Cyclic Stretch Magnitude and Duration Affect Rat Alveolar Epithelial Gene Expression

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Key Words

Cell injury • Ventilator-associated lung injury • Microarray

provocative evidence that the whole lung preparation may mask the response of individual cell types.

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Abstract

Mechanical ventilation with large tidal volumes can increase lung alveolar permeability and initiate inflammatory responses; but the mechanisms that regulate ventilator-associated lung injury and inflammation remain unclear. Analysis of the genomic response of the lung has been performed in intact lungs ventilated at large tidal volumes. This study is the first to study the genomic response of cultured primary alveolar epithelial cells undergoing large and moderate physiologic cyclic stretch. Responses were dependent on stretch magnitude and duration. Genomic expression was validated for 5 genes of interest: Amphiregulin, Glutamate-Cysteine Ligase Catalytic subunit, Matrix Metalloproteinase 7, Protein Phosphatase 1 regulatory inhibitor subunit 10, and Serpine-1, and protein expression mirrored genomic responses. Differences between results reported from homogenized intact lungs and monolayers of alveolar epithelial cells with type-I like phenotype provide

Introduction

Acute lung injury and acute respiratory distress syndrome occur in an estimated 190,000 cases in the United States every year with an associated mortality rate of 30 to 40%, despite recent advances in the treatment of the critically ill patients [1]. Mechanical ventilation is a cornerstone therapy used to support patients with respiratory insufficiency. However, mechanical ventilation with large tidal volumes can increase lung alveolar permeability and initiate inflammatory responses, but the mechanisms that regulate ventilator-associated lung injury and inflammation remain unclear [2]. Whole genome analysis approaches have been proposed as a feasible and efficient ways of identifying the molecular response to injury [3]. Genomic effects of large ventilation have been studied in intact rats [2, 4-9], mice [10], dogs [11], and isolated mouse lungs [12] exposed to large tidal volumes (V_T) with and without a concurrent sepsis

challenge. Gene knockout mice have also been used to determine responses to large tidal volumes with a secondary sepsis challenge or hyperoxia [10, 13, 14]. However, genomic analyses of homogenized intact lungs combine epithelial, endothelial, and infiltrating cells (e.g. neutrophils and macrophages) and blood in a single preparation, and thus may obfuscate tissue-specific molecular and genomic responses to large lung inflations [3].

To focus on epithelial cell response, monoculture preparations of A549 cells have been exposed to stretch with and without a sepsis challenge [15, 16]. However, in contrast to primary alveolar epithelial cells with type I or type II characteristics that demonstrate cytotoxic effects sensitive to stretch magnitude, duration, and rate effect [17, 18], A549 cell viability is not affected by stretch [17, 19, 20]. Thus, A549 cells may not be the most appropriate model for investigating genomic responses of the alveolar epithelium to stretch.

Large lung inflations have an adverse effect on the alveolar gas exchange, blood-gas barrier properties, and homeostasis [21] associated with large changes in surface area (ΔSA) of the alveolar epithelium [17, 22-25]. Our goal was to focus on the effects of large lung inflations on the alveolar epithelial type I cells, which cover 95-98% of surface area of the alveolus [26]. In a two-way design we stretched rat alveolar type I epithelial-like cells (RAEC) biaxially at 12% or 25% ΔSA , roughly corresponding to 64% and 86% of total lung capacity, respectively [17], to investigate physiological stretch magnitudes that have been shown to produce little cell death and permeability dysfunction, those associated with changes in permeability in rat alveolar cells with a type I phenotype [17, 22]. To capture acute and later-stage genomic responses, to mimic intact animal ventilator studies (typically 2-6 hours in duration), and for comparison with in vitro cell stretch studies (typically 1 hour in duration), cells were studied after 1 and 6 hours at each magnitude. Results were compared to unstretched cells to determine stretch magnitude and duration genomic responses.

