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Key Role of MicroRNA in the Regulation of Granulocyte Macrophage Colony-stimulating Factor Expression in Murine Alveolar Epithelial Cells during Oxidative Stress*

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Background: Accelerated mRNA turnover results in suppression of lung epithelial cell GM-CSF expression during oxidative stress.
Results: We found that microRNAs 133a and 133b, targeting GM-CSF, are both necessary and sufficient for this accelerated turnover.
Conclusion: MicroRNAs play a central role in determining cell-specific GM-CSF expression during stress.
Significance: Regulation of microRNA offers a new therapeutic approach to protect the lung during injury.

GM-CSF is an endogenous pulmonary cytokine produced by normal alveolar epithelial cells (AEC) that is a key defender of the alveolar space. AEC GM-CSF expression is suppressed by oxidative stress through alternations in mRNA turnover, an effect that is reversed by treatment with recombinant GM-CSF. We hypothesized that specific microRNA (miRNA) would play a key role in AEC GM-CSF regulation. A genome-wide miRNA microarray identified 19 candidate miRNA altered in primary AEC during oxidative stress with reversal by treatment with GM-CSF. Three of these miRNA (miR 133a, miR 133a*, and miR 133b) are also predicted to bind the GM-CSF 3′-untranslated region (UTR). PCR for the mature miRNA confirmed induction during oxidative stress that was reversed by treatment with GM-CSF. Experiments using a GM-CSF 3′-UTR reporter construct demonstrated that miR133a and miR133b effects on GM-CSF expression are through interactions with the GM-CSF 3′-UTR. Using lentiviral transduction of specific mimics and inhibitors in primary murine AEC, we determined that miR133a and miR133b suppress GM-CSF expression and that their inhibition both reverses oxidant-induced suppression of GM-CSF expression and increases basal expression of GM-CSF in cells in normoxia. In contrast, these miRNAs are not active in regulation of GM-CSF expression in murine EL4 T cells. Thus, members of the miR133 family play key roles in regulation of GM-CSF expression through effects on mRNA turnover in AEC during oxidative stress. Increased understanding of GM-CSF gene regulation may provide novel miRNA-based interventions to augment pulmonary innate immune defense in lung injury.

The pulmonary alveolar space is an enormous interface with the external environment. The alveolar epithelial cells (AEC)[2] that define this space express a large number of molecules that are critical for host defense and for homeostasis in the normal lung. Granulocyte-macrophage colony-stimulating factor (GM-CSF, also known as colony stimulating factor 2 or csf-2), an endogenous pulmonary growth factor, has important paracrine effects on immune and inflammatory cells, especially alveolar macrophages (1, 2) and autocrine effects on AEC (3, 4). Decreased or absent expression of GM-CSF in the lung results in important deficiencies in alveolar macrophages for clearance of surfactant and for the initial response to a variety of invading pathogens (1, 5, 6). Conversely, overexpression of GM-CSF in the lung protects against lung injury and limits susceptibility to a number of pathogens (1, 6–8). Thus GM-CSF is a key defender of the gas exchange sites in the lung.

A very large number of different kinds of insults can result in acute lung injury and its most severe form, the acute respiratory distress syndrome. Conditions that lead to acute lung injury/acute respiratory distress syndrome often involve oxidative stress, which plays a key role in the pathophysiology of lung injury. GM-CSF is an oxidant-sensitive gene. We have shown previously that AEC expression of GM-CSF is subject to suppression by exposure to hyperoxia, with important *in vivo* consequences, including increased susceptibility to lethal pneumonia and AEC apoptosis (8). AEC expression of GM-CSF during oxidative stress *in vitro* is regulated by changes in turnover of GM-CSF mRNA (9). MicroRNAs (miRNA) are a recently recognized class of non-coding short RNAs, ~22 nucleotides in length, which regulate gene expression through effects on mRNA stability or translation. miRNAs have been described in plants, worms, and mammals, with over 1100 putative human miRNAs. Wide ranging studies have determined that miRNAs play critical regulatory roles involved in development and dif-

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[2] The abbreviations used are: AEC, alveolar epithelial cell; miRNA, microRNA; PMA, phorbol 12-myristate 13-acetate.

ferentiation, inflammation, fibrosis, and neoplasia (10–14). We now report that a family of specific miRNA expressed in AEC plays a key role both in regulating constitutive GM-CSF expression at baseline and in suppressing GM-CSF expression during oxidative stress through interactions with the 3′-untranslated region of the GM-CSF mRNA. Detailed understanding of these mechanisms may afford a therapeutic opportunity for targeted manipulation of endogenous expression of GM-CSF in the lung.

EXPERIMENTAL PROCEDURES

Animals—Wild-type (WT) C57Bl/6 (Ly5.1; CD45.2) mice were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were housed under specific pathogen-free conditions and monitored daily by the veterinary staff. The animal care committee at the Salt Lake City Veterans Affairs Medical Center approved these experiments.

Exposure of Mice to Hyperoxia in Vivo—Mice were exposed to hyperoxia in shoebox-style cages within a 30-inch wide × 20-inch deep × 20-inch high Plexiglas chamber (15). This chamber was maintained at an oxygen concentration of >95% using a Pro-ox model 110 controller (Reming Bioinstruments). During the 4-day period of hyperoxia, mice remained unrestrained with free access to water and food. We based the duration of hyperoxia on our previous studies (8, 15), focusing on a period of hyperoxia that is injurious but not lethal for normal mice. At the conclusion of the hyperoxia exposure, mice were immediately anesthetized with Avertin and euthanized by transection of the abdominal aorta.

Immunofluoresence of Lung Sections—For immunofluorescence microscopy of whole lung sections, lungs from hyperoxia-exposed or control mice were gently inflated with 1 ml of freshly prepared 4% paraformaldehyde, dissected out of the thorax, and the heart, thymus, upper trachea, and esophagus were removed. The lungs were immersed in cold 4% paraformaldehyde + 3% sucrose for 16–24 h and then prepared for cryosection in OCT using standard methods. 20-μm sections were cut onto *Superfrost*™ Plus microscope slides. Sections were permeabilized and blocked with 1% Triton X-100 in 10% donkey serum for 1 h, washed, and then lung sections were stained with a rabbit polyclonal antibody against GM-CSF (sc-13101; 1:50) and a goat polyclonal antibody against podoplanin (T1α), a marker for Type I alveolar epithelium (16) (R&D AF 3244; 1:500). Primary antibodies were added for ~15 h at 4 °C in a humidified chamber. Alexa Fluor 647 (donkey anti-rabbit) and Alexa Fluor 488 (donkey anti-goat) secondary antibodies were used at 1:2000 for 1 h room temperature. Nuclei were counterstained with DAPI (50 ng/ml) for 3 min. Lungs were visualized using a Nikon A1 confocal microscope and analyzed with NIS Elements Advanced Research by Nikon.

Isolation and Purification of Primary Alveolar Epithelial Cells—Murine type II AEC were isolated and purified using a modification of published methods (17, 18). Lung cells were released by intratracheal instillation of dispase (BD Bioscience). Lung tissues were minced in DMEM with 0.01% DNase I (Sigma), and the resultant cellular suspension was filtered. The cells were incubated with biotinylated anti-CD32 and anti-CD45 (BD Bioscience) followed by streptavidin-coated magnetic particles (Promega) for magnetic removal of leukocytes. Mesenchymal cells were removed by overnight adherence to tissue culture-treated plastic (=day 0). The non-adherent cells were plated (=day 1) in DMEM with penicillin/streptomycin and 10% fetal calf serum in wells coated with fibronectin (Millipore, Temecula, CA). Primary AEC were allowed to attach for 48 h after which non-adherent cells were removed and the cell layers were washed gently with several changes of room temperature sterile PBS to remove all non-attached cells and debris. Fresh growth media was added and the primary AEC were utilized for experiments (=day 3). For each experiment, purity of the epithelial cell preparations was confirmed by staining with murine anti-vimentin to identify mesenchymal and bone marrow-derived cells (anti-vimentin, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary staining was performed with FITC-conjugated anti-mouse IgM. Routinely, fibroblast contamination was 3–6% on day 3 after isolation. All experiments were carried out in bronchial epithelial growth medium (Lonza, Walkersville, MD), unless otherwise indicated.

In Vitro Exposure of AEC to Hyperoxia—C57BL/6 mice exposed to an atmosphere of >95% oxygen die within ~6 days. We have shown previously that lung GM-CSF expression is significantly decreased by day 3 in hyperoxia (8). To model the stress of sublethal hyperoxia *in vivo* in an *in vitro* system, we exposed primary AEC to an atmosphere of 80% oxygen, 5% CO_2 for 48 h (9). Cells were placed in a sealed self-contained chamber (Billups-Rothenberg, Del Mar, CA). This chamber was humidified and maintained at 37 °C and was flushed daily with a commercially available gas mixture of CO_2 and oxygen, adjusted to maintain a fractional concentration of oxygen of 0.80 as measured in real time with an oxygen analyzer within the chamber ($maxO_2^+$, Maxtec, Salt Lake City, UT). Recombinant mouse GM-CSF (R&D Systems) was added immediately prior to the start of the 48-h period in 80% oxygen or room.

