miR-146a regulates mechanotransduction and pressure-induced inflammation in small airway epithelium

Yan Huang,[*,†] Melissa Crawford,[†,‡] Natalia Higuita-Castro,[*,†] Patrick Nana-Sinkam,[†,‡,1,2] and Samir N. Ghadiali[*,†,‡,1,2]

[*]Department of Biomedical Engineering, [†]Dorothy M. Davis Heart and Lung Research Institute, and [‡]Department of Internal Medicine, Division of Pulmonary, Allergy, Critical Care, and Sleep Medicine, The Ohio State University, Columbus, Ohio, USA

ABSTRACT **Mechanical ventilation generates biophysical forces, including high transmural pressures, which exacerbate lung inflammation. This study sought to determine whether microRNAs (miRNAs) respond to this mechanical force and play a role in regulating mechanically induced inflammation. Primary human small airway epithelial cells (HSAEpCs) were exposed to 12 h of oscillatory pressure and/or the proinflammatory cytokine TNF-α. Experiments were also conducted after manipulating miRNA expression and silencing the transcription factor NF-κB or toll-like receptor proteins IRAK1 and TRAF6. NF-κB activation, IL-6/IL-8/IL-1β cytokine secretion, miRNA expression, and IRAK1/TRAF6 protein levels were monitored. A total of 12 h of oscillatory pressure and TNF-α resulted in a 5- to 7-fold increase in IL-6/IL-8 cytokine secretion, and oscillatory pressure also resulted in a time-dependent increase in IL-6/IL-8/IL-1β cytokine secretion. Pressure and TNF-α also resulted in distinct patterns of miRNA expression, with miR-146a being the most deregulated miRNA. Manipulating miR-146a expression altered pressure-induced cytokine secretion. Silencing of IRAK1 or TRAF6, confirmed targets of miR-146a, resulted in a 3-fold decrease in pressure-induced cytokine secretion. Cotransfection experiments demonstrate that miR-146a's regulation of pressure-induced cytokine secretion depends on its targeting of both IRAK1 and TRAF6. MiR-146a is a mechanosensitive miRNA that is rapidly up-regulated by oscillatory pressure and plays an important role in regulating mechanically induced inflammation in lung epithelia.—Huang, Y., Crawford, M., Higuita-Castro, N., Nana-Sinkam, P., Ghadiali, S. N. miR-146a regulates mechanotransduction and pressure-induced inflammation in small airway epithelium. *FASEB J.* 26, 3351–3364 (2012). www.fasebj.org**

Key Words: acute respiratory distress syndrome · ventilation-induced lung injury · oscillatory transmural pressure · microRNA · TRAF6 · IRAK1 · toll-like receptor signaling pathway

As early as 1885, Samuel Meltzer recognized the importance of mechanical forces on both lung function and cell behavior (1, 2). It is now well established that mechanical forces can regulate important physiological processes (*i.e.*, lung development) and contribute to several lung disorders (3, 4). For example, the acute respiratory distress syndrome (ARDS) is a major cause of respiratory failure and mortality in critically ill patients (5). Although artificial ventilation is often required for survival during ARDS, the mechanical forces generated during ventilation can exacerbate lung inflammation and injury (6). This form of injury, known as ventilator-induced lung injury (VILI), may be due to excessive alveolar distension (7), high airway pressures (8), and/or cyclic airway closing and reopening (9). In addition to causing biophysical injury, such as cellular necrosis and increased alveolar-capillary permeability (10, 11), the mechanical forces generated during ventilation may also activate inflammatory signaling pathways and induce inflammatory cytokine secretion (12). For example, large cyclic stretching of the epithelium can induce cytokine secretion (13, 14), while compres-

Abbreviations: ARDS, acute respiratory distress syndrome; ECM, extracellular matrix; eNOS, endothelial nitric oxide synthase; FAK, focal adhesion kinase; HSAEpC, human small airway epithelial cell; IKK, inhibitor kinases of NF-κB; IκB-α, NF-κB inhibitor; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-8, interleukin-8; IRAK1, interleukin-1 receptor-associated kinase; LPS, lipopolysaccharide; miR, microRNA; miRNA, microRNA; mRNA, messenger RNA; NF-κB, nuclear factor-κB; qRT-PCR, quantitative reverse transcription–polymerase chain reaction; siRNA, silencing RNA; TLR, toll-like receptor; TNF-α, tumor necrosis factor α; TRAF6, tumor necrosis factor receptor-associated factor 6; UTR, untranslated region; VILI, ventilator-induced lung injury

[1] These authors contributed equally to this work.

[2] Correspondence: Wexner Medical Center at The Ohio State University, Dorothy M. Davis Heart and Lung Research Institute, 473 W. 12th Ave., Columbus, OH 43210, USA. E-mail: S.N.G., ghadiali.1@osu.edu; P.N.-S., patrick.nana-sinkam@osumc.edu

doi: 10.1096/fj.11-199240

This article includes supplemental data. Please visit *http://www.fasebj.org* to obtain this information.

sive forces, such as oscillatory pressure, can activate nuclear factor-κB (NF-κB) pathways in a cytoskeleton-dependent fashion (15). Although secretion of proinflammatory cytokines, such as interleukin-8 (IL-8), can recruit neutrophils to the lung, soluble factors can worsen VILI (16), and increased cytokine secretion into the circulation, especially IL-6, IL-8, and IL-1β, can produce a systemic inflammatory response that may result in multisystem organ failure (8, 17). Therefore, regulating the mechanotransduction events that lead to increased lung inflammation during ventilation is an important clinical issue.

Mechanotransduction is the process by which cells convert mechanical stimuli into biochemical responses, and several mechanisms for force sensing and mechanotransduction have been recently investigated. For example, stretch-activated ion channels and epithelial sodium channels have been shown to play a role in pressure-induced ATP release in urothelial cells (18). Experimental and mathematical studies from Tschumperlin *et al.* (19, 20) indicate that pressure-induced changes in interstitial geometry can increase the local ligand concentration and thus alter biochemical signaling in bronchial epithelial cells. Finally, the association of cell surface adhesion proteins (*i.e.*, integrins) with the extracellular matrix (ECM), actin filaments, Src kinase, and the focal adhesion kinase (FAK) may transmit mechanical signals from the ECM to signaling sites within the cells or at the cell surface (21). The integrin family can also regulate the action of NF-κB by degradation of inhibitor kinases of NF-κB (IKK), which in turn leads to the transcription of target cytokine genes IL-6 or IL-8 (22). Current evidence suggests that the activation and control of NF-κB plays a critical role in cytokine responses during ARDS and VILI (23, 24). Although our laboratory recently demonstrated that oscillatory transmural pressure can activate NF-κB pathways in epithelial cells (15), it is not known how mechanical forces influence the expression of important regulatory molecules such as microRNAs (miRNAs) in lung epithelia or whether mechanotransduction events responsible for lung inflammation can be regulated by specific miRNAs.

MiRNAs are noncoding, single-stranded RNAs of ~22 nt in length that regulate gene expression by suppression of translation or messenger RNA (mRNA) degradation. Recent human genomic studies revealed that ~30% of human genes can be potentially regulated by miRNAs (25). MiRNAs have emerged as key regulators of diverse biological processes (26, 27) and have been recently proposed as novel biomarkers or therapeutic targets for ARDS/VILI (28). Notably, several miRNAs have been shown to regulate inflammatory responses to proinflammatory cytokines or bacterial stimuli. For example, miR-155 has been shown to regulate inflammatory cytokine production in dendritic cells during microbial stimulation (29). MiR-146a has been shown to regulate tumor necrosis factor α (TNF-α) production during rheumatoid arthritis (30) and can also regulate IL-8 and RANTES production in epithelial cells during cytokine stimulation (31). In addition, other miRNAs, such as miR-214, miR-21, miR-223, and miR-224, are rapidly induced in the lung when treated with lipopolysaccharide (LPS; ref. 32). More recently, functional roles for miRNAs in mechanotransduction have been investigated. MiRNAs can mediate endothelial cell proliferation (33, 34) and modulate apoptosis and endothelial nitric oxide synthase (eNOS) activity in response to shear stress (33). However, the role of miRNAs in regulating the mechanotransduction processes responsible for inflammation in the pulmonary system remains largely unknown.

The goals of the present study were to investigate how oscillatory pressures influence both inflammatory cytokine secretion and miRNA expression in primary human lung epithelial cells and to determine whether miR-146a and its targets regulate mechanically induced inflammation in lung epithelia. We demonstrate that miR-146a is an early responsive miRNA that is up-regulated by oscillatory pressure and that miR-146a can regulate pressure-induced inflammation in lung epithelia by targeted suppression of interleukin-1 receptor-associated kinase (IRAK1) and tumor necrosis factor receptor-associated factor 6 (TRAF6), key proteins in the toll-like receptor (TLR) signaling pathway. To our knowledge, this is the first study both to describe miRNA responses to mechanical forces in lung epithelia and to demonstrate that specific miRNAs can regulate mechanically induced inflammation in lung epithelia. A better understanding of how mechanical forces influence miRNA expression and how miRNAs regulate the mechanotransduction processes responsible for lung inflammation may lead to novel biomarkers and/or innovative treatments for VILI and ARDS.

MATERIALS AND METHODS

Cell culture

Primary human small airway epithelial cells (HSAEpCs) were obtained from PromoCell GmbH (Heidelberg, Germany) and maintained in Airway Epithelial Cell Growth Medium and supplemented with SupplementPack (PromoCell). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. Polarized monolayers of HSAEpCs were obtained by seeding onto 23-mm-diameter 0.4-μm-pore polyester Transwell inserts (Costar, Corning, NY, USA) at a density of 1.9×10^5 cells/insert, and cells were grown to confluence prior to pressure and/or cytokine treatment. Cells were grown with 1.5 ml cell culture medium in the upper (apical) chamber and 2.5 ml in the lower (basal) chamber. Medium in the upper compartment was removed prior to the application of pressure.

Application of oscillatory pressure

Application of oscillatory pressure was performed as described previously (see Fig. 1 in ref. 15). Briefly, fitted stoppers were plugged tightly to the top of Transwell inserts to form a hermetically sealed pressure chamber in the apical compartment. Access ports in the plugs were connected to a manometer and a gas-tight syringe attached to a PHD2000 programmable syringe pump (Harvard Apparatus, Holliston, MA, USA). The syringe pump was programmed to execute repeated infusion and withdrawal at a constant flow rate for a given amount of time per cycle. This produced a triangular pressure waveform that varied from either 0 to 20 cmH_2O or 0 to 5 cmH_2O at a frequency of 0.2 Hz. Note that 20 cmH_2O is similar to the mean airway pressures observed during mechanical ventilation (35), and 0.2 Hz simulates normal breathing frequency.

