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Gene Expression of Rat Alveolar Type II Cells during Hyperoxia Exposure and Early Recovery

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Abstract

Alveolar epithelial cell (AEC) injury and repair during hyperoxia exposure and recovery have been investigated for decades, but the molecular mechanisms of these processes are not clear. To identify potentially important genes involved in lung injury and repair, we studied the gene expression profiles of isolated AEC II from control, 48-hour hyperoxia-exposed (>95% O₂) and 1-7 day recovering rats using a DNA microarray containing 10,000 genes. Fifty genes showed significant differential expression between two or more time points ($p < 0.05$, fold change >2). These genes can be classified into 8 unique gene expression patterns. Real-time PCR verified 14 selected genes in three patterns related to hyperoxia exposure and early recovery. The change in the protein level for two of the selected genes, *bmp-4* and *retnla*, paralleled that of the mRNA level. Many of these genes were found to be involved in cell proliferation and differentiation. In an *in vitro* AEC trans-differentiation culture model using AEC II isolated from control and 48 hrs hyperoxia-exposed rats, the expression of the cell proliferation and differentiation genes identified above were consistent with their predicted roles in the trans-differentiation of AEC. These data indicate that a coordinated mechanism may control AEC differentiation during *in vivo* hyperoxia exposure and recovery as well as during *in vitro* AEC culture.

Keywords

hyperoxia; alveolar type II cells; lung injury and repair; cell differentiation; DNA microarray

INTRODUCTION

Alveoli are the basic gas exchange units of the lung. Their epithelia consist of alveolar epithelial cells type I (AEC I) and type II (AEC II). AEC I are squamous and contribute to ~8% of the total cells in the lung. They occupy greater than 95% of the alveolar epithelial surface and function in O₂ and CO₂ exchange. AEC II are round and account for ~15% of the lung cells and less than 5% of the surface space. AEC II secrete surfactant lipid and proteins and also play roles in xenobiotic transformation, fluid clearance and the repair of injured alveolar epithelium.

Alveoli are constantly exposed to oxygen and environmental stresses (e.g. smoke or pollutant gases) and thus are susceptible to injury. AEC I are more susceptible to injury due to their large, flat shape and their inability to perform mitogenesis. Hyperoxia followed by recovery

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in room air is a well-established experimental model for AEC injury and repair [1]. Hyperoxia exposure results in the death of endothelial cells and AEC I, possibly through necrosis or apoptosis [2]. As a result of AEC I injury, these cells detach leaving behind denuded basement membranes. During the recovery stages, AEC II proliferate and differentiate to AEC I to replace the injured or dead AECs through a cascade of signaling transduction pathways [3].

Recently, genomic approaches have been applied for monitoring gene expression profiles of *in vitro* cultured AEC in response to glucocorticoids, tumor necrosis factor alpha (TNF α), lipopolysaccharide (LPS), keratinocyte growth factor (KGF), and diesel exhaust particle treatment [4-7]. The gene expression profile of the AEC II 7-day culture is different from that of freshly isolated AEC I [8]. Using a similar AEC II culture system, we have observed similar differences between cultured and freshly isolated AEC II, and have identified two AEC markers, the purinergic receptor P2X7 and the gamma-aminobutyric acid receptor pi subunit using a commercial DNA microarray [9]. However, potential phenotypic changes of AEC during cell culture limit the interpretation of the results.

Several groups have used whole mouse lung tissue for gene expression profiling [10-14]. One study has identified 385 genes changed in mouse whole lung tissue during hyperoxia for up to 48 hours [14]. Unexpectedly, little changes have been observed for hyperoxia-related genes, such as antioxidant enzymes (except heavy metal-binding proteins metallothionein) and proinflammatory genes. However, rats and mice respond to hyperoxia differently. The difference in gene expression among species has been observed between rats and mice in response to one week of chronic hypoxia [15]. During chronic hyperoxia, mice have shown less pulmonary vascular remodeling than rats, which is consistent with the expression levels of genes involved in endothelial cell proliferation in rats (up-regulation) and in mice (down-regulation). There are some advantages of using whole lung tissues for gene expression profiling. Whole lung tissues maintain its architecture and cell-cell interactions [16]. The profiling of whole lung tissue allows for the identification of genes that are changed in all of the cell types and avoid potential changes of gene expression induced by cell isolation procedure. However, there are also some limitations in this approach. The differential gene expression from whole lung tissue reflects the average effect of over 40 different cell types in the pulmonary system. This makes it difficult to assess changes in a particular cell type. Also, the average effect may significantly reduce the sensitivity of DNA microarray analysis. Furthermore, in whole tissue gene profiling, it is not possible to distinguish between the contributions of lung cells and infiltrating cells accompanied with inflammation induced by hyperoxia.

In the current studies, we isolated highly pure AEC II from hyperoxia-exposed (48 hours) and recovering (day 0, 1, 3, 5, and 7) rats and examined their gene expression profiles immediately after isolation using an in-house DNA microarray containing ~6,000 known rat genes and ~4,000 expression sequence tags (ESTs). Selected genes were verified by real-time PCR and Western blot. The global gene expression data systematically revealed the genes responding to hyperoxia exposure and recovery. Further studies of these genes will shed light on the mechanisms of alveolar epithelial cell injury and repair, including AEC proliferation and differentiation.

MATERIALS AND METHODS

Hyperoxia model

The hyperoxia model for the present study was used previously in our laboratory to investigate protein nitration [17] and to establish the method for isolating a highly pure AEC II preparation from injured lungs [18]. The Animal Care and Use Committee at the Oklahoma State University approved the animal protocols used in this study. Male Sprague-Dawley rats (~3 months,

Charles River Laboratories, Inc., Wilmington, MA) were housed for 1 week before experiments. Rats were exposed to >95% oxygen in a sealed Plexiglas chamber for 48 hours and then allowed to recover in normal room air for 1-7 days. The control rats were exposed to room air. The oxygen concentration was continuously monitored using an oxygen sensor (Vacu-Med, Ventura, CA) and the flow rate was maintained at 8 L/min using a flow meter. Rats had free access to food and water. The CO₂ in the chamber was removed by soda lime.