Cell Lines—Murine lung epithelial cell line 12 (MLE-12) cells, an immortal murine alveolar epithelial type-II cell line (19), C2C12 cells, a mouse muscle myoblast cell line, and EL4, a mouse T lymphocyte line, and H820, A549, and H441 human lung carcinoma cells and human lung fibroblasts (CCD 18Lu) were obtained from ATCC (Manassas, VA) and maintained in 10% DMEM. Human bronchial epithelial cells, small airway epithelial cells, and human lung microvascular endothelial cells were purchased from Lonza and maintained as directed in the appropriate cell media. Mouse microvascular endothelial cells were from Cell Biologics Inc. (Chicago, IL) and maintained in a specific mouse microvascular endothelial cell growth medium obtained from the supplier of the cells. All original cultures were expanded and stocks were frozen within 7 days of receipt to maintain a supply of close to original cells. Cultures were replenished every 4–6 weeks. Murine alveolar macrophages were obtained by whole lung lavage. Murine lung fibroblasts were isolated using standard procedures from lung digests.

Isolation of Murine Splenocytes—To obtain primary murine splenocytes, mice were euthanized and exsanguinated. The spleens were removed and smashed with the top of a 10-ml sterile syringe plunger in 5 ml of medium. The mixture was filtered through a 40-μm filter (Falcon) into a conical tube. The cells were centrifuged (12,000 × *g*) for 10 min at 4 °C and resus-

pended into 10 ml of hypotonic RBC lysis buffer (Imgenex, San Diego). After 5 min the solution was made isotonic by addition of 10 ml of 2× PBS. Splenocytes were precipitated, washed once with PBS, and resuspended in 10% DMEM, FCS at 5×10^6/ml (one mouse spleen = 1 data sample).

Real-time (RT)-PCR—Total cellular RNA was isolated from cultured cells using RNeasy (Qiagen, Valencia, CA). First strand cDNA was reverse transcribed from 1 μg of total RNA using a high capacity cDNA kit (Applied Biosystems, Foster City, CA). Gene-specific primers were designed using the Roche Applied Science Universal Probe Library Assay Design Center and synthesized/PC purified by the University of Utah DNA/Peptide Core. Specific PCR products were generated from cDNA (100 ng) using Brilliant SYBR Green qRT-PCR 2-step (Stratagene, La Jolla, CA) and an Mx3000P real-time computerized cycler from Stratagene. The two-step cycle program (T_m = 60 °C) with a dissociation analysis was used as recommended by Stratagene. Appropriate negative controls (no template control and Rox reference dye) were included in each experiment as recommended by Stratagene. Each biological sample was amplified in duplicate and the average of the duplicate taken for statistical analysis. The threshold cycle from GAPDH was used as a calibrator to normalize the specific RNA. Results are expressed as fold-change over control values after correcting for GAPDH and setting the first biological control at 100%.

GM-CSF Protein Measurement—After the appropriate perturbation (*e.g.* 48 h in hyperoxia) fresh media was added to the cultures for 18–24 h. Culture supernatants then were collected, centrifuged to remove cell debris, and either immediately assayed for cytokines levels by ELISA (R&D Systems), or frozen at −80 °C for later assay, careful to ensure only one freeze-thaw.

miRNA Isolation—For miRNA analysis, total RNA was isolated from cells *in vitro* using a Qiagen miRNeasy mini kit that combines phenol/guanidine lysis of samples. The method isolates all RNA molecules from 18 nucleotides upwards. Enrichment of miRNA and other small RNA (less than 200 nucleotides) was not performed. The RNA concentration and purity was determined by UV spectrophotometry. Only samples with an A_{260}:A_{230} ratio >1.7 and an A_{260}:A_{280} ratio >2.0 were taken for first strand amplified with RT^2 miRNA First Strand kit (SABiosciences/Qiagen). The same amount of RNA (0.5–2 μg of total RNA) was used for every sample in a given experiment.

Mouse miRNome Array—Total RNA, including miRNA, was collected by phenol/guanidine extraction with miRNeasy (Qiagen) from cultures under 4 experimental conditions (normoxia, hyperoxia, normoxia + GM-CSF, and hyperoxia + GM-CSF). The quality of each RNA sample was assessed by 260/280 and 260/230 ratios and was greater than 2 and 1.6, respectively. A custom array analysis of the 940 most abundantly expressed and best characterized miRNA sequences in the mouse miRNA genome was then performed by SABioscience/Qiagen (MAM3200). The RT^2 miRNA PCR Array is designed to analyze miRNA expression using real-time, reverse transcription PCR. The array uses a SYBR Green real-time PCR detection system that has been optimized for simultaneous analysis of expression of many mature miRNA sequences. The RT^2 miRNA PCR Arrays reflect miRNA sequences annotated by the Sanger miRBase Release 16 database. Fold-changes >3 between conditions were considered for further evaluation.

RT^2 miRNA Quantitative PCR Assays—Nineteen candidate miRNA demonstrating appropriate 3-fold changes in pre-miRNA in the array were investigated further. Using PCR primers for mature miRNA (SABiosciences/Qiagen), expression was normalized to expression of snord85. Snord family members are frequently chosen controls for the normalization of real-time PCR results in miRNA quantification studies. Within that family, snord85 showed the least degree of change due to experimental conditions. Thus, all miRNA data are presented normalized to snord85 expression.

Mouse GM-CSF 3′-UTR Reporter Assay—A dual reporter expression clone for murine GM-CSF 3′-UTR was obtained from GeneCopoeia. A reporter construct with a mutated miR133 target region was also generated by GeneCopoeia. The 3′-UTR sequences are inserted downstream of a firefly luciferase reporter gene driven by a SV40 enhancer. This construct allows use of firefly luciferase as the miRNA 3′-UTR target reporter, whereas a *Renilla* luciferase reporter driven by a CMV promoter provides an internal control for transfection efficiency. The mouse GM-CSF 3′-UTR reporter plasmid was transfected into C2C12 cells using Lipofectamine 2000 (Invitrogen). These experiments were performed in this cell line because it permitted efficient transfection with the 3′-UTR reporter plasmid and because we and others have found that it expresses members of the miR133 family. Cytokines were routinely added 24 h after transfection. Dual luciferase was measured using the Luc-Pair miR luciferase assay (GeneCopoeia).

miR133 Mimic and Inhibitor Treatment of Cell Lines—miR133a and miR133b were mimicked or inhibited in cell lines using Dharmacon miRIDIAN products (Thermo-Scientific Dharmacon). These included miRIDIAN double-stranded oligonucleotides designed to mimic the function of endogenous mature miRNA 133a and 133b, a universal miRIDIAN mimic negative and specific miR133a and -133b miRIDIAN miRNA hairpin inhibitors. The hairpin inhibitors are RNA oligonucleotides with a novel secondary structure designed to inhibit the function of endogenous miRNA. An appropriate miRIDIAN miRNA hairpin inhibitor control was used. Lipofectamine 2000 at 1 μl/ml was used to transfect the cells with miRIDIAN mimics and hairpin inhibitors. Preliminary experiments indicated a maximum effect plus minimum toxicity at a dose of 50 nM.

Lentiviral Transduction of miR133 Mimics and Inhibitors in Primary Murine AEC—Because of poor transfection efficiency in primary AEC we utilized Lentivirus miExpress and miArrest (GeneCopoeia) to mimic and inhibit miR133a and -133b in primary AEC. For miRNA studies, the precursor miRNAs of miR133a and miR133b were placed upstream of GFP in a lentiviral vector (GeneCopoeia), so that green fluorescence indicates transcription and translation of both GFP and transcription of the precursor miRNA. Lentivirus at an multiplicity of infection of 50 was transduced into the cells using 5 μg/ml of Polybrene (Sigma).

Statistical Analysis—Data are presented as mean ± S.E. Statistical analysis was carried out using GraphPad Prism version 4C software (GraphPad, Inc.). Differences between two groups were compared with the unpaired Student's *t* test. Two-tailed

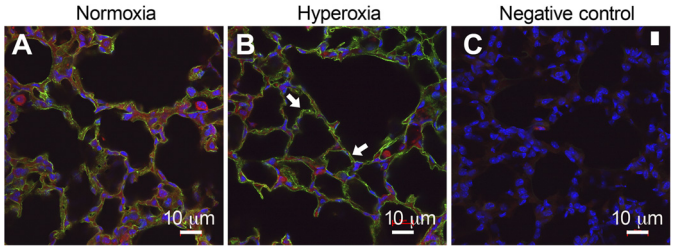

FIGURE 1. **Effect of hyperoxia on GM-CSF expression in the alveolar wall.** Lungs were harvested from mice in normoxia (*panels A* and *C*) or after 4 days in hyperoxia (*panel B*), frozen and then sectioned. Sections were dual stained for GM-CSF (*red*) or T1α (*green*) and examined using confocal microscopy as described under "Experimental Procedures." Control sections were exposed to secondary antibody alone (*panel C*). In *panel B, arrows* indicate areas of alveolar wall with diminished/absent GM-CSF staining compared with normoxia lungs. Nuclei were counterstained with DAPI (*blue*).