MiRNA profiling

Polarized HSAEpCs were exposed to oscillatory pressure (0–20 cmH_2O at 0.2 Hz) or 30 ng/ml TNF-α for 12 h. The combined effect of cytokines and mechanical force were also investigated by exposing HSAEpCs to both TNF-α and oscillatory pressure. Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) and measured using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The Nanostring nCounter system (NanoString Technologies, Seattle, WA, USA) was used to profile miRNA expression. Total RNA was mixed with pairs of capture and reporter probes tailored to each miRNA. After hybridization, excess reporters and capture probes were removed. and the purified ternary complexes were bound to the imaging surface, elongated, and immobilized. The surface was then imaged by the nCounter digital analyzer. To account for slight differences in hybridization and purification, data were normalized to the average counts for all control spikes in each sample.

Quantitative reverse transcription–polymerase chain reaction (qRT-PCR)

MiRNAs were quantitatively measured using TaqMan miRNA assay (Applied Biosystems, Carlsbad, CA, USA). All primers and probes were obtained from Applied Biosystems; sequences for the primers, gene ID numbers, and silencing RNA (siRNA) primers are provided in Supplemental Table S1. U18 was used as an internal control. For mRNA measurements, total RNA was extracted using the RNeasy kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. RNA concentrations were determined with a NanoDrop instrument (NanoDrop Technologies). qRT-PCR experiments were also performed using 300 ng total RNA input and IL-6, IL-8, and TNF-α TaqMan primer/probe sets (TaqMan Gene Expression Assays; Applied Biosystems). GAPDH was used as an endogenous control for normalization. Reverse transcription and real-time PCR were performed according to the manufacturer's protocols. RT reactions were run in a Bio-Rad MyCycler thermal cycler (Bio-Rad, Hercules, CA, USA), and PCR amplifications were performed on a 7900HT fast real-time PCR system (Applied Biosystems). Data were analyzed with the 7900HT SDS 2.3 software (Applied Biosystems). The relative miRNA/mRNA expression level was normalized to that of internal control by using the $2^{-\Delta\Delta Ct}$ cycle method.

MiRNA transfection

For gain of function or silencing of miRNA expression in HSAEpCs, Pre-miR miRNA Precursors (PM10722), Anti-miR miRNA Inhibitors (AM10722), and the negative control oligonucleotides (Applied Biosystems) were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were exposed to oscillatory pressure 48 or 72 h after transfection.

3′-Untranslated region (UTR) luciferase reporter assay

IRAK1, TRAF6, and their mutated 3′-UTR luciferase reporter constructs (pMiR-REPORT) were obtained from Addgene (Cambridge, MA, USA). HSAEpCs were cotransfected with 40 ng luciferase reporter plasmids, 40 ng of pRSV-β-Gal, pre-miR-146a, and negative control. Luciferase and β-galactosidase activities were measured using the Luciferase Reporter Assay (Promega, Madison, WI, USA) 48 h following transfection. All transfections were performed in triplicate using Lipofectamine 2000. The 3′ UTR for IRAK1 is 5′-AAAUCCG-GAAGUCAAAGUUCUCA-3′ and for TRAF6 is 5′-UGCUCUA-GAAAGUUGAGUUCUCA-3′, where underscored bases indicate the seed targeting site for miR-146a.

Preparation of cytosolic and nuclear lysates

After the appropriate treatments, HSAEpCs were washed twice with PBS and incubated in 0.1 ml ice-cold lysis buffer (10 mM HEPES, pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; and 0.5 mM PMSF) with freshly added protease inhibitor cocktail (Sigma, St. Louis, MO, USA) for 15 min, after which 10 μl of 10% Igepal CA-630 was added. Cells were scraped and then centrifuged. The supernatant was collected as a cytosolic lysate. The nuclear pellet was resuspended in nuclear extraction buffer (20 mM HEPES, pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM DTT; 0.5 mM PMSF; and 10% glycerol) with freshly added protease inhibitor cocktail. After vigorously vortexing, the lysates were placed on a shaker on ice and the samples were then centrifuged and the supernatant was used as the nuclear fraction.

ELISA

To measure NF-κB/p65 activation, the commercially available Trans-AM kit (Active Motif, Carlsbad, CA, USA) was used to detect and quantify NF-κB/p65 by ELISA. This system uses an oligonucleotide containing the NF-κB consensus binding site (5′-GGGACTTTCC-3′) that specifically binds to activated NF-κB in the nuclear extract. An NF-κB p65 antibody was used to detect p65 subunit activation. Absorbance of the final solution was read on a Synergy HT plate reader (Biotek, Winooski, VT, USA) within 5 min at 450 nm with a reference wavelength of 655 nm. Activation levels were normalized by folds when compared with control cells. Cytokine concentration in the medium (*i.e.*, cytokine secretion) was also determined with an ELISA kit (R&D Systems Minneapolis, MN, USA) following the vendor's protocol.

Western blot analysis

Cells were lysed and extracted in RIPA buffer [50 mM Tris, pH 8.0; 150 mM NaCl; 1% Nonidet P-40; 0.5% sodium deoxycholate; 0.1% sodium dodecyl sulfate (SDS); 1 mM PMSF; 2.5 mM sodium pyrophosphate; 1 mM β-glycerophosphate; 1 mM Na_3VO_4; and 1 μg/ml leupeptin]. The protein content was determined using the BCA protein assay (Pierce,

Rockford, IL, USA). Equal amounts of protein were resolved by electrophoresis on 8% SDS-polyacrylamide gel and electrotransferred to nitrocellulose membrane. After blocking in 5% BSA or milk in TBST (0.1% Tween-20) for 1 h, membranes were probed with primary antibodies against NF-κB inhibitor (IκB-α; Cell Signaling, Beverly, MA, USA), IRAK1, TRAF6, or TNF-α antibodies (Serotec, Raleigh, NC, USA) and HRP-conjugated secondary antibodies (Bio-Rad). Proteins were detected using the enhanced chemiluminescence (ECL) system (Pierce; Thermo Fisher Scientific, Rockford, IL, USA).

siRNA experiment

For RNA interference studies, the Silencer siRNA duplex targeting the mRNA sequences of human NF-κB p65, TNF-α, IRAK1 and TRAF6 (Applied Biosystems) was used. Cells were transfected with 50 or 100 nM targeted or negative control scrambled siRNA using Lipofectamine 2000 (Invitrogen), and transfected cells were directly plated on the apical side of cell culture inserts. Cells were then exposed to oscillatory pressure 48 or 72 h after transfection.

Statistical analysis

Data are presented as means ± SD. ANOVA and *post hoc* least significant difference analysis or Student *t* tests were performed, and values of $P < 0.05$ were considered statistically significant.

RESULTS

Oscillatory pressure activates the NF-κB pathway and induces IL-6, IL-8, and IL-1β secretion in HSAEpCs

Although cyclic stretching of A549 adenocarcinoma alveolar epithelial cells can induce cytokine secretion (13, 14), the inflammatory response to transmural pressures is not well characterized. We demonstrated previously that transmural pressure can activate NF-κB in A549 cells (15) and here sought to more fully characterize the inflammatory response of HSAEpCs to oscillatory transmural pressures. As shown in **Fig. 1*A***, exposing HSAEpCs to 0.2 Hz transmural oscillatory pressure (0–20 cmH_2O) for 1 or 4 h resulted in increased NF-κB DNA binding activity. Consistent with NF-κB activation, we also observed significant degradation of the inhibitory protein IκB-α *via* Western blot analysis (Fig. 1*B*). mRNA expression levels of inflammatory cytokines IL-6, IL-8, and TNF-α were also significantly increased in response to 4 h of oscillatory pressure (Fig. 1*C*). Figure 1*D*, *E* demonstrates that 12 h of oscillatory pressure can induce increased IL-6 and IL-8 cytokine secretion and that the amount of mechan-