AEC II isolation

AEC II were isolated from control, hyperoxia-exposed and recovering rats (age ~3 months) according to our recently improved method [18]. The purities of AEC II preparations were in the range of 93-97% as determined by the modified Papanicolaou staining procedure. The yields were $20\text{--}40 \times 10^6$ per rat with a peak on Day 1 ($40.00 \pm 4.08 \times 10^6$). The viability of these cells was over 92%. Four independent AEC II preparations from each time point [control (C), 48 hours-exposed (HOX), and recovering for 1, 3, 5, and 7 days (REC 1D, REC 3D, REC 5D, and REC 7D)], were used for RNA isolation and microarray hybridization.

RNA isolation

Total RNA was extracted using TRI reagents (Molecular Research Center, Cincinnati, OH). RNA quality and quantity was assessed with agarose gel electrophoresis and A₂₈₀/A₂₆₀ ratio with spectrophotometer (NanoDrop Technologies, Inc, Rockland, DE). The A₂₈₀/A₂₆₀ ratios for all RNA preparations were greater than 1.8.

Microarray Printing

The DNA microarray slides used in this study were printed in-house on glass slides with 50-mer aminated oligonucleotides from the Pan Rat 10K Oligonucleotide Set (MWG Biotech Inc., High Point, NC). It contained 6,221 known rat genes, 3,594 rat ESTs, and 169 *Arabidopsis* negative controls. The oligonucleotides were suspended in 3× SSC at 25 μM and printed on epoxy coated slides (CEL Associates, Pearland, Texas) with an OmniGrid 100 arrayer (GeneMachine, San Carlos, CA) per the manufacturer's instruction. Each oligonucleotide was spotted in triplicate on three identical 18 × 18 mm blocks: A, B, and C. The spot-spot distance was 180 μm and the space between blocks was 4 mm. After printing, the slides were incubated in 65% humidity overnight at room temperature. The slides were then dried and stored at room temperature. Printing spots were uniform as assessed by staining slides with SYTO 61 red fluorescent nucleic acid stain dye.

Microarray hybridization

The 2-step microarray hybridization was carried out with the 3DNA 50 Expression kit (Genisphere Inc., Hatfield, PA). Prior to hybridization, the slides were washed with 0.2% SDS once and with deionized water 4 times, and then dried by centrifugation. The 3 blocks on each slide were separated by two 2 × 25 × 1 mm thermostatic transparent tape strips to allow the hybridizations of 6 independent dual-color labeled biological samples. The strips were removed after hybridization for washing and scanning slides. Total RNA from each AEC II sample (C, HOX, REC 1D, REC 3D, REC 5D and REC 7D) was split into two aliquots; 5 μg each for reverse-transcription with Cy3- or Alexa 647-specific primers. cDNAs were purified with Microcom YM-30 columns (Millipore, Billerica, MA), adjusted to 0.5-0.6 μg/μl, and mixed with 2× formamide hybridization buffer (50% formamide, 6× SSC, and 0.2% SDS) in an equal volume. The DNA microarray slides were hybridized with paired cDNAs at 42°C for 48 hours. After being washed, the slides were re-hybridized with Cy3- and Alexa 647-specific capture reagents at 42°C for 2 hours. After the 2-step hybridizations, each of the hybridized slides was scanned twice with a laser confocal scanner, *ScanArray Express* (PerkinElmer Life and Analytical Sciences, Boston, MA): the first scan for the spot quantification with 90% laser

power and 70~80% PMT so that about 5% spots were saturated, and the other for the spot alignment with 90% laser power and 95% PMT.

Microarray data analysis

Raw data were extracted from the DNA microarray hybridization images with *GenePix* Pro 4 (Axon Instruments, Inc. Union City, CA). Spot image visualization, spot quality evaluation, data normalization and one-way ANOVA for the identification of the differentially expressed genes were performed as previously described using the software package *RealSpot*, which was developed in our laboratory [19]. The data set from *RealSpot* contained LOWESS normalized $\log_2$ ratios (HOX/C, REC 1D/HOX, REC 3D/REC 1D, REC 5D/REC 3D, REC 7D/REC 5D and C/REC 7D), globally normalized fluorescence intensity, quality index (QI), and one-way ANOVA p-value ($p < 0.05$, for all the statistical analyses in the present study). QI was calculated based on signal intensity and signal-to-background ratio. A QI of 0, 1, 2, 3, 4, and 5 represents negative, weak, middle, strong, saturated, and bad spots, respectively.

The differentially expressed genes among time points during hyperoxia exposure and recovery were identified based on both fluorescence intensities and normalized $\log_2$ ratios [7]. First, we filtered low quality spots with a mean QI of ≤ 1.0 (negative spot). Second, a statistical test was performed on fluorescence intensities using one-way ANOVA. The genes with a p-value of > 0.05 (no significant changes between any time points) were excluded. Third, the differentially expressed genes between any two adjacent time points were identified by t-test using $\log_2$ ratios. For instance, a gene was differentially expressed between HOX and REC 1D, if $\log_2$ (REC 1D/HOX) was significantly different from 0. Fourth, the remaining genes were further filtered by a fold change of > 2 to reduce the false-positive and to facilitate real-time PCR verification. The choice of 2-fold change as a cut-off value was arbitrary.

Finally, the genes from step 4 were classified into different gene expression patterns using a trajectory clustering-like algorithm [20], in which the gene expression directions, up-, down- and flat-regulation, were used to cluster in iterative steps. The mean $\log_2$ ratios of any two time points were replaced, based on the above p-values ($p < 0.05$) and $\log_2$ ratios, by a symbol “~” ($\log_2$ ratio > 1 , up-regulation), “v” ($\log_2$ ratio < -1 , down-regulation), or “-” (no significant change). By grouping 6 symbols of $\log_2$ ratios (HOX/C, REC 1D/HOX, REC 3D/REC 1D, REC 5D/REC 3D, REC 7D/REC 5D and C/REC 7D), the differentially expressed genes were classified into several unique gene expression patterns. For instance, a pattern, “~^~^~^”, means HOX=C, REC 1D>HOX, REC 3D=REC 1D, REC 5D=REC 3D, REC 7D=REC 5D and C=REC 7D. In other words, the genes in this pattern were expressed significantly higher in REC 1D than in HOX.