Materials and Methods

Primary Rat Alveolar Epithelial Cell Isolation

Alveolar type II cells were isolated from male Sprague-Dawley rats (N=7) based on a method reported by Dobbs et al. [27] with a slight modification reported earlier [18]. Type II cells were seeded onto fibronectin coated (10 $\mu\text{g}/\text{cm}^2$) flexible silastic membranes (Specialty Manufacturing, Saginaw, MI) mounted

in custom designed wells at a density of 10^6 cells/ cm^2 . The cells were cultured for 5 days with MEM supplemented with 10% fetal bovine serum, until they were flattened, formed domes and tight junctions [24], and demonstrated phenotypic traits associated with alveolar type I cells [28, 29]. Then these RAEC were serum-deprived with 20 mM Hepes supplemented with DMEM (CO_2 free buffering system) for 2 hours, subjected to biaxial cyclic stretch at 37°C , with a calibrated customized system with a hollow cylindrical post contacting the deformable membrane [17], at one of two amplitudes (12% or 25% change in surface area, ΔSA) for one of two durations (1 or 6 hours) at a frequency of 0.25 Hz (15 cycles/min). The four stretch groups were designated 12*1, 12*6, 25*1, and 25*6. All the samples were compared to unstretched control wells.

RNA Isolation and Microarray Analysis

Total RNA was extracted from the cells using Qiagen RNA isolation kit (cat# 74104, Qiagen Inc, Valencia, CA) according to the manufacturer's instructions. Two RNA samples were obtained for every isolation and every experimental group. The quantity and quality of the RNA samples was measured by using Agilent Bioanalyzer and Nanodrop spectrophotometer at the Penn Microarray Core. Samples with low RNA integrity number were discarded. Samples from all 7 rats were used: 2 rats for 1 experimental group, 3 rats for 4 groups, and 2 rats for all 5 groups, precluding a repeated measures analysis approach for the data. In summary, the final microarray data analyzed included 24 samples, with N=5 animals/group for each of the four stretched groups and N=4 animals for the controls.

The microarray protocols were conducted as described in the Illumina RNA Amplification protocol and Bead Station user manual (Illumina Inc., San Diego CA) to determine gene expression. Briefly, 50 ng of total RNA was converted to first-strand cDNA using reverse transcriptase primed by a poly (T) oligomer that incorporated the T7 RNA polymerase promoter. Second-strand cDNA synthesis was followed by in vitro transcription for linear amplification of each transcript, and the resulting biotinylated cRNA was assessed by bioanalyzer electrophoresis. Aliquots (500 ng) of cRNA were added to hybridization cocktails, heated at 65°C for 5 minutes and hybridized for 16 hours at 55°C to Sentrix Rat Ref-12 expression bead chips. The microarrays were then washed at low and high stringency and stained with streptavidin-Cy3 dye. Fluorescence at each microarray feature was measured using a bead array reader. The raw expression data for 22,523 rat gene probes was generated and stored digitally.

Data analysis

Data analysis was performed with Partek Genomic Suite (v6.4, Partek Inc. St. Louis, MO) and Spotfire Decisionsite (v9.1.1, Tibco Inc. Palo Alto, CA). The raw expression data, as exported from Illumina Bead Studio software was imported into Partek Genomics Suite (Partek Inc., St. Louis, MO). Probes were filtered to retain those that had a detection p-value of < 0.05 in at least 3 of the 24 samples, leaving 13,731 for subsequent analysis. The intensity values of the remaining probes were transformed ($\log_2$) and quantile-normalized.

Significance Analysis of Microarrays (SAM) is a powerful

statistical method for many microarray experiments, which includes calculation of a false discovery rate. However, our experimental design (including 4 factors - stretch magnitude, stretch duration, animal, array) was too complex for the operating modes of SAM. Instead, the expression patterns were analyzed for significant differential patterns using a 4-way mixed model ANOVA including terms for stretch magnitude, duration, animal, array and the interaction between stretch magnitude and duration. Pair-wise contrasts between control and each of the four stretch groups (12*1, 12*6, 25*1, and 25*6) were also determined in a post-hoc analysis. No significant array or animal effect was observed in the data.

To attribute significance to any of the p-values calculated in the ANOVA and pair-wise contrasts, we used a post-hoc correction of the p values generated in the ANOVA by determining the false discovery rate (Benjamini Hochberg, step-up), and using a 10 percent cutoff for analysis of the data [30]. Based on this criterion, there were a total of 4916 genes with at least one significant p-value in the 4 pair-wise comparisons. This gene set was further filtered to investigate only those genes with expression that was altered at least 2 fold up or down in at least one of the comparisons with controls, leaving 811 genes for subsequent analyses. Venn diagrams were used to show overlaps between groups.

