

Down-Regulation of miR-133a Contributes to Up-Regulation of RhoA in Bronchial Smooth Muscle Cells

Yoshihiko Chiba¹, Miki Tanabe¹, Kumiko Goto¹, Hiroyasu Sakai¹, and Miwa Misawa¹

¹Department of Pharmacology, School of Pharmacy, Hoshi University, Tokyo, Japan

Rationale: Augmented bronchial smooth muscle (BSM) contraction is one of the causes of bronchial hyperresponsiveness. The protein RhoA and its downstream pathways have now been proposed as a new target for asthma therapy. MicroRNAs (miRNAs) play important roles in normal and diseased cell functions, and a contribution of miR-133 to RhoA expression has been suggested in cardiomyocytes. **Objectives:** To make clear the mechanism(s) of up-regulation of RhoA observed in the BSMs of experimental asthma, the role of miR-133a in RhoA expression was tested.

Methods: Total proteins and RNAs (containing miRNAs) were extracted from cultured human BSM cells (hBSMCs) that were treated with antagomirs and/or IL-13, and bronchial tissues of BALB/c mice that were sensitized and repeatedly challenged with ovalbumin. RhoA protein and miR-133a were detected by immunoblotting and quantified real-time reverse transcriptase–polymerase chain reaction, respectively.

Measurements and Main Results: In hBSMCs, an up-regulation of RhoA was observed when the function of endogenous miR-133a was inhibited by its antagomir. Treatment of hBSMCs with IL-13 caused an up-regulation of RhoA and a down-regulation of miR-133a. In bronchial tissues of the repeatedly ovalbumin-challenged mice, a significant increase in RhoA was observed. Interestingly, the level of miR-133a was significantly decreased in BSMs of the challenged mice.

Conclusions: These findings suggest that RhoA expression is negatively regulated by miR-133a in BSMs. IL-13 might, at least in part, contribute to the reduction of miR-133a.

Keywords: microRNA (miRNA); miR-133a; RhoA; asthma; bronchial smooth muscle hyperresponsiveness

MicroRNAs (miRNAs) are a recently discovered class of small, noncoding, single-stranded RNAs that control expression of complementary target messenger RNAs (mRNAs) (1, 2). The mature miRNAs comprise approximately 22 nucleotides, derived from long transcripts of primary miRNAs (pri-miRNAs) and precursor miRNAs (pre-miRNAs). There is increasing evidence that the mature miRNAs negatively modulate gene expression primarily through base pairing to the 3' untranslated region of target mRNAs, resulting in mRNA cleavage and/or translation repression (1–3). Evidence also suggests a role for miRNAs in a variety of basic biological functions, including normal immune function (4) and lung development (5). In addition, an implication of miRNAs has been suggested in diverse diseases, including airway diseases such as chronic obstructive pulmonary disease (6) and asthma (4, 7–10). It is thus possible that miRNAs are attractive new drug targets.

(Received in original form March 3, 2009; accepted in final form July 24, 2009)

Supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan and The Pharmacological Research Foundation, Tokyo, Japan.

Correspondence and requests for reprints should be addressed to Yoshihiko Chiba, Ph.D., Department of Pharmacology, School of Pharmacy, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan. E-mail: chiba@hoshi.ac.jp

Am J Respir Crit Care Med Vol 180, pp 713–719, 2009

Originally Published in Press as DOI: 10.1164/rccm.200903-0325OC on July 30, 2009

Internet address: www.atsjournals.org

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

RhoA is one of the key proteins of bronchial smooth muscle (BSM) contraction and up-regulation of RhoA has been demonstrated in rodent BSMs of experimental asthma models. The mechanism(s) of up-regulation of RhoA is unclear, although the contribution of a microRNA, miR-133, to RhoA expression has been suggested in cardiomyocytes.

What This Study Adds to the Field

Our data show that miR-133a is a key regulator of BSM RhoA expression and provide new insights into the treatment of airway hyperresponsiveness in asthma.

Increased airway narrowing in response to nonspecific stimuli is a characteristic feature of human obstructive diseases, including bronchial asthma. One of the factors that contribute to the exaggerated airway narrowing in people with asthma is an abnormality of the properties of airway smooth muscle (11, 12). RhoA, a monomeric GTP-binding protein, is a key protein in the contraction of smooth muscles, including airway smooth muscles. In animal models of allergic bronchial asthma, an up-regulation of RhoA in association with the augmented contractility of bronchial smooth muscle (BSM) has been demonstrated (13, 14). Although the exact mechanism of up-regulation of RhoA is still unclear, the signaling of RhoA and its downstream Rho-kinases are now considered as a therapeutic target of asthma (15–18).

To date, the transcriptional/translational mechanism of RhoA is not well understood. However, a negative regulation of RhoA expression by miR-133 has recently been suggested in cardiomyocytes (19). Indeed, the RNAhybrid analyses (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/>) (20) of human and mouse mRNAs for RhoA revealed putative binding sites of miR-133a in their 3' untranslated regions (Figure 1). In the present study, we tested the hypothesis that down-regulation of miR-133 may be a cause of the up-regulation of RhoA in BSM. First, the effects of inhibitors of miRNA, called antagomirs (7), on the RhoA protein expression were examined in cultured human BSM cells (hBSMCs). The effects of IL-13, which is capable of induction of RhoA up-regulation (21, 22), on the expression of miR-133 in hBSMCs were also determined. Finally, the change in the expression level of miR-133 in BSM was investigated in a mouse model of allergic bronchial asthma.

METHODS

Cell Culture and Sample Collection

Normal hBSMCs (Cambrex Bio Science Walkersville, Inc., Walkersville, MD) were maintained in SmBM medium (Cambrex) supplemented with 5% fetal bovine serum, 0.5 ng/ml human epidermal growth factor, 5 µg/ml insulin, 2 ng/ml human fibroblast growth factor-basic, 50 µg/ml