The functional categories of each pattern were based on gene ontology annotation from Rat Genome Database gene association file (RGD, <http://rgd.mcw.edu>), gene ontology definitions (GO, <http://www.goontology.org>), and GeneRIF (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=Retrieve&dopt=Graphics>). Our microarray dataset has been submitted to GEO and can be accessed at the <http://www.ncbi.nlm.nih.gov/projects/geo/> (GSE 2707).

Real-time RT-PCR

The validation of selected targeted genes with QuantiTect SYBR Green PCR Kit (Qiagen Inc., Valencia, CA) was performed as previously described [9]. Total RNAs (1 μ g) were reverse-transcribed into cDNAs with 0.2 μ g/ μ l dT₁₇, 0.3 μ g/ μ l random hexamer primer, and 200 U MMLV reverse transcriptase (Invitrogen Inc., Carlsbad, CA). The real-time PCR thermal conditions for all genes were 95°C for 15 min, followed by 40 cycles each at 95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec and 77°C for 35 sec (signal collection temperature). The

primer pairs are listed in Table 1. The relative real-time PCR quantification was based on the delta delta Ct method [21]. The endogenous reference gene was β -actin (*actb*), which was not differentially expressed between any time points during hyperoxia exposure and recovery. One-way ANOVA tests were performed to compare the mRNA levels statistically among the samples from the different time points.

Western blot

SDS-PAGE and Western blot were performed as previously described [17]. The dilutions of antibodies were: mouse monoclonal anti-bmp-4 (Santa Cruz Biotech, Santa Cruz, CA), 1:250; mouse monoclonal anti-beta actin (Sigma, St. Louis, MO), 1:1000; horseradish peroxidase conjugated-rabbit anti-mouse (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), 1:2500.

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded lung tissue sections (C, HOX and REC 1) with a Vectastain ABC kit (Vector Labs., Burlingame, CA). The sections were treated in 20 mM citrate buffer, pH 6.0 in a microwave oven at 98°C for 15 min and then in 3% H₂O₂ at room temperature for 10 min. After blocking in 2.5% normal goat serum for 20 min, the sections were incubated with anti-retnla antibody (Chemicon, 1:100) overnight at 4°C, followed by incubation of goat-anti-rabbit secondary biotinylated antibody and ABC reagents. The sections were visualized with DAB and counter-stained with hematoxylin.

Cell culture

An *in vitro* cell culture system for the trans-differentiation of AEC II to AEC I-like cells was used to assess the expression of the identified genes [9]. AEC II used for this model were isolated from rats (~ 2 months of age). 48 hrs hyperoxic AEC II were isolated as described above. Both types of AEC II were cultured in the minimal essential medium with 1% fetal bovine serum in 37°C and 5% CO₂ in the plastic dishes for 1, 3, 5, and 7 days. At each time point, total RNAs were extracted as described above. T1 α (an AEC I marker), surfactant proteins A, B, and C (SP-A, SP-B and SP-C, AEC II markers), and selected genes from the DNA microarray data were monitored by real-time RT-PCR.

RESULTS

Hyperoxia model

We have previously observed that the hyperoxia exposure of rats leads to lung injury and the severity of injury depends on the duration of the exposure time [17]. Histological analysis has demonstrated the damage of alveolar epithelium and type II cell hyperplasia when rats are exposed to >95% O₂ for 48 hrs. During recovery at day 1 and day 3, the lung exhibits thick mesenchyme and almost no secondary septation. By day 7 of recovery, the lung regains its normal structure [22]. Coinciding with alveolar injury, rat body weight significantly decreased during hyperoxia exposure (control, 252.17 $\pm$ 3.98 gram, hyperoxia, 240.08 $\pm$ 6.22 gram) and increased during recovery for 1-7 days (Day 1, 257.00 $\pm$ 0.43 gram, Day 3, 253.50 $\pm$ 1.92 gram, Day 5, 277.50 $\pm$ 1.64 gram and Day 7, 286.00 $\pm$ 1.28 gram). To identify the genes in AEC II that are important for the hyperoxia response and early recovery, we performed a DNA microarray of the isolated AEC II from hyperoxic and recovering rats. AEC II were isolated from control (C), 48-hour hyperoxia-exposed (HOX), and recovering for 1, 3, 5, and 7 days (REC 1D, REC 3D, REC 5D, and REC 7D) rats. Despite the presence of inflammatory cells in the injured lungs, we were able to obtain >93% pure AEC II for all of the cell preparations using our recently improved method, in which inflammatory cells such as macrophages and lymphocytes were removed by negative immunomagnetic selection [18].

Differentially expressed genes

We used 4 independent AEC II preparations, each with 6 time points (C, HOX, REC 1D, REC 3D, REC 5D, and REC 7D). The samples from each biological replication were labeled with green or red dyes and simultaneously hybridized onto 3 different blocks on one single slide. The cDNA samples were paired in a loop-style, each with dye-flip to compensate the dye-specific bias (Fig. 1A). After image quantification and data normalization, we sequentially filtered 2,218 genes with a mean QI of <1.0, 5,405 genes with no significant changes based on fluorescence intensities (one-way ANOVA), 2,005 genes with no significant changes based on log₂ ratios (t-test), and 322 genes with a fold change of <2. (See Materials and Methods for detail on data analysis). The final 50 genes were listed in supplementary Table S1. Twenty-eight genes changed between C and HOX, or HOX and REC 1D and 20 genes changed between REC 5D and REC 7D, or REC 7D and C (Fig. 1B). Scatter plots between two time points were shown in Fig. 2.

Gene expression patterns during hyperoxia exposure and recovery

In order to elucidate the relationship between the changes in gene expression and the time points of alveolar injury and repair, the 50 identified genes were classified into 7 unique gene expression patterns A-F, each with 6 or more genes and 2 orphan genes (pattern O) (Table 2). The expression patterns were represented by three symbols: ~, no significant change; ^, P<0.05, up-regulation, and v, P<0.05, down-regulation. The bar graphs of patterns A-D are shown in Fig. 3A. The simplified corresponding diagrams are also illustrated in Fig. 3B, in which all of the time points were compared with control (C). Other patterns were not shown for the sake of clarity. The genes in pattern B (~~~~~), C (~v~~~~), and D (^v~~~~) may be involved in the early recovery after hyperoxia exposure since each gene in these three patterns showed a significant difference between HOX and REC 1D. Pattern A (v~~~~^) showed a more complex change in gene expression and thus may participate in multiple stages of lung injury and repair induced by hyperoxia exposure and recovery. The genes in patterns E (~~~~v~), F (~~~~^) and G (~~~~^) showed up or down regulations in the later stages of recovery and may play a role in the later stages of recovery.