TABLE 1
GM-CSF expression in lung cells and cell lines

	Constitutive GM-CSF expression	Preferential stimulus for GM-CSF expression
Murine AEC	4+	IL-1
MLE-12 cells	1+	TNF
Murine mvec	0	IL-1 and TNF
Murine lung fibroblasts	1+	TNF
Murine alveolar macrophages	0	TNF
Murine T cells (EL4)	0	PMA/A23187
Human mvec	1+	TNF
Human lung fibroblasts	0	TNF
Human small airway epithelial cells	1+	TNF
Human bronchial epithelial cells	2+	TNF
Human A549 cells	0	TNF
Human H441 cells	2+	TNF
Human H820	4+	IL-1 and TNF

tests of significance were used. Differences between multiple groups were compared with one-way analysis of variance. Comparisons were deemed statistically significant for $p < 0.05$.

RESULTS

Regulation of AEC GM-CSF Expression—We have shown previously that GM-CSF is expressed at baseline in primary murine AEC (1). GM-CSF mRNA is constitutively expressed in the normal alveolar wall (8). GM-CSF expression is sensitive to oxidative stress and is suppressed by hyperoxia in AEC *in vitro* (9). Expression of GM-CSF mRNA in whole lung and in the alveolar wall is also suppressed by exposure of mice to hyperoxia (8). Prior efforts to stain for GM-CSF protein in the lung have been unsuccessful. Using an approach described by Rauch *et al.* (20), we have now successfully stained for GM-CSF in mouse lung. As shown in Fig. 1, GM-CSF protein was found in the alveolar wall. Double staining for T1α, found on the surface of type I AEC, suggests that both type I (positive for T1α) and type II AEC (lacking T1α) express GM-CSF. Although it was not possible to distinguish intracellular AEC staining from staining of pulmonary endothelial cells or other interstitial cells using light microscopy, our *in vitro* survey of lung cell types (Table 1) indicated that mouse primary AEC express GM-CSF in greater abundance than other lung cells. This strongly suggests that the GM-CSF identified with staining was produced by AEC and that AEC are the major source of GM-CSF in the lung. Transient hyperoxia resulted in an appreciable decrease in GM-CSF staining, in parallel with previously demonstrated

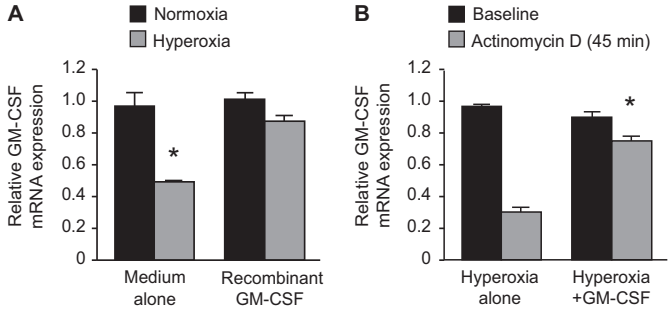

FIGURE 2. **Effect of treatment with GM-CSF on AEC GM-CSF mRNA expression during oxidative stress.** *Panel A,* primary AEC that had been in culture for 2 days were then placed in normoxia or hyperoxia for 48 h in control medium or in the presence of GM-CSF (5 ng/ml) (*gray bars*). The cells were then harvested and relative GM-CSF expression was determined by real-time PCR. Values are normalized to expression of GAPDH. Data are expressed as mean ± S.E., $n = 3$. *, $p < 0.01$ *versus* normoxia and <0.05 *versus* hyperoxia + GM-CSF. Hyperoxia + GM-CSF is not different from normoxia + GM-CSF or normoxia + medium alone. Data are representative of 3 independent experiments. *Panel B*, primary AEC were exposed to hyperoxia for 48 h in control medium or in the presence of GM-CSF. Transcription in AEC was stopped by addition of actinomycin D (5 μg/ml) for 45 min. Relative GM-CSF mRNA was determined by real-time PCR at 0 (*black bars*) and 30 min (*gray bars*). Data are expressed as mean ± S.E., $n = 4$. *, $p < 0.001$ *versus* hyperoxia alone + actinomycin D. Data are representative of 2 independent experiments.

(8) changes in mRNA expression *in vivo* and expression by AEC exposed to hyperoxia *in vitro*.

Regulation of AEC GM-CSF mRNA Turnover—Mechanistic studies have indicated that an increase in mRNA turnover is a key step in the suppression of GM-CSF expression during oxidative stress (9). We have found that that addition of recombinant GM-CSF protein during hyperoxia reversed this suppression (Fig. 2*A*). To determine whether treatment with exogenous GM-CSF reversed the effect of hyperoxia on GM-CSF mRNA turnover, we compared residual GM-CSF mRNA expression in AEC in hyperoxia 45 min after the addition of actinomycin D to block transcription (Fig. 2*B*). Treatment with exogenous GM-CSF resulted in stabilization of GM-CSF mRNA in hyperoxia, accounting for the preservation of GM-CSF expression by treatment with exogenous GM-CSF during hyperoxia. However, it is noteworthy that recombinant murine GM-CSF did not significantly alter the endogenous GM-CSF expression by AEC under normoxic conditions (Fig. 2*A*).

Candidate miRNA for Regulation of GM-CSF in AEC—Because miRNA are an important mechanism regulating mRNA stability for many genes, we used this model of hyperoxia-induced gene suppression to explore the potential role of miRNA in AEC GM-CSF regulation. miRNA was prepared from AEC exposed to normoxia or hyperoxia (80% oxygen + 5% CO_2 for 48 h) in the presence or absence of recombinant mouse GM-CSF. These four conditions were then screened using a mouse miRNA PCR array (Qiagen). This screen identified a number of pre-miRNA that changed in response to hyperoxia or exposure to GM-CSF. We hypothesized that miRNA directly involved in suppressing GM-CSF expression would be induced during hyperoxia and suppressed by treatment with GM-CSF during hyperoxia. Nineteen candidate miRNA showed reciprocal changes in the array, with at least 3-fold changes in pre-miRNA with hyperoxia that were reversed by exposure to recombinant GM-CSF (Table 2). These miRNA were then investigated further using quantitative PCR with primers specific for mature

miRNA (Qiagen). Among the 19 candidates, we found only three mature miRNA (miR133a, miR133a*, and miR133b) that were induced during hyperoxia with reversal in AEC treated with GM-CSF during hyperoxia. Interestingly, each of these three miRNA would be anticipated to bind the GM-CSF 3′-UTR, based on screening databases (miRGEN from the University of Pennsylvania and miRBase release 19, August 2012). Data for miR133a and miR133b are shown in Fig. 3*A*. Of note, these two miRNA were present at baseline and significantly induced during hyperoxia, with reversal when treated with GM-CSF during hyperoxia. Thus, based on both our experimental data and possible binding to the GM-CSF 3′-UTR, these miRNA are likely to play key roles in regulation of GM-CSF expression by primary AEC during oxidative stress. Because miR133a* levels in primary AEC were far lower than miR133a or miR133b, our studies focused on the latter two miRNAs.

TABLE 2
miRNA potentially influencing AEC GM-CSF mRNA expression based on microarray data

Let7a	miR133a	miR154*
Let7i*	miR133a*	miR196a
miR19a*	miR133b	miR376a
miR21*	miR134	miR377
miR29b	miR142	miR410
miR127*	miR146a	miR488
		miR683

GM-CSF Expression and Regulation in a Murine T Cell Line—We next determined whether the miR133 family of miRNAs might be involved in the regulation of GM-CSF expression in other cells. Because GM-CSF expression has been examined extensively in both human and murine T cells, we examined GM-CSF expression and the potential role of miR133 in its regulation in the EL4 murine T cell line. We confirmed previous reports (21) indicating that mouse T cells (EL4) do not produce GM-CSF in the absence of stimulation. Stimulation of EL4 cells with PMA and A23187 resulted in induction of GM-CSF expression (Fig. 3*B*). However, these cells did not produce GM-CSF in response to either IL-1β or TNFα (data not shown). In contrast to primary AEC, GM-CSF expression in EL4 cells was augmented greatly when the cells were exposed to hyperoxia (Fig. 3*B*).