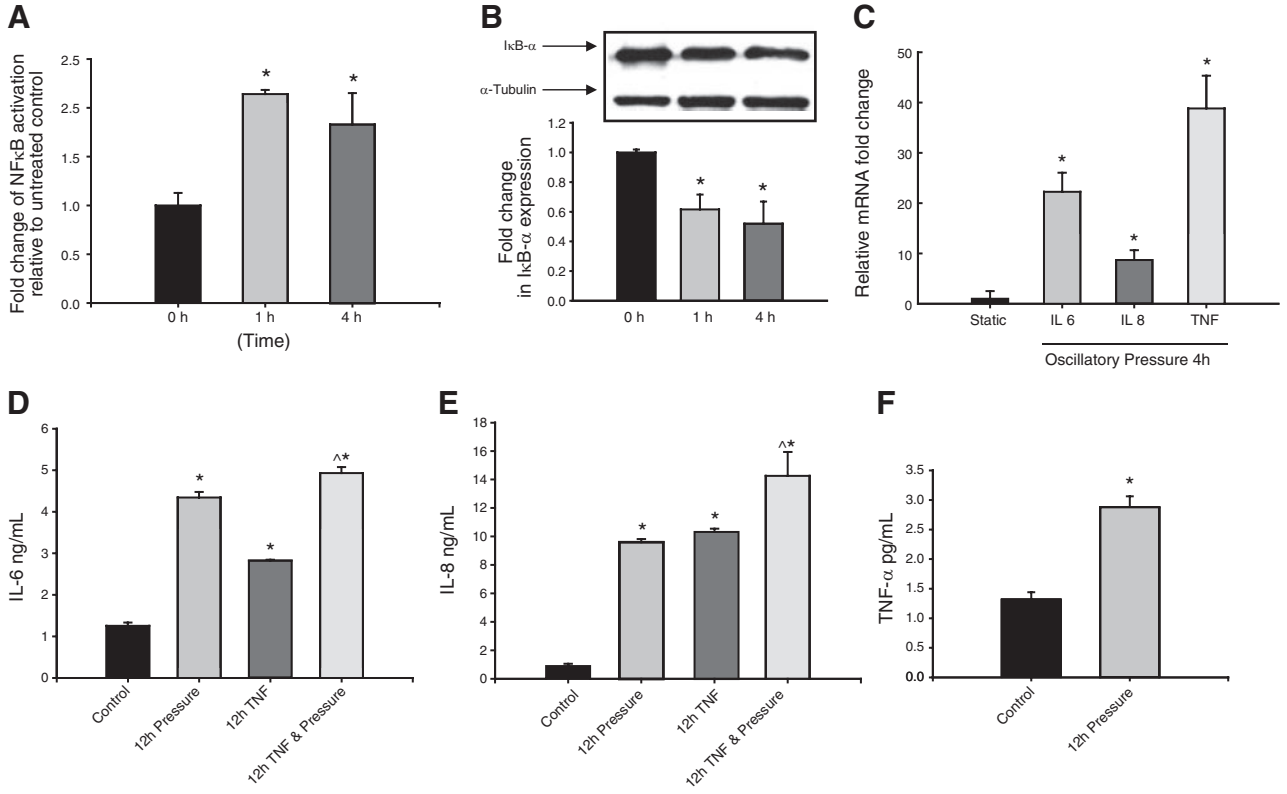

Figure 1. Oscillatory transmural pressure activates the NF-κB pathway and results in increased proinflammatory cytokine expression and secretion in HSAEpCs. *A, B*) Four hours of oscillatory pressure induced increased NF-κB DNA binding activity (*A*) and inhibition of IκB-α (*B*). IκB-α expression level was assessed by Western blot and densitometry. *C–F*) Four hours of oscillatory pressure also increased IL-6, IL-8, and TNF-α mRNA expression (*C*), while 12 h of pressure induced significant secretion of IL-6 (*D*) and IL-8 (*E*). Twelve hours of 30 ng/ml TNF-α also induced significant IL-6 (*D*) and IL-8 (*E*) secretion in HSAEpCs, but 12 h of pressure resulted in minimal TNF-α secretion (*F*). Combined TNF-α and pressure stimulation induced higher cytokine release than either stimulus alone (*D, E*). qRT-PCR data were normalized to GAPDH. HSAEpCs were exposed to 0.2-Hz, 0- to 20-cmH_2O oscillatory pressure. Results are presented as means ± SD; $n = 2$ (*A, C*), 3 (*B–F*). $*P < 0.05$ *vs.* 0 h or static control; $\wedge P < 0.05$ *vs.* 12 h pressure and 12 h TNF-α.

ically induced cytokine secretion is comparable to, and sometimes larger than, cytokine secretion during chemical stimulation; *i.e.*, 12 h of 30 ng/ml TNF-α. Interestingly, combined mechanical and chemical stimulation; *i.e.*, exposing HSAEpCs to 12 h oscillatory pressure in the presence of 30 ng/ml TNF-α, induced slightly higher cytokine release than either stimulus alone ($P<0.05$). However, this interaction between mechanically and chemically induced inflammation was less than additive. We also investigated the time course of proinflammatory cytokine production in response to oscillatory pressure. In addition to IL-6 and IL-8, for these studies we also investigated how oscillatory pressure alters IL-1β secretion. As shown in **Fig. 2**, IL-6 and IL-1β secretion levels continuously increase over the 12 h time course while increases in IL-8 saturate after 8 h of oscillatory pressure. We also show that IL-1β concentrations in response to oscillatory pressure are significantly lower than IL-6 or IL-8.

It is theoretically possible that increased IL-6/IL-8 secretion during oscillatory pressure is a secondary response to increased TNF-α secretion during pressure stimulation. However, as shown in Fig. 1*F*, 12 h of oscillatory pressure resulted in a very small increase in TNF-α secretion, with the concentration of TNF-α following pressure stimulation (~3 pg/ml) being negligible compared to the TNF-α concentration (30 ng/ml) required to elicit increased IL-6/IL-8 cytokine secretion. Since TNF-α does not have to be completely secreted to bind to its receptor on the same cell, we also conducted siRNA experiments to further investigate the role of TNF-α in pressure-induced cytokine production. For these experiments, 100 nM of siRNA TNF-α was used to knock down TNF-α expression, and both control cells treated with a scrambled siRNA and TNF-α-silenced cells were exposed to 12 h of 20 cmH_2O oscillatory pressure. As shown in Supplemental Fig. S1, no statistically significant difference was found in the amount of pressure-induced IL-6, IL-8, and IL-1β cytokine secretion between scramble controls and TNF-α-silenced cells. Therefore, pressure-induced increases in IL-6, IL-8, and IL-1β secretion does not depend on TNF-α expression or secretion.

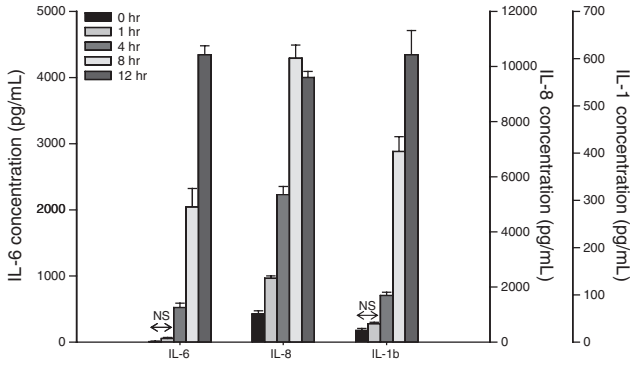

Figure 2. Oscillatory transmural pressure results in a time-dependent increase in proinflammatory cytokine secretion. IL-6 and IL-1β cytokine production continually increases during exposure to 1, 4, 8, and 12 h of oscillatory pressure. IL-8 cytokine production also increases but saturates after 8 h of oscillatory pressure. All means are statistically different ($P<0.01$) except as indicated ($n=4–6$).

Oscillatory pressure results in increased miR-146a expression in HSAEpCs

Although mechanical forces can alter inflammatory signaling in lung epithelia (13–15), to our knowledge, there is currently no information about how mechanical forces influence miRNA expression patterns in primary human lung epithelial cells. We therefore used the Nanostring nCounter system to profile miRNA expression patterns in HSAEpCs exposed to 12 h of 0.2 Hz oscillatory pressure (0–20 cmH_2O). Since proinflammatory cytokines may also alter miRNA expression patterns (36), HSAEpCs were also exposed to 12 h of 30 ng/ml TNF-α or 12 h of oscillatory pressure in the presence of 30 ng/ml TNF-α. All experiments were repeated in triplicate, and heatmaps of miRNAs that demonstrated statistically significant changes in miRNA expression ($P<0.05$) with fold changes >1.4 or <0.8 are shown in **Fig. 3*A*, *B***. Although TNF-α and oscillatory pressure both induced differential expression of several miRNAs, TNF-α resulted in a different pattern of miRNA expression compared with oscillatory pressure. Interestingly, only 3 miRNAs (miR-146a, miR-650, and miR-1260) exhibited differential expression following either TNF-α stimulation or oscillatory pressure (T or P) and only miR-146a and miR-1260 exhibited differential expression during all three conditions, including the combined chemical and mechanical stimulation condition (*i.e.*, T, P, and T+P). Specifically, we observed a ~1.6-fold increase in miR-146a expression and a ~0.75-fold decrease in miR-1260 expression during all three conditions. As shown in Supplemental Fig. S2*A*, increased miR-146a expression during oscillatory pressure was validated by qRT-PCR, but decreased miR-1260 expression was not validated. PCR was also used to investigate the time course of miR-146a expression during oscillatory pressure. As shown in Supplemental Fig. S2*B*, although miR-146a expression increases 1.2-fold at 4 or 8 h, 12 h of pressure induced a larger 1.8-fold increase in miR-146a expression. Note that since 146a expression and cytokine production were largest at 12 h, all other data related to cytokine production and miR146a expression in this manuscript were evaluated at the 12-h time point.

In addition to global profiling, we also used PCR to assess changes in a group of select miRNAs that have been implicated in inflammation and/or mechanotransduction (29, 37–39) and may not have been captured in the Nanostring system due to low counts (Fig. 3*C*). For these studies, cells were exposed to 12 h of TNF-α, oscillatory pressure, or both TNF-α and pressure, and expression levels were assessed *via* qRT-PCR. Similar to the Nanostring results, we observed a significant ($P<0.02$) ~2-fold increase in miR-146a expres-

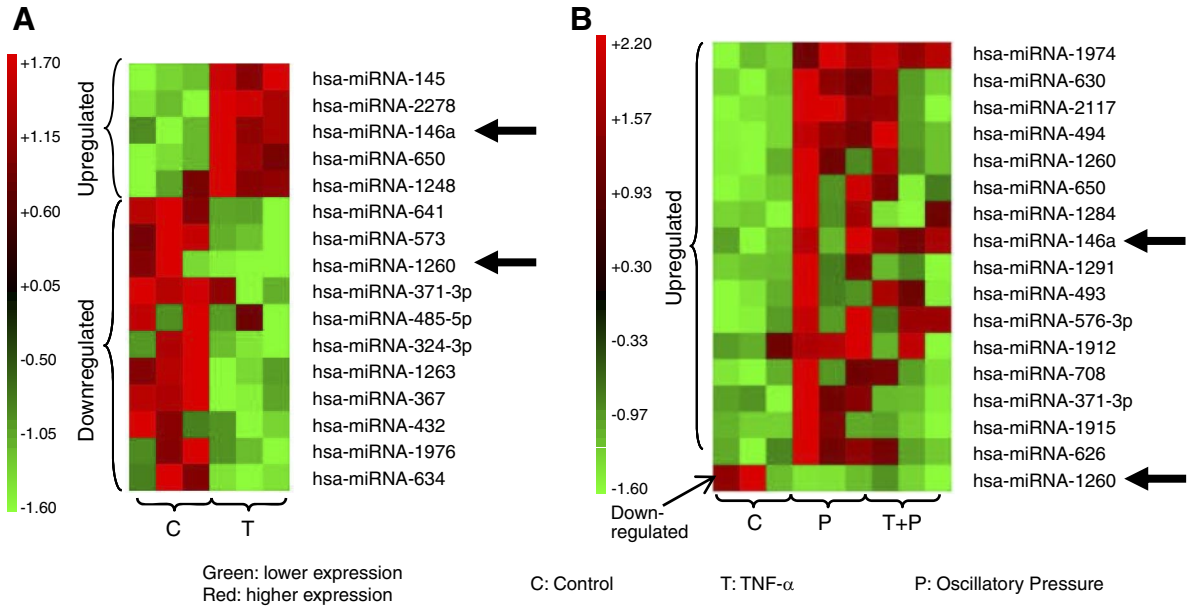

Figure 3. Effect of TNF-α, oscillatory pressure, and both TNF-α and pressure on miRNA expression. *A*, *B*) Heatmaps of miRNAs with statistically significant differences in expression levels ($P<0.05$) during TNF-α treatment (*A*) and pressure treatment (*B*). Data also shown for combined TNF-α and pressure treatment (*B*). Data generated in triplicate using Nanostring nCounter System with 12 h exposure time. *C*) Fold change of miRNA expression in HSAEpCs exposed to 12 h of 30 ng/ml TNF-α, 0.2-Hz oscillatory pressure, or both TNF-α and pressure compared to untreated cells; $n = 2–3$. Data were generated using RT-PCR. $*P < 0.05$ *vs.* untreated cells.

sion for all conditions. We also observed a significant ($P<0.05$) reduction in miR-181c and miR-27b during both TNF-α treatment and combined TNF-α/oscillatory pressure conditions and a significant ($P<0.05$) reduction in miR-33a for all three conditions. Finally, we observed a small but statistically significant ($P<0.01$) reduction in miR-21 for TNF-α and oscillatory pressure conditions. In summary, our miRNA screening experiments indicate that miR-146a expression is highly sensitive to both mechanical and chemical stimuli.