The gene functional categories (ontology) of 50 identified genes are listed in Table 2. Notably 11 genes are not functionally characterized. There were several genes related to cell growth and differentiation (cell cycle, differentiation and proliferation, and telomerase), indicating that there was an active alveolar repair during hyperoxia exposure and recovery.

Data validation

Next, we selected genes for verification with real-time PCR. Because of our research interests in lung injury and repair (especially cell proliferation and differentiation), we focused on the genes that changed during the early recovery stages between day 0 and 1 (Patterns B, C, and D). Consequently, 19 genes in patterns B, C and D were chosen. Real-time PCR data indicated that 14 genes were consistent with DNA microarray data (Fig. 4). Both microarray and real-time PCR revealed 5 genes with significantly higher expression in REC 1D samples than in HOX samples in pattern B. These genes were resistin-like molecule alpha (*retmla*), bone morphogenic protein-4 (*bmp4*), farnesyl pyrophosphate synthase (*fdps*), *hspe1*, and mesenchyme homeobox 2 (*meox2*) (Fig. 4A). In patterns C and D, 9 genes, delta-like homolog drosophila (*dlk1*), thiolase aa 1-397 (*aca2*), metallothionein 3 (*mt3*), ferritin light chain subunit (*ftl1*), cytosolic peroxisome proliferator-induced acyl-coa thioesterase (*bach*), urinary plasminogen activator receptor 2 (*plaur*), response gene to complement (*rgc32*), O-acetyltransferase milk fat globule membrane protein (*mfge 8*) and metallothionein 2a (*mt2a*), were consistently expressed higher in HOX samples than in REC 1D samples as seen by both real-time PCR and microarray assays (Fig. 4B & C). The known functions of the 14 genes are listed in Table 3. In most of the cases, the real-time PCR resulted in a higher fold change than

microarray. This was probably because (a) the real-time PCR calculations were based on the delta delta Ct method [21]; and (b) there are differences between solid-state (microarray) and solution hybridization (PCR).

The five genes that did not show consistent results may be due to low mRNA abundance, poor amplification (featured by high Ct), high variation among samples or differences in data normalization (LOWESS vs. housekeeping gene). The stearyl-coa desaturase (*scd1*) mRNA was not significantly changed between HOX and REC 1D by real-time RT-PCR, but the microarray assay indicated that *scd1* was expressed significantly higher in REC 1D than on HOX ($\log_2$ ratio of REC 1D/HOX = 1.59 ± 0.32). For ccaat binding transcription factor cbf subunit c (*Nfyc*) and *smad5*, microarray and real-time PCR showed the opposite changes between REC 1D and HOX (1.1 ± 0.28 and -1.6 ± 0.44 v.s. -3.4 ± 0.95 and 1.66 ± 0.38). The vimentin (*vim*) and preprocathepsin d (*ctsd*) expression had consistent $\log_2$ ratios between REC 1D/HOX by microarray (-1.2 ± 0.19 and -1.0 ± 0.18) and real-time PCR (-2.1 ± 0.66 and -4.0 ± 1.32), but inconsistent $\log_2$ ratios between C/HOX by microarray (1.73 ± 0.56 and 0.69 ± 0.12) and real-time PCR (-3.7 ± 0.85 and -1.4 ± 0.38).

Based on the availability of suitable antibodies, we further confirmed bmp-4 and *retnla* at the protein level in hyperoxic and recovering lungs by western blot and immunostaining, respectively. Western blot revealed that the expression of bmp-4 protein in AEC II was significantly higher in REC 1D than in HOX (Fig. 5A). Immunostaining showed that anti-*retnla* antibodies mainly stained the alveolar septa and the staining was much stronger in REC 1D than in HOX (Fig. 5B). These results were in consistent with the changes in mRNA expression (Fig. 4A and C).

Gene expression during *in vitro* trans-differentiation of AEC

It is well known that AEC II gradually trans-differentiate into AEC I-like cells when cultured on plastic dishes *in vitro* [23]. Using this model, we further studied 6 genes that are possibly involved in cell proliferation and differentiation. AEC II (D0) isolated from normal rats were cultured with 1% FBS for 1, 3, 5 and 7 days (D1, D3, D5 and D7). The morphology of AEC II (round) gradually changed to AEC I-like cells (flat) (data not shown). T1 α , an AEC I marker, was significantly higher on day 3 than on day 1 during in the *in vitro* culture (Fig. 6A). In contrast, SP-A and SP-C (AEC II markers) were markedly down-regulated in freshly isolated AEC II compared to day 1 and day 3, indicating the phenotypic change of AEC II to AEC I-like cells at day 3. *Dlk1* and *retnla* decreased significantly in freshly isolated AEC II after a 1 or 3 day culture (Fig. 6B). *Rgc-32*, *bmp4*, *meox2* and *fdps* were expressed lower on day 1 than freshly isolated AEC II. However, the expression of these genes increased on day 5 in comparison to day 3 (Fig. 6C). Among other genes identified above, *mt2a* has a change similar to *dlk1* and *retnla*. *Plaur*, *ftl1* and *bach* did not exhibit significant changes at any time points. *Mfg8* and *aca2* were expressed significantly higher on day 3 than on day 1. *Hspe1* and *mt3* were expressed lower on day 3 than on day 1 (data not shown).

