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Motile cilia harbor serum response factor as a mechanism of environment sensing and injury response in the airway

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[1]*VA Nebraska-Western Iowa Healthcare System, Research Service, Omaha, Nebraska;* [2]*Pulmonary, Critical Care, Sleep & Allergy Division of the Department of Internal Medicine, University of Nebraska Medical Center, Omaha, Nebraska; and* [3]*Department of Environmental, Agricultural, and Occupational Health, University of Nebraska Medical Center, Omaha, Nebraska*

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Nordgren TM, Wyatt TA, Sweeter J, Bailey KL, Poole JA, Heires AJ, Sisson JH, Romberger DJ. Motile cilia harbor serum response factor as a mechanism of environment sensing and injury response in the airway. *Am J Physiol Lung Cell Mol Physiol* 306: L829–L839, 2014. First published March 7, 2014; doi:10.1152/ajplung.00364.2013.—Nonmotile primary cilia are recognized as important sensory organelles during development and normal biological functioning. For example, recent work demonstrates that transcriptional regulators of the sonic hedgehog signaling pathway localize to primary cilia and participate in sensing and transducing signals regarding the cellular environment. In contrast, motile cilia are traditionally viewed as mechanical machinery, vital for the movement of solutes and clearance of bacteria and debris, but not participants in cellular sensing and signaling mechanisms. Recently, motile cilia were found to harbor receptors responsible for sensing and responding to environmental stimuli. However, no transcription factors are known to be regulated by cilia localization as a sensing mechanism in vertebrates. Using a mouse model of organic dust-induced airway inflammation, we found that the transcription factor serum response factor (SRF) localizes to motile cilia of airway epithelial cells and alters its localization in response to inflammatory stimuli. Furthermore, inhibition of SRF signaling using the small molecule CCG-1423 reduces organic dust-induced IL-8 release from bronchial epithelial cells and stimulates cilia beat frequency in ciliated mouse tracheal epithelial cells. Immunohistochemical analyses reveal that SRF localizes to the cilia of mouse brain ependymal and ovarian epithelial cells as well. These data reveal a novel mechanism by which a transcription factor localizes to motile cilia and modulates cell activities including cilia motility and inflammation response. These data challenge current dogma regarding motile cilia functioning and may lead to significant contributions in understanding motile ciliary signaling dynamics, as well as mechanisms involving SRF-mediated responses to inflammation and injury.

serum response factor; motile cilia; airway inflammation; organic dust

THE ROLE OF PRIMARY CILIA in acting as sensory organelles is well established (37). One mechanism by which primary nonmotile cilia appear to sense and transduce signals from the extracellular milieu is by harboring transcription factors along the ciliary axoneme. Specifically, sonic hedgehog (Shh) transcription factors Gli2 and Gli3 have been shown to localize to primary cilia and play a role in the regulation and transduction of Shh signaling during development and proper biological functioning (19, 41). Despite the well-appreciated role of primary cilia in modulating cell sensing and signaling, there is no clear evidence for a similar role for transcription factor regulation in motile cilia, which are traditionally considered to work as mechanical machines responsible for the movement of solutes, debris, and/or pathogens out of the environment (5, 24, 40). However, recent studies have revealed a surprising compilation of unexpected receptors on motile cilia, as well as evidence that motile cilia do indeed act as sensory organelles by harboring mechanoreceptive and chemoreceptive capacities, as reviewed by Bloodgood in 2010 (6). These sensory functions include the localization of bitter receptors on ciliated airway epithelium that regulate sensing and expelling of noxious substances in the milieu, and the expression of progesterone receptors on cilia in oviduct epithelium that assist in sensing and modulating cilia beat frequency (CBF) during the estrous cycle (8, 36, 40, 43). Furthermore, the Smoothened, FGFR1, and VANGL2 membrane protein receptors have been found to be localized to motile cilia and play roles in development in various different model systems (16, 31, 42). These data reveal that motile cilia do sense and respond to their environment. However, a direct connection between motile cilia function and regulation of cell signal transduction via transcription factor harboring has not been established.

Serum response factor (SRF) is a highly conserved transcription factor involved in a variety of cellular responses. SRF is vital for embryogenesis, in which mesoderm development is inhibited by SRF knockout (2). Furthermore, conditional cardiac-specific knockout of SRF leads to lack of cardiogenesis in developing embryos, whereas cardiac-specific overexpression of SRF in developed mice results in hypertrophic cardiomyopathy (25, 48). These observations suggest a dynamic role for SRF during cellular growth, maturation, and functioning in which SRF expression must be tightly regulated for appropriate cellular performance.

In addition to the role of SRF in development, this transcription factor is activated by a variety of stimuli, including serum, antioxidants, lipopolysaccharide, various proinflammatory cytokines, and viral-related proteins. Upon stimulation, SRF activates a variety of early-response genes including c-*fos*, *IL-2Rα*, and *HSP70*, among many others (12). These actions suggest that SRF plays a role in tissue injury response. Indeed, in vivo investigations focusing on ulcerative injuries reveal an important role for SRF in the reepithelialization of gastrointestinal tissue following injury, when the expression of SRF led to the formation of myofibroblasts from esophageal epithelial cells and fibroblasts residing around the injured tissues, allowing for tissue healing to occur (10, 11). Taken together, these data indicate that increased expression and activation of SRF may be important to tissue repair following injury; however,

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persistent or aberrantly augmented expression and activation of SRF could be ultimately detrimental to the tissue.

In the lung, SRF can be activated by TNF-α and TGF-β signaling. This activation leads to the development of myofibroblasts responsible for wound healing in response to lung tissue injury but is also associated with lung fibrosis (32, 33, 47). Previous studies from our laboratory have shown in vivo that inhalant exposures to noninfectious organic dust extracts (DE) derived from animal confinement facilities result in robust airway neutrophil influx and proinflammatory cytokine release (29). In vitro, we have shown that SRF signaling activation likely plays a role in this proinflammatory response, as shown by increased activation of SRF signaling correlating with proinflammatory cytokine production in submerged cultures of bronchial epithelial cells (BECs) (26). In addition, we have shown that BEC ciliary beating is modified by DE (46). Thus, to determine whether SRF signaling plays a role in these proinflammatory responses, particularly those mediated by the ciliated BECs, we examined SRF expression in those cell types, using human, bovine, and murine bronchial/tracheal epithelial cells. Furthermore, we assessed changes in SRF expression following airway inflammatory injury caused by single exposure of C57BL/6 mice to intranasal DE instillations. Additionally, inhibition of Rho kinase-related SRF signaling with CCG-1423 in mouse tracheal epithelial cells grown on air-liquid interface (MTEC-ALI) was performed to examine changes in CBF, and the effects of CCG-1423 on DE-induced proinflammatory IL-8 release in submerged cultures of bronchial epithelial cells (Beas-2B) was assessed.

Together, these experiments revealed previously uncharacterized localization of SRF to the motile cilia of bronchial and tracheal epithelial cells, where changes in localization correspond to different stages in lung inflammation and injury. Furthermore, when we assessed SRF expression in the ciliated ependymal cells of the brain and ciliated epithelium of the oviduct, SRF localized to the cilia of these cells as well, indicating that the location of this transcription factor is a mechanism for sensing and signaling transduction in motile cilia from disparate tissues. These data indicate that motile cilia act not only as mechanical machinery but also as sensing organelles harboring transcription factors that play an important role in ciliated cell signal transduction.

