of the targets of miR-140-3p.

Methods

Detailed METHODS are available in the data supplement. In animal experiments, male BALB/c mice were used. All animal experiments were approved by the Animal Care Committee of the Hoshi University (Approval No. 19–085; Tokyo, Japan). In the experiments of cultured human bronchial smooth muscle cells (hBSMCs), commercially available primary cells established from one normal donor (Cambrex Bio Science Walkersville, Inc.) were used at passages five through seven.

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This letter has a data supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

All the data are expressed as the mean $\pm$ SE. Statistical significance of difference was determined by unpaired Student's *t* test or one-way ANOVA with *post hoc* Bonferroni/Dunn (Prism 5 for Mac OS X; GraphPad Software, Inc.). A value of $P < 0.05$ was considered significant.

Results and Discussion

A computational simulation suggested strong interactions between miR-140-3p and 3'-untranslated region (3'-UTR) of RhoA mRNA both in human and mouse (see Figure E1 in the data supplement). To determine direct interaction between 3'-UTR of RhoA mRNA and miR-140-3p, 3 $\times$ FLAG-tagged human RhoA expression construct, which contains wild-type (WT) or mutated miR-140-3p seed-pairing and 3'-end-pairing sites (Mut) of human RhoA 3'-UTR (Figure 1A), and primary miR-140 expression construct (Figure 1B), which finally generates mature miR-140-3p and -5p in the cells, were prepared. Either the WT or Mut RhoA expression vector (or empty plasmid: mock) was cotransfected into human embryonic kidney cells 293 (HEK293) cells with the primary miR-140 expression or empty plasmid. As shown in Figure 1C, the expression of 3 $\times$ FLAG-fused RhoA (RhoA-3 $\times$ FLAG) was inhibited by cotransfection with WT RhoA 3'-UTR and EGFP (enhanced GFP)-mir-140 (compare lanes 4 with 3), whereas the inhibitory effect was not observed when cells were cotransfected with Mut RhoA 3'-UTR and EGFP-mir-140 (compare lanes 6 with 5). In another series of experiments, secreted placental alkaline phosphatase reporter bearing the WT (cytomegalovirus [CMV]/WT-RHOA 3'-UTR) and mutant miR-140-3p seed-pairing site of human RhoA 3'-UTR (CMV/Mut-RHOA 3'-UTR) were prepared, and either vector was cotransfected into HEK293 cells with β -galactosidase control vector and miR-140-3p mimic. As a result, the secreted placental alkaline phosphatase expression was reduced when cells were cotransfected with CMV/WT-RHOA 3'-UTR and miR-140-3p mimic, whereas its mutant CMV/Mut-RHOA 3'-UTR had no effect (Figure 1D). These findings indicate that miR-140-3p directly interacts with 3'-UTR of human RhoA and negatively regulates its protein expression.

To determine the role of miR-140-3p in RhoA expression of the ASM cells, cultured hBSMCs were transfected with its synthetic inhibitor, miR-140-3p inhibitor. Immunoblot analyses of these cells revealed that RhoA protein expression was significantly increased when the cells were transfected with miR-140-3p inhibitor (Figure 1E), indicating that miR-140-3p is an endogenous modulator of RhoA protein expression in hBSMCs. On the other hand, transfection with miR-140-3p mimic had no effect on the expression level of RhoA protein in the unstimulated cells (Figure 1E), indicating that endogenous miR-140-3p regulates effectively the basal RhoA protein level. Instead, in our preliminary study, the miR-140-3p mimic transfection resulted in a reduction of RhoA protein in the IL-13-stimulated hBSMCs, in which a downregulation of miR-140-3p concurrently with an upregulation of RhoA was observed (data not shown).

Finally, relevance between the miR-140-3p expression level and the BSM hyperresponsiveness was determined in a murine model of allergic airway disease. A well-established murine model of allergic asthma that has marked *in vivo* AHR (11) and hypercontractility of isolated BSM (12) was used. Immunoblot analyses showed that, as previously reported (12, 13), the RhoA protein expression was