Examining families of genes affected by stretch can provide insight into common signaling pathways, and reveal opportunities to modulate cellular responses. To compare gene ontologies emerging from our in vitro stretch model results were to those obtained after in vivo ventilation at large tidal volumes for extended periods, we focused our ontology analysis on the subset of genes that were changed significantly in the pair-wise comparison between 25*6 and controls (595 genes). These were examined for enrichment of gene ontology groups using the Database for Annotation Visualization, and Integrated Discovery (DAVID) [31, 32] functional annotation tool. The original set of 13,731 genes with detection values of $p < 0.05$ was used as the background population.

Pubmatrix mining

To identify published functional or disease relevance of the subset of the 595 genes that were up- or down-regulated at least two-fold at large, long stretch (25*6) relative to controls, we used Pubmatrix, a text-based data mining tool of Pubmed (NIH) [33]. Using the five search terms 1) acute lung injury ; 2) ventilation associated lung injury (VALI) or ventilation induced lung injury (VILI); 3) stretch; 4) barotraumas, biotrauma, or volutrauma; and 5) permeability, the co-occurrence of each search term was determined for each gene in this subset. Up-regulated genes were analyzed separately from down-regulated genes. The results were reported as the number of genes in the up- or down-regulated gene subset associated with that search term, normalized by to the total number of genes in the subset.

Because permeability dysfunction is associated with lung injury due to large tidal volumes, we performed an additional ontology analysis for the genes that Pubmatrix identified as associated with permeability. We identified the subset of our genes that were reported in the literature associated with permeability (108 genes), and examined them for enrichment of

gene ontology groups using DAVID [31, 32]. The genes that were more than two-fold up- or down-regulated at large stretch (25*1 and 25*6) when compared to controls were used as a background.

Real-time PCR Assay

To validate expression in five genes of interest (Amphiregulin, Glutamate-Cysteine Ligase Catalytic subunit, Matrix Metalloproteinase 7, Protein Phosphatase 1 regulatory inhibitor subunit 10, and Serpine-1), first-strand cDNA synthesis was performed in a 20 μ l reaction mixture using 1 μ g RNA SuperScript™ III reverse transcriptase (Invitrogen). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was evaluated and used as a housekeeping gene. Aliquots (2 μ l) of the cDNA (Rn00567471_m1, Rn00563101_m1, Rn00563467_m1, Rn00576196_m1, Rn00695641_m1, and Rn99999916_s1, respectively, from Applied Biosystems) were used in TaqMan gene expression assays to detect encoding in mRNAs obtained for each of the following experimental groups: 25*1, 25*6, and unstretched controls (N=3 rats/group), and mRNA levels were quantified in triplicate according to the supplier's recommendations. The densitometry values for each gene were normalized to that of Gapdh, and then to the unstretched control group. This normalized fold change in cT, or concentration of relative fluorescence over time, was expressed as mean plus or minus standard error.

Intracellular Serpine-1 Levels Determined from Western Blots

To determine the protein levels of intracellular Serpine-1, cells were assigned to 25*1, 25*6, or the unstretched control group (N=3 rats/group), and then scraped from the silastic membrane in the presence cell lysis buffer supplemented with the complete protease inhibitor cocktail (1:24; Boehringer Mannheim Biochemicals). This suspension was then sonicated and centrifuged at 15000x g for 15 minutes. The lysis supernatant was used for total protein quantification using the BioRad protein quantification kit. A 20 μ g aliquot of the cell lysate was analyzed by 10% SDS-polyacrylamide gel electrophoresis (200 V for 60 minutes), and the resolved proteins were transferred electrophoretically onto polyvinylidene difluoride membranes (PVDF, 70 V for 90 minutes). The membranes were incubated for 1 h in phosphate buffered saline (PBS) containing 5% powdered milk and 0.1% Tween-20 (PBS-MT) to block nonspecific binding. The PVDF membranes were then incubated overnight at 4°C in the presence of the rat Serpine-1 primary antibody (1:1500 in PBS-MT). After washing the membranes three times in 0.1% Tween-20 in PBS (PBS-T), the membranes were incubated for 1 hour with HRP-conjugated rabbit anti-goat secondary antibody (1:5000 Santa Cruz Biotech). The PVDF membranes were again washed 3 times with PBS-T, and developed using chemiluminescence (Pierce). The developed film from each experiment was digitized (Kodak Image Station), and average intensity of each band was determined using the commercial image analysis software package (Kodak). Blots were stripped and re-probed for GAPDH, as a housekeeping protein. The Serpine-1 densities were normalized by GAPDH, and then

normalized to unstretched cells from the same isolation. Experimental groups 25*1 and 25*6 were each compared to controls using Student's t test, and $p < 0.05$ was considered significant.