In order to better compare with the *in vivo* data, we also isolated AEC II from 48 hr hyperoxia-exposed rats, cultured for 1-7 days and determined gene expression levels of the 6 genes. Similar to normal AEC II, surfactant protein genes (SP-B and SP-C) decreased and T1 α increased when hyperoxic AEC II were cultured on plastic dishes (Fig. 7A), suggesting that these hyperoxic AEC II also trans-differentiate to AEC I-like cells. *Retnla* and *dlk1* had similar changes in hyperoxic AEC II in comparison with normal AEC II although the changes appear to be larger in the hyperoxic AEC II than normal AEC II (Fig. 7B). *Rgc 32* showed a decrease at day 1 in both hyperoxic and normal AEC II. However, *Rgc 32* restored its expression earlier in hyperoxic AEC II than normal AEC II (day 3 v.s. day 5) (Fig. 7C). *Bmp4* expression decreased at day 1 in both hyperoxic and normal AEC II, but normal AEC II exhibited an increase at day 5. The decrease in *meox2* expression delayed in hyperoxic AEC II in comparison

with normal AEC II (day 1 v.s. day 3). *Fdps* showed a distinct expression pattern between hyperoxic and normal AEC II. The difference may be due to the injury of AEC II by hyperoxia.

DISCUSSION

The gene expression profiles of freshly isolated AEC II from normal, hyperoxic, and recovering rats were investigated using a DNA microarray. We identified and verified 14 genes that changed significantly during hyperoxia exposure or early recovery stages. These genes are possibly involved in apoptosis, cell proliferation and differentiation. The mRNA expression of the six cell differentiation-related genes was determined in an *in vitro* trans-differentiation culture model of AEC. The information from this study may serve as a starting point for further investigation of alveolar injury and repair.

Hyperoxia injury and repair is a complex model composed of various biological processes, including hyperoxia-mediated lung injury during the exposure and subsequent lung tissue repair (cell proliferation, differentiation and apoptosis). Since these events are sequential, we used a loop design to compare adjacent time points in order to better probe gene expression in a particular event such as cell proliferation and differentiation. This method has several advantages over a reference design. In a reference design, each time point needs to hybridize to a common reference (i.e. control, or HOX, or pooled samples). Since any two time points must be indirectly compared through a common reference, the reference design results in accumulated errors from two hybridizations (i.e. each time point vs. reference). Also the reference design has a lower dynamic range. In the case of pooled samples as a reference, assuming that the expression level of a gene in C is 100 and that of any of the other 5 samples (HOX, REC 1D, REC 3D, REC 5D, and REC 7D) is 1, the ratio of C vs. the reference is $100/(105/6) = 5.7$. This is much lower than the ratio of C vs. another sample ($100/1 = 100$) in the loop design.

To analyze our data, two statistical tests were used based on fluorescence intensities and $\log_2$ ratios, which eliminated type I (false positive) and type II (false negative) errors resulting from the multiple comparisons of thousands of genes. For instance, there were about 389 false positive genes (5% of 7,782) after one-way ANOVA of fluorescence intensities. The false positive genes were reduced to about 19 (5% of 389) after a t-test of $\log_2$ ratios. Thus, the two-step tests greatly improved the reliability of the genes identified from the DNA microarray data. With an alternative one-step approach, Bonferroni correction, no significantly differential gene expression was detected because of the p-value adjustment to 6.43×10^{-6} ($=0.05/7782$). This correction insured that the global type I error was 0.05, but increased the type II error and excluded many truly differentially expressed genes.

Of 10,000 genes, we identified 50 genes that significantly changed between at least two time points based on statistical analyses and a 2-fold cut-off value. The number of the identified genes appears to be low compared to the results from a recent study in which 385 genes were identified from 48 hrs hyperoxia-exposed mouse lung tissues [14]. There are several possibilities for the difference: (a) we used more stringent criteria in data analysis. For example, we used a 2-fold cut-off value while the mouse study used a 1.5-fold cut-off value. (b) we used a single type of cells (AEC II) for gene expression profiling v.s. whole lung tissue in the mouse study. (c) rats and mice may respond to hyperoxia differently.

Surprisingly, of the 50 genes, we did not find “classical” antioxidant genes such as manganese superoxide dismutase and surfactant protein genes, although two genes, cytochrome p-450 isozyme 5 and metallothionein 2 are on the list. These genes were also not identified by a similar DNA microarray analysis of the hyperoxic mouse lungs [14]. However, these genes

have been reported to be changed in the lung exposed to hyperoxia [24-28]. The discrepancy may be due to the sensitivity of the detection methods or the absence of the genes on the arrays.

Six genes identified in this study are involved in cell proliferation and differentiation: *dlk1*, *bmp4*, *meox2*, *fdps*, *retnl* and *rgc-32*. Dlk1 (also known as preadipocyte factor 1, Pref-1) is a member of the notch/delta/serrate family and contains 6 EGF-like repeats and a transmembrane domain. It functions in cell fate determination and cell differentiation in various tissues and cells. For instance, Dlk1 inhibits the differentiation of adipocytes [29], bone marrow stromal cells [30], human hematopoietic cells [31], adrenal gland cells [32] and neuroendocrine cells [33]. Dlk1 also modulates the IGF-1 receptor and regulates the ERK/MAPK signaling pathway during cell differentiation [34]. Dlk1 may play dual roles during AEC II proliferation and differentiation. First, it may interact with notch receptors on the plasma membrane of adjacent cells (e.g. AEC I and AEC II) and regulate cell differentiation through the notch signaling pathway [35]. Second, Dlk1 or the notch-dlk1 complex may modulate the IGF1 receptor, which in turn affects cell proliferation through the IGF-ERK-MAPK pathway. The expression of Dlk1 in AEC II was increased during hyperoxia exposure but decreased during early recovery, indicating its role in AEC II proliferation and trans-differentiation to AEC I. This is also consistent with the observation that Dlk1 was highly expressed in the isolated AEC II, but was markedly less expressed in AEC I-like cells.

Bmp4, a member of the TGF- β superfamily, has been extensively studied during fetal lung development [16,36]. It is expressed highly in the distal tips of the fetal epithelium to a much lesser extent in the adjacent mesenchyme. Over-expression of bmp4 in the lung distal epithelium leads to the inhibition of cell proliferation and epithelial hypoplasia [37]. In the embryonic epithelium culture, the addition of bmp4 reduces epithelial growth and differentiation [38]. However, in fetal lung organ culture, the inclusion of bmp4 in the culture medium increases branching morphogenesis and cell proliferation, while the direct injection of bmp4 into the lung lumen has no effect [39]. A recent study has demonstrated that bmp4 up-regulates the FGF7/10 receptor, and thus acts at the upstream of the FGF signaling pathway [40]. However, another study has shown that the FGF7-MAPK and TGF- β signaling pathways are antagonized in the AEC II cell culture [41]. Therefore, bmp4 may play an essential role in the crosstalk between the FGF7 (KGF) and TGF- β pathways.