MATERIALS AND METHODS

Materials. Antibodies against SRF were obtained from Santa Cruz Biotechnology (G20 COOH-terminal peptide, polyclonal; Santa Cruz, CA) and Abcam (mono- and polyclonal; Cambridge, MA). The G20 blocking peptide for the Santa Cruz G20 SRF antibody was obtained from Santa Cruz Biotechnology. The monoclonal antibody against acetylated tubulin was obtained from Sigma-Aldrich (St. Louis, MO). The polyclonal antibody against tight junction protein 1 (ZO-1) was obtained from Invitrogen (Eugene, OR). The monoclonal antibody against tata-binding protein (TBP) was obtained from Abcam. Horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology. Alexa Fluor-488-, Alexa Fluor-594-, and Alexa Fluor-647-conjugated secondary antibodies were obtained from Life Technologies/Invitrogen (Eugene, OR). CCG-1423 (*N*-[2-[4(4-chlorophenyl)amino]-1-methyl-2-oxoethoxy]-3,5-bis(trifluoromethyl)-benzamide) was obtained through Cayman Chemical (Ann Arbor, MI). The Beas-2B cell line was purchased from American Type Culture Collection (Manassas, VA).

Animal care and housing. Six- to 8-wk-old male C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Feeding and care were overseen by University of Nebraska Medical Center Animal Care Facilities, and all experiments were regulated and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee, as well as that of the Omaha Veterans Affairs Medical Center, which follows NIH guidelines for the use of rodents.

Preparation of organic dust extract. The inflammatory stimulus, organic DE was prepared as previously described (30). Briefly, settled surface dust was obtained from local swine confinement facilities and placed in Hanks' balanced salt solution (Biofluids; Rockville, MD) at a concentration of 1 g of dust per 10 ml of solution. Following a 1-h incubation, solution was vortexed and centrifuged two times to eliminate large sediments. The final supernate was sterile filtered by use of a 0.2 μM filter (Nalgene; Rochester, NY), and stock DE was aliquoted and frozen at −80°C for use in future experiments. For use in the experiments, stock DE was diluted (vol/vol) in sterile phosphate-buffered saline [PBS; pH 7.4 (diluent)]. Prior work to understand the components within these extracts have been previously published (27), and these previous studies demonstrated by gas chromatography-tandem mass spectrometry the presence of a high concentration of muramic acid (component of peptidoglycan) and 3-hydroxy fatty acid (component of endotoxin) and a low concentration of ergosterol (component of fungi). In these studies, independent batches of DE were prepared and tested, but, because of the biological complexity of the samples, inherent variability in the samples was inevitable. The range of 100% stock DE samples contained 23–31 mg/ml of total protein as measured by NanoDrop spectrophotometry (NanoDrop Technologies, Wilmington, DE) and confirmed via a Bradford protein assay. Endotoxin levels in the stock DE samples ranged from 175–730 EU/ml when evaluated by a limulus amoebocyte lysate assay (according to manufacturer instructions; Sigma). Peptidoglycan levels were not quantitated, since commercially available measurement assays do not exist to the best of our knowledge. The DE is known to stimulate bronchial airway epithelial cells largely by activating TLR2 signaling, although other signaling mechanisms are indicated, including EGFR signaling, as previously described (3, 4, 13, 26, 28).

Animal exposure studies. Murine DE exposure studies were performed as previously described (29). Lightly sedated C57Bl/6 mice were intranasally treated with 50 μl of 12.5% DE or saline then euthanized 5 h following treatment. Lungs were inflated with formalin at a pressure of 20 cmH_2O for 24 h to allow for fixation and pulmonary architecture preservation, paraffin embedded, and sectioned for histological analysis.

Derivation of bovine trachea, bronchial epithelial cells, and axonemes. Bovine trachea and bronchial epithelial cells were derived as previously described (45). Briefly, cells were obtained from bronchi of bovine lung obtained from a local abattoir. Bronchi were incubated overnight in bacterial protease, then washed thoroughly to obtain the bronchial epithelial cells lining the lumen of the bronchi. Cells were then diluted in Dulbecco's modified Eagle's medium and cytospun onto slides with a Wescor Cytopro (Logan, UT). For preparation of bovine tracheal lysates, flash-frozen bovine tracheal tissues were homogenized in a Nonidet P-40-based cell lysis buffer. After 1 h incubation on ice with intermittent vortexing, lysates with centrifuged to pellet insoluble fractions, and supernates were used for Western blotting. For preparation of bovine ciliary axonemes, a previously developed and validated method was followed (18, 44). Trachea from bovine lungs were washed and vigorously shaken in detergent extraction buffer to remove the axonemes from the ciliated epithelial cells. This method isolates axonemes from the ciliary membranes and soluble cilia matrix. Axoneme solution was filtered and centrifuged. The pelleted axonemes were resuspended, aliquoted, and stored at −80°C for future experiments.

Derivation of mouse tracheal epithelial cells and air-liquid interface model. Mouse tracheal epithelial cells were isolated and grown on air-liquid interface as previously described (14, 23). Briefly, trachea from euthanized C57Bl6 or BALB/c mice were incubated in media with Pronase to dislodge the tracheal epithelial cells. Cells were grown in supplemented medium on Costar membrane inserts (Corning Life Sciences, Corning, NY). Upon reaching confluency, medium was removed from the top chamber and the cells were allowed to grow at the air-liquid interface. When cultures became ciliated, cells were scraped from the membranes and cytocentrifuged onto slides in a Wescor Cytopro for use in immunohistochemical analyses.

Derivation of human lung tissue samples. The institutional review board at the University of Nebraska Medical Center approved the studies using human tissue (no. 318-09-NH). The lung tissues used in these studies were obtained through the International Institute for the Advancement of Medicine from organ donors that did not meet the requirements for lung transplant donation. The tissues were received on ice in either University of Wisconsin solution or histidine-tryptophan-ketoglutarate solution 12–24 h after brain death. Tracheal biopsies were immediately taken on arrival and placed in paraformaldehyde, paraffin embedded, and sectioned. The donors used in this study were *1*) a 26-yr-old female who died of a cerebrovascular accident, *2*) a 57-yr-old male that died of a cerebrovascular accident and *3*) a 72-yr-old male that died of an intracranial hemorrhage. None of the donors had a history of lung disease or smoking.