Cell supernatant concentration of Matrix Metalloproteinase 7

To determine the protein levels of Matrix Metalloproteinase 7 (MMP7) released into the media by cells assigned to 25*1, 25*6 or unstretched control groups (N=3 rats/group), cellular supernatant was concentrated (Centricon tubes) and total protein was quantified using a BioRad protein quantification kit. Enzymatic levels of MMP7 were detected using Novex 4-16% Zymogram blue Cassiene gels (Invitrogen, Carlsbad, CA). A total protein of (50 μ g/lane) was loaded on to the gel, and the gel was run in Tris/glycine SDS running buffer under non-denaturing conditions. The gel was washed twice with zymogram renaturing buffer (BioRad) for 30 minutes at room temperature, and then developed in zymogram developing buffer (Biorad) for 48 hours. Then the gels were stained with Comassie Blue stain and destained. Enzymatic activity was visualized as a clear band against dark background of stained gelatin. Cells from the human colorectal cancer cell line SW620 were lysed and used as a positive control for MMP7 [34].

Immunofluorescence Aanalysis of intracellular Amphiregulin

To examine Amphiregulin levels qualitatively, cells assigned to 25*1, 25*6 or unstretched control groups (N=3 rats/group), were washed in PBS, fixed for 10 minutes with 1.5% paraformaldehyde and permeabilized with chilled methanol for 10 minute at 20° C, washing three times between steps with PBS. The cells were incubated with primary antibody (goat-anti-rat amphiregulin) overnight at -4°C. After three washes with PBS the secondary Alexa-488 antibody (donkey-anti-goat) was added for 45 minute and washed in phosphate buffered saline dried and mounted on to glass slides with DAPI mixed anti fade reagent (Invitrogen, Carlsbad, CA) The images were taken immediately with an epifluorescence microscope (Nikon, Melville, NY) using a 20x objective to view intracellular Amphiregulin (Alexa 488) and the cell nuclei (DAPI). Images were captured at randomly selected regions of interest in each well with a digital imaging system (Universal Imaging, West Chester, PA), and digital images were analyzed (Metamorph software; Universal Imaging).

Results

The microarray analysis revealed that 13,731 gene probes were present in at least 3 of the 24 samples evaluated. Multivariate analysis revealed that array and animal effects were not significant. In a pair-wise manner, the four stretch groups (12*1, 12*6, 25*1, and 25*6) were compared to unstretched controls. At a false discovery rate of 10% [30], there were no gene probes in the low

Fig. 1. Gene probes with at least 2-fold up or down regulation compared to unstretched controls. For magnitude effects (left panels), gene probes from the 12*6 and 25*6 groups that were significantly altered with respect to unstretched controls were compared for distinct and common gene probe responses. For duration effects (right panels) 25*1 and 25*6 group responses were compared. Up (top panels) and down (lower panels) regulation were evaluated separately.

stretch, short duration (12*1) group that were expressed at significantly higher or lower levels than unstretched controls, but larger stretch magnitudes for just 1 hour produced expression differences in 92 gene probes. Longer stretch durations stimulated responses in significantly more genes, with 3337 and 3681 gene probes in the 12*6 and 25*6 groups, respectively, experiencing significant up or down regulation compared to unstretched control cells. At closer examination of the cells stretched for 6 hours, 1230 gene probes were only significantly altered at low stretch, 1574 only at high stretch, but 2107 were altered significantly at both stretch magnitudes. At each stretch magnitude, longer durations of stretch altered more gene probes. Focusing on the higher stretch stretch magnitude (25% ΔSA), we found that 5 gene probes were altered only at 1 hour of stretch, 3594 altered only at long stretch durations, and 87 were significantly up or down regulated relative to unstretched controls at both stretch durations.

Further analysis focused exclusively on genes with 2-fold or more up or down regulation compared to unstretched controls (designated Large Responders) to limit the scope of our analysis to the genes with the largest responses to stretch. To isolate stretch magnitude and duration effects, and identify up and down regulated genes, we generated a set of Venn diagrams using Spotfire Decision Suite (Fig. 1). Most strikingly, many more genes were uniquely up-regulated (329) or down-regulated (232)

Table 1. Five largest up-regulated genes and five largest down-regulated genes, compared to unstretched controls. All values are reported as fold changes, with positive values defined as up-regulated responses, and negative values as down-regulated responses. When no value is reported, response was not significantly different from controls at a False Discovery Rate (FDR) less than or equal to 10 percent. Largest responders at 25% Δ SA stretch for 6 hours are indicated with an asterisk, and were selected for validation.

