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Key Developmental Regulators Change During Hyperoxia-Induced Injury and Recovery in Adult Mouse Lung

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Abstract Developmentally important genes have recently been linked to tissue regeneration and epithelial cell repair in neonatal and adult animals in several organs, including liver, skin, prostate, and musculature. We hypothesized that developmentally important genes play roles in lung injury repair in adult mice. Although there is considerable information known about these processes, the specific molecular pathways that mediate injury and regulate tissue repair are not fully elucidated. Using a hyperoxic injury model to study these mechanisms of lung injury and tissue repair, we selected the following genes based upon their known or putative roles in lung development and organogenesis: *TTF-1*, *FGF9*, *FGF10*, *BMP4*, *PDGF-A*, *VEGF*, *Ptc*, *Shh*, *Sca-1*, *BCRP*, *CD45*, and *Cyclin-D2*. Our findings demonstrate that several developmentally important genes (*Sca-1*, *Shh*, *PDGF-A*, *VEGF*, *BCRP*, *CD45*, *BMP4*, and *Cyclin-D2*) change during hyperoxic injury and normoxic recovery in mice, suggesting that adult lung may reactivate key developmental regulatory pathways for tissue repair. The mRNA for one gene (*TTF-1*), unchanged during hyperoxia, was upregulated late in recovery phase. These novel findings provide the basis for testing the efficacy of post-injury lung repair in animals genetically modified to inactivate or express individual molecules. J. Cell. Biochem. 100: 1415–1429, 2007.

Key words: hyperoxia-induced lung injury; lung repair; developmental regulators; adult mice; fibroblast growth factors

There is a limited understanding of the molecular regulation of cellular responses to injury and mechanisms of tissue repair in the lung. Hyperoxia is a reproducible and frequently used model to evaluate these processes, both in vitro and in vivo, and is a model relevant to the clinical management of critically ill patients who require oxygen supplementation. Although necessary for patient survival, oxygen in high concentrations is damaging to the lung and causes a nonspecific injury resembling acute respiratory distress syndrome (ARDS) [Kinnula et al., 1995 (in review)]. The resulting oxidative stress, with increased production of reactive oxygen species and free radicals, leads to lung injury through damaging effects on DNA, lipids, and other molecules [Kinnula et al., 1995].

The alveolar epithelial gas exchange surface is composed of cuboidal type II cells that produce surfactant and act as tissue stem cells, and highly flattened type I cells, which cover about 95% of the surface [Crapo et al., 1980]. In the lung, oxygen toxicity has been shown to result in DNA strand breakage and endothelial cell damage with capillary leak and alveolar edema [Crapo et al., 1980; Kinnula et al., 1995]. Electron microscopic studies of exposed lung have established that type I alveolar cells become damaged and detach from the basement membrane following prolonged exposures [Kistler et al., 1967; Crapo et al., 1980; Kinnula et al., 1995]. Alveolar type II cells are more resistant to oxidative stress and play key roles in tissue repair by dividing to produce cells that acquire a type I cell phenotype and repair

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the surface [Evans et al., 1975]. A general time course of these events has been described in several animal models, and it is well established that adult rodents can survive for 72 h in >95% oxygen [Evans et al., 1975; Crapo et al., 1980].

Despite cellular changes, the specific molecular pathways involved in lung repair processes are not well defined. In other adult or neonatal organs including skin, liver, pancreas, muscle, and vasculature, developmentally important genes have been linked to cellular repair [Steiling and Werner, 2003 (in review); Zhao and Hoffman, 2004; Beer et al., 2005]. Likewise other studies show that developmental genes can confer protection against injury [Panos et al., 1995] and are involved in organ regeneration [Steiling and Werner, 2003; Zhao and Hoffman, 2004]. In addition when specific regulatory pathways, such as the Ptc-Shh pathway, are overexpressed or when inhibitory controls are blocked, dysregulated proliferation and tumorigenesis can occur [Karhadkar et al., 2004].

We undertook these studies to survey the expression patterns of selected genes following hyperoxic lung injury to determine if expression of developmentally important regulators changes during injury or post-injury repair in adult mice. Many of the molecules selected for study are potent regulators of cell proliferation and differentiation during lung development as evidenced by severe lung phenotypes in null mutant animals. By QRT-PCR and Western blot we found significant increases in expression of some, but not all, of the genes studied. To determine if transcripts for selected growth factors could be upregulated by signaling pathways directly activated by hyperoxia, mRNAs were measured by QRT-PCR in a lung cell line cultured in normoxia or hyperoxia. In hyperoxic cells, mRNAs for FGF9, FGF10, and PDGF-A were markedly decreased in contrast to their increase in vivo. These findings suggest that changes in expression of these molecules in the intact mouse lung indirectly result from complex signaling pathways, rather than directly from activation by oxygen or its metabolites.

MATERIALS AND METHODS

Mouse Exposures

The hyperoxia exposure protocol has been published previously [Cao et al., 2003] and is summarized here. This exposure regimen has been widely used and is well characterized in terms of extent and sites of histologic damage in the lung. Adult C57BL mice (19–21 g) (Charles River, Wilmington, DE), a hyperoxia-sensitive strain, were housed under standard conditions with food and water ad libitum. Groups of five mice were placed in Plexiglass cages and exposed to >95% oxygen or room air (controls and hyperoxia recovery). Oxygen concentrations were checked daily with an O_2 monitor (J. H. Emerson, Cambridge, MA). Three animals at each time point were sacrificed at 0, 24, 48, and 72 h of oxygen or room air (control) exposure and after 1, 5, 14, and 28 days of post-exposure recovery in room air. Although by 72 h the animals were huddled together and lethargic with disordered fur, all animals survived the hyperoxic exposure with this protocol as expected. Previous studies have indicated that an additional 24-h exposure results in high mortality. Animals were anesthetized with a lethal intraperitoneal injection of sodium methohexital (Eli Lilly, Indianapolis, IN) and were then exsanguinated by severing of the abdominal aorta. The lungs were removed and frozen immediately on dry ice/100% ethanol for analysis as described below.

RNA Purification and cDNA Generation for QRT-PCR

Lungs were homogenized in 10 ml Trizol reagent (Gibco Invitrogen Corporation, Grand Island, NY) and total RNA was purified by standard methods (12-Cao) with sequential chloroform and isopropyl alcohol extractions to produce an RNA pellet. The pellet was washed with 75% ethanol in DEPC-treated water, air dried, and resuspended in 30 μl DEPC water. RNA concentrations were measured in 1 μl samples using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Samples were then treated with DNA-*free*™ DNase (Ambion, Inc., Austin, TX) for 30 min at 37°C, the reaction was terminated with DNase inactivation reagent (0.1 vol or 5 μl) (Ambion, Inc.) for 2 min at RT, followed by centrifugation at 10,000*g* to pellet the inactivation reagent. RNA concentrations were measured as before and samples were stored at −70°C.