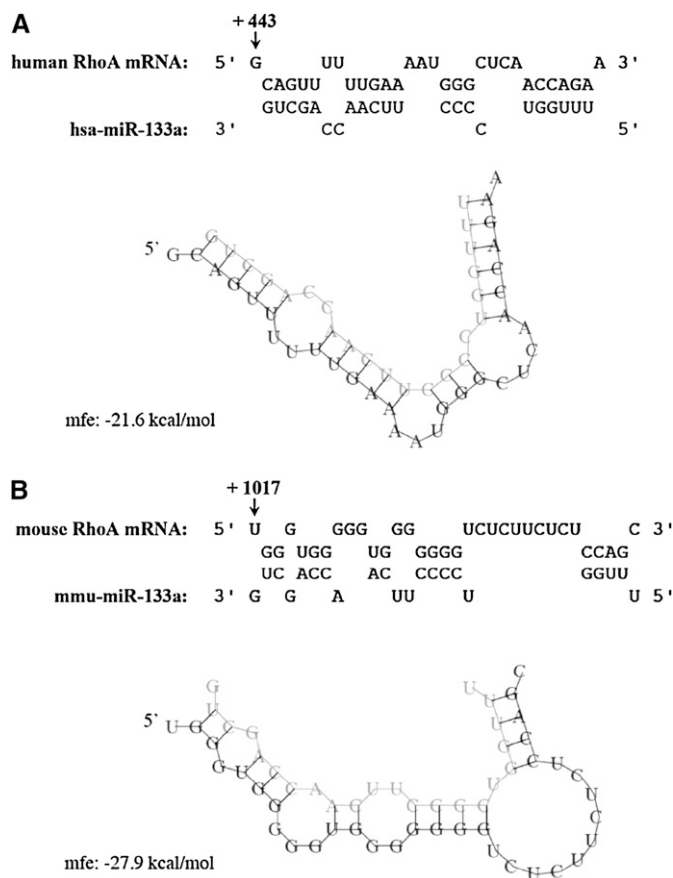


Figure 1. Computational modeling of the interaction between miR-133a and 3' untranslated region (UTR) of RhoA mRNA in (A) human and (B) mouse using the RNAhybrid software (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/>). The complementarity between miR-133a and 3'-UTR of RhoA mRNA is also shown in each panel. The (A) +443 and (B) +1017 indicate 443 and 1,017 bases downstream from the stop codon of RhoA, respectively. has = *Homo sapiens*; mfe = minimal free energy; mmu = *Mus musculus*.

gentamicin, and 50 ng/ml amphotericin B. Cells were maintained at 37°C in a humidified atmosphere (5% CO₂), fed every 48 to 72 hours, and passaged when cells reached 90 to 95% confluence. Then the hBSCs (passages five through seven) were seeded in 6-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) at a density of 3,500 cells/cm² and, when 80–85% confluence was observed, cells were cultured without serum for 24 hours before addition of recombinant human IL-13 (100 ng/ml; PeproTech EC, Ltd., London, UK). In some experiments, an inhibitor of STAT6, 4-(benzylamino)-2-[[2-(3-(chloro-4-hydroxyphenyl)ethyl]amino)pyrimidin-5-carboxamide (AS1517499, 100 nM; kindly provided from Astellas Pharma Inc., Tokyo, Japan) (22, 23), or its vehicle (0.3% dimethyl sulfoxide) was also treated 30 minutes before the addition of IL-13. At the indicated time after the IL-13 treatment, cells were washed with phosphate-buffered saline, immediately collected and disrupted with 1× sodium dodecyl sulfate sample buffer (125 μl/well), and used for Western blot analyses. Total RNAs containing microRNAs (miRNAs) were extracted using mirVana miRNA isolation kit (Ambion, Applied Biosystems, Austin, TX) according to the manufacturer's instructions.

Transfection of Antagomirs and Precursors

The hBSCs were plated at a density of 2 × 10⁵ cells/well in 6-well plates with SmBM medium. The next day, cells were transfected with 100 pmol (final 50 nM) of either inhibitor of miR-133a (antagomir-133a; Ambion; P/N: AM17000, Product ID: AM10413) or miR-133b (antagomir-133b; Ambion; P/N: AM17000, Product ID: AM10029), or precursor of miR-133a (pre-miR-133a; Ambion; P/N: AM17100, Product ID: PM10413) using the Lipofectamine 2000 transfection reagent

(Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. An inhibitor of let-7a (antagomir-let-7a; Ambion; P/N: AM17000, Product ID: AM10050) or a control precursor (negative precursor; Ambion; P/N: AM17110) was used as a negative control, respectively. Sham control cells that underwent the same transfection procedure without any of the antagomirs were also prepared. After a 4-hour transfection in Opti-MEM I reduced serum medium (Invitrogen), cells were cultured in SmGM medium for 20 hours and then in serum-free SmGM medium. Forty-eight hours after the transfection, IL-13 (100 ng/ml) or its vehicle (water) was administered to the cells, and total protein and RNA samples were prepared as described above.

Mouse Model of Allergic Bronchial Asthma

Male BALB/c mice were purchased from the Charles River Japan, Inc. (Kanagawa, Japan) and housed in a pathogen-free facility. All animal experiments were approved by the Animal Care Committee of the Hoshi University (Tokyo, Japan).

Preparation of a murine model of allergic bronchial asthma, which has an *in vivo* airway hyperresponsiveness (AHR) (24), was performed as described previously (14). In brief, BALB/c mice (8 wk of age) were actively sensitized by intraperitoneal injections of 8 μg ovalbumin (OA; Seikagaku Co., Tokyo, Japan) with 2 mg Imject Alum (Pierce Biotechnology, Inc., Rockford, IL) on Day 0 and Day 5. The sensitized mice were challenged with aerosolized OA-saline solution (5 mg/ml) for 30 minutes on days 12, 16, and 20. A control group of mice received the same immunization procedure, and inhaled saline aerosol instead of OA challenge. The aerosol was generated with an ultrasonic nebulizer (Nihon Kohden, Tokyo, Japan) and introduced to a Plexiglas chamber box (130 × 200 mm, 100 mm height) in which the mice were placed. Twenty-four hours after the last OA challenge, mice were killed by exsanguination from the abdominal aorta under urethane (1.6 g/kg, i.p.; Sigma and Aldrich, St. Louis, MO) anesthesia. About 3-mm length of the left main bronchial ring without epithelium was isolated (21) and homogenized directly in 300 μl of lysis/binding buffer provided by mirVana miRNA isolation kit (Ambion, Applied Biosystems). Total RNAs containing miRNAs were extracted as described above.