Immunohistochemistry and immunofluorescence. Immunohistochemistry/immunofluorescence procedures were performed similarly as previously described (29). For paraffin-embedded mouse lung tissue, specimens were cleared with Protocol Safeclear II (Fisher Scientific, Kalamazoo, MI) and re-hydrated through a decreasing series of ethanol concentrations. For frozen sectioned brain tissue samples, tissues were incubated with cold acetone for 5 min, then rinsed. For cell cytospins, cells were fixed for 10 min in 4% paraformaldehyde. Antigen retrieval for paraffin-embedded sections and cytospins was performed using 2 N HCl. Slides were blocked in milk solution then incubated for 2 h at room temperature, or overnight at 4°C in a humidified chamber, by using 1:500 polyclonal or 1:200 monoclonal antibodies against SRF and 1:250 primary antibody against acetylated tubulin. Immunohistochemistry of SRF shown in figures utilized the Abcam rabbit polyclonal Ab against SRF, except for Fig. 2, *A–C* and Fig. 3*B*, which utilized the Santa Cruz G20 rabbit polyclonal Ab against SRF. Slides were washed in PBS-Tween then incubated in a humidified chamber for 1 h in HRP-conjugated secondary antibodies or Alexa Fluor 488/594/647-conjugated secondary antibodies. Slides were washed in PBS and for HRP/immunohistochemistry developing, and antibody binding was detected by using the Chromagen substrate Developer ImmPACT DAB kit (Vector Labs, Burlingame, CA). Slides were counterstained in hematoxylin and blued with 0.1% sodium bicarbonate. Lung tissue sections were dehydrated and cleared before mounting and coverslipping. Slides were viewed by bright-field microscopy. For confocal microscopy, slides were washed in PBS following secondary incubation and coverslipped with Prolong Gold anti-fade reagent with DAPI (Invitrogen). Slides were viewed on a Zeiss LSM 510 meta laser scanning confocal microscope (Thornwood, NY). *Z*-stack analysis and compression of confocal images were performed with the Zeiss LSM Zen lite image browser software. The Fiji platform for image analysis was utilized to assess colocalization (35), by using the coloc 2 colocalization analysis and selecting for airway epithelium as a regions of interest for analyses.

Western blotting. Western blotting was performed with isolated demembranated bovine axoneme protein preparations or bovine tracheal lysates. Proteins were diluted in sample buffer containing β-mercaptoethanol and heated for 5 min prior to loading into a 4–20% acrylamide gradient Ready-Gel (Bio-Rad, Hercules, CA). Samples were separated at 100 V for 2 h then transferred onto a nitrocellulose membrane. Membranes were blocked for 1 h in milk solution, then incubated for 2 h at room temperature, or overnight at 4°C in 1:1,000 monoclonal antibody against SRF, 1:2,000 polyclonal Abcam antibody against SRF, 1:500 Santa Cruz G20 antibody against SRF, 1:2,000 polyclonal antibody to ZO-1, 1:2,000 monoclonal antibody to TBP, or 1:2,000 monoclonal antibody to acetylated tubulin. In antibody-blocking experiments, Santa Cruz G20 blocking peptide was preincubated for 30 min at a 1:250 concentration with the Santa Cruz G20 antibody prior to use, according to manufacturer's directions. Membranes were washed in PBS-Tween then incubated for 1 h at room temperature in 1:5,000 to 1:7,500 HRP-conjugated secondary antibody. After washing again, antibody binding was determined by use of SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL). Signal was developed via a LI-COR imaging system (Lincoln, NE).

CCG-1423-mediated inhibition of Rho kinase-related SRF signaling. For Beas-2B culture studies, cells were grown in serum-free LHC-9:RPMI as submerged, unciliated cultures, as previously described (26). Cells were pretreated for 1 h with 0 or 100 nM CCG-1423, followed by 5% DE for 24 h. Supernates were then collected and IL-8 levels analyzed via enzyme-linked immunosorbent assays (ELISAs), as previously described (30). ELISAs were performed three separate times (biological replicates) with two technical replicates performed for each experiment. Data were compiled to determine the means and SE.

For CBF measurements, MTEC-ALI cultures were treated with 0, 1, 10, or 100 nM CCG-1423 on the apical surface of the cultures in 30 μl fluid, and CBF measurements were taken hourly for 7 h by using the Sisson-Ammons Video Analysis software (SAVA) as previously described (38). CBF measurements were performed three separate times (biological replicates) with three technical replicates performed for each experiment and a minimum of six readings taken in separate well regions for each recording. Data were compiled to determine the means and SE.

Statistical methods. Significance was determined by the ANOVA method with Tukey's method for post hoc multiple comparisons (CBF and IL-8 data analyses), or Student's unpaired, two-tailed *t*-test method (colocalization/Pearson's correlation coefficient analyses). A significant result was defined as a difference resulting in a P value of less than 0.05. All statistical analyses were performed with GraphPad Prism Software.

RESULTS

SRF localizes to the cilia in bronchial/tracheal epithelial cells. To investigate the function and localization of SRF in ciliated BECs, we utilized three different model systems to assess the expression of SRF in these cell types. Specifically, we performed immunohistochemical staining to determine the presence and expression pattern of SRF in bovine BECs (BBECs), mouse tracheal epithelial cells grown on air-liquid interface (MTEC-ALI), and human lung tissue samples. Immunohistochemistry was performed on cytospins of BBECs and MTEC-ALI or slides of paraffin-embedded human lung tissue to stain for SRF expression by using three different commercially available antibodies against SRF (two polyclonal, one monoclonal). All samples revealed expression of SRF in the airway epithelial cells, with robust expression localizing to the cilia of the epithelial cells (Fig. 1, *A–C*). This localization has to our knowledge never before been identified in ciliated cells. In addition, confocal fluorescence microscopy of human lung tissue revealed colocalization of SRF with acetylated tubulin, confirming the localization of SRF to the cilia (Fig. 1, *D* and *E*). To further verify the localization of SRF to this cellular region was not artifact, detergent-isolated demembranated bovine axoneme preparations were probed by Western blotting for SRF using two different polyclonal antibodies. A single protein band of ~67 kDa corresponding to the SRF protein was detected in the axoneme preparations (Fig. 3*A*), confirming the localization of SRF to the cilia. In addition, when a blocking peptide to the G20 anti-SRF Santa Cruz antibody was preincu-