For use in QRT-PCR, cDNA was generated using 1 μg RNA, 1 μl primer poly dT (0.5 μg/μl) (Promega, Madison, WI) in a total volume of 10 μl DEPC H_2O. After annealing the following

reagents were added to the annealed primer/template (Promega): 5 μl AMV RT 5× buffer, 2.5 μl dNTP mix, 1 μl (25 units) RNasin (40 μ/μl), 2.5 μl sodium pyrophosphate (40 mM), 1 μl (15 units) AMV reverse transcriptase, and DEPC H_2O to final volume of 25 μl. The mixture was vortexed, centrifuged briefly (10,000*g* for 20 s, RT) and incubated for 1 h at 42°C.

QRT-PCR

Primers and probes for VEGF, PDGF-A, FGF9, FGF10, CD45, BCRP, Cyclin-D2, Shh, and Sca-1 mRNAs were created using Primer Express software (Applied Biosystems, Foster City, CA) (Table I) with specificity confirmed by BLAST. Oligonucleotide primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) and used in conjunction with Syber Green Master Mix (Applied Biosystems). Primers for TTF-1, Ptc, and BMP4 were obtained from Assays on Demand (Applied Biosystems) and used in conjunction with Taqman 2× PCR Master Mix (Applied Biosystems).

Optimal PCR conditions and dilutions for each gene were determined and a calibration curve spanning five logs was created for each gene using normal adult male mouse lung RNA. The minimum concentrations of forward and reverse oligonucleotide primers used in Syber Green reactions to obtain a single product were determined by generating a dissociation curve for each gene. cDNA (5 μl) was combined with either 25 μl Taqman 2× PCR Master Mix, the appropriate amount of Assay on Demand reagents (in μl of 20× solution for each assay: Ptc, 1.5; TTF-1, 2.5; BMP4, 2.5) and H_2O (final volume 50 μl) or with 25 μl Syber Green, primer oligonucleotides, and H_2O (final volume 50 μl). Each sample was run in triplicate. Controls lacking template were run for each gene. Means of C_T numbers from triplicate samples were calculated and used to determine amounts (ngs) of RNA from the reference calibration curve described above.

Normalization of mRNA Data

It is well established the genes such as GAPDH, β-actin, α-tubulin, and 18S ribosomal RNA, commonly used to normalize mRNA levels, are directly or indirectly regulated by altered oxygen concentrations [Nici et al., 1991; Ho et al., 1996; Carter et al., 1997; Graven et al., 1999; Perkowski et al., 2003]. Our preliminary QRT-PCR experiments on hyperoxic lung tissue generally confirmed that the magnitude of change of these mRNAs throughout the exposure period precluded their use as normalizing values. The general considerations related to normalization of QRT-PCR findings between two experimental groups have recently been discussed [Dheda et al., 2004; Khimani et al., 2005]. These reports recommend the use of one or more genes that does not change in the experimental conditions of interest to normalize levels of other genes that change. Our first QRT-PCR data showed that, of the >20 mRNAs studied, Ptc mRNA levels were the least variable (Supplemental Data Table I). Ptc mRNA expression was therefore used as the normalizing value. Because Ptc is expressed in lung cells but not in inflammatory cells that may enter the lung in response to hyperoxia and injury, this additionally normalizes changes in lung cell genes to baseline gene expression levels in the appropriate cell populations, and avoids the dilutional effects from influx of inflammatory cells to the lung.

Statistical Analysis

To eliminate statistical outliers for each time point the *Q*-test was utilized once per set of data, using a 90% confidence interval. The *t*-test was

TABLE I. Primers

Gene	Forward primer	Reverse primer
CD45	5′-GAC CAG TTT TGA GGT GGA AAGC-3′	5′-TGC ACT TCT CAG CAG TCC CAT-3′
Sca-1	5′-TGC TTC TGT TCA GCC TGA GCT-3′	5′-GGC TCC AGG AAG AAT TGT TGC-3′
BCRP	5′-CCA CGA CTG GTT TGG ACT CAA-3′	5′-GAA TAC CGA GGC TGA TGA ATGG-3′
Cyclin-D2	5′-AGC AGT GAA CCC AAG GAG GAA-3′	5′-GCG CTT CTT ACA TCC AAA ATG G-3′
VEGF	5′-GAG CGG AGA AAG CAT TTG TTT G-3′	5′-CGT TCG TTT AAC TCA AGC TGC C-3′
PDGF-A	5′-TAA CAC CAG CAG CGT CAA GTG-3′	5′-CTG GAC CTC TTT CAA TTT TGG C-3′
FGF9	5′-TGA TGG CTC CCT TAG GTG AAG T-3′	5′-GAC CCA GGT GGT CAT TTA GCA-3′
FGF10	5′-TGC ACA TAC ATG AGC CCT TTG T-3′	5′-TTT GCT CAG GTT AAG CCC CAG-3′
Shh	5′-ACT CAC CCC CAA TTA CAA CCC-3′	5′-TGC TCC CGT GTT TTC CTC A-3′

applied using a $P < 0.05$ to identify statistically significant results. All groups were analyzed including controls, combined and individual hyperoxia time points, and normoxic recovery time points. Data are presented as the mean of 3–5 individual experimental samples +/− the standard error of the mean (SEM).

Protein Purification

Protein was extracted from lungs homogenized in RIPA buffer containing 0.1% SDS and protein inhibitors (aprotinin 2 μg/ml, phenylmethylsulfonyl fluoride (PMSF) 100 μg/ml, and leupeptin 2 μg/ml). After centrifugation to pellet debris and DNA, SDS was added to the supernatant to 2%. After centrifugation, protein concentrations were measured with a modified Bradford method (BioRad, Hercules, CA) using bovine serum albumin as standard.

Western Analysis

Western blots of selected proteins based on QRT-PCR data were used to determine changes in protein levels during the injury phase (0, 24, 48, 72 h hyperoxia) and after 1-day normoxic recovery. Microarray analysis [Perkowski et al., 2003] shows altered expression of large groups of mRNAs between 8- and 48-h exposure, making it likely that important molecular pathways are activated quickly after initiation of lung injury. For each gene three independent experiments were performed with protein from all five time points. The protein loaded, primary antibody conditions, and duration of exposure were optimized for each protein. Depending on the protein, 50–100 μg of total protein was loaded per lane. Polyacrylamide gels were prepared by standard methods as follows: Ptc, 7.5% gels; BMP4, Sca-1, FGF10, 16% gels; FGF9, 12% gels; and electrophoresed using standard conditions. Because of the relatively low abundance of the growth factors, positive and negative controls were run when possible; positive controls: recombinant proteins rFGF10 and rFGF9 (Santa Cruz Biotechnology, Inc, Santa Cruz, CA); protein from MLE15 cells, expressing highly abundant Ptc and Sca-1 mRNAs by QRT-PCR, for Sca-1 and Ptc; embryonic (E15) mouse lung protein for BMP4; negative controls: FGF10 null mouse embryonic protein.