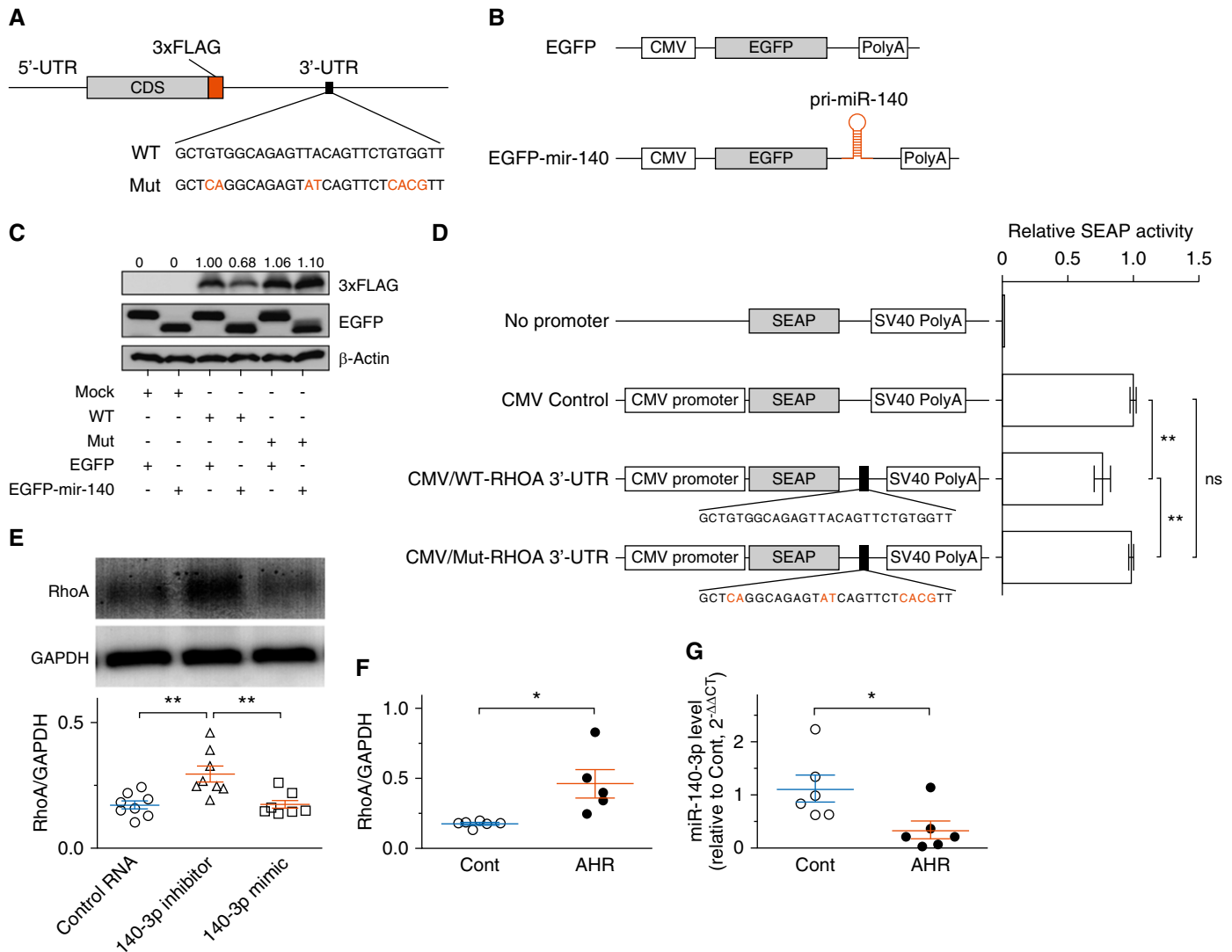


Figure 1. (A–C) The miR-140 expression vector represses RhoA expression by targeting the miR-140-3p binding site of 3'-untranslated region (3'-UTR) of RhoA mRNA. (A and B) Schematic representations of 3xFLAG-tagged human RhoA expression construct with wild-type (WT) or mutant (Mut) of miR-140-3p seed-pairing and 3'-end-pairing sequences of human RhoA 3'-UTR (A) and primary miR-140 expression construct (EGFP [enhanced GFP]-mir-140) (B). Either the WT or Mut RhoA expression vector (or empty plasmid: mock) was cotransfected into human embryonic kidney cells 293 (HEK293) cells with the primary miR-140 expression or empty plasmid. Twenty-four hours after the cotransfection, expression levels of 3xFLAG-fused RhoA (RhoA-FLAG), EGFP, and β -actin were determined by immunoblotting, and the data were summarized in C. The blots are representative of three independent experiments. The relative intensities of respective FLAG bands normalized by corresponding β -actin ones are also shown above. Note that the RhoA-FLAG expression was inhibited by cotransfection with WT RhoA 3'-UTR and EGFP-mir-140 (compare lanes 4 with 3), whereas the inhibitory effect was not observed when cells were cotransfected with Mut RhoA 3'-UTR and EGFP-mir-140 (compare lanes 6 with 5). (D) miR-140-3p directly represses RhoA expression by targeting the 3'-UTR of RhoA mRNA. Secreted placental alkaline phosphatase (SEAP) reporter bearing the WT or Mut miR-140-3p seed-pairing and 3'-end-pairing sequences of human RhoA 3'-UTR was cotransfected into HEK293 cells with β -galactosidase control vector and a synthetic miR-140-3p mimic. Twenty-four hours after the cotransfection, culture supernatants were subjected to the SEAP activity assay, and the results were normalized by β -galactosidase activity. Results are presented as mean $\pm$ SE from three independent experiments. (E) Inhibition of endogenous miR-140-3p causes an upregulation of RhoA protein in cultured human bronchial smooth muscle cells. Cells were transfected either with a nontargeting 20–25 nt RNA (control RNA) or a miR-140-3p inhibitor (140-3p inhibitor), total protein samples were prepared 72 hours after the transfection, and immunoblot analyses were performed. Representative Western blots are shown in the upper panel. The relative expression of RhoA to GAPDH proteins in each sample was calculated, and the data are summarized in the lower panel. Results are presented as mean $\pm$ SE from eight repeated experiments independently. $**P < 0.01$ versus the other groups by unpaired Student's *t* test. (F and G) Upregulation of RhoA protein and downregulation of miR-140-3p in bronchial smooth muscles of the antigen-induced airway hyperresponsive mice. The ovalbumin-immunized animals were repeatedly challenged with aerosolized ovalbumin solution, and