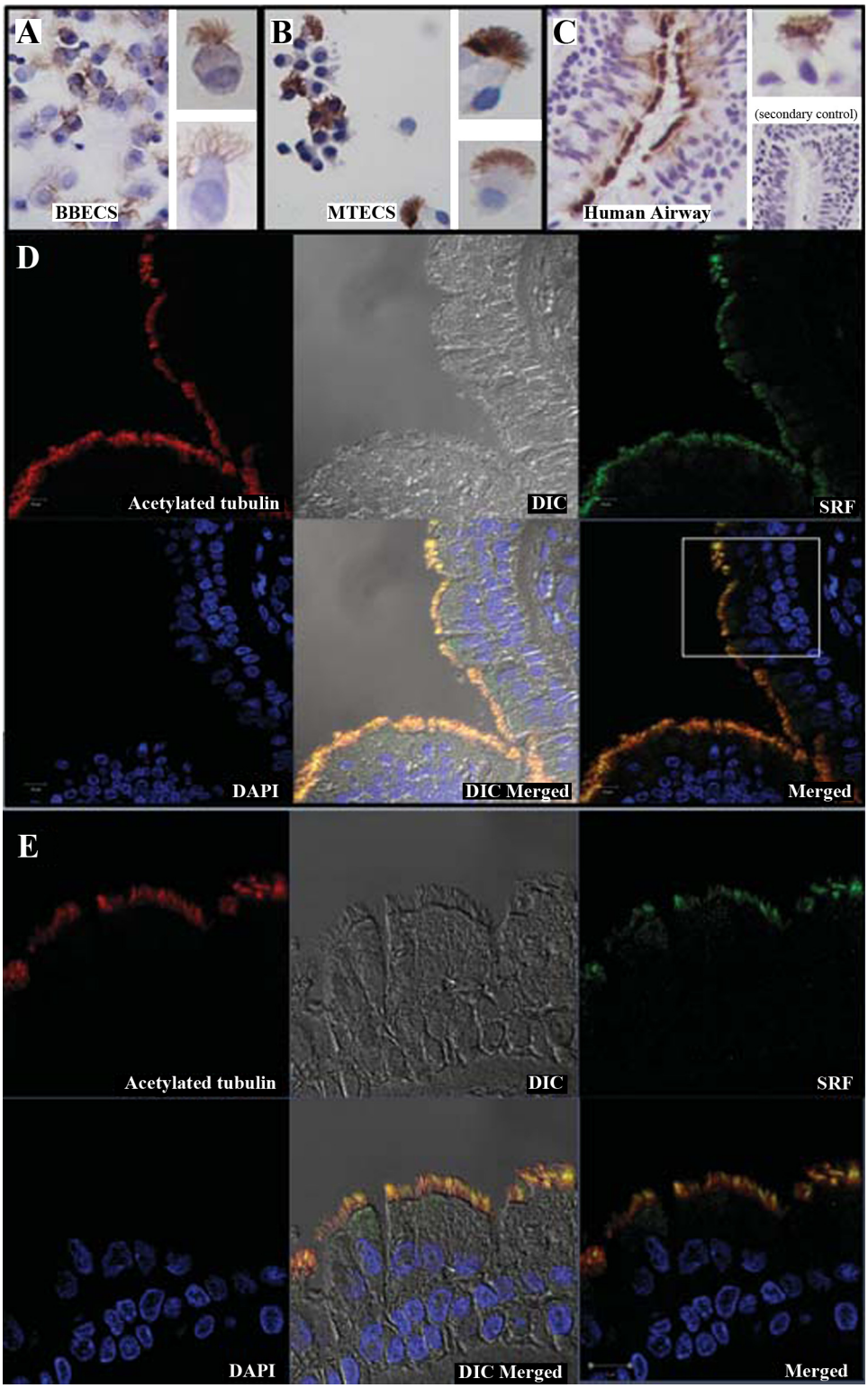

Fig. 1. Expression and localization of serum response factor (SRF) in unstimulated ciliated airway epithelial cells. Cytospins of bovine bronchial epithelial cells (BBECs; *A*), mouse tracheal epithelial cells (MTECs; *B*), and paraffin-embedded and sectioned human lung tissue (*C*) were utilized in immunohistochemical staining to assess SRF protein expression and localization in ciliated airway epithelium. For *A–C*, images on left are at ×400, with magnified images to the right to illustrate staining (and secondary control *inset* in *C*, as indicated). In *D*, paraffin-embedded and sectioned human lung tissue was analyzed via confocal microscopy for dual staining of SRF (green) and acetylated tubulin (red). DIC, differential interference contrast. The inset indicated by the box in the non-DIC Merged panel of *D* is zoomed in and rotated for greater clarity and magnification of SRF subcellular localization in *E*.

bated with the primary antibody, staining of ciliated epithelial cells via immunohistochemistry in serial mouse lung sections was completely abrogated (Fig. 2, *A–C*), further confirming SRF staining specificity. Probing of the isolated, demembranated bovine axoneme preparations with the G20 anti-SRF Santa Cruz antibody preincubated with the G20 blocking peptide also abrogated SRF detection (Fig. 3*B*). Furthermore, when the bovine axoneme preparations were centrifuged to pellet axonemes out of any cell or membrane debris in the supernate, then probed for SRF, only the pelleted fraction (and not the supernate containing any debris) was positive for SRF protein expression (Fig. 3*C*). Probing the bovine axoneme preparations for the cytoplasmic protein zonula adherens-1 (ZO-1) and the nuclear protein tata-binding protein (TBP) revealed no banding in the axoneme prep-

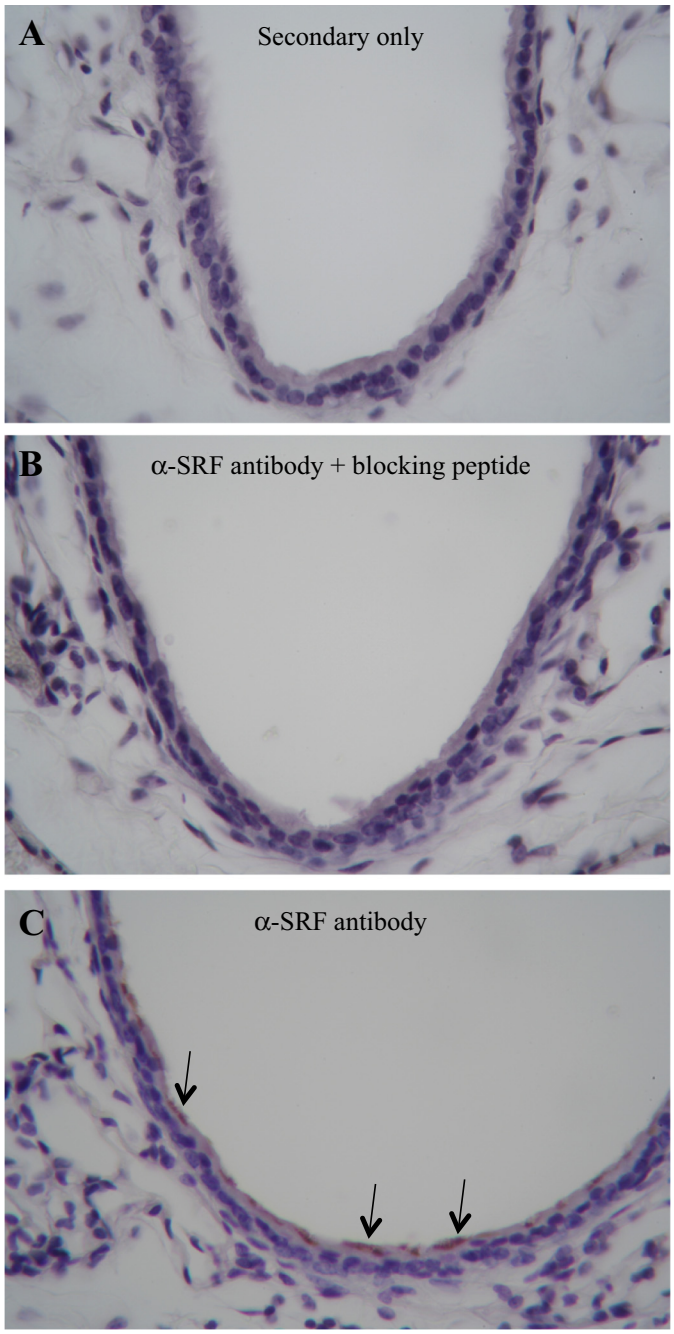

Fig. 2. Expression of SRF protein on the cilia of airway epithelial cells SRF staining in unstimulated ciliated airway epithelial cells of serial mouse lung sections is blocked when Santa Cruz G20 anti-SRF antibody is preincubated with G20 blocking peptide. *A*: secondary only control ×400. *B*: anti-SRF Ab + blocking peptide ×400. *C*: anti-SRF Ab ×400.

arations, while banding was evident in lysates of bovine trachea (Fig. 3*D*). To confirm proper loading of axoneme preparations, blots were also probed for acetylated tubulin, and axoneme preparations exhibited evident acetylated tubulin staining (Fig. 3*D*).