EL4 cells expressed miR133a and miR133b, but at lower levels compared with primary AEC (Fig. 3*C*). Treatment with PMA and A23187 did not influence expression of these miRNAs in normoxia. Expression of miR133a (but not miR133b) was increased in hyperoxia, under conditions in which GM-CSF mRNA expression was induced. These results demonstrate that

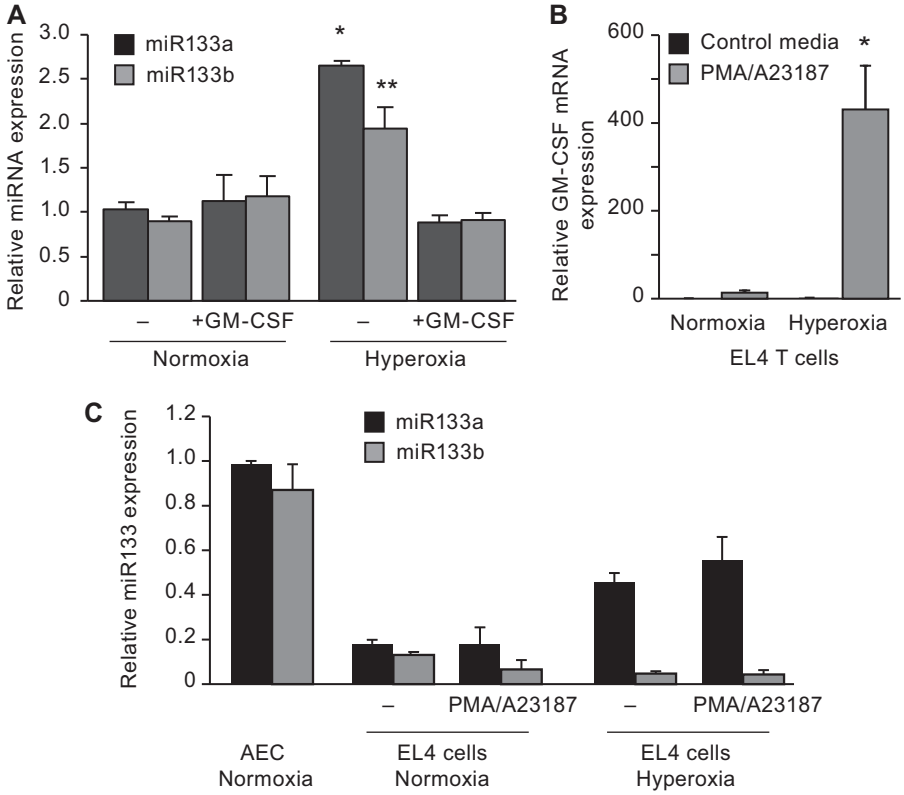

FIGURE 3. **Induction of miR133a and miR133b in primary AEC and EL4 murine T-cells, in response to hyperoxia.** *Panel A,* AEC were exposed to normoxia or hyperoxia in the absence (−) or presence (+*GM-CSF*) of GM-CSF (20 ng/ml). miRNA was purified and quantitative expression of individual miRNA species was determined by real-time PCR, normalized to snord85. miR133a (*black bars*); *, $p < 0.001$ for hyperoxia *versus* both normoxia and hyperoxia + GM-CSF. miR133b (*gray bars*): **, $p < 0.05$ for hyperoxia *versus* both normoxia and hyperoxia + GM-CSF. Data shown are representative of 3 independent experiments. *Panel B,* EL4 cells were exposed to normoxia or hyperoxia (48 h in room air or 80% oxygen) in the presence or absence of PMA/A23187 for the last 15 h of oxygen exposure. Relative GM-CSF mRNA expression was determined by real-time PCR. Values are relative to unstimulated EL4 cells in normoxia and are normalized to expression of GAPDH. Data are expressed as mean ± S.E., $n = 3$. *, $p < 0.01$ *versus* normoxia + PMA/A23187. The experiment shown is representative of 3 independent experiments. *Panel C,* miRNA was purified from primary murine AEC in normoxia and from EL4 cells in normoxia or hyperoxia (with or without stimulation with PMA/A23187) and quantitative expression of individual miRNA species was determined by real-time PCR, normalized to snord85. Relative values are normalized to the value of miR133a in AEC exposed to normoxia. Data are expressed as mean ± S.E., $n = 3$. Data are representative of 3 independent experiments.

GM-CSF expression and regulation differ widely between primary murine AEC and murine EL4 T cells and suggest that miR133 family members do not have a significant role in GM-CSF regulation in hyperoxia in the latter cells.

Impact of Hyperoxia in Vivo on Expression of GM-CSF and miR133 ex Vivo in AEC and Splenocytes—To explore the consequences of *in vivo* exposure to hyperoxia on cell type-specific expression of GM-CSF, mice were placed in hyperoxia for 4 days, as in our previous work (8). Primary AEC and splenocytes were then isolated and RNA was extracted immediately. Control mice were maintained in normoxic conditions. Freshly isolated AEC from mice exposed to hyperoxia demonstrated significantly decreased GM-CSF mRNA expression compared with AEC from control mice in normoxia (Fig. 4*A*). GM-CSF mRNA was far more abundant in AEC compared with that in splenocytes. The low level expression of GM-CSF by splenocytes was not altered by hyperoxia. In AEC, miR133a and miR133b were significantly induced following *in vivo* hyperoxia, whereas expression of these miRNAs in splenocytes was not affected by hyperoxia. In comparison, expression of Let7a, another miRNA active in the lung, but not predicted to bind the GM-CSF 3′-UTR, was not influenced by *in vivo* hyperoxia in either cell type. Thus these data from cells isolated from mice exposed to hyperoxia *in vivo* recapitulate our observations from cells exposed to hyperoxia *in vitro*.

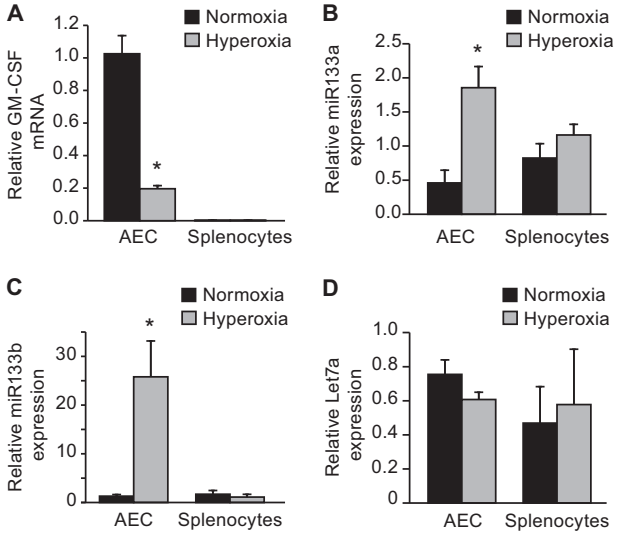

FIGURE 4. **Effect of *in vivo* hyperoxia on GM-CSF and miRNA expression in freshly isolated AEC or splenocytes.** Mice were exposed to ambient air or an atmosphere of >95% oxygen for 4 days. Primary murine AEC and splenocytes were then isolated and RNA was prepared immediately for analysis. *Panel A,* relative GM-CSF mRNA expression was determined by real-time PCR. Values are normalized to expression of GAPDH. The relative expression of GM-CSF mRNA in splenocytes = 0.0031 in both normoxia and hyperoxia groups. *, $p <$ 0.001 *versus* AEC from normoxia mice. Data are expressed as mean ± S.E., $n =$ 4 mice. *Panels B* and *C,* relative expression of miR133a (*panel B*) and miR133b (*panel C*) was determined by real-time PCR, with values normalized to expression of snord85. Data are expressed as mean ± S.E., $n = 4$ mice. In each instance, *, $p < 0.01$ *versus* normoxia AEC. *Panel D,* relative Let7a miRNA expression was determined by real-time PCR, normalized to expression of snord85. Data are expressed as mean ± S.E., $n = 4$ mice. The differences were not significant. Data in all panels are representative of 2 independent experiments.

miR133 Regulates GM-CSF 3′-UTR Reporter Expression—We next investigated the role of miR133 in GM-CSF regulation in more detail, using specific mimics and inhibitors. Initial experiments using a 3′-UTR GM-CSF reporter construct were carried out in the murine C2C12 (myoblast) cell line, which we and others (23) have found expresses members of the miR133 family. As shown in Fig. 6*A*, activity of the GM-CSF 3′-UTR reporter in C2C12 cells was reduced by addition of mimics of

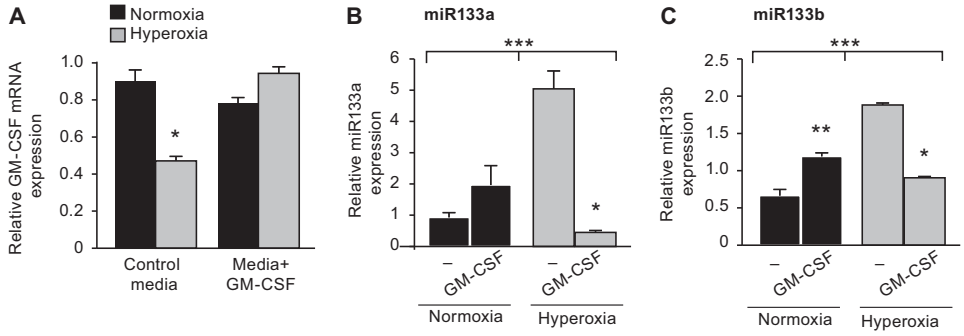

FIGURE 5. **Expression of GM-CSF and miR133 family members in human H820 cells in normoxia and hyperoxia.** Human H820 cells were exposed to normoxia (*black bars*) or hyperoxia (*gray bars*) for 48 h in control medium or in the presence of human GM-CSF (5 ng/ml). The cells were harvested and relative expression of GM-CSF mRNA (*panel A*), miR133a (*panel B*), or miR133b (*panel C*) was determined by real-time PCR, normalized to expression of GAPDH (for GM-CSF) or snord85 (for miRNA). Data are expressed as mean ± S.E., $n = 3$. *Panel A,* *, $p < 0.001$ *versus* normoxia and $p < 0.01$ *versus* hyperoxia + GM-CSF. *Panel B,* *, $p < 0.001$ *versus* normoxia (with or without GM-CSF) and *versus* hyperoxia + GM-CSF; ***, $p < 0.001$ comparing normoxia (−) with hyperoxia (−). *Panel C,* *, $p < 0.001$ *versus* normoxia (with or without GM-CSF) and *versus* hyperoxia + GM-CSF; **, $p < 0.001$ *versus* normoxia alone. ***, $p < 0.001$ comparing normoxia (−) with hyperoxia (−).