After transfer PVDF membranes were blocked in 1× TBST (Tris-buffered saline with 0.1% Tween-20) containing 5% milk for 1 h, washed three times for 5 min with 1× TBST, and exposed overnight at 4°C to FGF9 (1:500), Ptc (1:200), Sca-1 (1:500), and BMP4 (1:100) primary antibodies in 1× TBST containing 1% dry milk. For FGF10 the membrane was exposed to primary antibody (1:400) in 1× TBST containing 1% dry milk for 36 h. For FGF9, BMP4, Ptc, and FGF10 after exposure to the primary antibody, membranes were washed in 1× TBST for 10 min × 6 washes followed by incubation in secondary antibody [anti-rabbit (FGF9, Ptc), or anti-goat (FGF10, Sca-1, and BMP4) at 1:10,000, 1 h, RT] and washed ×6 in 1× TBST for 10 min each. This protocol was also used for Sca-1 immunoblots, except that 1× TBST with 0.2% TWEEN-20 was used for all washes. HRP-labeled secondary antibodies were detected using the Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) with signal intensities such that densitometry could be used for quantification. Equal protein loading was assessed by Coomassie blue staining of the acrylamide gel after transfer of proteins to the PVDF membrane as has been used in other publications [Baker et al., 2005; Wilson et al., 2005]. As typical housekeeping genes *GAPDH*, β-actin, α-tubulin have been shown to change in hyperoxia [Nici et al., 1991; Ho et al., 1996; Carter et al., 1997; Graven et al., 1999; Perkowski et al., 2003], Western blots were not restained for these genes as loading controls.

The following primary antibodies were used: goat anti-human FGF10 (c-17; sc-7375) and rabbit anti-human Patched (H-267, sc-9016) from Santa Cruz Biotechnology, Inc.; rabbit anti-mouse FGF9 (#5057), and goat anti-BMP4 (#5674) from Biovision (Mountain View, CA); goat anti-mouse Sca-1/Ly6 (#AF1226) from R & D Systems, Inc. (Minneapolis, MN). Secondary antibodies were horseradish peroxidase labeled anti-rabbit IgG (Ptc, FGF9) and anti-goat IgG (for FGF10, Sca-1, BMP4) (Vector Laboratories, Burlingame, CA).

Densitometry was performed using Quantity One software (Version 4.0, Bio-Rad). Signals from hyperoxia-exposed animals were normalized to normoxia controls. A minimum of three blots were analyzed for each protein with samples obtained from three separate exposure experiments.

Despite attempts with several antibodies, we were unable to detect PDGF-A protein (100 μg/lane), although PDGF-A protein was detectable

from mouse epididymal protein (positive control).

Gene Expression in Hyperoxia-Exposed Cell Lines

MLG cells, a neonatal lung fibroblast cell line established from normal lung of a ddY strain mouse, were plated on 35-mm culture plates and maintained in DMEM medium (Gibco-BRL, Gaithersburg, MD), with 10% fetal bovine serum (Gibco-BRL), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco-BRL) at 37°. At confluency, cells were exposed to 95%O_2/5% CO_2 for 2 and 4 h. Similar control cultures were maintained in 95% air/5% CO_2. RNA was extracted from 2 plates (pooled) at each time point and from controls. Three individual sets of exposures were run for each time point plus controls.

Procedures for RNA extraction and DNase treatment were as described above. cDNA was generated using 1 μg of DNA-free RNA in 2 μl 10× buffer, 4.4 μl $MgCl_2$ (25 mM), 4.0 μl deoxyNTPs (2.5 mM), 1.0 μl random hexamers (50 μM), 0.4 μl RNase Inhibitor (20 U/μl), 1.25 μl Multiscribe Reverse Transcriptase (50 U/μl) diluted to total volume of 20 μl (Taqman Reverse Transcriptase Reagents kit, Applied Biosystems). Samples were reverse transcribed (1 PCR cycle with the hexamer incubation hold [10 min, 25°C]; RT hold [60 min, 37°C]; RT inactivation hold [5 min, 95°C]).

QRT-PCR was performed as before. Results were normalized to 18S ribosomal RNA which was unchanged between 2 and 4 h of hyperoxic exposure.

RESULTS

Genes Selected for Analysis

Table II lists the genes selected for study, a description of their roles in lung development, and the characteristics of their null phenotypes, if known. The majority of these genes, growth factors and signaling molecules, are regulators of cell proliferation and differentiation during lung development. Targeted deletions of many are embryonic lethal or result in a severely altered phenotype of the lung or other organs. Two genes are putative markers of stem cells, progenitor cells from either bone marrow or lung.

TABLE II. Table of Genes

Developmental genes		
Thyroid transcription factor 1 (TTF-1, Nkx2.1)	Tissue-specific transcription factor expressed in lung epithelium and required for secondary bud formation in embryos	Lethal at term with failed formation of lung parenchyma [Kimura et al., 1996]
Fibroblast growth factor 10 (FGF10)	Critical regulator of branching morphogenesis	Neonatal lethal with failure of branching beyond trachea [Sekine et al., 1999]
Fibroblast growth factor 9 (FGF9)	Pleural gene	Neonatal lethal with lung hypoplasia [Colvin et al., 2001]
Platelet-derived growth factor-A (PDGF-A)	Essential for alveolus formation and growth	Neonatal or postnatal lethal with failed alveologenesis [Bostrom et al., 1996]
Vascular endothelial growth factor (VEGF)	Regulates endothelial cell proliferation	Mid-gestation lethal with abnormal angiogenesis [Ferrara et al., 1996]
Bone morphogenetic protein-4 (BMP4)	TGFβ superfamily growth factor that inhibits FGF10-induced branching and overall proliferation	Early embryonic lethal [Dunn et al., 1997]
Sonic hedgehog (Shh)	Regulates proliferation, differentiation, branching morphogenesis, and patterning of mesenchyme	Late fetal or term lethal with severe growth retardation, esophageal atresia and stenosis, tracheal and lung hypoplasia [Chiang et al., 1996]
Patched (Ptc)	Sonic hedgehog receptor that suppresses the signaling molecule Smoothened (Smo)	Mid-gestation lethal with lack of complete neural tube closure, overgrown headfolds, and abnormal cardiac formation [Goodrich et al., 1997]
Stem cell markers		
Breast cancer resistance protein (BCRP)	ABC transporter expressed in some stem cells including lung SP cells. Involved in mesoderm induction in development	Fewer bone marrow SP cells; non-lethal [Zhou et al., 2002]
Stem cell antigen-1 (Sca-1)	Membrane glycoprotein expressed by hematopoietic cells and pulmonary endothelium [Kotton et al., 2003]	Mild thrombocytopenia and decreased multipotent bone marrow cells; non-lethal [Ito et al., 2003]
Inflammatory cell marker		
CD45	Expressed by bone marrow—derived cells of hematopoietic lineages	Inhibition of T and B lymphocyte maturation; non-lethal [Martin et al., 2001]
Cell proliferation gene		
Cyclin-D2	Cell cycle entry and regulation of G_1/S progression	Knockout phenotype unknown; functional redundancy with other D-type cyclins