Real-Time Reverse Transcriptase–Polymerase Chain Reaction for miRNAs

Mature miRNAs were quantified by real-time reverse transcriptase–polymerase chain reaction (RT-PCR) using TaqMan microRNA assays (Applied Biosystems, Foster City, CA). In brief, 10 ng of total RNA prepared as described above was reverse transcribed with stem-loop RT primer specific for human and mouse miR-133a (P/N: 4395357; Applied Biosystems), mouse miR-133a* (P/N: 4381060), or human and mouse miR-133b (P/N: 4395358) using TaqMan microRNA reverse transcription kit (P/N: 4366596) according to the manufacturer's instructions. Real-time PCR was performed on the resulting complementary DNA using miR-133a-, miR-133a*, or miR-133b-specific TaqMan primers and TaqMan universal PCR master mix in a 7500Fast real-time PCR system (Applied Biosystems) at standard 7500 mode. The reactions were incubated in a 96-well optical plate at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The expression of let-7a was used as endogenous control for data normalization (25–27): stem-loop RT primer and TaqMan primer specific for human and mouse let-7a (P/N: 4373169; Applied Biosystems). Under these conditions, excellent linearity between the log of target input and comparative threshold (C_T) value in each target miRNA was observed with C_T range of 38 to 15 when 10^{−18} to 10^{−11} M concentrations of respective synthetic pre-miRNAs (Ambion; which are also detected by the assay systems) were used as standard miRNAs.

Real-Time RT-PCR for RhoA mRNA

The mRNA levels of RhoA were also examined by real-time RT-PCR. Briefly, complementary DNAs were prepared from the total RNA (10 ng) using QuantiTect reverse transcription kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. Then the RT reaction mixture (1 μl) was subjected to real-time PCR analyses using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The thermal cycle profile

used was (1) denaturing for 15 seconds at 95°C, (2) annealing primers for 30 seconds at 55°C, and (3) extending the primers for 30 seconds at 72°C. The PCR amplification was performed at 40 cycles with monitoring fluorescence. The primer sets used were: QuantiTect Primer Assay, QT00044723 for human RhoA, QT00197568 for mouse RhoA, QT00079247 for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH), QT0019388 for mouse GAPDH (Qiagen Inc.).

Western Blot for RhoA Protein

Protein samples were subjected to 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and the proteins were then electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 3% skim milk, the PVDF membrane was incubated with polyclonal rabbit anti-RhoA (1:2,500 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibody. Then the membrane was incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:2,500 dilution; Amersham Biosciences, Co., Piscataway, NJ), detected by an enhanced chemiluminescent system (Amersham Biosciences, Co.) and analyzed by a densitometry system. Detection of housekeeping gene was also performed on the same membrane by using monoclonal mouse anti-GAPDH (1:10,000 dilution; Chemicon International, Inc., Temecula, CA) to confirm the same amount of proteins loaded.

Data and Statistical Analyses

In the real-time PCR analyses, the comparative threshold cycle (C_T) method was used for relative quantification of the target genes. Differences in the C_T values (ΔC_T) between the tested gene and the let-7a or GAPDH were calculated to determine the relative expression levels, using the following formula: $\Delta\Delta C_T = (\Delta C_T \text{ of the treated sample}) - (\Delta C_T \text{ of the control sample})$. The relative expression level between the samples was calculated according to the equation $2^{-\Delta\Delta C_T}$.

All the data are expressed as the mean $\pm$ SE. Statistical significance of difference was determined by unpaired Student *t* test, Bonferroni/Dunn test, or one-way analysis of variance with *post hoc* Bonferroni/Dunn (Stat-View for Macintosh version 5.0; SAS Institute, Inc., Cary, NC). A value of *P* less than 0.05 was considered significant.

RESULTS

Expression of miRNAs in Cultured hBSMCs and Mouse BSM Tissues

The sequences of mature miRNAs referred from the database, miRBase (<http://microrna.sanger.ac.uk/>) (28), are shown in Table 1. There is no species difference between human and mouse in the mature sequence of miR-133a, miR-133b, or let-7a, but the expression of mature miR-133a*, a minor miRNA derived from precursors of miR-133a-1 and miR-133a-2, is not suggested in humans (28). As shown in Table 2, the real-time RT-PCR analyses using miRNA-specific stem-loop RT primers and TaqMan primers successfully detected the targeted miRNAs, indicating expressions of these genes both in hBSMCs and mouse BSM tissues. Although the possibility of contamination of miRNAs derived from cells other than the BSM cells is not excluded in the mouse tissues, the expressions of miR-133a and miR-133b in the hBSMCs (Table 2) suggest that these genes are also expressed in the mouse BSM cells.

Effects of Inhibition of miR-133a or miR-133b on RhoA Protein Expression in Cultured hBSMCs

To determine the roles of miR-133a and miR-133b in RhoA expression, hBSMCs were treated with their inhibitors, named antagomir-133a and antagomir-133b. An inhibitor of let-7a, antagomir-let-7a, was also used as negative control, because the gene was used as endogenous control in the real-time RT-PCR analyses currently. As shown in Figure 2, a significant increase in the expression level of RhoA protein was observed in the cells treated with antagomir-133a. On the other hand,

TABLE 1. MATURE SEQUENCE OF MICRORNAS

ID	Accession	Sequence
hsa-miR-133a	MIMAT0000427	5'-UUUGGUCCCUUUAACACGACUG-3'
mmu-miR-133a	MIMAT0000145	5'-UUUGGUCCCUUUAACACGACUG-3'
mmu-miR-133a* [†]	MIMAT0003473	5'-GCUUGGUAUUAUGGAACCAAU-3'
hsa-miR-133b	MIMAT0000770	5'-UUUGGUCCCUUUAACACGACUA-3'
mmu-miR-133b	MIMAT0000769	5'-UUUGGUCCCUUUAACACGACUA-3'
hsa-let-7a	MIMAT0000062	5'-UGAGGUAGUAGGUUGUAUAGUU-3'
mmu-let-7a	MIMAT0000521	5'-UGAGGUAGUAGGUUGUAUAGUU-3'

Definition of abbreviations: hsa = *Homo sapiens*; mmu = *Mus musculus*.

The sequences are from the miRBase, Release 12.0 (<http://microrna.sanger.ac.uk/>) (28).

[†] Expression of mature miR-133a* (a minor miRNA) is not suggested in humans (28).

neither antagomir-let-7a nor antagomir-133b affected the RhoA expression. The findings indicate that miR-133a, but not miR-133b, is an endogenous modulator of RhoA protein expression. Under these conditions, antagomir-133a had no effect on the expression level of miR-133a measured by real-time PCR (data not shown), indicating that the antagomir-133a used in the present study might inhibit base pairing of miR-133a to target gene rather than degradation of miR-133a itself.