Influence of pressure magnitude on cytokine production and miR-146a expression

In addition to investigating high oscillatory pressure magnitudes of 20 cmH_2O, we also investigated how a lower 5-cmH_2O, 0.2-Hz pressure magnitude influences cytokine production and miR-146a expression. As shown in Supplemental Fig. S3*A*, 5-cmH_2O pressure does induce a small, statistically significant increase in IL-6/IL-8/IL-1β secretion. However, 20-cmH_2O pressure induced significantly more cytokine production than 5-cmH_2O pressure. Supplemental Fig. S3*B* indicates that 5-cmH_2O pressure does not induce any statistically significant change in miR-146a expression. Therefore, increased miR-146a expression and cytokine production is clearly dependent on the magnitude of pressure applied to HSAEpCs.

Influence of IL-1β on miR-146a expression in HSAEpCs

Figure 2 demonstrates that pressure induces increased IL-1β cytokine production. IL-1β has been shown to induce expression of miRNA-146a in primary bronchial epithelial cells (40). We therefore exposed HSAEpCs to 12 h of 10 ng/ml of recombinant IL-1β and monitored miR-146a expression. As shown in Supplemental Fig. S4*A*, IL-1β does not induce any statistically significant

change in miR-146a. Perry *et al.* (40) also demonstrated that IL-1β can induce IL-8 secretion from A549 alveolar epithelial cells, and we demonstrate that IL-1β also induces increased IL-8 secretion from HSAEpCs. We therefore conclude that IL-1β is a potent inducer of IL-8 cytokine production in HSAEpCs but does not alter miR-146a expression in these cells.

Effect of silencing NF-κB on miR-146a expression and cytokine secretion

As shown in Fig. 1*A*, oscillatory pressure leads to the rapid activation of NF-κB in primary HSAEpCs. MiR-146a has been previously reported to be a NF-κB-dependent gene in monocytes (41). Therefore, we conducted siRNA experiments to investigate whether increased miR-146a expression during oscillatory pressure is dependent on NF-κB activation. Intracellular NF-κB was effectively silenced using either 50 or 100 nM siRNA NF-κB p65 (**Fig. 4*A***). Silencing NF-κB at 50 nM resulted in a small decrease in miR-146a expression during oscillatory pressure conditions ($P=0.064$ with respect to scrambled siRNA control; Fig. 4*B*). Since NF-κB is a key mediator of inflammation, we also examined how silencing NF-κB influences pressure-induced cytokine release. Silencing NF-κB at 50 nM significantly ($P<0.05$) attenuated pressure-induced increases in IL-6 and IL-8 secretion (Fig. 4*C*). These results indicate that, although pressure-induced increases in miR-146a expression are only weakly dependent on NF-κB, pressure-induced cytokine production and secretion are strongly NF-κB dependent.

MiR-146a regulates pressure-induced cytokine secretion in HSAEpCs

Although miR-146a has been shown to regulate inflammatory responses to bacterial and chemical stimuli in alveolar and bronchial epithelial cells (40), it is not known whether miR-146a can regulate the mechanotransduction events responsible for increased IL-6 and IL-8 secretion in primary human airway epithelial cells. We therefore conducted a set of miRNA transfection studies to investigate how silencing and overexpressing miR-146a influences both baseline (*i.e.*, no mechanical stimulation) and mechanically induced cytokine production in HSAEpCs. As shown in Supplemental Fig. S5*A*, transfection with 50 or 100 nM of the anti-miR-146a inhibitor significantly reduced baseline miR-146a expression levels and led to a small but statistically significant increase in baseline IL-6 secretion ($P<0.05$). Conversely, transfection with the pre-miR-146a precursor resulted in a concentration dependent overexpression of miR-146a and a reduction in baseline IL-6 secretion for concentrations > 0.08 nM (Supplemental Fig. S5*B*). Therefore, miR-146a negatively modulates baseline cytokine secretion in HSAEpCs.

To determine whether miR-146a expression regulates mechanically induced inflammation and cytokine secretion, we manipulated miR-146a levels in HSAEpCs and exposed cells to either 4 or 12 h of oscillatory pressure. First, suppression of miR-146a expression levels with 100 nM anti-miR-146a resulted in no statistically significant change in pressure-induced NF-κB activation (**Fig. 5*A***). However, suppression of miR-146a expression did result in a statistically significant ($P<0.05$) increase in pressure-induced IL-6 and IL-8 secretion (Fig. 5*B*, *C*). Conversely, overexpression of miR-146a with 0.5 nM pre-miR-146a resulted in significant ($P<0.05$) reductions in pressure-induced NF-κB activation (Fig. 5*D*) and dramatically reduced IL-6 and IL-8 secretion during oscillatory pressure (Fig. 5*E*, *F*). These results indicate that changes in miR-146a expression can regulate mechanically induced inflammation and cytokine production in HSAEpCs.

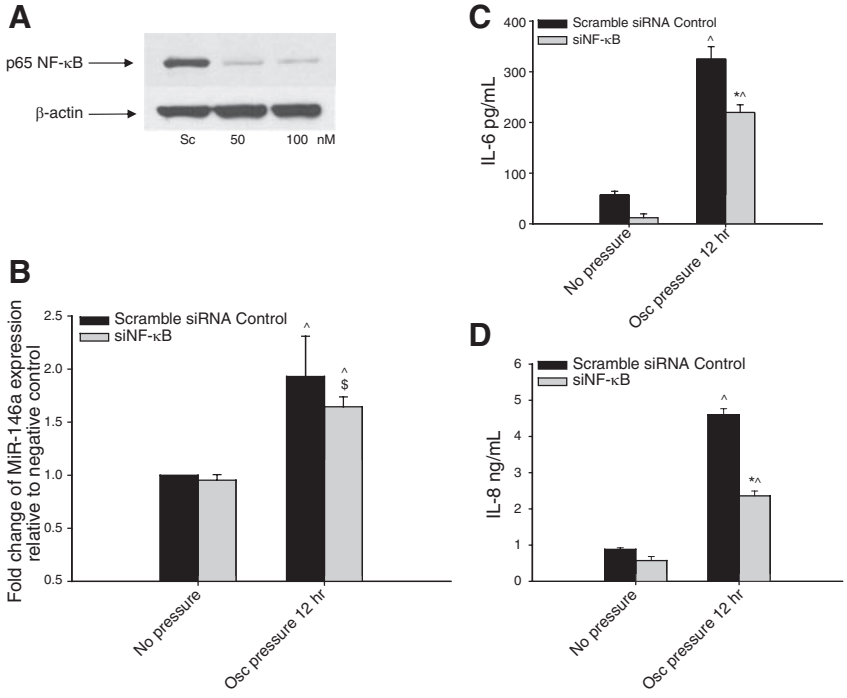

Figure 4. Effect of silencing NF-κB on miR-146a expression and cytokine release. *A*) NF-κB expression was silenced by transfection with 50 nM or 100 nM NF-κB p65 siRNA. *B*, *C*) Silencing NF-κB results in a small decrease in miR-146a expression during oscillatory pressure (*B*) and significantly reduces pressure-induced increases in IL-6 and IL-8 secretion (*C*). HSAEpCs were transfected with scramble siRNA (control) or NF-κB p65 siRNA and subjected to 12 h of oscillatory pressure 48 h after transfection. $n = 4$ (*B*); 6 (*C*).*$P < 0.05$ *vs.* control; ^$P < 0.05$ *vs.* no pressure; $^{\$}P = 0.064$ *vs.* control.