proteins and total RNAs including microRNAs were extracted from the main bronchi 24 hours after the last challenge. (F) RhoA expression level determined by immunoblotting. Representative Western blots are shown in the upper panel. The relative expression of RhoA to GAPDH proteins in each sample was calculated, and the data are summarized in lower panel. (G) miR-140-3p expression level determined by real-time qRT-PCR. The relative expressions of miR-140-3p to U6 small nuclear RNA (snRNA) was calculated by the $2^{-\Delta\Delta CT}$ methods. Results are presented as mean $\pm$ SE from 5 to 6 different animals, respectively. $*P < 0.05$ versus control animal by unpaired Student's *t* test. AHR = airway hyperresponsive; CDS = coding sequence; CMV = cytomegalovirus; Cont = control animal; PolyA = polyadenylic acid; UTR = untranslated region.

markedly and significantly increased in BSMs of the diseased mice (Figure 1F). The real-time qRT-PCR analyses revealed that, in this animal model of asthma, the miR-140-3p expression was significantly reduced in BSMs ($P < 0.05$ by unpaired Student's t test; Figure 1G). Although the mechanism of reduction of miR-140-3p is unclear now, our preliminary study also revealed a reduction of its 5p strand, miR-140-5p, which suggests an idea that the transcription of *mir-140* gene might be reduced.

In conclusion, the current findings suggest that RhoA protein expression is negatively regulated by miR-140-3p in BSMs. In BSMs of the repeatedly antigen-challenged mice, a downregulation of miR-140-3p might cause an upregulation of RhoA, presumably resulting in an augmentation of the contraction. The crucial roles of RhoA and its downstream Rho-kinases were also demonstrated in contraction of human BSM (14). The RhoA/Rho-kinase pathway has now been proposed as a new target for the treatment of AHR in asthma (15). Thus, the current study might provide us a new insight into the treatment of the AHR; however, further studies, such as those using different animal models and human specimens, are required to fully validate the role of miR-140-3p in allergic asthma. ■

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TBX4 Transcription Factor Is a Positive Feedback Regulator of Itself and Phospho-SMAD1/5

To the Editor:

TBX4 (T-box transcription factor 4) encodes an early embryonic transcription factor that plays an essential role in pulmonary vascular and airway branching (1, 2). *TBX4* mutations are associated with pulmonary arterial hypertension (PAH); however, the cellular and molecular mechanisms behind this remain unknown (3–6). Decreased BMP (bone morphogenetic protein)–Smad1/5/8 phosphorylation and increased TGF β (transforming growth factor β)–SMAD2/3 phosphorylation is linked to PAH in humans and animal models, highlighting the

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