Effects of IL-13 on Expression Levels of miR-133a and RhoA mRNA in Cultured hBSMCs

Because IL-13 is capable of induction of RhoA protein up-regulation in hBSMCs (21, 22), both miR-133a and RhoA mRNA were monitored in the IL-13-treated cells. Figure 3A shows time-course changes in the levels of these genes after treatment with IL-13 (100 ng/ml). IL-13 caused an increase in RhoA mRNA and a decrease in miR-133a in hBSMCs: the peaks with statistical significance were observed at 12 to 24 and 3 to 6 hours, respectively (Figure 3A). IL-13 also caused a decrease in miR-133a in a concentration-dependent manner, but a significant decrease was observed only at 100 ng/ml of IL-13 (Figure 3B). The IL-13-induced up-regulation of RhoA mRNA was abolished by the coinubation with a STAT6 inhibitor AS1517499 (100 nM), whereas, interestingly, this treatment had no effect on the down-regulation of miR-133a induced by IL-13 (Figure 4).

Effects of Antagomir-133a and Pre-miR-133a on IL-13-induced Up-Regulation of RhoA Protein

To determine the effect of inhibition of endogenous miR-133a on the IL-13-induced up-regulation of RhoA protein, hBSMCs transfected with antagomir-133a were also stimulated by IL-13. Consistent with our previous studies (21, 22), an up-regulation

TABLE 2. EXPRESSION OF MATURE MICRORNAS IN CULTURED HUMAN BRONCHIAL SMOOTH MUSCLE CELLS AND MOUSE BRONCHIAL SMOOTH MUSCLE TISSUES DETERMINED BY QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

	Human BSM Cells (n = 6)		Mouse BSM Tissues (n = 6)	
	C_T	ΔC_T	C_T	ΔC_T
miR-133a	32.8 $\pm$ 0.1	10.1 $\pm$ 0.1	15.8 $\pm$ 0.6	-3.6 $\pm$ 0.5
miR-133a*	ND	ND	29.0 $\pm$ 0.5	9.5 $\pm$ 0.4
miR-133b	33.6 $\pm$ 0.2	10.9 $\pm$ 0.3	17.9 $\pm$ 0.5	-1.6 $\pm$ 0.4
let-7a	22.7 $\pm$ 0.1	—	19.4 $\pm$ 0.1	—

Definition of abbreviations: BSM = bronchial smooth muscle; C_T = threshold cycle; $\Delta C_T = (C_T \text{ of target gene}) - (C_T \text{ of let-7a})$; ND = not determined because the expression of mature miR-133a* (a minor miRNA) is not suggested in humans (28).

Data are shown as mean $\pm$ SEM.

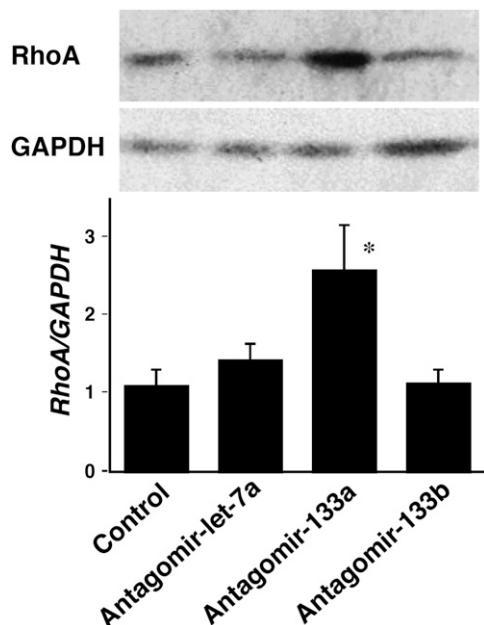


Figure 2. Effects of inhibition of miRNAs by specific antagonirs for let-7a (antagomir-let-7a), miR-133a (antagomir-133a), and miR-133b (antagomir-133b) on the level of RhoA protein in cultured human bronchial smooth muscle cells. Cells were transfected with any of the antagonirs (final 50 nM) or vehicle (water) as described in METHODS section. Total protein samples were prepared 72 hours after the transfection and immunoblot analyses were performed. (Upper panel) Representative Western blots. The bands were analyzed by a densitometer and normalized by the intensity of corresponding glyceraldehyde 3-phosphate dehydrogenase band, and the data are summarized in the lower panel. Results are presented as mean $\pm$ SE from five independent experiments. * $P < 0.05$ versus the other groups by Bonferroni/Dunn test.

of RhoA protein was observed when cells were incubated with IL-13 for 24 hours (Figure 5; control vehicle- vs. IL-13-treated groups). Antagomir-let-7a had no significant effect on the IL-13-induced up-regulation of RhoA protein (Figure 5; antagomir-let-7a IL-13-treated vs. control IL-13-treated). IL-13 caused a further, but not statistically significant, increase in the level of RhoA protein in the antagomir-133a-treated cells (Figure 5; antagomir-133a IL-13-treated vs. control or antagomir-let-7a IL-13-treated).

The effect of pre-miR-133a on the IL-13-induced up-regulation of RhoA was also tested. Although the negative precursor had no effect on basal expression or IL-13-induced up-regulation of RhoA protein, the basal expression of RhoA protein was significantly decreased by the pre-miR-133a treatment (Figure 5; pre-miR-133a vehicle-treated vs. control vehicle-treated). In addition, the IL-13-induced up-regulation of RhoA protein was inhibited by the pre-miR-133a treatment, almost completely (Figure 5).

Effects of Antagomir-133a and Pre-miR-133a on IL-13-induced Increase in RhoA mRNA

Next, the expression level of RhoA mRNA was measured in cells treated with antagomir-133a or pre-miR-133a. As shown in Figure 6A, both antagomir-133a and pre-miR-133a had no effect on the basal expression of RhoA mRNA. Neither antagomir-133a nor pre-miR-133a affected the IL-13-induced increase in RhoA mRNA (Figure 6A). Western blot analyses also revealed that neither antagomir-133a nor pre-miR-133a affected phosphorylation of STAT6 induced by IL-13 (Figure 6B). In each