GENE NAME	25% Δ SA 6h	12% Δ SA 6 h
*Protein Phosphatase1 receptor antagonist 10	6.7	8.8
Zinc finger, AN1 -type domain 2A	6.5	5.5
Kruppel like factor 4	5.6	3.7
Neuropeptide Y	5.2	
Fos -like antigen 1	5.1	2.5
Extracellular peptidase inhibitor	-3.4	
Phospholipase A2,group IIA	-3.8	
Resistin like gama	-3.8	-5.5
Lysosomal Phospholipase A2	-4.3	-2.7
*Matrix Metalloproteinase 7	-5.3	

Table 2. Eight up- and down-regulated genes with more than two-fold expression changes compared to unstretched controls, that have been reported in the literature as having significant responses in intact animal, humans and lungs. All values are reported as fold changes, with positive values defined as up-regulated responses, and negative values as down-regulated responses. When no value is reported, response was not significantly different from controls at a False Discovery Rate (FDR) less than or equal to 10 percent. Those indicated with an asterisk were selected for validation.

GENE	SYMBOL	12*6	25*1	25*6
Allograft inflammatory factor 1	Aif1			-2.5
Arginase 2	Arg2			2.3
*Glutamate-cysteine ligase catalytic subunit	GCLC			2.9
IL1receptor antagonist	ILrn			-2.2
*Serine (or Cysteine) proteinase inhibitor, Clade E member 1	Serpine-1	-2.04		-2.13
*Amphiregulin	Areg	4.28	4.18	3.84
Methionine adenosyltransferase II, alpha	Mat2a			2
Ribosomal protein, large P2	Rplp2			2.8

only after 6 hours of stretch (designated Late in Fig. 1) at the higher magnitude than those altered only after 1 hour of stretch (Early, Fig. 1), where only 1 gene probe was uniquely up-regulated and none were down regulated (right panels, Fig. 1). Thus, we conclude that large genomic responses occur after 1 hour of stretch. Focusing on stretch magnitude effects at longer stretch durations (left panels, Fig. 1), higher magnitude stretch (High, Fig. 1) uniquely down-regulated a larger pool of genes (199) than it up-regulated (160), whereas the low magnitude stretch (Low, Fig. 1) for the same long duration uniquely up-regulated considerably more (165) gene probes than were down-regulated (50).

Typically, the largest fold responses in the Large Responders were at 6 hours duration, and at 25% Δ SA. The five largest up-regulated genes in the 25*6 group compared to controls were Protein phosphatase-1 receptor antagonist 10 (Ppp1r10), Zinc finger, AN1-type domain 2A (Zfand2a), Kruppel like factor 4 (KLF4), neuropeptide Y (NPY), and Fos -like antigen 1 (Fra1), all with more than five-fold increases in expression (Table

1, 25% Δ SA column). The five largest down-regulated genes in that group were extracellular peptidase inhibitor (EXPI), phospholipase A2-group IIA (pla2g2a), Resistin like gamma (retnlg), Lysosomal Phospholipase A2 (Lysosomal phosphatase A2) and Matrix Metalloproteinase 7 (MMP-7), all with more than three-fold decreases in expression (Table 1). Many of these genes were Large Responders at both stretch magnitudes (Table 1 12% Δ SA column, and All Stretch in Fig. 1, left). We selected the gene with the largest up-regulation in the 25*6 Large Responder group (Ppp1r10) and largest down-regulation (MMP7) for validation (indicated with asterisks in Table 1).

We identified eight genes in the Large Responder group that also had been reported by others [3, 4, 12, 15] in the literature as having a significant response to large lung inflations (without concurrent chemical or septic challenge) in intact animals and lungs (Table 2). Three of these genes were selected for validation (indicated with asterisks in Table 2). First, we selected amphiregulin (AREG) and glutamate-cysteine ligase catalytic subunit

Fig. 2. Quantitative PCR amplification of mRNA. Black bars are the unstretched controls, the dotted bars are the 25*1 stretch group and dashed are the 25*6 stretch group. The numbers below are microarray expression fold change values for the corresponding group (also provided in Tables 1 and 2). * indicates the values are significantly different from unstretched controls.