Gene Expression of Key Regulators by QRT-PCR

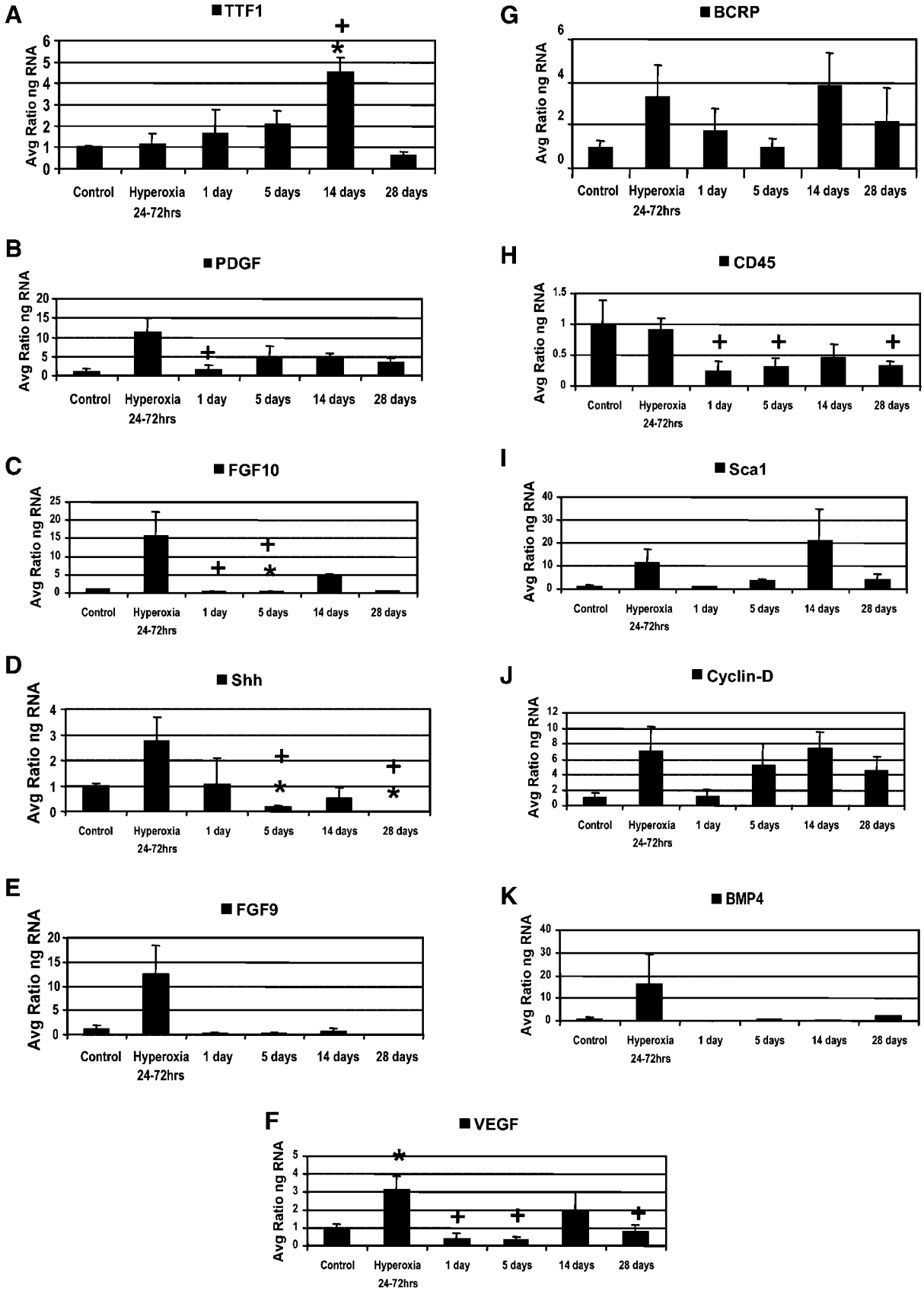
Figure 1 shows the time course of changes in mRNA abundance for each gene as measured by QRT-PCR. Expression levels are shown as a normalized ratio of the average ng RNA for the gene of interest compared to *Ptc*, a gene whose level of mRNA expression changed the least throughout hyperoxic exposure and post-exposure recovery as described in the Materials and Methods. The hyperoxia time points are grouped together and displayed as the average of the combined results for all animals exposed for 24–72 h. Given our small sample size (n = 3) and the known biologic variability between individual animals, by combining these time points, we more clearly demonstrate the impact of hyperoxia on gene expression. The changes described below are categorized as "exposure or injury" and "recovery" phases but it is likely that molecular repair commences as soon as the injury is initiated and that injury is ongoing and persists for some time after returning the animals to normoxia. Statistical analysis using the *t*-test was also performed comparing all time points to individual hyperoxia points (24, 48, and 72 h). Graphs showing the QRT-PCR results for the individual hyperoxia time points and the statistically significant changes ($P < 0.05$) from those comparisons are provided in Supplementary Material Table II and Supplementary Material Figure 1.

Gene expression changes from normoxia (control) to hyperoxia (24–72 h). Expression levels of several genes increase during hyperoxia. As is evident in Figure 1, there are increases in mRNAs for *PDGF-A*, *FGF10*, *Shh*, *FGF9*, *VEGF*, *BCRP*, *Sca-1*, *Cyclin-D2*, and *BMP4*. The magnitude of change for each gene is variable, however, with several genes having fold increases of 10–15× (*FGF9*, *FGF10*, *PDGF-A*, *BMP4*, and *Sca-1*), while others increase 3–7-fold. With the exception of VEGF and PDGF-A, these changes do not reach statistical significance. For FGF10, the *P*-value of the combined hyperoxia time points compared to control has borderline statistical significance (*P*-value 0.06); however, FGF10 demonstrates a statistically significant increase between control and 72 h of hyperoxia (*P*-value 0.03) (Supplementary Material Fig. 1C). FGF9 (Fig. 1E) demonstrates a similar trend of increasing in hyperoxia (a 12-fold peak at 72 h, not significant [N.S.]). Sca-1 (Fig. 1I) and BCRP (Fig. 1G) also trend towards an increase in hyperoxia with Sca-1 expression increasing 30-fold at 72 h in hyperoxia (Supplementary Material Fig. 1I). BMP4, shown in a recent publication to be sensitive to oxygen sensing pathways (NO regulation) [Shinkai et al., 2005], increases 15-fold in hyperoxia (Fig. 1K). The transcription factor TTF-1 (Nkx2.1) and CD45 mRNAs do not change during hyperoxia (Fig. 1A and H, respectively).