Meox2 is a transcription factor of the homeobox family with a helix-turn-helix motif, regulating cell proliferation, differentiation, and migration. Meox2 is also known as growth arrest-specific homeobox (Gax) [42]. Meox2 is down-regulated in vascular smooth muscle cells following mitogen activation *in vitro* and *in vivo* [43,44]. It inhibits cell proliferation in a p21^{WAF/CIP}-dependent manner [45]. Over-expression of meox2 also inhibits cardiomyocyte proliferation [46]. The deletion of meox2 leads to defective differentiation and morphogenesis of limb muscles [47]. In the placenta, meox2 may regulate the interaction of epithelial and mesenchymal cells [48]. Meox2 may have an inhibitory effect in the G0/G1 transition of the cell cycle and is down-regulated by ERK1/2-MAPK-p38 δ activation [42].

Fdps is an enzyme in the isoprene biosynthetic pathway for the synthesis of the cholesterol and nonsterol metabolites in the peroxisome [49]. Fdps plays an important role in colon cancer [50] and prolongs the activation of FGF-ERK-MAPK signaling by binding to the FGF receptor [51]. It has dual roles in cell proliferation and differentiation by controlling lipid synthesis and stabilizing FGF receptors. During the hyperoxia exposure and early recovery stages, an increased expression of Fdps may enhance AEC II proliferation through the FGF-induced signaling pathways.

Rgc32 promotes cell proliferation by translocating to nuclei and stimulating quiescent cells to enter into S-phase in aortic smooth muscle cells upon complement activation [52,53]. In

cytosol, it is a regulatory subunit of the M-phase promoting factor (MPF) for the G2/M phase transition. In the nucleus, it directly interacts with cyclin—dependent kinase p34^{cdc2} and is essential for G1/S phase transition. Cyclin-dependent kinase 2 (cdc2) inhibits the ERK-MAPK signaling pathway during mitosis through the modulation of the meiosis-specific serine/threonine-protein kinase (MEK1) [54].

The last gene, *retnla*, is a cytokine with an unknown cellular receptor. In adipose tissue, Retnla has an inhibitory effect on the differentiation of 3T3-L1 preadipocytes to adipocytes [55]. The expression of Retnla in AEC II is also stimulated in the bleomycin-induced pulmonary fibrosis model. Using co-cultures of AEC II and fibroblasts, Retnla has been found to promote myofibroblast differentiation independent of TGF- β [56]. Additionally, Retnla stimulates the proliferation of pulmonary microvascular smooth muscle cells [57]. An increase of *retnla* during the initial recovery of AEC II after hyperoxia exposure may indicate its role in promoting the differentiation of AEC II to AEC I via an autocrine regulation.

A working model illustrating the relationship between 6 genes during AEC proliferation and differentiation is shown in Fig. 8. Bmp4 secreted from mesenchymal cells binds with the BMP receptor (BMPR) in AEC II initiates the TGF β signaling pathway and cross-talks with the FGF signaling via up-regulation of the FGF receptor (FGFR). Fdps directly interacts with FGFR and modulates the FGF signaling. The activation of FGF signaling leads to the activation of ERK, which regulates Meox2, while rgc32 inhibits ERK via interacting with cdc2. Rgc 32 also regulates the G2/M phase transition via MPF. Dlk 1 on the plasma membrane of AEC II regulates differentiation of an adjacent AEC I or AEC II by interacting with the notch receptor on the cell membrane of these cells. Dlk1 also modulates the IGF receptor (IGFR) and thus the ERK pathway. The receptor of retnla or its down stream signaling is unknown, but it may regulate AEC differentiation in an autocrine manner. Our working model has two features. First, most of the genes are linked to the MAPK signaling and modulate the components of MAPK pathways. Therefore, these genes may fine-tune the MAPK signaling pathway. Second, cell-cell communication or interaction plays a pivotal role in the proposed model. Signaling molecules (IGF, FGF, Bmp4 and Retnla) secreted from adjacent cells or AEC II itself may directly stimulate or inhibit AEC II proliferation and differentiation. Dlk1-Notch may induce AEC II differentiation by a direct cell-cell contact via the dlk1-notch signaling pathway.

In summary, we identified 14 genes changed in AEC II during the hyperoxia exposure and the early recovery phase in a rat model using a DNA microarray and real-time PCR. We propose a model for AEC II proliferation and differentiation, in which the 6 genes modulate the MAPK and TGF- β signaling pathways to fine-tune the balance of AEC II proliferation and differentiation. Future studies on these 6 genes will improve the understanding of AEC II proliferation and differentiation through the modulation and integration of MAPK, TGF- β and other signaling pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A

B

Fig 1. DNA microarray analysis of AECII during hyperoxia exposure and recovery

(A) Loop hybridization design. Six AEC II samples were isolated from the normal control (C), 48 hour-hyperoxia exposed (HOX), and subsequent recovery in room air for 1, 3, 5, and 7 days (REC 1D, REC 3D, REC 5D, and REC 7D). The samples were paired in a loop style, each with dye-flipping: C-HOX, HOX-REC 1D, REC 1D-REC 3D, REC 3D-REC 5D, REC 5D-REC 7D, and REC 7D-C. There were 4 biological replications (4 independent cell preparations). (B) Differentially expressed genes. Two statistical analyses, one-way ANOVA and paired t-test, identified 50 genes that had a fold change of more than 2 between at least two time points. The numbers of the genes that had a fold change of >2 (increase) or <2 (decrease) were counted. The percentage is the sum of the increase and the decrease count divided by total gene count.