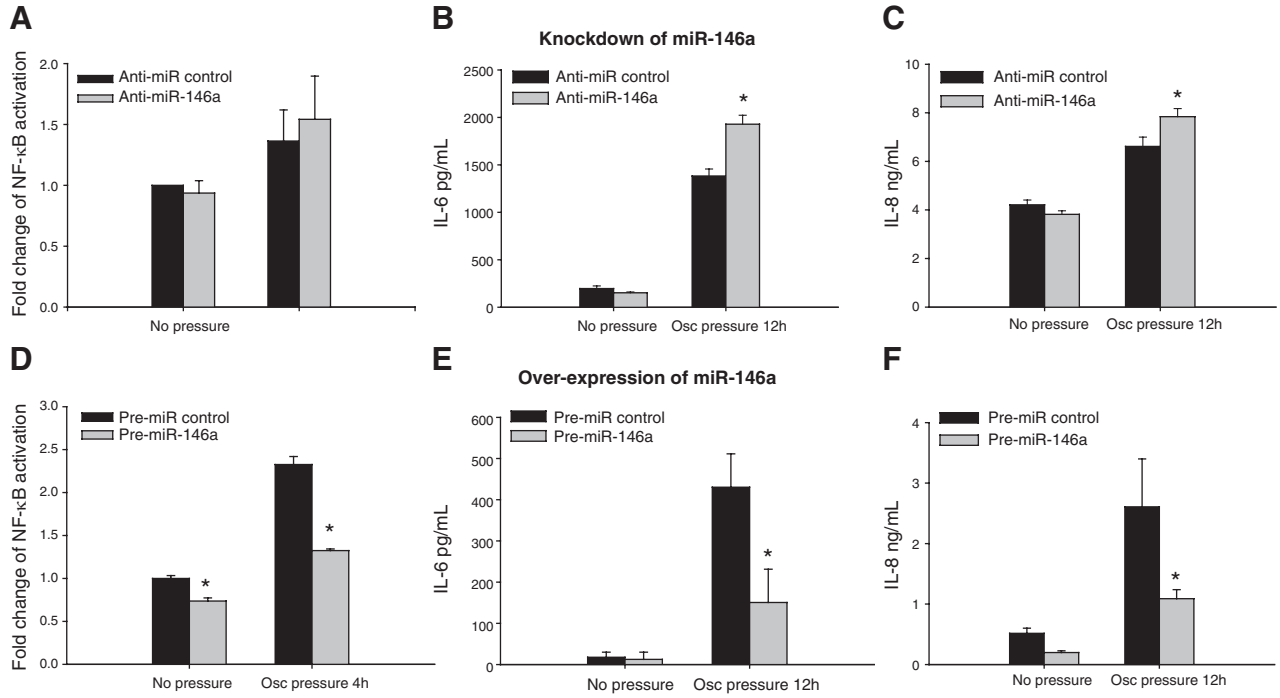

Figure 5. Effect of miR-146a knockdown and overexpression on NF-κB activation (*A*, *D*), IL-6 secretion (*B*, *E*), and IL-8 secretion (*C*, *F*). NF-κB activation was assessed after 4 h of oscillatory pressure, while cytokine secretion was assessed after 12 h of pressure. *A–C*) Knocking down miR-146a with 100 nM anti-miR-146a resulted in minimal changes in pressure induced NF-κB activation (*A*) and a statistically significant increase in IL-6 (*B*) and IL-8 (*C*) secretion during oscillatory pressure. *D–F*). Overexpression of miR-146a with 0.5 nM pre-miR-146a resulted in a statistically significant decrease in NF-κB activation (*D*) and dramatic reductions in pressure-induced IL-6 (*E*) and IL-8 (*F*) secretion. Experiments were conducted 2 or 3 d after transfection; $n = 2$ (*A*, *D*), 6 (*B*, *C*, *E*, *F*). $*P < 0.05$ *vs.* anti-miR or pre-miR control.

MiR-146a targets IRAK1 and TRAF6 in HSAEpCs

Previous studies indicate that miR-146a may regulate inflammatory responses to microbial components and proinflammatory cytokines by targeting key proteins in the TLR signaling pathway (41), including IRAK1 and TRAF6. However, the potential role of IRAK1 and TRAF6 in regulating mechanically induced inflammation in primary human lung epithelial cells has not been investigated. Since miRNA targeting can vary by cell type, we first confirmed that overexpression of miR-146a in HSAEpCs results in reduced IRAK1 and TRAF6 protein levels. As shown in Supplemental Fig. S6*A*, *B*, overexpression of miR-146a suppressed IRAK1 protein expression in a concentration-dependent fashion and also suppressed TRAF6 expression. To validate that IRAK1 and TRAF6 are direct targets of miR-146a in HSAEpCs, reporter plasmids containing the IRAK1 and TRAF6 3′ UTR with the binding site for miR-146a and the mutated internal control were cotransfected with pre-miR-146a into HSAEpCs. As shown in Supplemental Fig. S6*C*, *D*, transfection with the IRAK1 or TRAF6 reporters resulted in lower luciferase activity for cells treated with 2 nM pre-miR-146a ($P<0.05$), while no change in luciferase activity was observed in cells transfected with the mutated internal control. These data indicate that miR-146a regulates IRAK1 and TRAF6 expression through specific 3′-UTR binding site.

IRAK1 and TRAF6 are required for the mechanotransduction of pressure into cytokine secretion

To elucidate further the mechanotransduction mechanisms by which oscillatory pressure induces cytokine production in lung epithelia, we silenced the miR-146a targets, IRAK1 and TRAF6, using siRNA and measured IL-6 and IL-8 cytokine secretion after exposure to 12 h of oscillatory pressure. First, as shown in **Fig. 6*A***, siRNA efficiently knocked down IRAK1 and TRAF6 protein expression levels. As shown in Fig. 6*B*, silencing IRAK1 with 50 nM siRNA resulted in a significant ($P<0.05$) reduction in both baseline cytokine production and pressure-induced IL-6/IL-8 secretion. Similarly, silencing of TRAF6 using 50 nM siRNA resulted in a significant ($P<0.05$) reduction in baseline cytokine production and pressure-induced IL-6 secretion as well as a reduction in pressure-induced IL-8 secretion ($P=0.06$; Fig. 5*C*). These data indicate that the mechanotransduction of oscillatory pressure into cytokine secretion depends on key enzymes and proteins in the TLR signaling pathway; *i.e.*, IRAK1 and TRAF6.

MiR146a's regulation of pressure-induced cytokine secretion depends on its targeting of IRAK1 and TRAF6

To determine the relative importance of IRAK1 and TRAF6 on miR-146a's ability to regulate pressure-induced cytokine secretion, we investigated how miR-

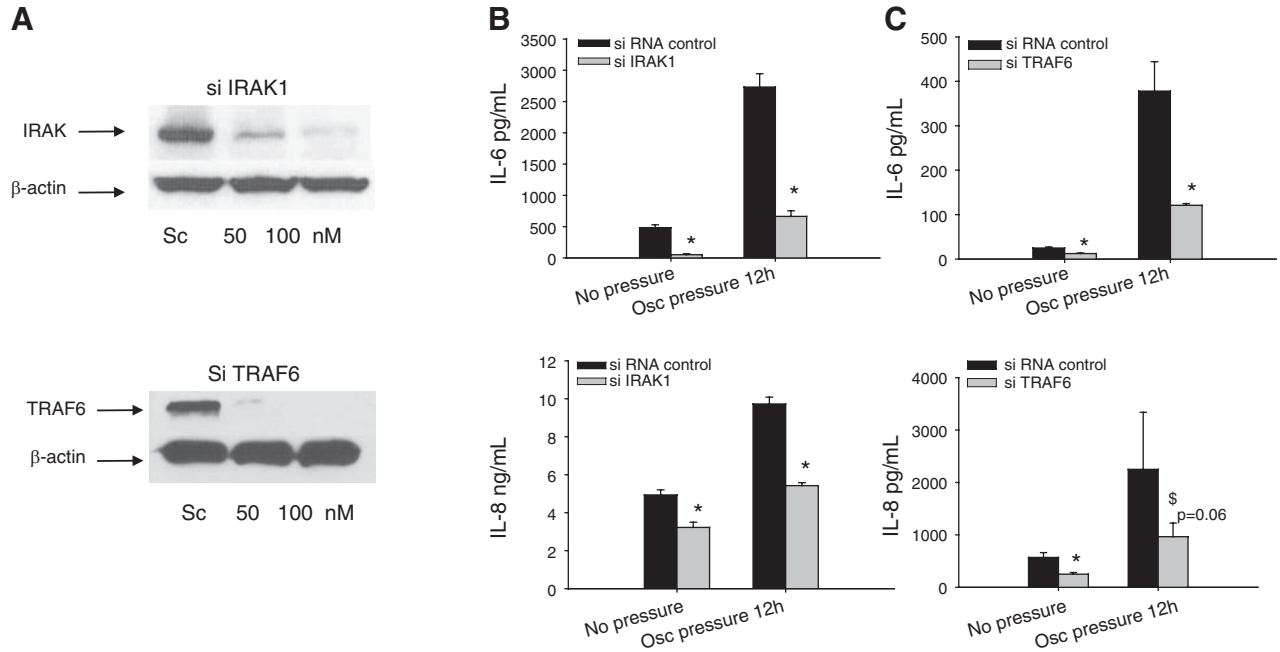

Figure 6. Effect of silencing miR-146a targets IRAK1 and TRAF6 on pressure-induced cytokine secretion. *A*) IRAK1 and TRAF6 protein expression was efficiently silenced using either 50 or 100 nM IRAK1 and TRAF6 siRNA. *B*) Silencing IRAK1 significantly reduced both baseline cytokine levels and pressure-induced IL-6 and IL-8 cytokine secretion. *C*) Silencing TRAF6 also significantly reduced baseline and pressure-induced IL-6 and IL-8 cytokine secretion. $n = 2$–5. $^{*}P < 0.05$ *vs.* siRNA control; $^{\$}P = 0.06$ *vs.* siRNA control.

146a inhibitors influence pressure-induced cytokine secretion in cells with silenced IRAK1 or TRAF6. HSAEpCs were cotransfected with siRNA against IRAK1 or TRAF6 (or a scrambled siRNA control); and miR-146a inhibitor (anti-miR-146a) (or the negative anti-miR control). IL-6 and IL-8 cytokine secretion were then measured in both cells exposed to no pressure and 12 h of oscillatory pressure. As shown in **Fig. 7**, oscillatory pressure induced an increase in IL-6 and IL-8 secretion for all cotransfection combinations. Consistent with single-transfection experiments (Fig. 5*B, C*), treatment with anti-miR-146a and scrambled siRNA resulted in a statistically significant increase in IL-6 and IL-8 cytokine secretion (white bars) with respect to cells treated with the negative anti-miR and scrambled siRNA (black bars). In addition, treatment with IRAK1 siRNA or TRAF6 siRNA and negative anti-miR resulted in a dramatic reduction in both IL-6 and IL-8 secretion ($P < 0.01$), also consistent with single-transfection siRNA experiments (Fig. 6*B, C*). However, treatment with IRAK1 siRNA and anti-miR-146a resulted in no change in IL-6 and IL-8 secretion with respect to cells treated with IRAK1 siRNA and the negative anti-miR. Similarly, treatment with TRAF6 siRNA and anti-miR-146a also resulted in no change in IL-6 and IL-8 secretion with respect to cells treated with TRAF6 siRNA and the negative anti-miR. If the regulation of pressure-induced cytokine secretion by miR-146a occurred *via* non-IRAK1 or non-TRAF6 targets, we would have expected an increase in cytokine production during cotransfection with siRNA IRAK1 or siRNA TRAF6 and anti-miR-146a. Since this increase was not observed, these data indicate that miR-146a's ability to regulate pressure-induced cytokine secretion depends on its modulation of both the IRAK1 and TRAF6 targets.