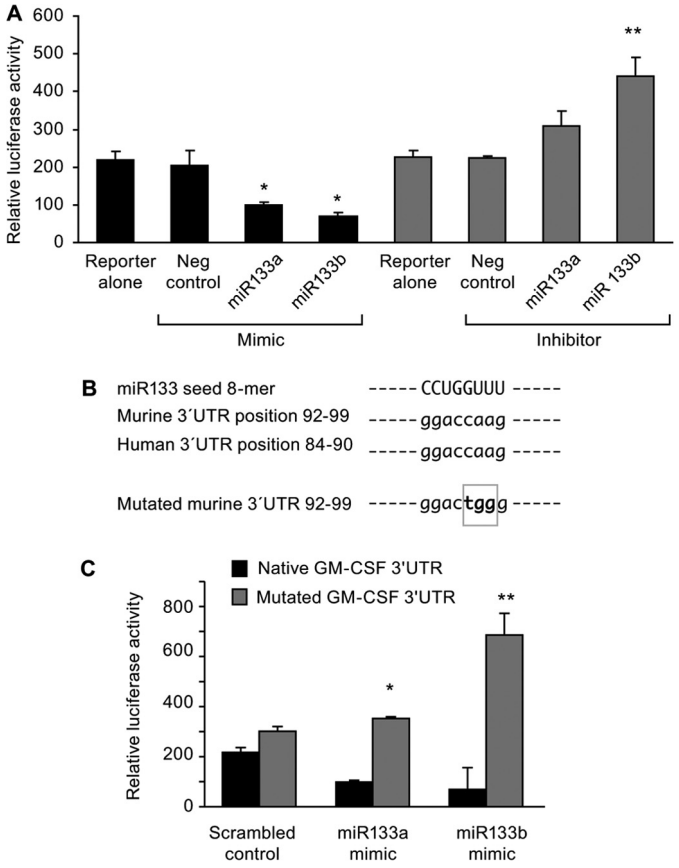

FIGURE 6. **miR133 mimics and inhibitors modulate activity of a GM-CSF 3′-UTR reporter.** *Panel A,* C2C12 myoblasts were transfected with 1 μg of GM-CSF 3′-UTR reporter plasmid in the presence or absence of 50 nM miR133 mimic or 25 nM miR133 short-hairpin inhibitor (miRidian) as described under "Experimental Procedures." Cells were incubated for 48 h after transfection, lysed, and firefly luciferase was determined as a measure of the GM-CSF 3′-UTR level. Values were controlled for transfection efficiency by normalization to *Renilla* luciferase activity. Data are expressed as mean ± S.E., $n = 3$. *, $p < 0.05$ *versus* mimic negative control. **, $p < 0.01$ *versus* inhibitor negative control. Data are representative of 3 independent experiments. *Panel B*, the miR133 seed sequence (which is conserved between murine and human) and its targets in the murine and human GM-CSF 3′-UTR are shown. A reporter construct as created with a mutated murine target for miR133 in the GM-CSF 3′-UTR. In *panel C*, the effect of miR133 mimics on reporter activity for the native and mutated reporter are shown. *, $p < 0.01$ *versus* native GM-CSF 3′UTR; **, $p < 0.001$ *versus* native GM-CSF 3′UTR. Data are expressed as mean ± S.E., $n = 3$. Data are representative of 3 independent experiments.

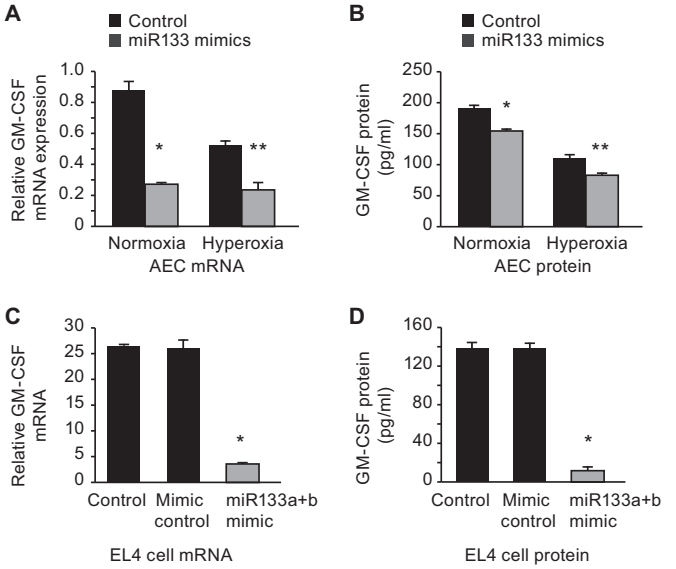

FIGURE 7. **Suppression of AEC and EL4 GM-CSF expression by miR133 mimics.** AEC (*panels A* and *B*) and EL4 (*panels C* and *D*) were transduced overnight with lentiviral precursors to miR133a or miR133b as described under "Experimental Procedures" before exposure to hyperoxia for 48 h (AEC) or stimulation with PMA/A23187 (EL4 cells). Relative GM-CSF mRNA expression in AEC (*panel A*) and EL4 (*panel C*) was determined by real-time PCR. Values are normalized to expression of GAPDH. GM-CSF protein expression by AEC (*panel B*) and EL4 (*panel D*) was determined by ELISA. Data are expressed as mean ± S.E., $n = 3$. Data are representative of 3 (AEC) or 2 (EL4) independent experiments. *Panel A*, *, $p < 0.001$ *versus* normoxia control. **, $p < 0.01$ *versus* hyperoxia control. *Panel B,* *, $p < 0.001$ *versus* normoxia control. **, $p < 0.01$ *versus* hyperoxia control. *Panel C,* *, $p < 0.001$ *versus* control and mimic control. *Panel D,* *, $p < 0.001$ *versus* control and mimic control.

both miR133a and miR133b, indicating 3′-UTR-mediated degradation of the message. Conversely, short hairpin inhibitors of miR133a and miR133b resulted in increased GM-CSF 3′-UTR reporter activity, reflecting inhibition of endogenous miR133 in C2C12 cells leading to decreased turnover of the reporter message (Fig. 6*A*). These data confirmed that miR133 family members acted in the anticipated manner on the 3′-UTR of GM-CSF mRNA.

The miR133 seed sequence is complementary to 8 nucleotide sequences in the GM-CSF 3′-UTR that are fully conserved between mouse and human (Fig. 6*b*). To confirm the specificity of these effects for GM-CSF regulation, the miR133 target site in the GM-CSF 3′-UTR was mutated in our reporter construct. Disruption of the binding of miR133 to the reporter construct by this mutation resulted in loss of responsiveness to miR133a and miR133b mimics (Fig. 6*C*).

miR133 Regulates Endogenous GM-CSF in Primary AEC Cells—To confirm the influence of miR133 family members on GM-CSF expression in primary murine AEC, we utilized a lentivirus system to introduce mimics and inhibitors of the miRNA into the cells. Transduction of primary AEC with lentiviral mimics of miR133a and miR133b resulted in a suppression of GM-CSF mRNA (Fig. 7*A*) and protein expression (Fig. 7*B*). Of interest, we also found that miR133 mimics suppressed expression of GM-CSF in PMA/A23187-stimulated EL4 cells (Fig. 7, *C* and *D*). Because EL4 cells do not express GM-CSF unless stimulated and express low levels of the miR133 family members, it is not surprising that preincubation of these cells with miR133 mimics prior to stimulation resulted in a more profound suppression compared with primary AEC that both constitutively express GM-CSF and express more abundant miR133. Together these data indicate that this family of miRNA has significant activity to suppress GM-CSF expression in both AEC (that express miR133 constitutively) and bone marrow-derived cells.