Fig 2. Scatter plots

Each plot represents normalized fluorescence intensities of AEC II isolated from the normal control (C), 48 hour-hyperoxia exposed (HOX), and subsequent recovery in room air for 1, 3, 5, and 7 days (REC 1D, REC 3D, REC 5D, and REC 7D). Gray dots are 10K features (genes) on each microarray, and black dots highlight the significantly changed 50 genes with 2 or higher fold-change during hyperoxia exposure and recovery. The X- and Y-axes are the normalized fluorescence intensity average in arbitrary units. Dash-lines outline the 2-fold changes between the X- and Y-axes. The 50 genes were identified based on the changes between at least two time points. Some genes did not change at some time points and thus some of the black dots fell between two dash-lines at the different time points.

Fig 3. Gene expression patterns in AECII during hyperoxia exposure and recovery

(A) Changes in Log₂ ratio between two time points. Fifty genes, identified by statistical tests and a 2-fold change cut-off value were classified into 7 groups (A-G) and one orphan group (O, 2 genes) based on significant changes at two or more time points. For the sake of clarity, only patterns A-D are shown. The Y-axis is the log₂ ratio. The values shown are means $\pm$ S.D. of all the genes in each pattern. C, normal alveolar type II cells (AEC II), HOX, REC 1, REC 3D, REC 5D, REC 7D: AEC II from 48 hour hyperoxia exposure and subsequent recovery for 0, 1, 3, 5, and 7 days. *P<0.05, log₂ ratio of each gene in each pattern vs. 0. The expression pattern symbol: ~, no significant change; ^, P<0.05, up-regulation and v, P<0.05, down-regulation. (B): Simplified plots showing the change trends of gene expression at each time point in comparison with the control cells. A change with a log₂ ratio of >1 or <-1 was scaled to 100-200 or 0-100. The lines between other two points had a -45 or 45-degree slope. Y-axis

is relative mRNA abundance (scale 0-200 with the control cells being 100). X-axis represents all of the time points listed at the bottom of the graph. * $p < 0.05$; HOX or REC 7D vs. C; # $p < 0.05$, HOX vs. REC 1D, or REC 5D vs. REC 7D.

Fig 4. Comparisons of log₂ ratios from DNA microarray and real-time PCR

The genes from pattern B (Panel A), Pattern C (Panel B) and Pattern D (Panel C) were selected for verification by quantitative real-time PCR (qPCR). The microarray data was LOWESS-normalized and real-time PCR data was normalized to β -actin. Error bars represent standard errors. All of the log₂ ratios were significantly different from 0 ($p < 0.05$). There were 4 biological replications for both microarray and real-time PCR analyses. C: normal alveolar type II cells (AEC II); HOX and REC 1D; 48-hour hyperoxia exposure and recovery for 0 and 1 day.

Fig 5. Bmp-4 and retlna protein expression in hyperoxic and recovering lungs

(A) The AEC II were from 48-hour hyperoxia-exposed (HOX) and recovering for 1 day (REC 1D) rats. Bmp-4 protein level was detected by Western blot using anti-bmp-4 antibodies, quantified by densitometry, and normalized to β -actin. The results were expressed as a percentage of HOX. Data shown are means $\pm$ SE (n=5). *P<0.05 v.s. HOX. (B) The lung tissue sections from control (C), 48-hour hyperoxia-exposed (HOX) and recovering for 1 day (REC 1D) rats were stained with anti-retlna antibodies and ABC reagents. An increased staining was observed in REC 1D in comparison with C and HOX.

Fig 6. Expression patterns of 6 cell differentiation-related genes in an AEC culture model
 AEC II isolated from control rats were cultured on plastic dishes for 1, 3, 5, and 7 days (D0, D1, D3, D5, and D7). The mRNA level was measured by quantitative real-time PCR. The results were expressed as log₂ ratios between two time points. The values are mean log₂ ratio, and the error bars are the standard errors. *P<0.05 vs. D0; #P<0.05 vs. D0. Data were from 4 independent cell preparations, each measured in duplicate. (A) SP-A, SP-C and T1 α ; (B) retnl and dlk1; (C) rgc32, bmp4, meox2 and fdps.

Fig 7. Expression patterns of 6 cell differentiation-related genes in cultured AEC II isolated from 48 hr hyperoxia-exposed rats

AEC II isolated from 48 hr hyperoxia-exposed rats were cultured on plastic dishes for 1, 3, 5, and 7 days (D0, D1, D3, D5, and D7). The mRNA level was measured by quantitative real-time PCR. The results were expressed as log₂ ratios between two time points. The values are mean log₂ ratio, and the error bars are the standard errors. *P<0.05 vs. D0; #P<0.05 vs. D0. Data were from 3 independent cell preparations, each measured in duplicate. (A) SP-B, SP-C and T1α; (B) retlna and dlk1; (C) rgc32, bmp4, meox2 and fdps.

Fig 8. Functional scheme of the 6 genes during AEC II proliferation and differentiation
 The middle double line oval represents an AEC II during proliferation and differentiation, and the other two smaller double line ovals are adjacent cells *in vivo* or *in vitro*. The gray circles inside cells are nuclei. Round text boxes represent the proteins. The 6 genes are highlighted. Proteins in cylinders are plasma membrane receptors. Highlighted arrows indicate protein-protein interactions related to 6 genes, and gray arrows indicate other protein-protein interactions. Signaling pathways or interactions are showed in gray ovals. Main abbreviations: MPF, M-phase promoting factor; ERK, extracellular receptor kinase.