DISCUSSION

Although mechanical ventilation is a life-saving therapy for patients with ARDS, the mechanical forces generated during ventilation exacerbate lung injury, promote lung inflammation, and can lead to distant organ failure (6, 8). Notably, ARDS is a heterogeneous disease where different lung regions experience different types of mechanical forces (*e.g.*, cyclic stretching, shear stress, and high transmural pressure). In addition to causing physical injury, these mechanical forces can be sensed by lung epithelial cells and converted into biochemical signals related to inflammation. In particular, mechanically induced secretion of proinflammatory cytokines may be important, since these cytokines may contribute to systemic inflammatory responses and multisystem organ failure (8, 17). However, the molecular mechanisms that regulate mechanotransduction in lung epithelia are not well established. In this study, we explored the novel hypothesis that mechanical forces can induce differential expression of miRNAs in HSAEpCs and that miRNAs play an important role in regulating mechanically induced inflammation in lung epithelia. To our knowledge, this is the first study to identify an early responsive miRNA, miR-146a, that is differentially expressed in response to compressive pressure forces and can also regulate pressure-induced cytokine secretion from lung epithelial cells. In addition to being a mechanosensitive miRNA that regulates mechanically

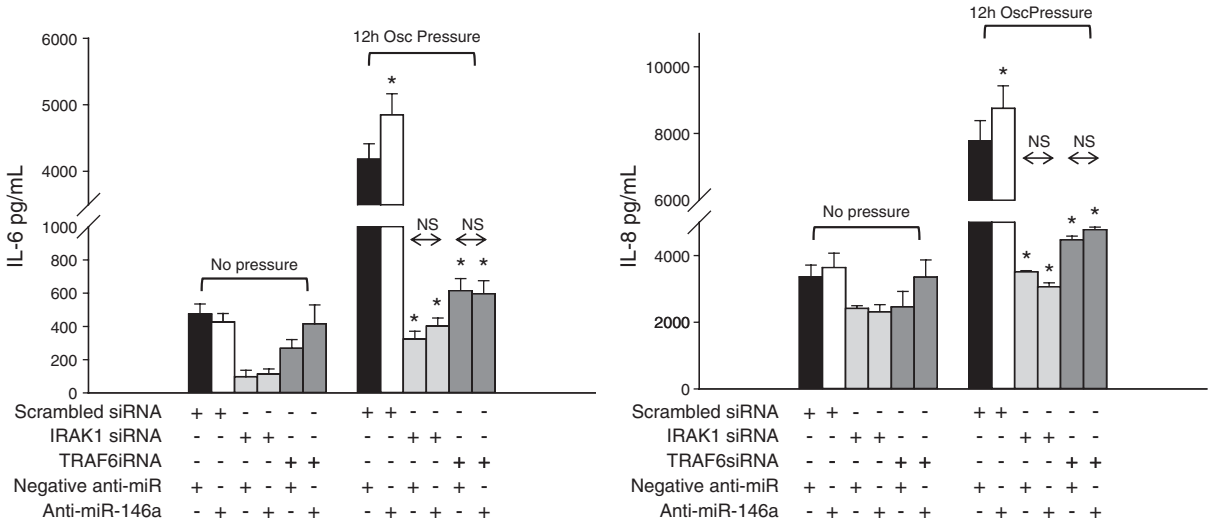

Figure 7. Effect of simultaneously knocking down IRAK1 or TRAF6 and miR-146a on pressure-induced cytokine secretion. Oscillatory pressure induced more cytokine production in cells treated with anti-miR-146a + scrambled siRNA (open bars) than in control cells treated with negative anti-miR + scrambled siRNA (solid bars). Cells treated with siIRAK1 or siTRAF6 exhibited significant reductions in IL-6 and IL-8 secretion compared to the negative anti-miR + scrambled siRNA control. However, no statistically significant difference was found in cytokine production between the anti-miR146a + siIRAK1 and the negative anti-miR + siIRAK1 samples (light shaded bars) or between the anti-miR146a + siTRAF6 and the negative anti-miR + siTRAF6 samples (dark shadedbars). These data indicate that regulation of pressure-induced cytokine secretion by miR-146a depends on its targeting of IRAK1 and TRAF6. $n = 4–6$. $^*P < 0.01$ *vs.* scrambled siRNA and negative anti-miR controls (solid bars).

induced inflammation, miR-146a can also regulate cytokine induced inflammation in lung epithelia (31). MiR-146a may, therefore, represent a novel biomarker and/or treatment target for lung inflammation during mechanical ventilation and ARDS.

Although cyclic stretching of lung epithelial cells can result in cytokine production (14), the inflammatory response of lung epithelial cells to transmural pressure is not as well characterized. In this study, we demonstrated that 0.2-Hz, 20-cmH_2O oscillatory pressure can activate NF-κB inflammatory pathways in HSAEpCs within 1 to 4 h (Fig. 1*A*, *B*). This rapid activation is consistent with LPS stimulation, which activities NFκB within 1 to 2 h (42). We also demonstrate that oscillatory pressure induces a time-dependent increase in IL-6, IL-8, and IL-1β cytokine production (Fig. 2) and a negligible amount of TNF-α secretion (Fig. 1*F*) and that 20-cmH_2O pressure induces significantly more cytokine production than 5-cmH_2O pressure (Supplemental Fig. S3*A*). We also show *via* siRNA experiments that pressure-induced increases in IL-6, IL-8, and IL-1β are not dependent on TNF-α expression (Supplemental Fig. S1) and that pressure-induced increases in IL-6 and IL-8 cytokine production are strongly NF-κB dependent (Fig. 4*C*). Although we recognize that factors other than NF-κB might regulate cytokine production, data in this report indicate that NF-κB inflammatory signaling pathways play a key role in the mechanotransduction processes responsible for excessive inflammation during mechanical ventilation.

Mechanotransduction is the process by which cells convert mechanical forces into biochemical signals and has been shown to play a critical role in diverse biological processes (4, 21). Recently, the role of miRNAs as both mechanosensitive agents and regulators of mechanotransduction have been investigated. For example, laminar flow or shear stress can alter miRNA expression in endothelial cells, and manipulation of specific miRNAs can alter shear-stress-induced changes in endothelial cell growth, apoptosis, and inflammation (33, 34, 37, 39, 43). In addition, cyclic mechanical stretching has been shown to alter miRNA expression in human airway smooth muscle cells (44), chondrocytes (45), and human trabecular meshwork cells (46). However, to our knowledge, no information is available about how mechanical forces, such as oscillatory pressure, influence miRNA expression in primary HSAEpCs. Also, there is limited information about how specific miRNAs regulate the mechanotransduction processes responsible for lung inflammation. In this study, we profiled miRNA expression patterns in HSAEpCs in response to chemical (TNF-α) and mechanical (oscillatory pressure) stimuli and showed that both stimuli, as well as a combination of chemical and mechanical stimuli, significantly increased miR-146a expression (Fig. 3). qRT-PCR measurements validated increased miR-146a expression for all three conditions (Fig. 3*C*) and also indicated that pressure-induced increases in miR-146a are time dependent (Supplemental Fig. S2*B*). Therefore, miR-146a is an early responsive, mechanosensitive miRNA that was selected for further analysis in this study. We note that in our qRT-PCR array (Fig. 3*C*) oscillatory pressure, TNF-α and combined pressure/TNF-α significantly down-regulated miR-33a expression. Recently, miR-33a was shown to target the serine/threonine kinase Pim-1 (47) and Pim-1 has been shown to regulate NF-κB activity and IL-6 production (48). Therefore, future studies that investigate the role of

miR-33a and Pim-1 in regulating mechanically induced inflammation are warranted.

MiR-146a is a well-studied miRNA that has an important role in regulating the innate and adaptive immune response, inflammation (49), fundamental cellular processes such as differentiation, and proliferation and cancer metastases (50). Previous studies have demonstrated that miR-146a expression may be regulated by several factors, including microbial agents, LPS, and proinflammatory cytokines and NF-κB (40, 41, 51). Consistent with those studies, we demonstrated that 30 ng/ml TNF-α increases miR-146a expression in primary HSAEpCs. Interestingly, although Perry *et al.* (40) have shown that IL-1β can induce the expression of miRNA-146a in primary bronchial epithelial cells, we demonstrated that IL-1β does not alter miR-146a expression in HSAEpCs (Supplemental Fig. S4*A*). More important, the novel finding of this study is that a mechanical stimulus, *e.g.*, oscillatory pressure, can increase miR-146a expression (Fig. 3). In previous studies (40, 41), increased miR-146a expression was induced *via* proinflammatory stimuli that act through TLR pathways. Although oscillatory pressure is not normally considered to be an inflammatory stimulus, we clearly demonstrate that oscillatory pressure can activate NF-κB inflammatory pathways and induce proinflammatory cytokine secretion (Figs. 1 and 2). It is therefore interesting that this mechanical force up-regulates a miRNA that has been shown to be a counterregulator of chemically induced inflammation that acts through NF-κB pathways. However, oscillatory pressure only induces a ~2-fold increase in miR-146a expression (Fig. 3), which is clearly not large enough to regulate inflammation in normal, nontransfected HSAEpCs, since we also observe significant increases in cytokine secretion during oscillatory pressure (Figs. 1 and 2). However, experiments that increased miR146a expression levels *via* pre-miR transfection clearly demonstrate that overexpression of miR146a can negatively regulate pressure-induced cytokine production (Fig. 5*E*, *F*). Therefore, overexpression of this miR may have therapeutic value as a way to regulate pressure-induced cytokine production. In addition, our studies indicate that increased expression of miR-146a may represent a biomarker of "excessive" pressure during ventilation, since increased miR-146a expression was not observed at lower pressures (Supplemental Fig. S3*B*). Identifying biomarkers of mechanically induced inflammation might be important, since a significant amount of inflammation during ventilation occurs after the initial bacterial or viral insult has been cleared. However, developing miRNA biomarkers for mechanically induced inflammation will require an understanding of how forces other than transmural pressure influence miR expression.

Previous studies indicate that chemically induced changes in miR-146a expression are predominately driven by NF-κB (41). However, in this study, silencing NF-κB had only a minor effect on pressure-induced increases in miR-146a expression (Fig. 4*B*). It is therefore possible that miR-146a expression in the setting of oscillatory pressure may be regulated by other transcription factors, such as Krüppel-like factor (KLF; ref. 52) or E-26 transcription factor (ETS; ref. 53). Bhaumik *et al.* (50) demonstrated that overexpressing miR-146a can regulate cytokine production in breast cancer cells by inhibiting the expression of IRAK1 and TRAF6 and reducing NF-κB activity. Similarly, in this study, we demonstrated that overexpression of miR-146a significantly attenuates pressure-induced increases in NF-κB activation and IL-6/IL-8 cytokine production (Fig. 5*D*–*F*). Interestingly, silencing miR-146a resulted in a statistically significant increase in IL-6 and IL-8 cytokine secretion (Fig. 5*B*, *C*), but changes in cytokine levels during miR-146a knockdown were not as large as the changes observed during miR-146a overexpression. These data suggest that overexpression and knockdown of a miRNA may not necessarily lead to opposite physiological effects. Although most previous studies have implicated IRAK1 or TRAF6 targeting in the regulation of miR-146a on inflammation, Perry *et al.* (40) demonstrated that miR-146a can negatively regulate chemically induced cytokine production in alveolar epithelial cells without significantly altering IRAK1 and TRAF6. In this study, we clearly demonstrate that overexpressing miR-146a down-regulates the IRAK1 and TRAF6 targets in HSAEpCs (Supplemental Fig. S6) and that silencing IRAK1 and TRAF6 significantly reduces pressure-induced cytokine production (Fig. 6). We also investigated the relative importance of IRAK1 and TRAF6 targeting on the ability of miR-146a to regulate pressure-induced cytokine secretion by cotransfecting cells with the miR-146a inhibitor and a siRNA against IRAK1 or TRAF6. Under these conditions, recovery of pressure-induced cytokine production in siRNA treated cells would indicate that the regulation of miR-146a on cytokine production occurs by a non-IRAK1 or non-TRAF6 mechanism. However, as shown in Fig. 7, we observed no difference in cytokine production between cells treated with siRNA IRAK1/TRAF6 and anti-miR-146a and cells treated with siRNA IRAK1/TRAF6 and a negative anti-miR control. Therefore, miR-146a's ability to regulate pressure-induced cytokine secretion is dependent on its targeting of both IRAK1 and TRAF6.