In primary murine AEC, miR133 family members are induced when GM-CSF expression is suppressed in the face of oxidative stress. To determine whether the miRNA are in fact actively involved in the regulation of GM-CSF expression during oxidative stress, we carried out studies involving specific inhibition of miR133 family members in primary cells exposed to hyperoxia. Inhibition of miRNA activity was achieved using miArrest (GeneCopoeia) lentiviral particles specific for miR133a and miR133b. Inhibition of miR133 resulted in a significant increase in AEC GM-CSF mRNA expression in both normoxia

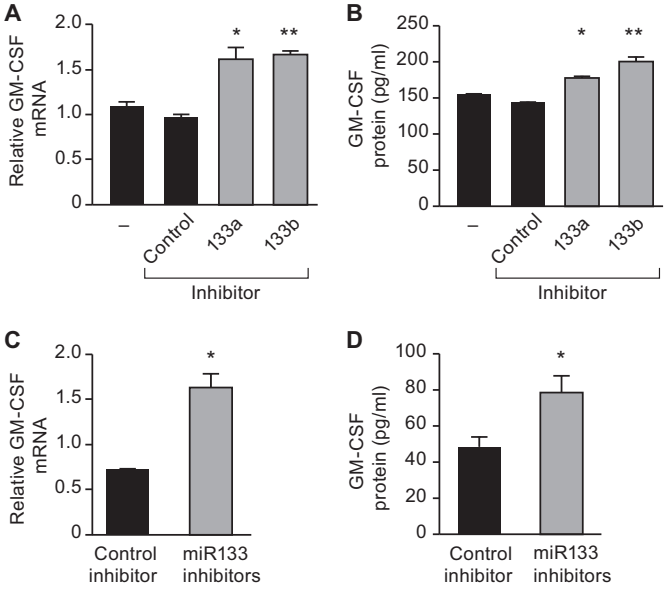

FIGURE 8. **Effect of inhibition of miR133 on GM-CSF expression in primary AEC in normoxia and hyperoxia.** AEC cells in normoxia (*panels A* and *B*) or hyperoxia (*panels C* and *D*) were untreated (−) or transduced with lentiviral control inhibitor or inhibitors specific for miR133a and miR133b in the presence of 5 μg/ml of Polybrene at a multiplicity of infection of 50. Relative GM-CSF mRNA expression was determined by real-time PCR, normalized to expression of GAPDH (*panels A* and *C*). Expression of GM-CSF protein was determined by ELISA (*panels B* and *D*). Data are expressed as mean ± S.E., $n = 4$. The data shown are representative of 3 independent experiments. *Panel A*, *, $p < 0.01$ *versus* inhibitor control; **, $p < 0.001$ *versus* inhibitor control. *Panel B*, *, $p < 0.01$ *versus* inhibitor control; **, $p < 0.001$ *versus* inhibitor control. *Panel C*, *, $p = 0.001$. *Panel D*, *, $p < 0.05$.

(Fig. 8*A*) and hyperoxia (Fig. 8*C*). Although in these short term experiments the effects on protein expression were less profound than for mRNA, inhibition of miR133a and miR133b resulted in a modest increase in GM-CSF protein in cells in normoxia (Fig. 8*b*) and a much greater increase in GM-CSF protein expression in hyperoxia (Fig. 8*D*). Together, these data indicate that miR133a and miR133b play key roles both in regulating basal expression and in the hyperoxia-induced suppression of GM-CSF expression in primary murine AEC.

DISCUSSION

Although its name might suggest that GM-CSF would play a role in leukocyte proliferation and maturation in the bone marrow, initial studies involving GM-CSF mutant mice and targeted replacement of GM-CSF within the lung clearly demonstrated a critical role for this GM-CSF in pulmonary homeostasis (24, 25). In the absence of GM-CSF, even for short periods, alveolar macrophages express an immature phenotype and are significantly impaired in their ability to clear pulmonary surfactant, leading to accumulation of surfactant phospholipid and protein in the lungs and alveolar proteinosis (24–26). Subsequent work expanded the physiologic importance of GM-CSF; in its absence, mice are more susceptible to pneumonia from a variety of pathogens (1, 5, 6) and demonstrate impaired healing and more severe fibrosis after acute injury with bleomycin or intratracheal fluorescein isothiocyanate (FITC) (27–30). It is now known that GM-CSF has direct effects on AEC as a mitogen and anti-apoptotic factor, and that overexpression of GM-CSF in the lung provides significant protection in models of acute lung injury (4, 31). However, it is apparent that the location and extent of GM-CSF expression must be tightly regulated. Aberrant expression may lead to unbridled inflammatory responses and has been postulated to contribute to processes such as rheumatoid arthritis and chronic obstructive pulmonary disease (32, 33). These contrasting effects strongly support a detailed study of cell-specific GM-CSF regulation.

In most cells, GM-CSF expression is induced by inflammatory signals, especially TNF. In contrast, we have found that GM-CSF is significantly expressed in resting primary murine AEC *in vitro* and that this expression is further induced in response to LPS or IL-1β, but with little response to TNF. GM-CSF expression in the lung is significantly impaired during environmental stress due to hyperoxia or acute injury with bleomycin (8, 28). Similarly, in primary AEC GM-CSF expression is greatly reduced during oxidative stress. Our recent studies have shown that this decreased expression is due to accelerated turnover of GM-CSF mRNA (9). In the present study we have provided new information concerning the mechanisms by which expression is suppressed in response to oxidative stress. We have identified a family of miRNA (miR133) whose expression is induced in AEC exposed to hyperoxia (when GM-CSF mRNA expression is reduced) and is suppressed when those cells are treated with recombinant GM-CSF (which preserves AEC GM-CSF expression in the face of hyperoxia). Similar effects were observed in primary AEC exposed to hyperoxia *in vitro*, in murine AEC isolated from mice exposed to hyperoxia *in vivo*, and in a human lung epithelial cell line that models the GM-CSF expression pattern of primary murine AEC. miR133 sequences are predicted to bind the 3′-UTR of GM-CSF. Transduction of AEC with miR133 mimics is sufficient to reduce GM-CSF expression, whereas transduction with miR133 inhibitors restores GM-CSF expression and limits mRNA turnover despite hyperoxia. Interestingly, the miR133 miRNA appear to be active in primary AEC but not in a T cell line that expression of GM-CSF is not subject to suppression in response to hyperoxia. This contrast further emphasizes the distinctive regulation of GM-CSF in the alveolar epithelium and the critical importance of studying this regulation in AEC.

miRNAs are a recently recognized class of non-coding short RNAs, ~22 nucleotides in length, which regulate gene expression through effects on mRNA stability or translation. The recognized scope of effects of miRNA is expanding exponentially. It is now estimated that over 30% of human genes may be regulated in part by miRNAs (34). miRNAs are transcribed as larger pri-miRNA and processed first to pre-miRNA and subsequently to mature miRNA. miRNA act as endogenous short hairpin RNA (shRNA) to negatively regulate target genes by binding through nucleotide complementarity to sequences in the 3′-UTR of target genes to decrease both mRNA and protein expression (13). There has been debate about the precise contributions of three potential mechanisms by which miRNA reduce gene expression. Some have argued that the initiating step is destabilization of mRNA resulting in a decreased steady state of mRNA and thus in decreased protein expression (35). For some messages, deadenylation may be an important step. Others have argued that repression of translation is the initial

event, which in turn leads to mRNA decay (36). Despite uncertainty about the initiating event for change in expression in response to a miRNA interacting with its target, it is clear that mRNA and protein expression are consistently linked in these experiments, with miRNA inducing "concordant" reductions in both mRNA and protein (36). We have consistently found parallel changes in GM-CSF mRNA and protein expression in primary AEC.

As interest in the biology of miRNA has expanded, a number of prominent miRNA species have been identified in the lung. In particular, Let7 family members are expressed in relative abundance and are postulated to play a role in pulmonary fibrosis (37). Interestingly, Let7 was identified in our microarray experiments as induced by hyperoxia and suppressed by treatment with recombinant GM-CSF during hyperoxia; however, Let7 does not appear to target GM-CSF 3′-UTR directly. Its potential indirect involvement in regulation of GM-CSF expression has not yet been determined. Our data from AEC isolated from mice exposed to hyperoxia *in vivo* suggests that Let7a has no direct role in regulating GM-CSF expression during oxidative stress. In contrast, the miR133 family is expressed less in AEC and has not previously been identified as important in the lung. Our experiments clearly indicate that these miRNA are active in regulation of GM-CSF in AEC at baseline and in the context of oxidative injury.

Much of the literature concerning miR133 involves its role in muscle cells, especially in the heart and vasculature (for review see Refs. 38 and 39). It is enriched in cardiac myocytes (40) and vascular smooth muscle cells (41). In each instance, miR133 appears to play an important role suppressing cell growth (42, 43) and expression of extracellular matrix constituents (44). Cardiac hypertrophy and fibrosis are associated with cell-specific decreases in miR133 expression (40, 45). Transfection with miR133 mimics is protective against fibrosis-associated atrial fibrillation in some models (47), suggesting a causal role for alterations in miR133 expression in this condition. Conversely, suppression of miR133 is necessary for normal growth factor-dependent appendage regeneration in zebrafish (48). Thus miR133 has an important role as an endogenous inhibitor of growth in the normal adult organ.

There are some data suggesting that miR133 may also influence epithelial cell growth in the lung. A recent report concerning non-small cell lung cancer describes a relationship between the tumor stage and expression of miR133b (49, 50). Acting through effects on epidermal growth factor receptor, miR133b appears to limit proliferation and invasion of cancer cells. Together, these data are consistent with our observation that miR133a and miR133b act to modulate growth factor expression both at baseline and in response to oxidative stress.