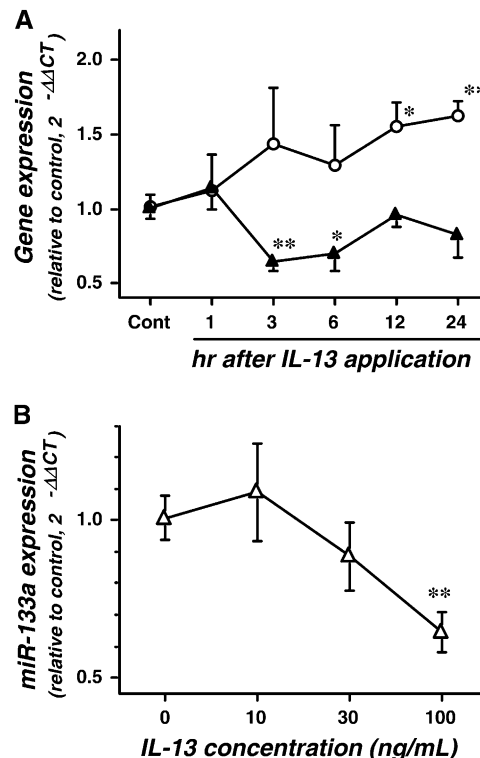


Figure 3. Effects of IL-13 on the expressions of RhoA mRNA and miR-133a in cultured human bronchial smooth muscle cells. (A) Time-course changes in the expression levels of RhoA mRNA (open circles) and miR-133a (solid triangles) after the application of IL-13 (100 ng/ml). (B) A concentration-dependent decrease in the expression level of miR-133a by IL-13 (for 3 h). The gene expressions were determined by quantitative real-time reverse transcriptase-polymerase chain reaction. The relative gene expressions of RhoA to glyceraldehyde 3-phosphate dehydrogenase mRNAs and miR-133a to let-7a were calculated by the $2^{-\Delta\Delta CT}$ methods as described in METHODS section. Results are presented as mean $\pm$ SE from four to six independent experiments. * $P < 0.05$ and ** $P < 0.01$ versus respective control (Cont; no IL-13 treatment in A or 0 ng/ml IL-13 in B) by Bonferroni/Dunn test.

group of the cells, the increase in RhoA mRNA induced by IL-13 was inhibited by AS1517499, almost completely (Figure 6A).

Effects of Repeated Antigen Exposure on the Levels of RhoA mRNA and miR-133 in Mouse BSM Tissues

Finally, expression levels of RhoA mRNA and miR-133 were measured in BSM tissues of mouse asthma model, which has BSM hyperresponsiveness (14, 21, 22). As shown in Figure 6, an up-regulation of RhoA mRNA and down-regulations of miR-133a and miR-133a* (a minor miRNA) were observed in BSM tissues of the OA-challenged mice.

DISCUSSION

The current study revealed that inhibition of endogenous miR-133a by antagomir-133a caused an up-regulation of RhoA protein in cultured hBSMCs (Figure 2). Conversely, the hBSMCs transfected with pre-miR-133a showed a down-regulation of RhoA protein (Figure 5). Treatment of hBSMCs with IL-13 caused up-regulations of RhoA protein (21, 22) and mRNA (Figure 3A) together with a down-regulation of miR-133a (Figure 3). These observations indicate that the expression level of RhoA is negatively regulated by endogenous miR-

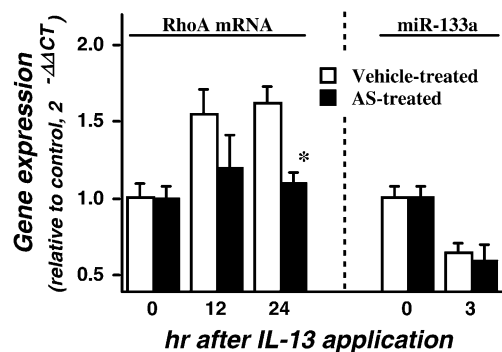


Figure 4. Effects of AS1517499 (AS; 100 nM), an inhibitor of STAT6 activation, on the IL-13 (100 ng/ml)-induced up-regulation of RhoA mRNA and down-regulation of miR-133a in cultured human bronchial smooth muscle cells. AS1517499 (solid columns) or its vehicle (0.3% dimethyl sulfoxide; open columns) was applied 30 minutes before the application of IL-13. The gene expressions were determined as in Figure 3. Results are presented as mean $\pm$ SE from four to five independent experiments. * $P < 0.05$ versus respective vehicle-treated group by unpaired Student *t*-test.

133a and that IL-13 could induce RhoA up-regulation via an inhibition of miR-133a expression at least in part. In the repeatedly antigen-challenged mice that possess BSM hyperresponsiveness together with up-regulations of RhoA protein and IL-13 (14, 22), a decrease in the level of miR-133a in BSM tissue was also found (Figure 7). Taken together, the reduced expression of miR-133a might cause an up-regulation of RhoA in BSM cells, resulting in an augmentation of the contraction, which is one of the causes of AHR in people with asthma (11, 12).

Airway smooth muscle is an important effector tissue regulating bronchomotor tone. It has been suggested that modulation of airway smooth muscle by inflammatory mediators such as cytokines may play an important role in the development of AHR (29), one of the characteristic features of patients with allergic bronchial asthma. In the BALB/c strain of mice that were actively sensitized and repeatedly challenged using the same procedures as in the current study, an *in vivo* AHR accompanied by the increased IgE production and pulmonary eosinophilia has been demonstrated (24). In this animal model of allergic bronchial asthma, an increased contractility of isolated BSM to contractile agonists has also been found (14, 30). The augmented BSM contraction induced by antigen challenge has reportedly been associated with an up-regulation of RhoA (14), a small GTPase that is involved in the agonist-induced Ca^{2+} sensitization of smooth muscle contraction (31, 32). Although the mechanism of up-regulation of RhoA is not fully understood, the current findings that expression level of RhoA protein was increased in hBSMCs treated with antagomir-133a (Figure 2), but not antagomir-133b, suggest that inhibition of miR-133a is involved in the RhoA up-regulation. The observation that the level of miR-133a was decreased in the BSM tissues of mice with allergic asthma (Figure 7), in which an up-regulation of RhoA protein was found (14, 22), might also support the idea.

In general, the miRNA gene is transcribed into a pri-miRNA, and then processed into pre-miRNA. The pre-miRNA is processed into a short RNA duplex termed miRNA duplex, and mature single-stranded miRNA is then generated. The RNA strand of the miRNA duplex that is complementary to the miRNA, termed minor miRNA and depicted with star symbol (miRNA*), is also generated, whereas the miRNA* is usually degraded (33, 34). MiR-133 family of miRNAs is the most