We summarize the pathways this study implicates in pressure-induced cytokine production in **Fig. 8**. Specifically, this study clearly demonstrates that pressure-induced cytokine production is strongly dependent on NFκB, IRAK1, and TRAF6. We also demonstrate that pressure can induce increased miR146a expression but that this increase is minor (2-fold) and likely occurs *via* a non-NFκB dependent pathway (Fig. 8, dashed line). Finally, this study demonstrated that miR-146a's ability to regulate pressure-induced cytokine production is dependent on its targeting of IRAK1 and TRAF6.

In addition to the novel findings that mechanical forces can alter miR-146a expression levels and that miR-146a can regulate mechanically induced inflammation, this study has identified the TLR pathway as a

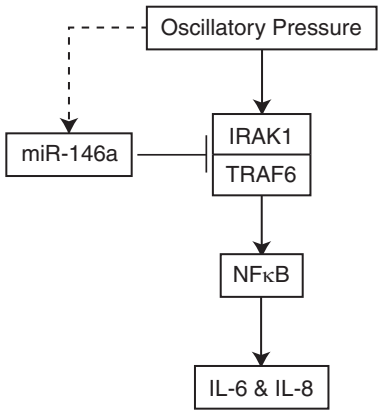

Figure 8. Schematic diagram of pathways involved in pressure-induced cytokine production in HSAEpCs and mechanisms of miR146a regulation. Dashed line indicates minor levels of activation.

novel mechanotransduction pathway in lung epithelia. Specifically, silencing of the key TLR proteins IRAK1 and TRAF6 had a dramatic effect on pressure-induced cytokine secretion (Figs. 6 and 7). It is well established that microbial components can activate TLR pathways in lung epithelial cells and induce cytokine production (54). In addition, recent studies indicate that mechanical stretching can up-regulate the expression of the TLR2 receptor (55). However, to our knowledge, this is the first study to demonstrate that mechanically induced increases in cytokine production from lung epithelia are dependent on downstream components of the TLR signaling cascade (*e.g.*, IRAK1 and TRAF6). This finding has important clinical implications, because it suggests that targeting the TLR pathway may be effective in mitigating both bacterially and virally induced inflammation and mechanically induced inflammation during ventilation. In support of this concept, Vaneker *et al.* (56) ventilated normal and TLR4-knockout mice with a low-tidal-volume ventilation protocol and observed increased plasma levels of IL-6 in normal mice but no increase in plasma IL-6 in the TLR4-knockout mice. Although our study indicates that the TLR signaling pathways plays a role in the mechanotransduction of pressure into inflammatory signaling, the mechanisms responsible for this conversion are not clear. For example, it is not known whether upstream components, such as MyD88, and/or the TLR receptors themselves play a role in mechanotranduction, and future studies could use selective siRNA techniques to further investigate the mechanisms by which TLR signaling influences mechanotransduction.

Although this study clearly demonstrated that oscillatory pressure can activate NF-κB inflammatory pathways, increase proinflammatory cytokine production, and alter miRNA expression patterns, the epithelium likely experiences other types of mechanical forces during ventilation. For example, the reopening of collapsed airways has been shown to exert shear stress and complex surface tension forces on the epithelium (9), while nonoccluded regions likely experience large stretching deformations (7). However, it is not known how shear stress, surface tension forces, or stretching deformation influence miRNA expression patterns in lung epithelia. Future *in vitro* studies could be conducted to evaluate the miRNA responses to these mechanical forces. Future studies could also investigate how changing the magnitude and frequency of mechanical stimulation influence miRNA expression patterns and inflammatory responses. Finally, although *in vitro* studies are useful in determining the miRNA and inflammatory response to specific, well-controlled mechanical stimuli, future *in vivo* studies will be required to fully ascertain the role of miRNAs in regulating ventilator-associated injury and inflammation.

In summary, we have demonstrated that oscillatory pressures influence inflammation and miRNA expression in primary human lung epithelial cells and that miR-146a is a mechanosensitive miRNA that plays an important role in regulating mechanically induced inflammation in lung epithelia. In particular, miR-146a was found to be an early responsive miRNA that is sensitive to mechanical forces and may therefore be a potential biomarker for mechanically induced inflammation during ventilation. Furthermore, we demonstrated that overexpression of miR146a can regulate pressure-induced cytokine secretion by targeting key proteins (IRAK1 and TRAF6) in the TLR signaling pathway. These data, combined with the known role of miR-146a in regulating bacterially and virally induced inflammation, indicate that miR-146a may represent a potential therapeutic for reducing both microbial and mechanically induced inflammation during ventilation. Finally, the identification of the TLR signaling pathway as an important component of mechanotransduction in lung epithelia is a novel finding. Our data suggest that targeting this pathway even after the initial bacterial or viral infection has been cleared, might be an effective way to reduce lung inflammation and ventilation induced lung injury. FJ

This work was supported by U.S. National Science Foundation CAREER grant 0852417 (S.N.G.), U.S. National Institutes of Health grant CA150297-01(S.P.N.) and an American Heart Association postdoctoral fellowship to Y.H. The authors thank the Nucleic Acid Shared Resource (NASR) at the Ohio State University Comprehensive Cancer Center for providing access to the Nanostring nCounter System, and Dr. Carlo Croce for critically reading and reviewing the manuscript.

REFERENCES

1. Meltzer, S. J., and Welch, W. H. (1885) The behaviour of the red blood-corpuscles when shaken with indifferent substances. *J. Physiol.* **5,** 255–260
2. Meltzer, S. J., and Auer, J. (1909) Continuous respiration without respiratory movements. *J. Exp. Med.* **11,** 622–625
3. Tschumperlin, D. J., Boudreault, F., and Liu, F. (2010) Recent advances and new opportunities in lung mechanobiology. *J. Biomech.* **43,** 99–107
4. Orr, A. W., Helmke, B. P., Blackman, B. R., and Schwartz, M. A. (2006) Mechanisms of mechanotransduction. *Dev. Cell.* **10,** 11–20