Based on chromosomal location, miRNAs are often transcriptionally co-regulated in clusters. In fact, miR133a has been found in a cluster with miR1, whereas miR133b has been placed in a cluster with miR206 (51). We found that both miR1 and miR206 are expressed in murine primary AEC and induced by hyperoxia *in vitro* (data not shown). However, in contrast to miR133 family members, expression of neither of these other miRNAs was suppressed when cells in hyperoxia were treated with GM-CSF. We speculate that post-transcriptional mechanisms account for the effect of GM-CSF on expression of miR133a and miR133b in hyperoxia (52).

Oxidative stress is a common feature of human acute respiratory distress syndrome due to a variety of different processes, including acute inflammatory insults such as sepsis, pneumonia, and acid aspiration. Our studies have focused on injury induced by exposure to hyperoxia. Although supplemental oxygen is a lifesaving measure that is a key tool in the clinical armamentarium, prolonged exposure of rodents to high concentrations of oxygen causes oxidative stress, pulmonary epithelial cell and endothelial injury, acute lung injury, and death. Thus we have chosen to use exposure to hyperoxia as an animal model that is relevant to human acute lung injury/acute respiratory distress syndrome due to a range of processes beyond exposure to toxic concentrations of oxygen.

There are several important features of our model system. GM-CSF expression by AEC differs in important ways from that in other cell types, including bone marrow-derived cells and many commonly studied cell lines. Thus it was important that studies such as these reflect the behavior of primary AEC. Major portions of this work were carried out using primary murine AEC exposed to hyperoxia *in vitro*. We also found similar patterns of expression in murine AEC isolated from mice exposed to hyperoxia *in vivo*, strongly suggesting that the response of AEC to *in vitro* oxidative stress *in vitro* is representative of *in vivo* effects. We also extended our observations to human cells, using a cell line whose behavior recapitulates many of the characteristics of type II alveolar epithelial cells (22). Of particular importance for these studies, the pattern of expression of GM-CSF in H820 cells closely resembles that in primary murine AEC, including constitutive expression, IL-1-stimulated expression, and hyperoxia-induced suppression of GM-CSF expression.

The miRNAs of interest were identified in experiments in which GM-CSF expression was suppressed by exposure to hyperoxia and restored/preserved by the addition of recombinant GM-CSF to the culture medium. Database searches to determine whether specific miRNA might interact with the 3′-UTR of GM-CSF were only carried out subsequently, based on experimental data. It is of interest that GM-CSF itself suppresses the induction of miR133a and miR133b by hyperoxia. We have shown previously that GM-CSF has important protective effects for the alveolar epithelium. The preservation of GM-CSF synthesis in the face of injury due to hyperoxia is further evidence of the key defensive role of GM-CSF in the lung. However, it appears that this effect of GM-CSF on GM-CSF mRNA expression is only evident in the face of hyperoxia. The impact of GM-CSF on miR133a and miR133b expression in normoxia is quite limited, suggesting that GM-CSF does not participate in an autocrine positive feedback loop that might stimulate unbridled AEC proliferation.

In conclusion, we have identified a family of miRNA (miR133a and miR133b) that has a key role in regulating basal expression and in determining the pathologic suppression of GM-CSF expression during oxidative stress. Targeted manipulation of these miRNAs *in vivo* may offer a new approach to preservation of GM-CSF expression in the alveolar epithelium

in vivo in lung injury and thus a new approach to limiting injury and promoting normal repair.

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REFERENCES

1. Paine, R., 3rd, Preston, A. M., Wilcoxen, S., Jin, H., Siu, B. B., Morris, S. B., Reed, J. A., Ross, G., Whitsett, J. A., and Beck, J. M. (2000) Granulocyte-macrophage colony-stimulating factor in the innate immune response to *Pneumocystis carinii* pneumonia in mice. *J. Immunol.* **164,** 2602–2609
2. Shibata, Y., Berclaz, P. Y., Chroneos, Z. C., Yoshida, M., Whitsett, J. A., and Trapnell, B. C. (2001) GM-CSF regulates alveolar macrophage differentiation and innate immunity in the lung through PU.1. *Immunity* **15,** 557–567
3. Huffman Reed, J. A., Rice, W. R., Zsengellér, Z. K., Wert, S. E., Dranoff, G., and Whitsett, J. A. (1997) GM-CSF enhances lung growth and causes alveolar type II epithelial cell hyperplasia in transgenic mice. *Am. J. Physiol.* **273,** L715–L725
4. Paine, R., 3rd, Wilcoxen, S. E., Morris, S. B., Sartori, C., Baleeiro, C. E., Matthay, M. A., and Christensen, P. J. (2003) Transgenic overexpression of granulocyte macrophage-colony stimulating factor in the lung prevents hyperoxic lung injury. *Am. J. Pathol.* **163,** 2397–2406
5. Ballinger, M. N., Paine, R., 3rd, Serezani, C. H., Aronoff, D. M., Choi, E. S., Standiford, T. J., Toews, G. B., and Moore, B. B. (2006) Role of granulocyte macrophage colony-stimulating factor during Gram-negative lung infection with *Pseudomonas aeruginosa*. *Am. J. Respir. Cell Mol. Biol.* **34,** 766–774
6. LeVine, A. M., Reed, J. A., Kurak, K. E., Cianciolo, E., and Whitsett, J. A. (1999) GM-CSF-deficient mice are susceptible to pulmonary group B streptococcal infection. *J. Clin. Investig.* **103,** 563–569
7. Huang, F. F., Barnes, P. F., Feng, Y., Donis, R., Chroneos, Z. C., Idell, S., Allen, T., Perez, D. R., Whitsett, J. A., Dunussi-Joannopoulos, K., and Shams, H. (2011) GM-CSF in the lung protects against lethal influenza infection. *Am. J. Respir. Crit. Care Med.* **184,** 259–268
8. Baleeiro, C. E., Christensen, P. J., Morris, S. B., Mendez, M. P., Wilcoxen, S. E., and Paine, R., 3rd. (2006) GM-CSF and the impaired pulmonary innate immune response following hyperoxic stress. *Am. J. Physiol. Lung Cell Mol. Physiol.* **291,** L1246–L1255
9. Sturrock, A., Vollbrecht, T., Mir-Kasimov, M., McManus, M., Wilcoxen, S. E., and Paine, R., 3rd (2010) Mechanisms of suppression of alveolar epithelial cell GM-CSF expression in the setting of hyperoxic stress. *Am. J. Physiol. Lung Cell Mol. Physiol.* **298,** L446–L453
10. Nana-Sinkam, S. P., Hunter, M. G., Nuovo, G. J., Schmittgen, T. D., Gelinas, R., Galas, D., and Marsh, C. B. (2009) Integrating the MicroRNome into the study of lung disease. *Am. J. Respir. Crit. Care Med.* **179,** 4–10
11. Sotillo, E., and Thomas-Tikhonenko, A. (2011) Shielding the messenger (RNA). MicroRNA-based anticancer therapies. *Pharmacol. Ther.* **131,** 18–32
12. Jiang, X., Tsitsiou, E., Herrick, S. E., and Lindsay, M. A. (2010) MicroRNAs and the regulation of fibrosis. *FEBS J.* **277,** 2015–2021
13. Chen, K., and Rajewsky, N. (2007) The evolution of gene regulation by transcription factors and microRNAs. *Nat. Rev. Genet.* **8,** 93–103
14. Tomankova, T., Petrek, M., and Kriegova, E. (2010) Involvement of microRNAs in physiological and pathological processes in the lung. *Respir. Res.* **11,** 159
15. Baleeiro, C. E., Wilcoxen, S. E., Morris, S. B., Standiford, T. J., and Paine, R., 3rd (2003) Sublethal hyperoxia impairs pulmonary innate immunity. *J. Immunol.* **171,** 955–963
16. Gonzalez, R. F., and Dobbs, L. G. (1998) Purification and analysis of RTI40, a type I alveolar epithelial cell apical membrane protein. *Biochim. Biophys. Acta* **1429,** 208–216
17. Corti, M., Brody, A. R., and Harrison, J. H. (1996) Isolation and primary culture of murine alveolar type II cells. *Am. J. Respir. Cell Mol. Biol.* **14,** 309–315
18. Mendez, M. P., Morris, S. B., Wilcoxen, S., Greeson, E., Moore, B., and Paine, R., 3rd. (2006) Shedding of soluble ICAM-1 into the alveolar space in murine models of acute lung injury. *Am. J. Physiol. Lung Cell Mol. Physiol.* **290,** L962–L970
19. Wikenheiser, K. A., Vorbroker, D. K., Rice, W. R., Clark, J. C., Bachurski, C. J., Oie, H. K., and Whitsett, J. A. (1993) Production of immortalized distal respiratory epithelial cell lines from surfactant protien C/simian virus 40 large tumor antigen transgenic mice. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 11029–11033
20. Rauch, P. J., Chudnovskiy, A., Robbins, C. S., Weber, G. F., Etzrodt, M., Hilgendorf, I., Tiglao, E., Figueiredo, J. L., Iwamoto, Y., Theurl, I., Gorbatov, R., Waring, M. T., Chicoine, A. T., Mouded, M., Pittet, M. J., Nahrendorf, M., Weissleder, R., and Swirski, F. K. (2012) Innate response activator B cells protect against microbial sepsis. *Science* **335,** 597–601
21. Osborne, C. S., Vadas, M. A., and Cockerill, P. N. (1995) Transcriptional regulation of mouse granulocyte-macrophage colony-stimulating factor/IL-3 locus. *J. Immunol.* **155,** 226–235
22. O'Reilly, M. A., Gazdar, A. F., Clark, J. C., Pilot-Matias, T. J., Wert, S. E., Hull, W. M., and Whitsett, J. A. (1989) Glucocorticoids regulate surfactant protein synthesis in a pulmonary adenocarcinoma cell line. *Am. J. Physiol.* **257,** L385–L392
23. Rao, P. K., Kumar, R. M., Farkhondeh, M., Baskerville, S., and Lodish, H. F. (2006) Myogenic factors that regulate expression of muscle-specific microRNAs. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 8721–8726
24. Dranoff, G., Crawford, A. D., Sadelain, M., Ream, B., Rashid, A., Bronson, R. T., Dickersin, G. R., Bachurski, C. J., Mark, E. L., and Whitsett, J. A. (1994) Involvement of granulocyte-macrophage colony-stimulating factor in pulmonary homeostasis. *Science* **264,** 713–716
25. Reed, J. A., and Whitsett, J. A. (1998) Granulocyte-macrophage colony-stimulating factor and pulmonary surfactant homeostasis. *Proc. Assoc. Am. Phys.* **110,** 321–332
26. Ikegami, M., Jobe, A. H., Huffman Reed, J. A., and Whitsett, J. A. (1997) Surfactant metabolic consequences of overexpression of GM-CSF in the epithelium of GM-CSF-deficient mice. *Am. J. Physiol.* **273,** L709–L714
27. Charbeneau, R. P., Christensen, P. J., Chrisman, C. J., Paine, R., 3rd, Toews, G. B., Peters-Golden, M., and Moore, B. B. (2003) Impaired synthesis of prostaglandin E2 by lung fibroblasts and alveolar epithelial cells from GM-CSF$^{-/-}$ mice. Implications for fibroproliferation. *Am. J. Physiol. Lung Cell Mol. Physiol.* **284,** L1103–L1111
28. Christensen, P. J., Bailie, M. B., Goodman, R. E., O'Brien, A. D., Toews, G. B., and Paine, R., 3rd (2000) Role of diminished epithelial GM-CSF in the pathogenesis of bleomycin-induced pulmonary fibrosis. *Am. J. Physiol. Lung Cell Mol. Physiol.* **279,** L487–L495
29. Moore, B. B., Coffey, M. J., Christensen, P., Sitterding, S., Ngan, R., Wilke, C. A., McDonald, R., Phare, S. M., Peters-Golden, M., Paine, R., 3rd, and Toews, G. B. (2000) GM-CSF regulates bleomycin-induced pulmonary fibrosis via a prostaglandin-dependent mechanism. *J. Immunol.* **165,** 4032–4039
30. Piguet, P. F., Grau, G. E., and de Kossodo, S. (1993) Role of granulocyte-macrophage colony-stimulating factor in pulmonary fibrosis induced in mice by bleomycin. *Exp. Lung Res.* **19,** 579–587
31. Beck, J. M., Preston, A. M., Wilcoxen, S. E., Morris, S. B., Sturrock, A., and Paine, R., 3rd (2009) Critical roles of inflammation and apoptosis in improved survival in a model of hyperoxia-induced acute lung injury in *Pneumocystis murina*-infected mice. *Infect. Immun.* **77,** 1053–1060
32. Vlahos, R., Bozinovski, S., Chan, S. P., Ivanov, S., Lindén, A., Hamilton, J. A., and Anderson, G. P. (2010) Neutralizing granulocyte/macrophage colony-stimulating factor inhibits cigarette smoke-induced lung inflammation. *Am. J. Respir. Crit. Care Med.* **182,** 34–40
33. Hamilton, J. A. (2002) GM-CSF in inflammation and autoimmunity. *Trends Immunol.* **23,** 403–408
34. Bartel, D. P. (2004) MicroRNAs. Genomics, biogenesis, mechanism, and function. *Cell* **116,** 281–297
35. Guo, H., Ingolia, N. T., Weissman, J. S., and Bartel, D. P. (2010) Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature* **466,** 835–840
36. Djuranovic, S., Nahvi, A., and Green, R. (2011) A parsimonious model for gene regulation by miRNAs. *Science* **331,** 550–553
37. Pandit, K. V., Milosevic, J., and Kaminski, N. (2011) MicroRNAs in idio-