Gene expression changes from hyperoxia to normoxia (recovery). When the hyperoxia-exposed animals are put back into a normoxic environment, which is hypoxic relative to the preceding environment, mRNA levels of the increased genes returned to control values (Fig. 1). Many of these changes are statistically significant (FGF10, PDGF-A, VEGF, TTF-1, Shh, CD45). There is a significant ~20-fold decrease in FGF10 during days 1–5 of recovery (Fig. 1C). Likewise VEGF expression decreases significantly between hyperoxia and 1and 5 days of normoxic recovery (Fig. 1F). Other developmental genes demonstrate a similar trend, with the exception of TTF-1, that increases late in recovery (Fig. 1A). Shh demonstrates a trend toward increasing in hyperoxia (N.S.) but is significantly lower than baseline controls in the 5–28 days recovery period (Fig. 1D). CD45 mRNA levels are significantly decreased during the recovery phase (Fig. 1H). Cyclin-D2 mRNA decreases (N.S.) during recovery with a late sevenfold increase between 5 and 28 days (Fig. 1J).

Late gene expression changes. The time course of TTF-1 (Nkx2.1) expression is notable

Fig. 1. QRT-PCR was performed for developmentally important genes during hyperoxic injury and repair using total lung RNA from control and experimental animals. Graphs of each gene are displayed: (**A**) TTF-1; (**B**) PDGF-A; (**C**) FGF10; (**D**) Shh; (**E**) FGF9; (**F**) VEGF; (**G**) BCRP; (**H**) CD45; (**I**) Sca-1; (**J**) Cyclin-D2; (**K**) BMP4. Each graph plots the relative ratio of the average ng RNA of the noted gene compared to Ptc (n = 3 samples/group except in D [see below]) for the groups noted on the X-axis. Groups on the a-axis are: control (in normoxia), hyperoxia (combined for 24–72 h), and post-exposure recovery (1, 5, 14, and 28 days). Bars represent standard error of the mean (SEM). In D, n = 5/group for control, hyperoxia, and 1 day recovery and n = 3/group for 5, 14, and 28 days of recovery. * denotes a statistically significant result ($P < 0.05$) for the group marked compared to control. $^{+}$ denotes a statistically significant result ($P < 0.05$) for the group marked compared to the combined hyperoxia group.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FGF9
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VEGF
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BCRP
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CD45
I
Sca1
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Cyclin-D
K
BMP4

Fig. 1.

in that this transcription factor is known to regulate a number of genes that characterize the peripheral lung epithelium. After a gradual increase during post-hyperoxia recovery, TTF-1 transcripts increase significantly ~4.5-fold on day 14 (Fig. 1A). Several other mRNAs are elevated on day 14 (BCRP, Sca-1, Cyclin-D2) but these changes are not significant.

Many of the time points demonstrate statistical significance. Others show trends, yet do not reach $P < 0.05$. This is most likely the result of variability between individual animals, a small sample size ($n = 3$), and arbitrary time points selected for analysis. Although we analyzed lungs at 8 time points in contrast to the 3–4 time points more conventionally studied, the timing of true peak levels of expression of each gene would be expected to be variable and may have been missed. As these genes are tightly regulated during lung development it is likely that they are similarly under tight regulatory controls in this model.

Expression Patterns for Selected Proteins by Western Blot Analysis

Figures 2 and 3 show patterns of expression for selected proteins. Most changes in gene expression shown by QRT-PCR occur during the hyperoxic exposure period with notable decreases in expression at 1 day of normoxic

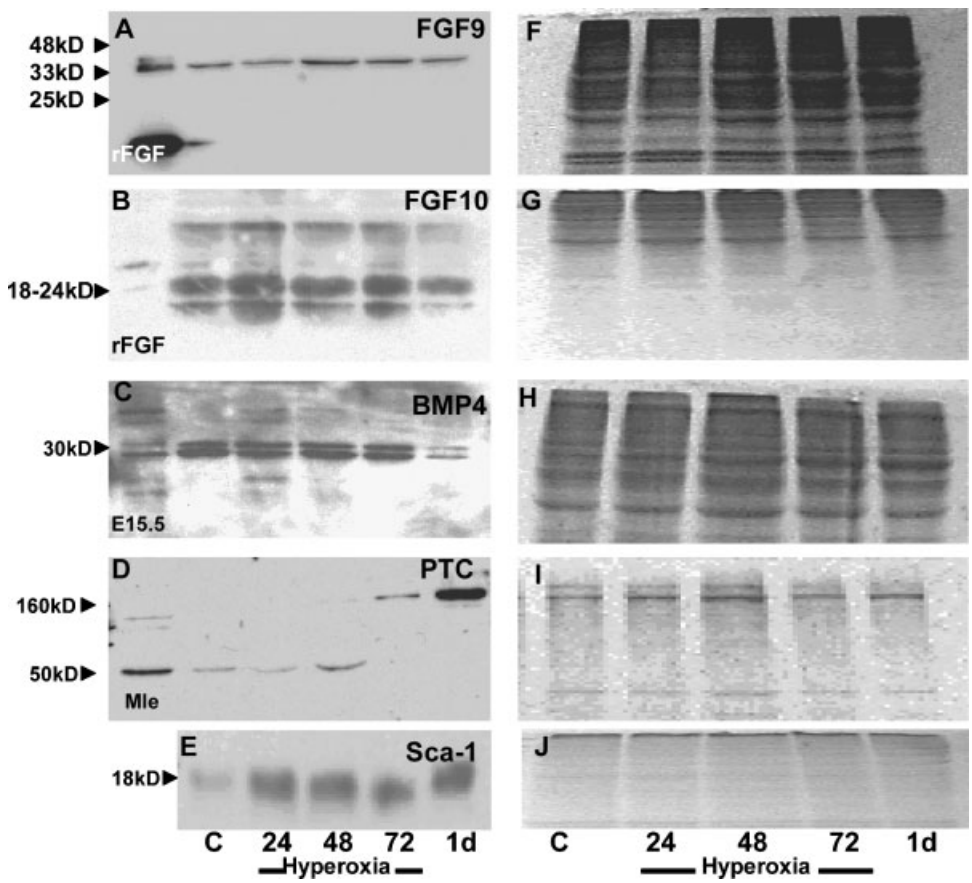

Fig. 2. Protein expression analysis in hyperoxia and normoxic recovery was performed for FGF9, FGF10, BMP4, Ptc, and Sca-1. A representative Western blot (**A**–**E**) for each protein with matched Coomassie blue stained gel after transfer (**F**–**J**) is shown. Lanes: C, normoxic control; 24, 48, and 72 h, hyperoxia; and 1 day, normoxic recovery. A: FGF9, 32.5 kDa, peaks between 24 and 48 h of hyperoxic exposure. Recombinant FGF9 protein (rFGF9), as shown as positive control, has two bands. Only one band is seen in mouse lung. B: FGF10, detected at 18–24 kDa, shows a trend toward increasing in hyperoxic exposure, with peak intensity at 72 h. Recombinant FGF10 protein (rFGF10) in lane 1, is a positive control. C: BMP4, at 30 kDa, trends toward decreasing in late hyperoxia and by 1 day or normoxic recovery. As a positive control, mouse embryonic day 15.5 lung tissue (E15.5) was used, shown in lane 1. Two bands are seen for BMP4, most likely representing the two promoters of varying molecular weights [Feng et al., 1995]. D: Ptc, 154–172 kDa, increases in intensity in late hyperoxia and 1 day of post-exposure recovery. MLE15 cells (Mle), an epithelial cell line, were used as a positive control, displayed in lane 1. E: Sca-1, 18 kDa is increased at all time points compared to control.