Table 1**Real-time PCR primers**

Gene name	Sequence (forward and reverse)
Heat shock 10 kd protein 1 (hspe 1)	AAG GAG TGC CGC TGA AAC TGT GCT GAA TCT CTC CAC CCT TTC C
Ccaat binding transcription factor cbf subunit c (nfyc)	TCC AGA CCC TTG CTA CCA ATG GCT GGT TGG CTG ACT GAA TAA A
Mesenchyme homeobox 2 (meox2)	CCC GAG AAA AGG AAC TGG TGA A TAT CGC GAC TGT CGT CAT TTG C
Stearyl-coa desaturase (scd1)	GGA GGA CAC GCT GAA ACT CTC A GGA AAA CCT CTG CCA TGC ATC T
Testis-specific farnesyl pyrophosphate synthetase (fdps)	GCA GAC TCT CGA CCT CAT CAC A CCC ATC AAT TCC AGC CAT G
Resistin-like molecule alpha (retnlα)	GCA ACT GCC TGT GCT TAA TCG T TGC GCA TGA GTC AGA TTT CCA
Thiolase aa 1-397 (acaa2)	AGA ATC ACC GCA CAC CTG GTT AAT CCC TTC AGG CTG TGT TCT G
Ferritin light chain subunit (ftl1)	GTC TTC GCG GTT AGC TCC ATA CAC GTC ATC CCG ATC AAA AAA G
Preprocathepsin d (ctsd)	TGA AGA ATG GCA CAT CCT TCG A TGG CTT CCC CAA AGA TCT GTT
Metallothionein 3 (mt3)	TGC ACC TGC TCG GAC AAA T CCC TCT TCG CCT TTG CAA A
Vimentin (vim)	CAG GAT TTC TCT GCC TCT TCC A CCT GTC CGT CTC TGG TTT CAA C
Delta-like homolog drosophila (dlk1)	CCC AGG CCC TTC TAC ATT ATC G GCA GCT TGT CAC ACA GCA ACA C
O-acetyltransferase milk fat globule membrane protein (mfge8)	CTC AGA GCA ACA GTG CCA AGG A TCC ACT GCA CAC CAT CAT CAC
Rgc32 protein	CCC TGG CAA AAT CGG CTA CTA TTG AAT ATG GTG CAG GCA GGT A
Urinary plasminogen activator receptor 2 (plaur)	TTC AGA GCT TAC CAC CGA ATG G GCC TCC AAG CAC TGA TTC ATT G
Cytosolic peroxisome proliferators -induced acyl-coa thioesterase (bach)	CAA ACC CCG ATT TCC TTT GAG AGT GTG CTC TGG TGT CCA CGA T
Metallothionein 2a (mt2a)	CGC TTC CTA GGT AAG CGC TCT A TCG CAA CAT ATT CCC CAC TCA T
Bone morphogenetic protein 4 (bmp4)	CCC TGG TCA ACT CCG TTA ATT CT CAC CTT GTC GTA CTC GTC CAG AT
Beta actin (actb)	ACC GTG AAA AGA TGA CCC AGA T CAG TGG TAC GAC CAG AGG CAT A
Surfactant protein A (SP-A)	TAA CTA AGT GCT GCC CTC TGA ACT AGG CTT TCC CAG AAA TCC
Surfactant protein B (SP-B)	AAT GAC CTG TGC CAA GAG TGT G AGG ACC AGC TTG TTC AGC AGA G
Surfactant protein C (SP-C)	AGC CCA CCG GAT TAC TCG AC AGG ACT ACC ACC ACA ACC ACG
T1 alpha (T1α)	GGC AGG TGC TAG AAA CTG GTT GCC GTT GAC GAA CCA AGA AC

Table 2 Gene ontology of the significantly changed genes in AEC II during hyperoxia exposure and subsequent recovery [†]

Pattern Symbol Meaning	Total	A	B	C	D	E	F	G	O
Cancer	1	1							
Cell cycle	5	2	1	1		1			
Cell differentiation	2				1			1	
Cell proliferation	1		1						
Chaperone	1					1			
Histone	2	2							
Hormone	1		1						
Intermediate	3	1			1			1	
Filament	1								
Ion binding	1								
Ion channel	2	1				1		1	
Lamin	1								
Metabolism	6		1	1	1	1	1	1	
mRNA export	1								
Peptidase	1				1				
Phagocytosis	1			1					
Phosphorylation	3		1				2		
Telomerase	1						1		
Transcription	6	1	1	1		1	1		1
Unclear	11	1	1	2	2	2	1	1	1
Total	50	9	7	6	6	7	7	6	2

[†] The significantly changed genes were identified by two statistical tests (one-way ANOVA and paired t-test) and using a fold change cut-off value of 2. Symbol meanings, ^, up-regulation (p<0.05, log2 ratio>1.0), v, down-regulation (p<0.05, log2 ratio<-1.0), ~, not significantly changed. The symbols were arranged in the order of HOX/C, REC 1D/HOX, REC 3D/REC 1D, REC 5D/REC 3D, REC 7D/REC 5D and C/REC 7D.

Table 3 The known functions of 14 verified genes [†]

Genbank	Gene name	Function
Pattern B (~~~~~): <u>REC 1D > HOX</u>		
NM_053333	Resistin-like molecule alpha (retnl)	May act as a hormone; lung inflammation
NM_012827	Bone morphogenetic protein 4 (bmp4)	Plays a role in induction of cell proliferation
NM_031840	Testis-specific farnesyl pyrophosphate synthetase (fdps)	Isoprene biosynthesis; cell membrane synthesis
NM_012966	Heat shock 10 kd protein 1(hspe1)	Stress-induced mitochondrial matrix protein and molecular chaperone
NM_017149	Mesenchyme homeobox 2(meox2)	Homeobox gene; may have a regulatory function in the G0-to-G1 transition of the cell cycle in vascular smooth muscle cells
Pattern C (~~~~~): <u>HOX > REC 1D</u>		
NM_013214	Cytosolic peroxisome proliferator-induced acyl-coa thioesterase (bach)	Involved in fatty acyl-CoA oxidation
NM_017350	Urinary plasminogen activator receptor 2 (plaur)	Involved in plasminogen activation
NM_054008	Rgc32 protein (rgc32)	Increased expression is detected in oligodendrocytes in response to complement activation; may play a role in cell cycle activation
NM_012811	O-acetyltransferase milk fat globule membrane protein(mfge8)	Involved in the o-acetylation of gd3 ganglioside sialic acid
M11794	metallothionein 2a (mt2a)	Neuroprotective proteins, may provide a cellular defense strategy in response to DNA damage
Pattern D (~~~~~): <u>HOX > C and REC 1D</u>		
NM_053744	Delta-like homolog drosophila(dlk1)	Involved in maintaining the undifferentiated status of the preadipocyte
X05341	Thiolase aa 1-397 (acca2)	Mitochondrial matrix protein with acetyl-coenzyme A acyltransferase 2 activity
NM_053968	Metallothionein 3 (mt3)	May inhibit cell growth in nervous system
NM_022500	Ferritin light chain subunit 1 (ftl1)	Iron storage

[†]The function descriptions are from GeneRIF (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=Retrieve&dopt=Graphics>).