pathic pulmonary fibrosis. *Transl. Res.* **157,** 191–199
38. Han, M., Toli, J., and Abdellatif, M. (2011) MicroRNAs in the cardiovascular system. *Curr. Opin. Cardiol.* **26,** 181–189
39. Townley-Tilson, W. H., Callis, T. E., and Wang, D. (2010) MicroRNAs 1, 133, and 206. Critical factors of skeletal and cardiac muscle development, function, and disease. *Int. J. Biochem. Cell Biol.* **42,** 1252–1255
40. Carè, A., Catalucci, D., Felicetti, F., Bonci, D., Addario, A., Gallo, P., Bang, M. L., Segnalini, P., Gu, Y., Dalton, N. D., Elia, L., Latronico, M. V., Høydal, M., Autore, C., Russo, M. A., Dorn, G. W., 2nd, Ellingsen, O., Ruiz-Lozano, P., Peterson, K. L., Croce, C. M., Peschle, C., and Condorelli, G. (2007) MicroRNA-133 controls cardiac hypertrophy. *Nat. Med.* **13,** 613–618
41. Torella, D., Iaconetti, C., Catalucci, D., Ellison, G. M., Leone, A., Waring, C. D., Bochicchio, A., Vicinanza, C., Aquila, I., Curcio, A., Condorelli, G., and Indolfi, C. (2011) MicroRNA-133 controls vascular smooth muscle cell phenotypic switch *in vitro* and vascular remodeling *in vivo*. *Circ. Res.* **109,** 880–893
42. Zhang, D., Li, X., Chen, C., Li, Y., Zhao, L., Jing, Y., Liu, W., Wang, X., Zhang, Y., Xia, H., Chang, Y., Gao, X., Yan, J., and Ying, H. (2012) Attenuation of p38-mediated miR-1/133 expression facilitates myoblast proliferation during the early stage of muscle regeneration. *PLoS One* **7,** e41478
43. Tao, J., Wu, D., Xu, B., Qian, W., Li, P., Lu, Q., Yin, C., and Zhang, W. (2012) MicroRNA-133 inhibits cell proliferation, migration and invasion in prostate cancer cells by targeting the epidermal growth factor receptor. *Oncol. Rep.* **27,** 1967–1975
44. Duisters, R. F., Tijsen, A. J., Schroen, B., Leenders, J. J., Lentink, V., van der Made, I., Herias, V., van Leeuwen, R. E., Schellings, M. W., Barenbrug, P., Maessen, J. G., Heymans, S., Pinto, Y. M., and Creemers, E. E. (2009) miR-133 and miR-30 regulate connective tissue growth factor. Implications for a role of microRNAs in myocardial matrix remodeling. *Circ. Res.* **104,** 170–178
45. Villar, A. V., Merino, D., Wenner, M., Llano, M., Cobo, M., Montalvo, C., García, R., Martín-Durán, R., Hurlé, J. M., Hurlé, M. A., and Nistal, J. F. (2011) Myocardial gene expression of microRNA-133a and myosin heavy and light chains, in conjunction with clinical parameters, predict regression of left ventricular hypertrophy after valve replacement in patients with aortic stenosis. *Heart* **97,** 1132–1137
46. Deleted in proof
47. Li, H., Li, S., Yu, B., and Liu, S. (2012) Expression of miR-133 and miR-30 in chronic atrial fibrillation in canines. *Mol. Med. Rep.* **5,** 1457–1460
48. Yin, V. P., Lepilina, A., Smith, A., and Poss, K. D. (2012) Regulation of zebrafish heart regeneration by miR-133. *Dev. Biol.* **365,** 319–327
49. Moriya, Y., Nohata, N., Kinoshita, T., Mutallip, M., Okamoto, T., Yoshida, S., Suzuki, M., Yoshino, I., and Seki, N. (2012) Tumor suppressive microRNA-133a regulates novel molecular networks in lung squamous cell carcinoma. *J. Hum. Genet.* **57,** 38–45
50. Liu, L., Shao, X., Gao, W., Zhang, Z., Liu, P., Wang, R., Huang, P., Yin, Y., and Shu, Y. (2012) MicroRNA-133b inhibits the growth of non-small-cell lung cancer by targeting the epidermal growth factor receptor. *FEBS J.* **279,** 3800–3812
51. Chen, J. F., Mandel, E. M., Thomson, J. M., Wu, Q., Callis, T. E., Hammond, S. M., Conlon, F. L., and Wang, D. Z. (2006) The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat. Genet.* **38,** 228–233
52. Obernosterer, G., Leuschner, P. J., Alenius, M., and Martinez, J. (2006) Post-transcriptional regulation of microRNA expression. *RNA* **12,** 1161–1167