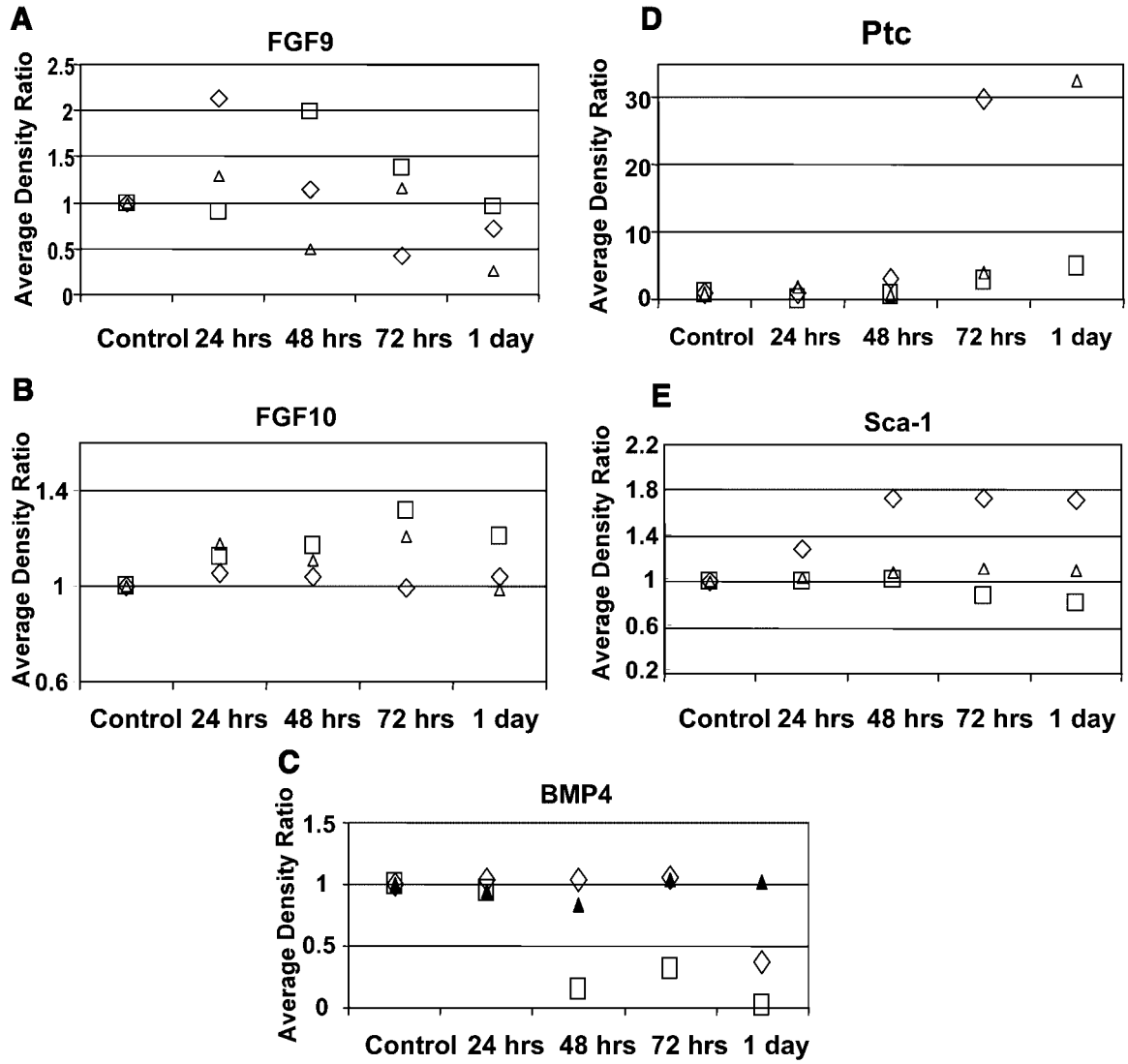

Fig. 3. A–E: Scattergrams of the densitometric values for changes in the protein concentrations compared to control (normoxia) for time points 24, 48, and 72 h (hyperoxia) and for 1 day (normoxic recovery) (n = 3 samples/time point). The different symbols represent three different Western blots. These figures display trends in changing amounts of protein while demonstrating the variability in time and fold difference for the peak change for each gene for the different animals: (A) FGF9; (B) FGF10; (C) BMP4; (D) Ptc; E, Sca-1. Note that in D, the densitometric value obtained from one Western blot for recovery day 1 was unusually high, at 185 density units, and was not included in the graph.

recovery. Therefore, only the normoxia control, hyperoxia time points (24, 48, and 72 h), and day 1 of recovery were analyzed by Western blot. Many of these proteins, for example, growth factors and transcription factors, are produced in low abundance even though they are potent developmental regulators, and there are few published Western blots of their tissue expression patterns. We maximized detection by loading 50–100 μg protein per lane and by optimizing binding conditions for each antibody, most of which directed against the human homologue of the mouse protein of interest.

A representative Western blot for each protein and the Coomassie blue-stained acrylamide gel after transfer are shown in Figure 2. Figure 3 shows scattergrams of the densitometric values from three blots (n = 3 samples/time point) for each protein. These graphs demonstrate the overall trends in change in protein expression but display also the variability in the time and fold difference of peak change for the different animals. Trends in changes in protein expression correlate well with changes in mRNAs (Fig. 1) for the various genes. FGF9 increases ~2× early in hyperoxia

with a peak at 24–48 h (Figs. 2A, and 3A). FGF10 protein demonstrates a trend toward increasing during hyperoxic exposure, with peak increase occurring between 24 and 72 h of hyperoxia (Figs. 2B, and 3B). BMP4 decreases to varying degrees (maximum decrease by 0.8-fold) late in hyperoxic exposure and on day 1 (Figs. 2C, and 3C). Although Ptc mRNA remains relatively unchanged during the hyperoxia exposure, Ptc protein increases late in hyperoxia and early post-exposure recovery with increases ranging from 4 to 180× (Figs. 2D, and 3D). The translational regulation of Ptc is complex. There are at least five isoforms of murine Ptc generated by varying use of five exons as the first exon [Nagao et al., 2005]. The mRNA undergoes alternative splicing generating multiple proteins of molecular weights ranging from 32.2 to 172 kDa [Nagao et al., 2005]. Sca-1 protein increases (nearly 2×) at all time points compared to control, most notably in late hyperoxia, although the peak time points differ for different animals (Figs. 2E, and 3E).