SRF localization changes during single exposures to DE. We hypothesized that a change in localization of SRF to the cilia occurs during DE-induced airway inflammation. To test this hypothesis we performed immunohistochemistry on lung tissues from C57BL/6 mice that were given a single DE instillation and then euthanized 5 h following the exposure. As seen in Fig. 4, *A–C*, after a single exposure to DE, SRF axonemal localization is decreased, implicating SRF modulation, and corroborating our previously published in vitro results indicating SRF activation in submerged BEC cultures follow-

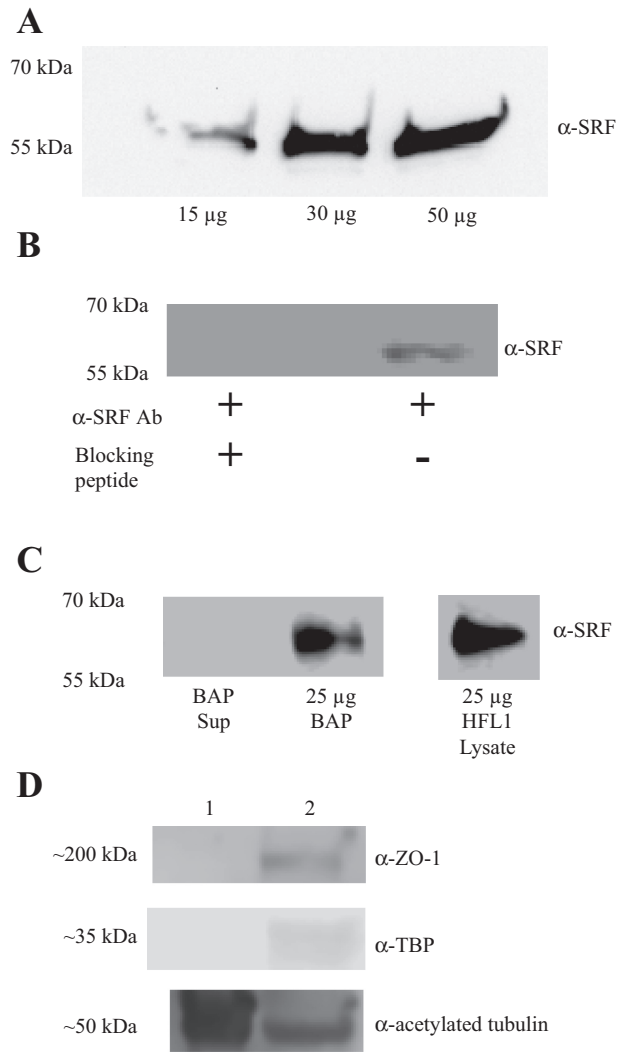

Fig. 3. Confirmation of SRF protein found in isolated, demembranated bovine axoneme preparations. *A*: load-dependent protein expression of SRF in isolated demembranated bovine axoneme preparations. Protein (15, 30, or 50 μg) was loaded in each lane from a prestandardized axoneme sample of 1 mg/ml. *B*: to confirm primary antibody specificity to SRF, 25 μg protein was loaded in each lane from a prestandardized isolated, demembranated bovine axoneme sample of 1 mg/ml. The blot was divided and treated with either 1:500 Santa Cruz G20 anti-SRF antibody or the G20 antibody preincubated for 30 min with the G20 blocking peptide. *C*: as a negative control against cell or membrane debris contaminants in the bovine axoneme preparations (BAP), detergent-isolated and demembranated bovine axoneme preparations were centrifuged to pellet the axonemes out of any debris. The axoneme pellet and the supernate (Sup) following centrifugation were run on a gel, transferred to a nitrocellulose membrane, and probed for SRF by using the Abcam antibody against SRF. On a separate gel, human lung fibroblast cell line (HFL1) lysates were also probed for SRF to confirm band/antibody specificity. *D*: as a control for cytoplasmic or nuclear protein contamination of bovine axoneme preparations, 15 μg of the bovine axoneme proteins (*lane 1*) or bovine tracheal lysates (*lane 2*) were run on a gel, transferred to a nitrocellulose membrane, and probed for the cytoplasmic protein zonula adherens-1 (ZO-1), the nuclear protein TATA binding protein (TBP), and the axonemal/microtubule-associated protein acetylated tubulin.