Fig. 3. Large magnitude-long duration (25*6) genes with two-fold or more changes in expression that are reported in the literature associated with search terms related to large tidal volume acute lung injury. Analysis performed using Pubmatrix. Separate analyses were performed for up- and down-regulated genes, and results are reported as a percent of the 317 up-regulated and 210 down-regulated genes in the 25*6 group that were available in the Pubmatrix database.

(GCLC), because they are both strongly up-regulated in intact, heterogeneous intact lung preparations as well as in our monoculture stretch model. Second we selected serine proteinase inhibitor (Serpine 1) because it has been reported as up-regulated in whole lung microarray analyses, but is down-regulated in our monoculture preparation.

Microarray analysis results for these five genes of interest (Ppp1r10, MMP7, AREG, GCLC, and Serpine-1) were validated for the 25*1, 25*6, and control groups using quantitative real-time PCR, a standard of measure for validating RNA expression data (Fig. 2). The real-time PCR results showed good agreement in magnitude and direction (up or down regulation) with the microarray data (compare bar values with the Table 1 and 2 values presented as numbers below bars in Fig. 2).

The functional significance of the 595 genes in 25*6 group with significant expression alterations (further restricted to FDR $\leq$ 10 percent) compared to controls were evaluated with DAVID [31, 32] applying medium

stringency, using the 13,731 genes that were significantly altered ($p < 0.05$) as a background population. Not all genes were available in the database. In those genes that were down-regulated at 25*6, we identified four genes that were enriched significantly with respect to our background populations that are members of the cell surface receptor-linked signal transduction gene family and four more that would be characterized as part of the protein kinase activity gene family. For those genes that were up-regulated at 25*6, eight functional families were significantly enriched with respect to the background population. Over 40 genes belonged to the family that regulates cellular metabolic processes, 10-20 to either protein phosphatase or protein kinase activity families, and 4-10 to each of the cellular biosynthesis, RNA splicing/processing, ubiquitin, protein termination, or transmembrane transport functional gene families.

Of the 595 genes that were altered two-fold or more in the 25*6 group relative to controls, 527 (317 up-regulated and 210 down-regulated) were available in the

Fig. 4. Serpine 1 Western blot (top) and blot intensity values bottom. Black bars are unstretched controls, dotted bars represent 25*1, and dashed bars indicate 25*6 groups. N=3 isolations/group. All stretched groups normalized to GAPDH, and then to unstretched controls from that isolation. Asterisk indicates significant reduction relative to controls.

Fig. 5. Top: MMP7 Cassiene Zymogram demonstrating reduction the white regions associated with enzyme activity. The lower band is 18 kDa active MMP7 in the supernatant. Each 100 µg sample was pooled from the 6-8 wells of the same isolation. Bottom: The quantization of the active MMP7 intensities (N=3 isolation/group) values. Black bar is control, dotted bar is 25*1, and dashed bar is 25*6.

Pubmatrix (NIH Pubmed) text-based data mining tool [33] to search the reported literature for their association with our five search terms (see list in Methods). Results (Fig. 3) are reported as the percent of the 25*6 up-regulated or down-regulated genes that were reported in literature associated with that search term. Very few (<10%) of these genes have been previously associated with VALI, VILI or stretch in the published literature, but approximately 10% of the up regulated and 18% down regulated genes have been reported as associated with acute lung injury. One of the largest co-occurrence was with the search terms biotrauma, barotrauma, or volutrauma, accounting for 26% of the up regulated genes and 36% of the down-regulated genes. Similarly, permeability was associated in the literature with 24% of the up-regulated and 43% of the down-regulated genes.

Eight genes had reported Pubmatrix associations with the all five of the search terms: oxidative stress induced protein, macrophage expressed gene 1, extracellular peptidase inhibitor, interferon gamma inducible protein,

thyrotropin releasing hormone, glutathione peroxidase 3, ICAM 2, beta 2- adrenergic receptor. Furthermore seven additional genes (RNA polymerase 1-2, MMR_HSR1 domain containing protein RGD1359460, Placental growth factor, CREB binding protein, TLR2, collagen type1 alpha, amphiregulin) are involved in all the search categories except barotrauma/biotrauma/volutrauma. Finally, Arginase 2 is reported in all categories except VALI/VILI. These genes may warrant further examination in the future.