Cell Line Studies

Changes in oxygen tension can regulate expression of many genes via direct activation of signaling pathways, responses that occur readily in cultured cells in the absence of changes in organ physiology or complex tissue changes. To determine if hyperoxia exposure could increase FGF10, FGF9, and PDGF-A mRNAs in vitro, MLG cells were exposed to 95% O_2/5% CO_2 for 2 and 4 h and compared to control cells maintained in 95% air/5% CO_2. These growth factors are highly expressed by this mesenchymal cell line. We have previously shown that hyperoxia increases binding of transcription factors to a lung promoter within 2–4 h resulting in a ~2.5× increase in mRNA abundance by 24 h [Cao et al., 2003]. Table III shows mRNA levels measured by QRT-PCR for these growth factors normalized to 18S ribosomal RNA and displayed as the average ratio (n = 4–5/group). FGF10, FGF9, and PDGF-A relative mRNAs all decreased when the cells were exposed to hyperoxic conditions for varying periods of time. These decreases could be the result of hyperoxia-induced cell death; however, this is unlikely because the cells appeared normal with few detached, dead cells, and the relative expression of 18S increased in hyperoxia when compared to controls (54×, 61× at 2 and 4 h, respectively). This suggests that increases in FGF10, FGF9, and PDGF-A expression in the lung may result from changes in signaling pathways, mRNA stability, and/or cell populations rather than by direct effects of oxygen.

TABLE III. Gene Expression in Hyperoxia-Exposed MLG Cells

Gene	C	2 h	4 h
FGF10	100	0.135	0347
FGF9	100	64.6	1.37
PDGF	100	0.079	0.103

MLG cells, mouse lung fibroblast cells; C, control; maintained in 95% air/5% CO_2.

DISCUSSION

Little is known about the details of molecular regulation of post-injury lung repair. To understand these complex processes, a number of investigators have used gene expression microarrays to characterize the molecular composition of hyperoxia-injured lung in rodents and report significant changes in a large number of mRNAs at various times after initiation of lung injury. These include upregulation of antioxidant enzymes and overall increases in cell cycle inhibitory genes [Perkowski et al., 2003; Cho et al., 2005], findings consistent with previous studies using other analytical methods. Except for upregulation of BMP4 mRNA, the microarray findings have not shown statistically significant changes in the mRNAs we measured [Perkowski et al., 2003]. The importance of microarray data in terms of specific mechanisms of injury and repair is unclear and, for many genes, the function of the encoded protein in the normal lung is not known. Although these expression profiling surveys identify genes that potentially contribute to injury-repair, the microarray data are limited by the small number of time points sampled, their non-quantitative nature, false negatives, and the inability to detect low-abundance genes.

Given the low-expression levels in adult lungs of many molecules of interest, we chose to use QRT-PCR and Western blot analyses to quantify mRNA and characterize changes in protein abundance as used by others [Li et al., 2005]. Many of the selected molecules have not been reported in adult lung previously, but all were detected by these sensitive methods. During

normal development, these growth factors and signaling molecules are under tight temporal and spatial regulation, leading us to analyze lungs at seven time points. However, this too may miss peak expression levels, especially during early hyperoxia when there may be very rapid changes in gene expression.

Hyperoxia is frequently used as a model of moderate to severe lung injury, and the pathogenesis of injury and the time course of tissue and cellular changes have been examined in some detail [Crapo et al., 1980; Barry and Crapo, 1985; Narasaraju et al., 2005]. Initial injury is thought to result from direct cellular damage caused by reactive oxygen species (ROS); by 24–48 h there is an early injury phase with endothelial cell injury [Crapo et al., 1980; Barry and Crapo, 1985] resulting in capillary leakage, alveolar edema, endothelial cell death. This is followed by type I cell injury death and desquamation [Crapo et al., 1980; Barry and Crapo, 1985]. There is a modest influx of inflammatory cells that is not apparent in rodents until about 60 h or later (100% oxygen exposure) and is not significant in 85% oxygen exposure [Barry and Crapo, 1985]. Thus we would not expect inflammatory cell mRNAs to contribute significantly to molecular changes we observed until late in the exposure period. CD45, a marker of inflammatory cells did not increase significantly during hyperoxia but decreased (N.S.) during the normoxic recovery period.

Type II cell proliferation may commence after about 48 h of hyperoxia and continue for several weeks into recovery [Bachofen and Weibel, 1982; Narasaraju et al., 2005]. In hyperoxic human lung, type II-like cuboidal cells are present by ~2 weeks [Bachofen and Weibel, 1982]; in the rat, total type II cell number does not increase during 60 h of hyperoxia. Surfactant protein A, B, and C mRNAs produced by these cells are downregulated however [Allred et al., 1999] in contrast with the upregulation of genes we studied. Another study in rats shows a ~6-fold increase over control in type II cells on recovery day 3 [Narasaraju et al., 2005; published online]. Collectively, the studies suggest that type II cell proliferation can commence during the hyperoxic exposure but is more prominent during the recovery phase.

We provide new information on the behavior of important developmental genes in the injured adult lung. The changes in mRNA levels are quite rapid, most occur during hyperoxia exposure, most mRNAs are upregulated, and most increases decline during normoxic recovery. Of considerable interest are changes in expression of FGF10 and FGF9, 2 of the 25 known fibroblast growth factors [Steiling and Werner, 2003] of which FGF1, 2, 7, 9, 10, 18, and others are expressed in developing and adult lung. Of those that have been knocked-out, only FGF 9 and 10 are critical regulators of lung organogenesis [Sekine et al., 1999; Colvin et al., 2001], and their null mutations, lethal at birth, result in lung hypoplasia [Colvin et al., 2001] and failure of branching morphogenesis distal to the trachea, respectively [Sekine et al., 1999]. These two FGFs have been implicated in oncogenesis of human tumors [Theodorou et al., 2004; Hendrix et al., 2006].

Hyperoxia increases FGF10 and FGF9 mRNAs and proteins in mouse lung. During development, FGFs influence diverse cellular functions including early patterning, regulation of growth and differentiation, cell migration, angiogenesis, and survival [Steiling and Werner, 2003], all processes likely involved in tissue repair. Earlier studies suggest the involvement of FGFs in lung repair or protection against injury [Panos et al., 1995; Hokuto et al., 2004]. In animals genetically engineered to produce a soluble, inhibitory FGFR in the lung, hyperoxic injury is prolonged and survival time decreased [Hokuto et al., 2004]. Although this study demonstrates the overall importance of FGF signaling in injury-repair, it does not identify which FGF ligands are involved.