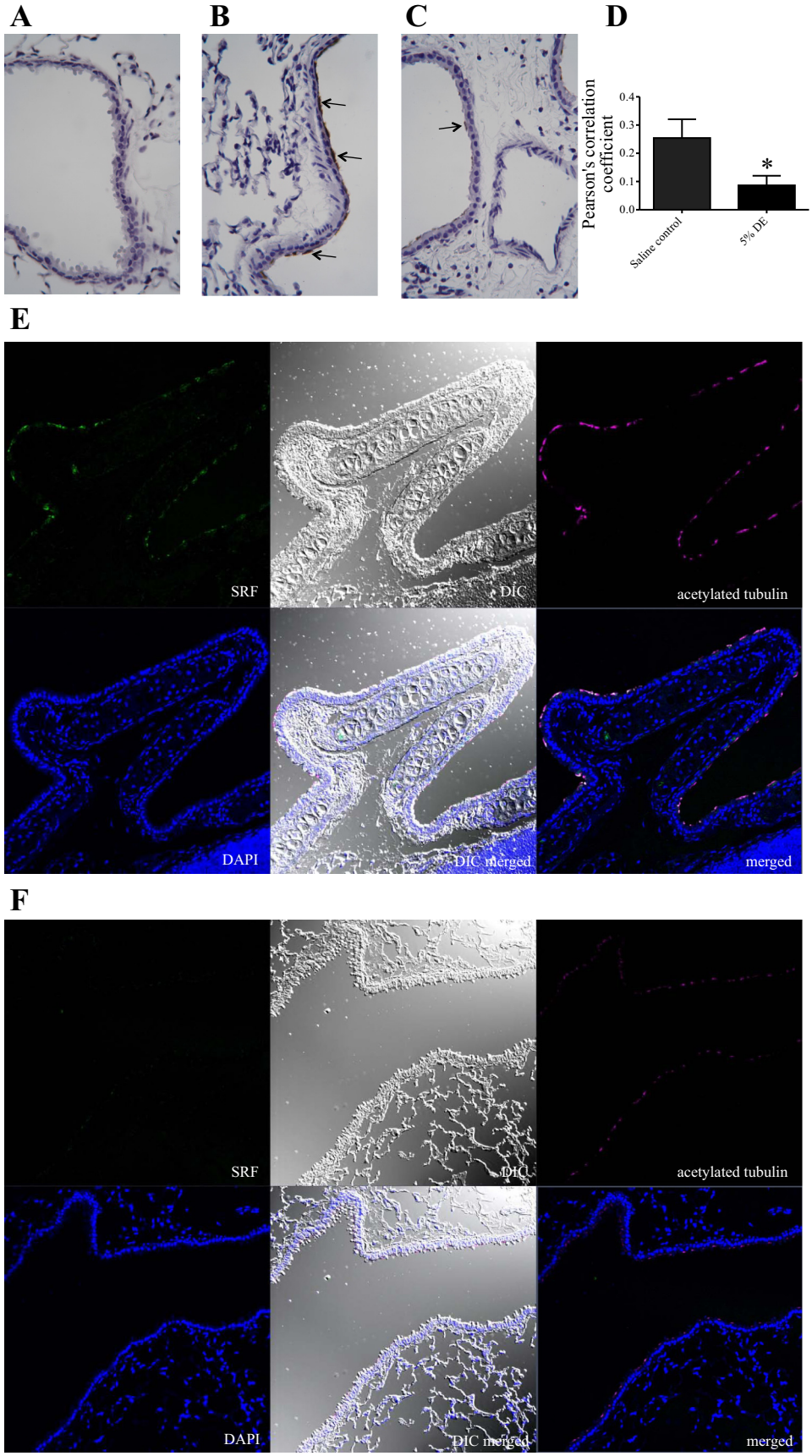

Fig. 4. Effect of inhalant dust extract (DE) on the expression and localization of SRF in airway ciliated epithelial cells in mice. Mice were intranasally treated with DE or saline. At 5 h following DE or saline treatment, mice were euthanized and lungs were inflated with formalin, paraffin embedded, and sectioned. Immunohistochemistry (*A–C*) and immunofluorescence staining (*E* and *F*) were performed to examine the expression and localization of SRF in the airway epithelium. *A*: secondary antibody control. *B*: ×400 view of SRF expression and localization in airway epithelium of saline-instilled mice. *C*: ×400 view of SRF expression and localization in airway epithelium of DE-instilled mice. *D*: Pearson's correlation coefficient indicating the degree of colocalization of SRF and acetylated tubulin in immunofluorescence-stained sections of the airway epithelium, based on analysis of a minimum of 10 airway epithelium sections from medium to large airways in 3 saline- or DE-instilled mice each. *E*: ×200 view of SRF expression (green) and colocalization with acetylated tubulin (magenta) in airway epithelium of saline-instilled mice. *F*: ×200 view of SRF expression (green) and colocalization with acetylated tubulin (magenta) in airway epithelium of DE-instilled mice. Data are represented as means ± SE. $*P < 0.05$ compared with saline control group, based on unpaired, 2-tailed Student's *t*-test.

ing DE stimulation (26). Furthermore, airway sections from saline- and DE-treated mice were assessed for SRF and acetylated tubulin colocalization by confocal microscopy. As shown in Fig. 4, *E* and *F*, acetylated tubulin staining (magenta) is present at the ciliated surface of the airway epithelium (as confirmed by differential interference contrast imaging of the ciliated surface). In saline-treated control samples, SRF colocalizes with the acetylated tubulin (Fig. 4*E*), as quantified using the Pearson's correlation coefficient [a measure indicating the degree of colocalization between SRF (green) and acetylated tubulin (magenta) (1, 22); Fig. 4*D*]. The Pearson's correlation coefficient for SRF colocalization with acetylated tubulin was significantly decreased in DE-treated mice vs. saline controls, corroborating an apparent decrease in SRF staining at the ciliated surface of the airway epithelium in the DE-treated group (Fig. 4*F*). These data indicate reduced colocalization of SRF with acetylated tubulin found on the ciliated airway epithelium in the DE-treated mice.

SRF signaling pathway influences IL-8 release in DE-exposed BECs and modulates CBF in MTEC-ALI. To assess the role of SRF-related signaling in the proinflammatory response of BECs to DE, we utilized CCG-1423, a small molecule inhibitor that prevents Rho kinase-related signaling by preventing the formation and DNA binding/signaling of the SRF/megakaryoblastic leukemia-1 (MKL) cofactor complex (17). Submerged (unciliated) BECs (Beas2B) were treated with 0 or 100 nM CCG-1423 for 1 h prior to stimulation with 5% DE. At 24 h following DE treatment, IL-8 release was significantly reduced in BEC culture supernates that were pretreated with CCG-1423 vs. those receiving DE treatment alone (Fig. 5*A*). Furthermore, to investigate the role of SRF-related signaling in modulating CBF, MTEC-ALI cultures were treated with 0, 1, 10, or 100 nM CCG-1423 and CBF measurements were taken over a 7-h time period. CCG-1423 exhibited a dose-dependent effect on resting CBF (Fig. 5*B*). Over time, cells exposed to 100 nM CCG-1423 had increased CBF, reaching significance by 5 h postexposure. These data indicate a functional role for SRF-related signaling in regulating the proinflammatory response of BECs to DE, while also modulating resting CBF.

Expression and localization of SRF in motile cilia of ependymal cells and ciliated oviduct epithelium. Because of the importance of our findings regarding the role of SRF in motile cilia of BECs, we sought to determine whether this mechanism of motile cilia signaling regulation might be present in motile cilia of other nonairway tissues. To do so, we performed immunohistochemistry on frozen, sectioned mouse brain tissues and paraffin-embedded mouse oviduct tissues. As shown in Fig. 6, SRF staining in these tissues dramatically localized to the ependymal cell cilia (Fig. 6*B*) and cilia of the oviduct epithelium (Fig. 6*D*), strikingly similar to what we found in the motile cilia of the airway epithelium. Staining of mouse sperm did not reveal expression of SRF in the flagella/modified cilia of these cells (data not shown). Taken together, these data suggest that the localization of the SRF transcription factor to motile cilia and subsequent regulatory control of signal transduction is not unique to the airway epithelium. Rather, sensing and signaling via this unique subcellular localization of SRF may be a common theme for signaling in the regulation of many cells that express motile cilia.