The search term with the largest combined representation in the published literature was permeability, associated with 108 of the 527 genes that were altered at least 2 fold up or down with 25% ΔSA cyclic stretch for 6 hours duration. So we subjected this more focused set of genes for functional annotation by DAVID, using the entire list of genes with two-fold or greater changes in expression (595 genes) as the background population. We found 47 genes demonstrated enriched expression relative to the background population, representing seven different

Fig. 6. Qualitative immunofluorescence reveals higher intensity of amphiregulin relative to background in the 25*1 (center) and 25*6 (right) groups, relative to unstretched controls (left).

functional families of genes: signal transduction, cation transport, G-protein mediated signaling, cell surface receptor mediated signal transduction, ion transport, cholesterol metabolism and anion transport. Interestingly, chromosome 6 contains 11 of these 47 genes and chromosome 10 contains 18 of these 47 genes, showing a relationship in the permeability specific activity.

Protein expression for Serpine 1 decreased two-fold with either 1 or 6 hours of cyclic stretch at 25 % Δ SA compared to controls (Fig. 4), corroborating the 1.49-2.13 down-regulation in gene expression (Table 2, Fig. 2). Enzymatic activity of active MMP7 (18 kDa) in the cellular supernatant of stretched cells decreased two- and three-fold after 1 and 6 hours (Fig. 5), respectively, of 25% Δ SA cyclic stretch with respect to unstretched controls, consistent with a marked reduction in MMP7 gene expression (Table 1, Fig. 2). Finally, we found a qualitative increase in the fluorescence intensity of amphiregulin in both 25*1 and 25*6 stretch groups compared to unstretched controls (Fig. 6), supporting the up-regulation of amphiregulin at the genomic level (Table 2, Fig. 2).

Discussion

Primary alveolar epithelial cells with a type I-like phenotype demonstrate a genomic response to cyclic stretch that is magnitude and duration dependent. At larger physiologic stretch magnitudes, many more genes were uniquely regulated at longer stretch durations than shorter. At these longer stretch durations for prolonged periods, cellular metabolic processes, protein degradation pathways, transmembrane transport activity, and kinases are up-regulated, and cell surface receptor signaling is

down regulated. The gene with the largest up-regulation was Ppp1r10, an inhibitory protein of protein phosphatase 1. Because protein phosphatase 1 stimulates cellular apoptosis [35], an increase in Ppp1r10 may reflect a protective response. We also found significant up-regulation of epithelial growth factor receptor ligand amphiregulin, which is upstream of ERK and NF- κ B, in agreement with data obtained from heterogeneous cell populations from minced intact lungs exposed to large tidal volumes [2, 3, 8, 10, 12]. Genes involved in maintaining glutathione homeostasis (GCLC) are also up-regulated in both our monoculture primary epithelial cell stretch preparation, and in heterogeneous cell populations from minced intact lungs after large tidal volume inflations [2, 3, 8, 12]. Glutathione is a major cellular antioxidant, and its up-regulation to stretch is a protective mechanism, associated with scavenging reactive oxygen species generated with large stretch [14].

The gene with largest decrease in expression was MMP7, which regulates lung inflammation and repair by promoting re-epithelialization, but is also associated with increases in transepithelial neutrophil influx [36]. Thus, down-regulation of MMP7 with stretch may have both adverse and beneficial effects.

Serpine-1, a serine proteinase inhibitor, was also significantly down-regulated with stretch in our monolayers, but has been reported to be up-regulated in intact lungs at large tidal volumes [2, 3, 8, 37]. We agree with others who have proposed that different cells resident in the lung may have unique primary and secondary responses, and that when assaying the homogenized lung, individual cell type responses may be masked [3]. For example, because down-regulation of serpine-1 in knockout mice is associated with protection from

mechanical ventilation induced injury [38], we might expect that marked down regulation of serpine 1 in the type I epithelium may be a protective mechanism to decrease the membrane breakdown in the presence of repeated stretch. However, serpine-1 may be up-regulated in the intact lungs via secondary pathways triggered by the response of other resident cell types. Thus, while studying a monoculture preparation may be an advantage of our investigation, it may also represent a major limitation if the response of other cell types initiate changes in the type I alveolar epithelium at the genomic level. Further

investigation is recommended to delineate the cell-specific from global responses to large cellular deformations associated in large lung inflations to enhance our understanding of mechanisms associated with ventilator associated lung injury.

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