FGFs and their receptors appear to serve a general repair function in many organs including skin, muscle, and elsewhere [Ortega et al., 1998; Steiling and Werner, 2003; Zhao and Hoffman, 2004; Kawai et al., 2005]. FGFs1 and 2 are expressed in skin wounds and stimulate repair when administered exogenously [Ortega et al., 1998; Kawai et al., 2005]. FGFR4 mRNA, required for embryonic muscle cell differentiation [Marics et al., 2002], is not detectable in normal adult muscle, but increases transiently after injury during post-natal skeletal muscle regeneration [Zhao and Hoffman, 2004]. This transient increase and time of onset [Ortega et al., 1998; Zhao and Hoffman, 2004; Kawai et al., 2005] are similar to those of FGF9 and FGF10 mRNAs and proteins in the injured lung. Although FGF7 (KGF, keratinocyte growth factor) protects the lung in vivo against

hyperoxic and other injuries [Panos et al., 1995], we did not include FGF7 in our survey because FGF7 null mutations do not influence neonatal survival or result in apparent lung abnormalities [Guo et al., 1996].

PDGF-A mRNA expression increased significantly ($P < 0.05$) during hyperoxia. A null mutation in PDGF-A results in defects in elastic fiber production and emphysema postnatally due to failed septation [Bostrom et al., 1996]. Whether alveoli can be recreated by septation after adult lung injury is debated, but repair of alveoli and of elastic fibers after hyperoxic injury may require PDGF signaling.

While changes in oxygen tension can upregulate many genes directly in single cell populations, we did not observe increases in FGF10, FGF9, or PDGF-A in MLG cells exposed to hyperoxia. The expression of these genes relative to 18S was decreased compared to controls. From this we speculate that increases in FGF10, FGF9, and PDGF-A expression in the hyperoxic lung are more likely to be indirect, perhaps in response to changes in cell–cell signaling pathways and/or cell populations, rather than direct effects of oxygen.

TTF-1 (Nkx2.1) mRNA is markedly increased in late recovery, and TTF-1 protein has been previously localized to sites of lung injury [Stahlman et al., 1996]. This transcription factor, a member of the Nkx2 family of homeodomain proteins, is expressed in the endodermal foregut upon induction by FGF signaling [Serls et al., 2004], and its activity is essential for branching morphogenesis and type II cell differentiation. In adult animals, however, overexpression of TTF-1 in type II cells results in emphysema, epithelial cell hyperplasia, and abnormal alveolar morphology [Wert et al., 2002]. The large increases in TTF-1 that we observed may be responsible for the mild to moderate hyperoxia-induced emphysema, the enhanced expression of differentiation genes for epithelial repair, or for both, as discussed by others [Wert et al., 2002; Kohno et al., 2004; Serls et al., 2004].

Both increases and decreases in VEGF expression have been shown in response to hyperoxia [Klekamp et al., 1999; Corne et al., 2000; D'Angio and Maniscalco, 2002]. This variability appears to depend on the age and animal species, the experimental conditions, the tissue sampled (e.g., lung, placenta, etc.), and the method of normalizing expression levels [Khaliq et al., 1999; Klekamp et al., 1999; Corne et al., 2000; D'Angio and Maniscalco, 2002]. We noted both an increase and a decrease, depending on whether the lungs are analyzed during exposure or recovery. VEGF mRNA increases significantly (about threefold) during hyperoxia exposure but post-exposure levels are significantly decreased for at least 5 days. This decrease is particularly interesting, given that rhVEGF treatment to newborn rats is protective against hyperoxic injury [Kunig et al., 2005].

The Shh-Ptc pathway is a critically important developmental signaling system that regulates patterning, branching morphogenesis, and cell proliferation, partly by induction of Cyclin-D2 [Mill et al., 2003]. Constituents of this pathway increase in response to hyperoxia but at different time points. Shh mRNA increases by 24 h of hyperoxia, but decreases during recovery. Ptc mRNA does not change during exposure but Ptc protein is variably increased on recovery day 1 (2 of 3 animals). These genes are expressed by different cell types, Ptc by mesenchymal cells and Shh by epithelial cells. Epithelial cells are preferentially damaged by hyperoxia, perhaps accounting for decreased Shh expression at later times. Several Shh pathway targets, for example, FGFs, Cyclin-D2, and BMP4, also increase during hyperoxia at similar times, suggesting that the pathway may be activated functionally.

Shh, known to be expressed at low levels in normal mouse lung, is enhanced during repair of damaged airway epithelium, in lung fibrotic diseases, and in fibrosis-associated inflammatory processes [Stewart et al., 2003; Watkins et al., 2003], but little is known about its importance in lung diseases. However, in other organs (ovary, skin, and prostate), Shh pathway dysregulation leads to hyperproliferation and tumorigenesis [Wetmore, 2003; (in review)]. This pathway is likely activated in hyperoxia, as we have shown. That changes in the molecular constituents of the pathway can be readily detected with standard biochemical assays supports substantial changes in expression levels, given that expression of these molecules is low abundance in normal lung.

Whether hematopoietic stem cells or tissue-specific stem cells participate in lung repair is controversial. We used two putative stem cell markers, BCRP and Sca-1, to identify possible large influxes of stem cells or rapid proliferation

of local cells that could participate in repair. Sca-1 is expressed by bone marrow stem cells, cardiac side population (SP) cells, lung SP cells, and others, and has been demonstrated by immunohistochemistry in normal mouse lung endothelial cells [Kotton et al., 2003]. The ABC-type half-transporter BCRP1 has been reported in airway epithelial cells, capillary endothelium, pulmonary smooth muscle, and lung SP cells [Summer et al., 2003]. Since both Sca-1 and BCRP proteins mark well-differentiated cells in the normal adult lung it is not clear how meaningful they are as markers of stem cells. However, both stem cell markers decreased during normoxic recovery when increased epithelial cell differentiation and proliferation occurs. Although we cannot rule out important functions related to changes in expression of these genes, neither changes with a consistent time course that would suggest large increases in stem cell populations.

In summary, this survey of selected genes shows that expression levels of molecules that play fundamental roles in lung development are altered during hyperoxia and normoxic recovery. We view these new observations as the first in a sequence of steps to identify sequential and causal changes in gene expression during lung injury and subsequent repair, a very important clinical issue related to exposure of humans to toxins, noxious gases, pathogenic bacteria and viruses, and other insults. It is important now to establish which cell populations produce the identified molecules and ultimately to test their functional importance using conditional gene knock-outs, specific inhibitors, or other methods to suppress specific gene expression in the adult lung. Given the high mortality of acute respiratory failure in human patients [Rubenfeld et al., 2005], a syndrome treated clinically with high concentrations of inhaled oxygen, understanding the molecular changes that ensue and identifying ways to trigger more rapid healing responses seem worthwhile.

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