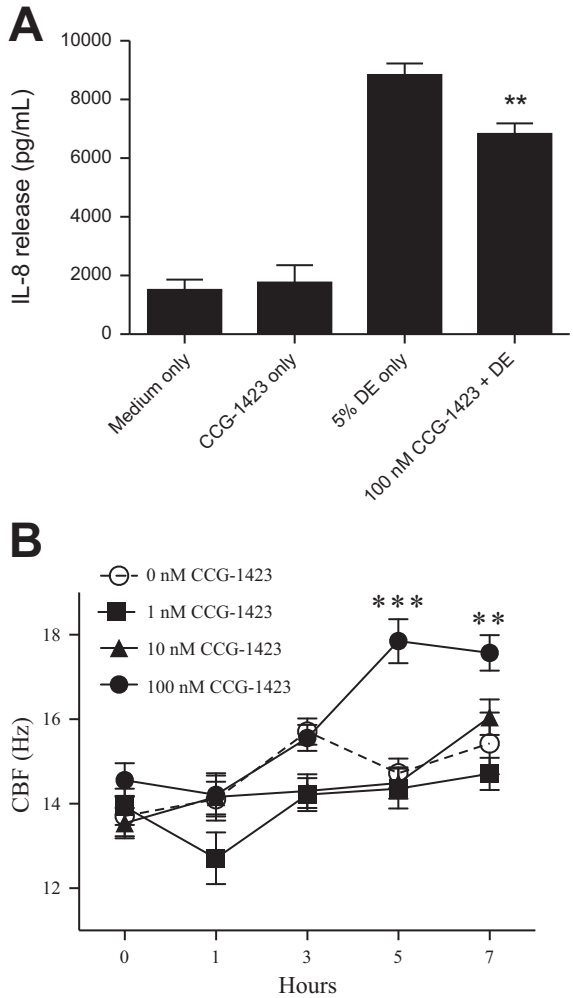

Fig. 5. Effect of inhibition of Rho kinase-related SRF signaling on IL-8 release in DE-stimulated bronchial epithelial cells (BECs), and cilia beat frequency (CBF) in mouse tracheal epithelial cells grown on air-liquid interface (MTEC-ALI) cultures. *A*: submerged, unciliated BECs (Beas2B) cultures were treated with 0 or 100 nM CCG-1423 for 1 h prior to stimulation with 5% DE. At 24 h post-DE, supernates were collected and analyzed for IL-8 levels by ELISA. *B*: ciliated MTEC-ALI cultures were treated with 0, 1, 1, or 100 nM CCG-1423 on the apical surface of the cultures. At 1, 3, 5, and 7 h, CBF measurements of cultures were taken with SAVA software (38). Data are represented as means ± SE. $***P < 0.001$, $**P < 0.01$ compared with 0 nM CCG-1423 group for each respective time point, based on ANOVA statistical analysis with Tukey's method of post hoc comparisons among groups.

DISCUSSION

Our data demonstrate a novel role for motile cilia in sensing and regulating signal transduction in ciliated cells. The localization of SRF to the cilia of unstimulated airway epithelial cells suggest a unique mechanism of SRF organelle sequestration in these cells and a previously undescribed function of SRF in regulating ciliary motility. Our data reveal changes in SRF localization in the airway epithelium corresponding with acute exposures to an inflammatory insult, indicating that SRF plays a role in reacting to these insults that is regulated by its localization to the motile cilia of these cells.

We recently found that SRF signaling is activated in submerged cultures of BECs in response to exposures to DE (26). Therefore, we sought to determine what role SRF plays in the

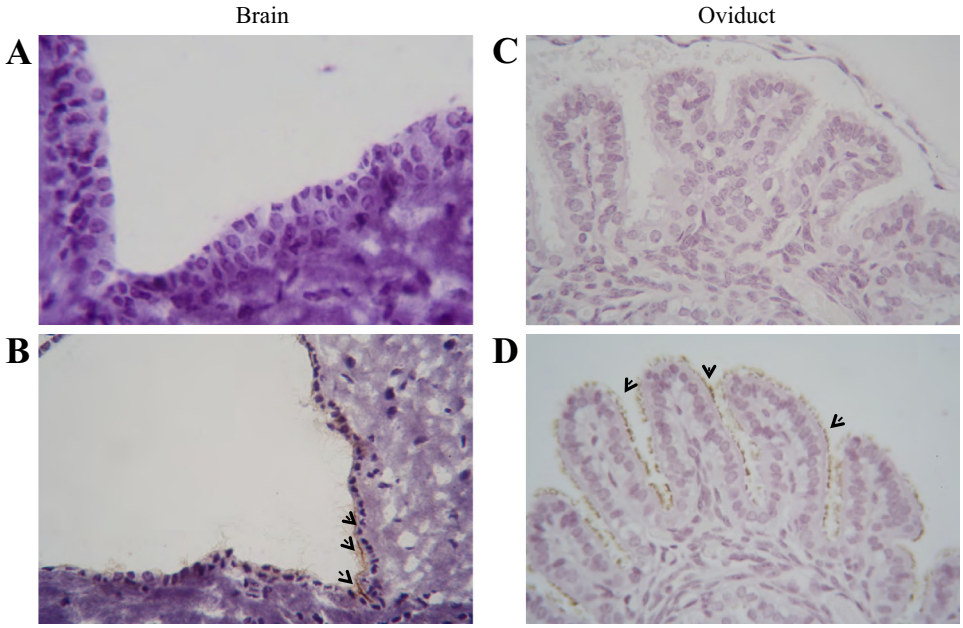

Fig. 6. Expression and localization of SRF to motile cilia of other organ systems. Frozen mouse brain tissue sections and paraffin-embedded mouse oviduct tissues were stained for SRF protein expression. *A*: secondary antibody control (shown in brain tissue). *B*: ×400 view of SRF expression and localization to cilia of brain ependymal cells. *C*: secondary antibody control in oviduct tissue. *D*: ×400 view of SRF expression and localization to cilia in mouse oviduct epithelium.

response of ciliated BECs to acute lung injury, using a murine model of organic dust-induced airway inflammation. Previous studies by our laboratory have shown that single exposures to DE in mice lead to acute airway inflammation characterized by increased proinflammatory cytokines (TNF-α, IL-6, CXCL1, CXCL2) and neutrophilic influx in the bronchial alveolar lavage fluid (29). The data reported in these new investigations imply that modulation of SRF in BECs in vivo is reliant on a change in SRF's subcellular localization during the response to acute inflammatory insult, where SRF cilia localization is decreased during acute exposure to DE. SRF has known roles in immediate early gene activation in response to such activators as proinflammatory cytokines and lipopolysaccharide (12, 20). Additionally, SRF is regulated by cytoskeletal dynamics including Rho kinase-related signaling and localization restriction for functioning (9, 21, 39). Thus it is possible that SRF activation occurs during acute inflammatory insult, leading to decreased ciliary localization, although it is important to note that no apparent SRF nuclear accumulation occurs following DE exposures that would indicate SRF nuclear activation and signaling. Therefore, it is also plausible that SRF may be serving an additional, unique role in the cilium that is currently undefined and could be regulated by SRF degradation and/or relocalization. This being considered, our data do reveal SRF signaling activities to be involved with proinflammatory responses; inhibition of Rho kinase/SRF signaling with the small molecule inhibitor CCG-1423 reduces the proinflammatory response of submerged, unciliated BECs to DE by significantly decreasing IL-8 release compared with cells stimulated with DE alone. Although these unciliated, submerged cell cultures are not an adequate model for studying the role of SRF ciliary localization in regulating SRF signaling, these investigations do shed light on the importance of this signaling pathway in the inflammatory response of BECs. Because IL-8 functions to activate and recruit neutrophils into the airway (7), the SRF-dependent modulation of IL-8 release in these studies is likely of functional significance in the context of airway inflammation. Although IL-8 release in cells receiving CCG-1423 was still significantly upregulated, we have shown that multiple pathways in addition to Rho kinase/SRF signaling, including NF-κB and MAPK, are upregulated following DE (26). The activation of these pathways likely accounts for the remaining increase in IL-8 release following CCG-1423 inhibition of Rho kinase/SRF signaling. Furthermore, inhibition of Rho kinase/SRF pathway signaling with CCG-1423 modifies CBF in MTEC-ALI cultures, whereby inhibition of SRF signaling leads to increased CBF over time. The control of mucociliary clearance by cilia beating in the airways is vital to maintaining lung homeostasis and a pathogen-free environment, with CBF rate having a significant effect on clearance efficiency (34). The change of several Hz between control and CCG-1423-treated MTEC-ALI cultures is likely to convey functional significance, as these differences in CBF have functional effects (15). Particularly since SRF signaling inhibition caused increased CBF, understanding the role of SRF signaling in BECs could be revealing in regards to the role of SRF in regulating the response of BECs to inflammatory stimuli. Together, these experiments shed light on a unique role for the SRF transcription factor in the regulation of ciliated BEC in response to inflammatory stimuli likely related to regulation of mucociliary clearance.