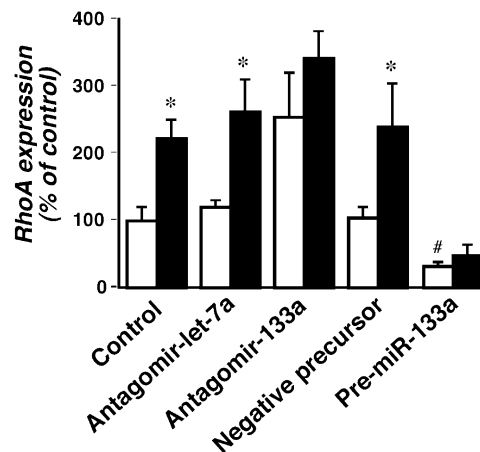


Figure 5. Effect of inhibition or up-regulation of miR-133a by its antagomir (antagomir-133a) or precursor (pre-miR-133a), respectively, on IL-13 (100 ng/ml)-induced up-regulation of RhoA protein in cultured human bronchial smooth muscle cells. Cells were transfected with antagomir-133a, a control antagomir-let-7a, pre-miR-133a, a control precursor (negative precursor) (final 50 nM, respectively), or vehicle (water) as described in METHODS section. IL-13 (solid columns) or its vehicle (water; open columns) was applied 48 hours after the transfection. Total protein samples were prepared 24 hours after the IL-13 application and immunoblot analyses were performed. The expression level of RhoA protein was expressed as percentage of the vehicle-treated control. Results are presented as mean $\pm$ SE from five independent experiments. * $P < 0.05$ versus respective vehicle-treated group by unpaired Student *t*-test. Note that the basal expression of RhoA protein was significantly decreased when cells were transfected with pre-miR-133a (# $P < 0.05$ vs. the control, vehicle-treated group).

highly expressed miRNA in cardiac myocytes (35). Mature miR-133a is generated by processing of its pre-miRNAs, miR-133a-1 and miR-133a-2, that are transcripts of different genes. Reportedly, miR-133a-1 and miR-133a-2 are specifically expressed in skeletal muscle and cardiac myocytes (19). Although its expression in BSM was not determined by the investigators, the current real-time PCR analyses revealed an expression of mature miR-133a in hBSMCs and mouse BSM tissues (Table 2). As suggested in the miRBase (28), miR-133a* was also expressed in the mouse BSM tissues (Table 2). But the expression level of miR-133a* was extremely lower than that of mature miR-133a (Table 2), indicating that miR-133a* generated in BSM might undergo some degradation process (33).

The current real-time PCR analyses also suggested a difference in the expression levels of miR-133a between hBSMCs and mouse BSM tissues: miR-133a is expressed at very low levels in hBSMCs but at high levels in mouse BSM tissues (Table 2). Nevertheless, the finding that the inhibition of endogenous miR-133a by antagomir-133a caused an up-regulation of RhoA protein (Figure 2) indicates that miR-133a is functionally relevant in hBSMCs even in the apparently low expression. Some species difference in the generation process, such as the transcription of pri-miR-133a and the processing into pre-miR-133a (miR-133a-1 and miR-133a-2) and miR-133a duplex, and/or difference in the stability of mature miR-133a in the cells may be involved in the difference observed. Making clear the mechanism of the difference might provide us the way that controls the expression of miR-133a *in vivo*. Alternatively, if miR-133a level in BSM cells could be increased *in vivo*, it may act more effectively in humans.

Our previous findings that both the increased BSM contractility and the up-regulation of RhoA protein observed in the mouse model of allergic asthma were reproduced by IL-13 (21)

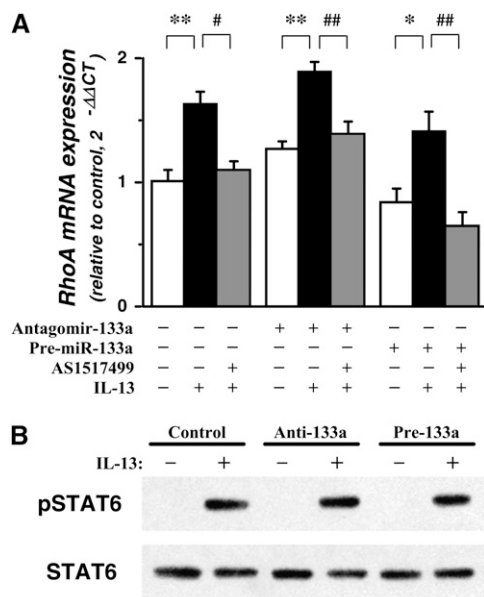


Figure 6. Effect of inhibition or up-regulation of miR-133a by its antagomir (antagomir-133a) or precursor (pre-miR-133a), respectively, on IL-13 (100 ng/ml)-induced (A) up-regulation of RhoA mRNA and (B) phosphorylation of STAT6 in cultured human bronchial smooth muscle cells. Cells were transfected with antagomir-133a, a control antagomir-let-7a, pre-miR-133a, a control precursor (negative precursor) (final 50 nM, respectively), or vehicle (water) as described in METHODS section. Cells were also treated with IL-13 and/or AS1517499, an inhibitor of STAT6 activation, as indicated. (A) Total RNA samples were prepared 24 hours after the IL-13 application. The RhoA mRNA expression was determined by quantitative real-time reverse transcriptase-polymerase chain reaction and the relative expression of RhoA to glyceraldehyde 3-phosphate dehydrogenase mRNAs was calculated by the $2^{-\Delta\Delta CT}$ methods as described in METHODS section. Results are presented as mean $\pm$ SE from five to six independent experiments. * $P < 0.05$ and ** $P < 0.01$ versus respective control by unpaired Student *t*-test. # $P < 0.05$ and ## $P < 0.01$ between the solid and shaded columns, respectively. (B) Total protein samples were prepared 1 hour after the IL-13 application, and immunoblot analyses for phosphorylated (pSTAT6) and total STAT6 proteins were performed. The blots shown are representative of three independent experiments, respectively. Anti-133a = antagomir-133a.

suggest that IL-13, one of the major cytokines up-regulated in the airways of people with asthma (36, 37), is a key for the induction of AHR. Consistent with our previous study (21, 22), IL-13 caused an up-regulation of RhoA protein in the control hBSMCs (Figure 5). Previously, the IL-13-induced up-regulation of RhoA protein was inhibited by a STAT6 inhibitor AS1517499 or a small interfering RNA (siRNA) for STAT6 (22). However, inhibitory effects of these treatments were only partial, whereas complete inhibition of STAT6 activation or protein expression was observed (22), suggesting that mechanism(s) other than the STAT6-dependent mechanism might also contribute to the IL-13-induced up-regulation of RhoA protein. We show here for the first time that IL-13 induced a down-regulation of miR-133a in hBSMCs (Figure 3). Based on the results that antagomir-133a caused an up-regulation of RhoA protein (Figure 2), the IL-13-induced down-regulation of miR-133a could be one of the causes of the IL-13-induced up-regulation of RhoA protein. One of the important findings of the current study is that AS1517499 had no effect on the IL-13-induced down-regulation of miR-133a, whereas the up-regulation of RhoA mRNA was inhibited almost completely (Figure 4). The