5. Notter, R. H., Finkelstein, J. N., and Holm, B. A., eds. (2005) *Lung Injury: Mechanisms, Pathophysiology, and Therapy. Lung Biology in Health and Disease, Vol. 196*, Taylor & Francis Group, Boca Raton, FL, USA
6. Plataki, M., and Hubmayr, R. D. (2010) The physical basis of ventilator-induced lung injury. *Expert Rev. Respir. Med.* **4,** 373–385
7. Lionetti, V., Recchia, F. A., and Ranieri, V. M. (2005) Overview of ventilator-induced lung injury mechanisms. *Curr. Opin. Crit. Care.* **11,** 82–86
8. Slutsky, A. S. (2005) Ventilator-induced lung injury: from barotrauma to biotrauma. *Respir. Care* **50,** 646–659
9. Ghadiali, S. N., and Gaver, D. P. (2008) Biomechanics of liquid-epithelium interactions in pulmonary airways. *Respir. Physiol. Neurobiol.* **163,** 232–243
10. Yalcin, H. C., Perry, S. F., and Ghadiali, S. N. (2007) Influence of airway diameter and cell confluence on epithelial cell injury in an in vitro model of airway reopening. *J. Appl. Physiol.* **103,** 1796–1807
11. Cohen, T. S., Gray Lawrence, G., Khasgiwala, A., and Margulies, S. S. (2010) MAPK activation modulates permeability of isolated rat alveolar epithelial cell monolayers following cyclic stretch. *PLoS One* **5,** e10385
12. D'Angelo, E., Koutsoukou, A., Della Valle, P., Gentile, G., and Pecchiari, M. (2008) Cytokine release, small airway injury, and parenchymal damage during mechanical ventilation in normal open-chest rats. *J. Appl. Physiol.* **104,** 41–49
13. Jafari, B., Ouyang, B., Li, L. F., Hales, C. A., and Quinn, D. A. (2004) Intracellular glutathione in stretch-induced cytokine release from alveolar type-2 like cells. *Respirology* **9,** 43–53
14. Vlahakis, N. E., Schroeder, M. A., Limper, A. H., and Hubmayr, R. D. (1999) Stretch induces cytokine release by alveolar epithelial cells in vitro. *Am. J. Physiol.* **277,** L167–173
15. Huang, Y., Haas, C., and Ghadiali, S. (2010) Influence of transmural pressure and cytoskeletal structure on NF-B activation in respiratory epithelial cells. *Cell. Mol. Bioeng.* **3,** 415–427
16. Jaecklin, T., Engelberts, D., Otulakowski, G., O'Brodovich, H., Post, M., and Kavanagh, B. P. (2011) Lung-derived soluble mediators are pathogenic in ventilator-induced lung injury. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **300,** L648–L658
17. Halbertsma, F. J., Vaneker, M., Scheffer, G. J., and van der Hoeven, J. G. (2005) Cytokines and biotrauma in ventilator-induced lung injury: a critical review of the literature. *Neth. J. Med.* **63,** 382–392
18. Olsen, S. M., Stover, J. D., and Nagatomi, J. (2011) Examining the role of mechanosensitive ion channels in pressure mechanotransduction in rat bladder urothelial cells. *Ann. Biomed. Eng.* **39,** 688–697
19. Kojic, N., Chung, E., Kho, A. T., Park, J. A., Huang, A., So, P. T., and Tschumperlin, D. J. (2010) An EGFR autocrine loop encodes a slow-reacting but dominant mode of mechanotransduction in a polarized epithelium. *FASEB J.* **24,** 1604–1615
20. Kojic, N., Huang, A., Chung, E., Ivanovic, M., Filipovic, N., Kojic, M., and Tschumperlin, D. J. (2010) A 3-D model of ligand transport in a deforming extracellular space. *Biophys. J.* **99,** 3517–3525
21. Schwartz, M. A. (2010) Integrins and extracellular matrix in mechanotransduction. *Cold Spring Harb. Perspect. Biol.* **2,** a005066
22. Antonov, A. S., Antonova, G. N., Munn, D. H., Mivechi, N., Lucas, R., Catravas, J. D., and Verin, A. D. (2011) alphaVbeta3 integrin regulates macrophage inflammatory responses via PI3 kinase/Akt-dependent NF-kappaB activation. *J. Cell. Physiol.* **226,** 469–476
23. Liu, Y. Y., Liao, S. K., Huang, C. C., Tsai, Y. H., Quinn, D. A., and Li, L. F. (2009) Role for nuclear factor-kappaB in augmented lung injury because of interaction between hyperoxia and high stretch ventilation. *Transl. Res.* **154,** 228–240
24. Li, H., Su, X., Yan, X., Wasserloos, K., Chao, W., Kaynar, A. M., Liu, Z. Q., Leikauf, G. D., Pitt, B. R., and Zhang, L. M. (2010) Toll-like receptor 4-myeloid differentiation factor 88 signaling contributes to ventilator-induced lung injury in mice. *Anesthesiology* **113,** 619–629
25. Taft, R. J., Pang, K. C., Mercer, T. R., Dinger, M., and Mattick, J. S. (2010) Non-coding RNAs: regulators of disease. *J. Pathol.* **220,** 126–139
26. Urbich, C., Kuehbacher, A., and Dimmeler, S. (2008) Role of microRNAs in vascular diseases, inflammation, and angiogenesis. *Cardiovasc. Res.* **79,** 581–588
27. Sonkoly, E., Stahle, M., and Pivarcsi, A. (2008) MicroRNAs and immunity: novel players in the regulation of normal immune function and inflammation. *Semin. Cancer Biol.* **18,** 131–140
28. Zhou, T., Garcia, J. G., and Zhang, W. (2011) Integrating microRNAs into a system biology approach to acute lung injury. *Transl. Res.* **157,** 180–190
29. Ceppi, M., Pereira, P. M., Dunand-Sauthier, I., Barras, E., Reith, W., Santos, M. A., and Pierre, P. (2009) MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 2735–2740
30. Pauley, K. M., Satoh, M., Chan, A. L., Bubb, M. R., Reeves, W. H., and Chan, E. K. (2008) Upregulated miR-146a expression in peripheral blood mononuclear cells from rheumatoid arthritis patients. *Arthritis Res. Ther.* **10,** R101
31. Perry, M. M., Williams, A. E., Tsitsiou, E., Larner-Svensson, H. M., and Lindsay, M. A. (2009) Divergent intracellular pathways regulate interleukin-1beta-induced miR-146a and miR-146b expression and chemokine release in human alveolar epithelial cells. *FEBS Lett.* **583,** 3349–3355
32. Moschos, S. A., Williams, A. E., Perry, M. M., Birrell, M. A., Belvisi, M. G., and Lindsay, M. A. (2007) Expression profiling in vivo demonstrates rapid changes in lung microRNA levels following lipopolysaccharide-induced inflammation but not in the anti-inflammatory action of glucocorticoids. *BMC Genomics* **8,** 240
33. Wang, K. C., Garmire, L. X., Young, A., Nguyen, P., Trinh, A., Subramaniam, S., Wang, N., Shyy, J. Y., Li, Y. S., and Chien, S. (2010) Role of microRNA-23b in flow-regulation of Rb phosphorylation and endothelial cell growth. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 3234–3239
34. Weber, M., Baker, M. B., Moore, J. P., and Searles, C. D. (2010) MiR-21 is induced in endothelial cells by shear stress and modulates apoptosis and eNOS activity. *Biochem. Biophys. Res. Commun.* **393,** 643–648
35. Brower, R. G., Lanken, P. N., MacIntyre, N., Matthay, M. A., Morris, A., Ancukiewicz, M., Schoenfeld, D., and Thompson, B. T. (2004) Higher versus lower positive end-expiratory pressures in patients with the acute respiratory distress syndrome. *N. Engl. J. Med.* **351,** 327–336
36. Suarez, Y., Wang, C., Manes, T. D., and Pober, J. S. (2010) Cutting edge: TNF-induced microRNAs regulate TNF-induced expression of E-selectin and intercellular adhesion molecule-1 on human endothelial cells: feedback control of inflammation. *J. Immunol.* **184,** 21–25
37. Zhou, J., Wang, K. C., Wu, W., Subramaniam, S., Shyy, J. Y., Chiu, J. J., Li, J. Y., and Chien, S. (2011) MicroRNA-21 targets peroxisome proliferators-activated receptor-α in an autoregulatory loop to modulate flow-induced endothelial inflammation. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 10355–10360
38. Bhaumik, D., Scott, G. K., Schokrpur, S., Patil, C. K., Orjalo, A. V., Rodier, F., Lithgow, G. J., and Campisi, J. (2009) MicroRNAs miR-146a/b negatively modulate the senescence-associated inflammatory mediators IL-6 and IL-8. *Aging (Albany, NY)* **1,** 402–411
39. Qin, X., Wang, X., Wang, Y., Tang, Z., Cui, Q., Xi, J., Li, Y. S., Chien, S., and Wang, N. (2010) MicroRNA-19a mediates the suppressive effect of laminar flow on cyclin D1 expression in human umbilical vein endothelial cells. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 3240–3244
40. Perry, M. M., Moschos, S. A., Williams, A. E., Shepherd, N. J., Larner-Svensson, H. M., and Lindsay, M. A. (2008) Rapid changes in microRNA-146a expression negatively regulate the IL-1beta-induced inflammatory response in human lung alveolar epithelial cells. *J. Immunol.* **180,** 5689–5698
41. Taganov, K. D., Boldin, M. P., Chang, K. J., and Baltimore, D. (2006) NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 12481–12486
42. Fakler, C. R., Wu, B., McMicken, H. W., Geske, R. S., and Welty, S. E. (2000) Molecular mechanisms of lipopolysaccharide induced ICAM-1 expression in A549 cells. *Inflamm. Res.* **49,** 63–72
43. Ni, C. W., Qiu, H., and Jo, H. (2011) MicroRNA-663 upregulated by oscillatory shear stress plays a role in inflammatory

response of endothelial cells. *Am. J. Physiol. Heart Circ. Physiol.* **300,** H1762–H1769
44. Mohamed, J. S., Lopez, M. A., and Boriek, A. M. (2010) Mechanical stretch up-regulates microRNA-26a and induces human airway smooth muscle hypertrophy by suppressing glycogen synthase kinase-3beta. *J. Biol. Chem.* **285,** 29336–29347
45. Guan, Y. J., Yang, X., Wei, L., and Chen, Q. (2011) MiR-365: a mechanosensitive microRNA stimulates chondrocyte differentiation through targeting histone deacetylase 4. *FASEB J.* **25,** 4457–4466
46. Luna, C., Li, G., Qiu, J., Epstein, D. L., and Gonzalez, P. (2009) Role of miR-29b on the regulation of the extracellular matrix in human trabecular meshwork cells under chronic oxidative stress. *Mol. Vis.* **15,** 2488–2497
47. Thomas, M., Lange-Grunweller, K., Weirauch, U., Gutsch, D., Aigner, A., Grunweller, A., and Hartmann, R. K. (2011) The proto-oncogene Pim-1 is a target of miR-33a. *Oncogene* **31,** 918–928
48. Nihira, K., Ando, Y., Yamaguchi, T., Kagami, Y., Miki, Y., and Yoshida, K. (2010) Pim-1 controls NF-kappaB signalling by stabilizing RelA/p65. *Cell Death Differ.* **17,** 689–698
49. Li, L., Chen, X. P., and Li, Y. J. (2010) MicroRNA-146a and human disease. *Scand. J. Immunol.* **71,** 227–231
50. Bhaumik, D., Scott, G. K., Schokrpur, S., Patil, C. K., Campisi, J., and Benz, C. C. (2008) Expression of microRNA-146 suppresses NF-kappaB activity with reduction of metastatic potential in breast cancer cells. *Oncogene* **27,** 5643–5647
51. Ghose, J., Sinha, M., Das, E., Jana, N. R., and Bhattacharyya, N. P. (2011) Regulation of miR-146a by RelA/NFkB and p53 in STHdh(Q111)/Hdh(Q111) cells, a cell model of Huntington's disease. *PLoS One* **6,** e23837
52. Sun, S. G., Zheng, B., Han, M., Fang, X. M., Li, H. X., Miao, S. B., Su, M., Han, Y., Shi, H. J., and Wen, J. K. (2011) miR-146a and Kruppel-like factor 4 form a feedback loop to participate in vascular smooth muscle cell proliferation. *EMBO Rep.* **12,** 56–62
53. Curtale, G., Citarella, F., Carissimi, C., Goldoni, M., Carucci, N., Fulci, V., Franceschini, D., Meloni, F., Barnaba, V., and Macino, G. (2010) An emerging player in the adaptive immune response: microRNA-146a is a modulator of IL-2 expression and activation-induced cell death in T lymphocytes. *Blood* **115,** 265–273
54. Guillot, L., Medjane, S., Le-Barillec, K., Balloy, V., Danel, C., Chignard, M., and Si-Tahar, M. (2004) Response of human pulmonary epithelial cells to lipopolysaccharide involves Toll-like receptor 4 (TLR4)-dependent signaling pathways: evidence for an intracellular compartmentalization of TLR4. *J. Biol. Chem.* **279,** 2712–2718
55. Charles, P. E., Tissieres, P., Barbar, S. D., Croisier, D., Dufour, J., Dunn-Siegrist, I., Chavanet, P., and Pugin, J. (2011) Mild-stretch mechanical ventilation up-regulates toll-like receptor 2 and sensitizes the lung to bacterial lipopeptide. *Crit. Care* **15,** R181
56. Vaneker, M., Joosten, L. A., Heunks, L. M., Snijdelaar, D. G., Halbertsma, F. J., van Egmond, J., Netea, M. G., van der Hoeven, J. G., and Scheffer, G. J. (2008) Low-tidal-volume mechanical ventilation induces a toll-like receptor 4-dependent inflammatory response in healthy mice. *Anesthesiology* **109,** 465–472

Received for publication November 4, 2011.
Accepted for publication May 1, 2012.