In addition, our findings reveal a novel role for SRF and motile cilia in participating in regulation of nonreceptor mediated, genomic-associated signal transduction, which has not been previously described. Traditional dogma dictates that primary cilia play a role in sensing and regulating signal transduction. For example, the Gli2 and Gli3 transcription factors associated with the Shh signaling pathway are now well recognized to associate with primary cilia as a vital part of their appropriate biological actions (19, 41). Until recently, however, it was thought that these types of sensory and signaling actions were unique to primary cilia and lacking in motile cilia, which were though to have solely mechanical functions. Recent reports have shed light on potential sensory and response roles of the motile cilia on airway epithelium, including the finding that bitter receptors are expressed on motile cilia and

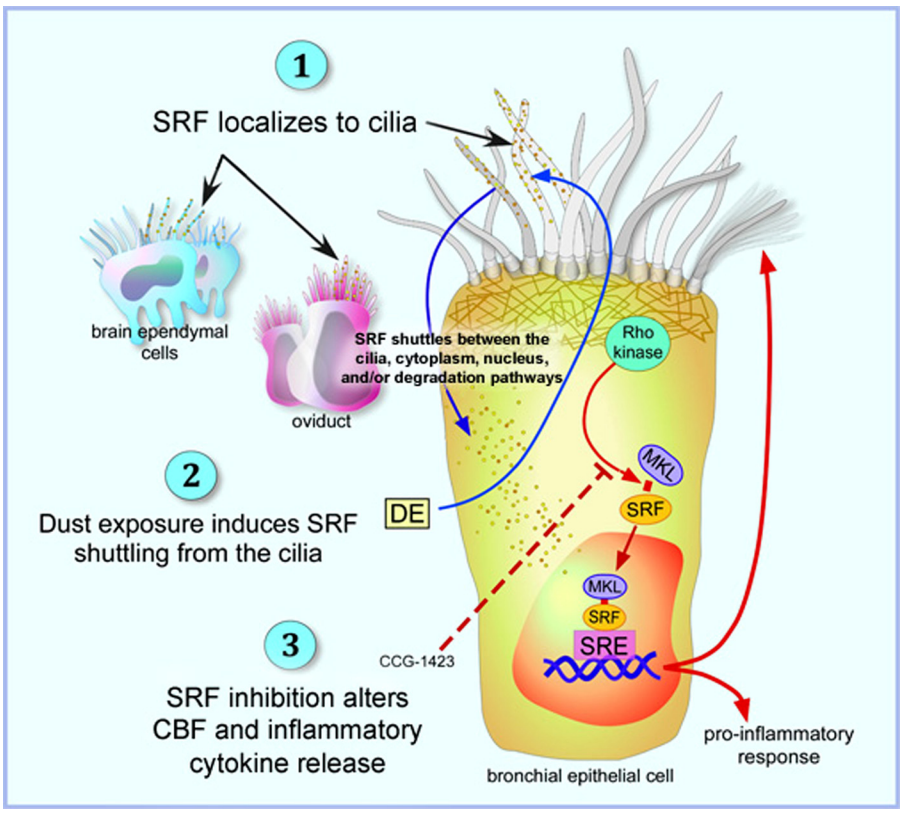

Fig. 7. Proposed model of SRF action in ciliated airway epithelial cells exposed to organic dust. The data presented here indicate that SRF is localized on the cilia of bronchial airway epithelial cells, as well as brain ependymal and oviduct ciliated cells. We hypothesize that exposure to DE causes SRF to be shuttled from the cilia to the cytoplasm, nucleus, or degradation pathways (i.e., through intraflagellar transport and/or other shuttling mechanisms). Inhibition of SRF in ciliated cells abrogates the inflammation response in the airway potentially due to interference in Rho kinase and/or MAPK signaling pathways. Furthermore, our data indicate that Rho kinase/SRF inhibition modifies cilia beat frequency, which we hypothesize to be a result of abrogated cytoskeletal dynamics and homeostatic signaling mechanisms that are controlled by the Rho kinase signaling pathway. MKL, megakaryoblastic leukemia (SRF coactivator); SRE, serum response element (DNA binding site of SRF).

assist in the response of cilia beat to noxious substances (36). Additionally, progesterone receptors have been found on the motile cilia of oviduct epithelium and play a role in quickly responding to progesterone in the milieu (8, 43). In fact, it has recently been found that in sea urchins the hedgehog signaling receptor Smoothened is located on motile cilia during development and motile cilia are required for hedgehog signaling (42), although no connection has been made to motile cilia in modulating signal transduction via transcription factor regulation at the cilia. Our findings reported here of the localization of SRF to the motile cilia of airway epithelium further challenge the traditional concept of motile cilia having merely a mechanical function for the cell and ascribe a role for these organelles that is similar to that of primary cilia in sensing and regulating signal transduction in response to injurious stimuli. Furthermore, the expression and localization of SRF to the motile cilia of ependymal cells in the brain and oviduct epithelium suggest that this is not a lung epithelium-specific phenomenon; the regulation of SRF activation by motile cilia may be a conserved biological function of these organelles.

In conclusion, our data identify a novel role for motile cilia in regulating SRF-related signal transduction in the cell. In Fig. 7, we have presented a working model of our presented data and our hypotheses regarding SRF functioning in cells having motile cilia. Future experiments examining what specific stimuli, including cytokines or other injurious stimuli modulate the localization and activation of SRF in cells having motile cilia are warranted. In addition, investigations pertaining to the trafficking of SRF to the cilia, and the potential role of intraflagellar transport in these mechanisms will be valuable to understanding how SRF interacts with the cilia. We speculate that the active, motor-driven transport from the cilia to the cell body can account for the regulation of SRF localization to the cilia in unstimulated and inflammatory environments. These experiments will prove highly valuable in determining the role of SRF in the activation of these cells, as well as the role of its cilia-related localization. Because of SRF's known role in wound healing responses associated with nonciliated epithelial injury (11), future in vivo investigations examining the role of SRF in ciliated BEC wound healing models are also warranted and will be valuable to understanding the role of this protein in ciliated BECs.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

T.M.N., T.A.W., K.L.B., J.A.P., J.H.S., and D.J.R. conception and design of research; T.M.N., J.S., and A.J.H. performed experiments; T.M.N., T.A.W., J.S., K.L.B., A.J.H., and D.J.R. analyzed data; T.M.N., K.L.B., J.A.P., and J.H.S. interpreted results of experiments; T.M.N. and A.J.H. prepared figures; T.M.N. and A.J.H. drafted manuscript; T.M.N., T.A.W., J.S., K.L.B., J.A.P., A.J.H., J.H.S., and D.J.R. edited and revised manuscript; T.M.N., T.A.W., J.S., K.L.B., J.A.P., A.J.H., J.H.S., and D.J.R. approved final version of manuscript.

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