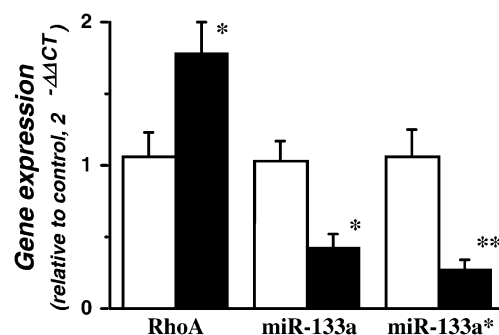


Figure 7. Effects of repeated ovalbumin antigen (OA) exposure to sensitized mice on the expression levels of RhoA mRNA (left), miR-133a (middle), and miR-133a* (right) in bronchial tissues. The gene expressions were determined by quantitative real-time reverse transcriptase-polymerase chain reaction. Open columns = control animals; solid columns = OA-challenged. The relative gene expressions of RhoA to glyceraldehyde 3-phosphate dehydrogenase mRNAs, miR-133a to let-7a, and miR-133a* to let-7a were calculated by the $2^{-\Delta\Delta CT}$ methods as described in the METHODS section. Results are presented as mean $\pm$ SE from five to six independent experiments. * $P < 0.05$ and ** $P < 0.01$ versus respective control by unpaired Student *t*-test.

findings indicate that miR-133a negatively modulates the expression of RhoA gene by a translation repression rather than by an mRNA cleavage. This might also be supported by the findings that both antagomir-133a and pre-miR-133a had no effect on the basal expression or the IL-13-induced up-regulation of RhoA mRNA (Figure 6). Indeed, significant increases in the levels of RhoA proteins were observed from 6 hours after the IL-13 treatment (21), whereas significant increases in RhoA mRNAs were detected at 12 to 24 hours (Figure 3A). In addition, evidence in cardiomyocytes that overexpression of miR-133 caused a decrease in RhoA protein level without affecting its mRNA level (19) might also support the idea. We thus conclude that IL-13 is capable of induction of RhoA protein up-regulation both by increasing RhoA mRNA via STAT6-dependent mechanism(s) and by increasing RhoA translation via the STAT6-independent down-regulation of miR-133a in hBSMCs.

We postulated that inhibition of endogenous miR-133a could further augment the up-regulation of RhoA protein induced by IL-13. To test the hypothesis, the hBSMCs treated with antagomir-133a were incubated with IL-13 for 24 hours. However, IL-13 did not show a significant increase in the level of RhoA protein in the antagomir-133a-treated cells, although a tendency to increase was observed (Figure 5). Similarly, no significant difference in the RhoA protein level was observed between the antagomir-let-7a- and IL-13-treated cells and antagomir-133a- and IL-13-treated ones (Figure 5). These are probably due to the ceiling effect on the RhoA protein expression in the cells.

Previous evidence that IL-13 is a key factor for induction of BSM hyperresponsiveness and up-regulation of RhoA protein in mouse model of allergic bronchial asthma (21) reminds us that miR-133a might also be down-regulated in BSMs of the diseased animals. To test the idea, the expression level of miR-133a was measured in BSM tissues of the repeatedly antigen-challenged mice. As expected, a down-regulation of miR-133a was observed in BSM tissues of the antigen-challenged mice (Figure 7). The current study also revealed an increase in the level of RhoA mRNA, but the increment of mRNA (~ 1.8 -fold increase from the control; Figure 7) was much less than that of protein (~ 4.3 -fold increase) (22). These findings also suggest that miR-133a might negatively modulate the expression of RhoA gene by a translation repression rather than by an

mRNA cleavage in mouse BSM as in the case of IL-13-treated hBSMCs described above. In addition, the results that the expression level of miR-133a* was also decreased to almost the same extent as that of miR-133a (Figure 7) suggest that a gene transcription of miR-133a-1 and/or miR-133a-2 may be reduced in BSMs of the antigen-challenged mice, because mature miR-133a is generated from miR-133a/miR-133a* duplex as described above. Further studies are needed to make clear the mechanism of up-regulation of RhoA in BSMs of the murine model of allergic bronchial asthma.

In conclusion, the current findings suggest that RhoA protein expression is negatively regulated by miR-133a in BSMs. IL-13 is capable of reducing the miR-133a expression in BSMs. In BSMs of the repeatedly antigen-challenged mice, the miR-133a down-regulation causes an up-regulation of RhoA, presumably resulting in an augmentation of the contraction. An importance of RhoA and its downstream Rho-kinases was also demonstrated in contraction of human BSM (38). The RhoA/Rho-kinase pathway has now been proposed as a new target for the treatment of AHR in asthma (15–18). Thus, the current study might provide us a new insight into the treatment of the AHR.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

References

- Ambros V. The functions of animal microRNAs. *Nature* 2004;431:350–355.
- Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;116:281–297.
- Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell* 2009;136:215–233.
- Rodriguez A, Vigorito E, Clare S, Warren MV, Couttet P, Soond DR, van Dongen S, Grocock RJ, Das PP, Miska EA, *et al.* Requirement of bic/microRNA-155 for normal immune function. *Science* 2007;316:608–611.
- Williams AE, Moschos SA, Perry MM, Barnes PJ, Lindsay MA. Maternally imprinted microRNAs are differentially expressed during mouse and human lung development. *Dev Dyn* 2007;236:572–580.
- Schembri F, Sridhar S, Perdomo C, Gustafson AM, Zhang X, Ergun A, Lu J, Liu G, Zhang X, Bowers J, *et al.* MicroRNAs as modulators of smoking-induced gene expression changes in human airway epithelium. *Proc Natl Acad Sci USA* 2009;106:2319–2324.
- Mattes J, Yang M, Foster PS. Regulation of microRNA by antagonists: a new class of pharmacological antagonists for the specific regulation of gene function? *Am J Respir Cell Mol Biol* 2007;36:8–12.
- Yang M, Mattes J. Discovery, biology and therapeutic potential of RNA interference, microRNA and antagonists. *Pharmacol Ther* 2008;117:94–104.
- Miller RL, Ho SM. Environmental epigenetics and asthma: current concepts and call for studies. *Am J Respir Crit Care Med* 2008;177:567–573.
- Sonkoly E, Pivarski A. Advances in microRNAs: implications for immunity and inflammatory diseases. *J Cell Mol Med* 2009;13:24–38.
- Seow CY, Schellenberg RR, Pare PD. Structural and functional changes in the airway smooth muscle of asthmatic subjects. *Am J Respir Crit Care Med* 1998;158:S179–S186.
- Martin JG, Duguet A, Eidelman DH. The contribution of airway smooth muscle to airway narrowing and airway hyperresponsiveness in disease. *Eur Respir J* 2000;16:349–354.
- Chiba Y, Takada Y, Miyamoto S, Mitsui-Saito M, Karaki H, Misawa M. Augmented acetylcholine-induced, Rho-mediated Ca^{2+} sensitization of bronchial smooth muscle contraction in antigen-induced airway hyperresponsive rats. *Br J Pharmacol* 1999;127:597–600.
- Chiba Y, Ueno A, Shinozaki K, Takeyama H, Nakazawa S, Sakai H, Misawa M. Involvement of RhoA-mediated Ca^{2+} sensitization in antigen-induced bronchial smooth muscle hyperresponsiveness in mice. *Respir Res* 2005;6:4.
- Gosens R, Schaafsma D, Nelemans SA, Halayko AJ. Rho-kinase as a drug target for the treatment of airway hyperresponsiveness in asthma. *Mini Rev Med Chem* 2006;6:339–348.
- Schaafsma D, Gosens R, Zaagsma J, Halayko AJ, Meurs H. Rho kinase inhibitors: a novel therapeutic intervention in asthma? *Eur J Pharmacol* 2008;585:398–406.
- Schaafsma D, Roscioni SS, Meurs H, Schmidt M. Monomeric G-proteins as signal transducers in airway physiology and pathophysiology. *Cell Signal* 2008;20:1705–1714.
- Kume H. RhoA/Rho-kinase as a therapeutic target in asthma. *Curr Med Chem* 2008;15:2876–2885.
- Carè A, Catalucci D, Felicetti F, Bonci D, Addario A, Gallo P, Bang ML, Segnalini P, Gu Y, Dalton ND, *et al.* MicroRNA-133 controls cardiac hypertrophy. *Nat Med* 2007;13:613–618.
- Rehmsmeier M, Steffen P, Hochsmann M, Giegerich R. Fast and effective prediction of microRNA/target duplexes. *RNA* 2004;10:1507–1517.
- Chiba Y, Nakazawa S, Todoroki M, Shinozaki K, Sakai H, Misawa M. Interleukin-13 augments bronchial smooth muscle contractility with an up-regulation of RhoA protein. *Am J Respir Cell Mol Biol* 2009;40:159–167.
- Chiba Y, Todoroki M, Nishida Y, Tanabe M, Misawa M. A novel STAT6 inhibitor AS1517499 ameliorates antigen-induced bronchial hypercontractility in mice. *Am J Respir Cell Mol Biol* 2009; (in press).
- Nagashima S, Yokota M, Nakai E, Kuromitsu S, Ohga K, Takeuchi M, Tsukamoto S, Ohta M. Synthesis and evaluation of 2-[[2-(4-hydroxyphenyl)-ethyl]amino]pyrimidine-5-carboxamide derivatives as novel STAT6 inhibitors. *Bioorg Med Chem* 2007;15:1044–1055.
- Kato Y, Manabe T, Tanaka Y, Mochizuki H. Effect of an orally active Th1/Th2 balance modulator, M50367, on IgE production, eosinophilia, and airway hyperresponsiveness in mice. *J Immunol* 1999;162:7470–7479.
- Stanczyk J, Pedrioli DM, Brentano F, Sanchez-Pernaute O, Kolling C, Gay RE, Detmar M, Gay S, Kyburz D. Altered expression of MicroRNA in synovial fibroblasts and synovial tissue in rheumatoid arthritis. *Arthritis Rheum* 2008;58:1001–1009.
- Laios A, O'Toole S, Flavin R, Martin C, Kelly L, Ring M, Finn SP, Barrett C, Loda M, Gleeson N, *et al.* Potential role of miR-9 and miR-223 in recurrent ovarian cancer. *Mol Cancer* 2008;7:35.
- Lee JW, Choi CH, Choi JJ, Park YA, Kim SJ, Hwang SY, Kim WY, Kim TJ, Lee JH, Kim BG, *et al.* Altered microRNA expression in cervical carcinomas. *Clin Cancer Res* 2008;14:2535–2542.
- Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ. miRBase: tools for microRNA genomics. *Nucleic Acids Res* 2008;36:D154–D158.
- Amrani Y, Panettieri RA Jr. Modulation of calcium homeostasis as a mechanism for altering smooth muscle responsiveness in asthma. *Curr Opin Allergy Clin Immunol* 2002;2:39–45.
- Chiba Y, Ueno A, Sakai H, Misawa M. Hyperresponsiveness of bronchial but not tracheal smooth muscle in a murine model of allergic bronchial asthma. *Inflamm Res* 2004;53:636–642.
- Somlyo AP, Somlyo AV. Ca^{2+} sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol Rev* 2003;83:1325–1358.
- Chiba Y, Misawa M. The role of RhoA-mediated Ca^{2+} sensitization of bronchial smooth muscle contraction in airway hyperresponsiveness. *J Smooth Muscle Res* 2004;40:155–167.
- Liu X, Fortin K, Mourelatos Z. MicroRNAs: biogenesis and molecular functions. *Brain Pathol* 2008;18:113–121.
- Nana-Sinkam SP, Hunter MG, Nuovo GJ, Schmittgen TD, Gelinas R, Galas D, Marsh CB. Integrating the microRNome into the study of lung disease. *Am J Respir Crit Care Med* 2009;179:4–10.
- Duisters RF, Tjissen AJ, Schroen B, Leenders JJ, Lentink V, van der Made I, Herias V, van Leeuwen RE, Schellings MW, Barenbrug P, *et al.* miR-133 and miR-30 regulate connective tissue growth factor: implications for a role of microRNAs in myocardial matrix remodeling. *Circ Res* 2009;104:170–178.
- Berry MA, Parker D, Neale N, Woodman L, Morgan A, Monk P, Bradding P, Wardlaw AJ, Pavord ID, Brightling CE. Sputum and bronchial submucosal IL-13 expression in asthma and eosinophilic bronchitis. *J Allergy Clin Immunol* 2004;114:1106–1109.
- Saha SK, Berry MA, Parker D, Siddiqui S, Morgan A, May R, Monk P, Bradding P, Wardlaw AJ, Pavord ID, *et al.* Increased sputum and bronchial biopsy IL-13 expression in severe asthma. *J Allergy Clin Immunol* 2008;121:685–691.
- Yoshii A, Iizuka K, Dobashi K, Horie T, Harada T, Nakazawa T, Mori M. Relaxation of contracted rabbit tracheal and human bronchial smooth muscle by Y-27632 through inhibition of Ca^{2+} sensitization. *Am J Respir Cell Mol Biol* 1999;20:1190–1200.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.