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Potential therapeutic targets in Nrf2-dependent protection against neonatal respiratory distress disease predicted by cDNA microarray analysis and bioinformatics tools

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Abstract

Hyperoxia exposure of newborn rodents has served as a model for bronchopulmonary dysplasia (BPD) phenotypes found in a sub-population of human premature infants. We previously demonstrated that Nrf2 modulates molecular events during saccular-to-alveolar lung maturation and also has a protective role in the pathogenesis of hyperoxia-induced acute lung injury, mortality, and arrest of saccular-to-alveolar transition using *Nrf2*-deficient and wild-type neonate mice. In this review, we describe how whole-genome transcriptome analyses can identify the means through which Nrf2 transcriptionally modulates organ injury and morphology, cellular growth/proliferation, vasculature development, and immune response during BPD-like pathogenesis. We illustrate how recently developed bioinformatics tools can be used to identify sets of Nrf2-dependently modulated genes in the BPD model, and elucidate direct Nrf2 downstream targets and chemicals/drugs that may act on them. These approaches will provide significant insights into promising therapeutic agents for Nrf2-dependent treatments of complications of preterm birth like BPD.

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Introduction

Respiratory distress syndrome is a breathing disorder that affects a subpopulation of infants with preterm birth (prior to 37 weeks of completed gestation). Premature infant lungs are underdeveloped (saccular phase at 24–36 weeks of gestation) and are unable to make enough surfactant to maintain alveolar ventilation and blood oxygenation until they enter into the alveolar phase. Critical morphologic processes of the saccular phase include expansion of distal airways for subsequent alveolar formation, differentiation of type 1 and 2 pneumocytes, and thinning of the air-blood barrier. Treatments for lung prematurity include surfactant replacement therapy, mechanical ventilation, oxygen, and other strategies to reduce further lung injury, provide nutrition and other support for lung growth and recovery, and prevent lung infections. If premature infants still require oxygen therapy by the time they reach normal delivery dates, they're diagnosed with bronchopulmonary dysplasia (BPD). Although BPD has multifactorial causes, it has been recently defined as a result from aberrant development of immature lungs exposed to the *ex-uterine* milieu [1] because the disease is mostly concentrated in very-low-birth weight (<1000 g) premature infants [2]. The risk of BPD is indeed inversely proportional to the gestational age at birth [3]. Unfortunately, permanent arrest of lung growth in BPD may cause lifelong functional abnormalities [4].

Due to lack of a safe and effective treatment, BPD remains the most significant pulmonary complication of preterm birth. Excessive oxygen use can paradoxically increase the risk of BPD because it can lead to oxidant injury while low oxygen saturation targets may increase the likelihood of death [5]. In addition, studies with high frequency ventilation have led to potentially less invasive care strategies [6]. Inhaled nitric oxide (iNO) has been approved clinically for more than a decade while efficacy to prevent or treat BPD is controversial [7]. Moreover, the National Institutes of Health Consensus Development Conference (https://consensus.nih.gov/2010/inofinalstatement.htm) concluded with avoidance of routine iNO use for premature infants. In addition to clinical investigations, experimental BPD models in rodents have been widely examined for pathogenesis and therapeutic intervention

because neonatal rodents are normally born with premature lungs and develop pulmonary changes similar to BPD after hyperoxia exposure. More recent studies with BPD models demonstrated a novel stem cells therapeutic option. These studies demonstrated that prophylactic administration of mesenchymal stromal cells before hyperoxia exposure showed marked mitigation of alveolar injury, inflammation, pulmonary hypertension, and lung compliance, while administration of stem cells after hyperoxia exposure did not provide convincing results (see review [8*]).

In utero expression of airway antioxidant enzymes is known to increase toward term gestation to prepare for birth into an oxygen (O_2)-rich (from 3% to 21% O_2) environment [9]. Therefore, preterm infants with low birth weight are not only more sensitive to increased oxygen concentrations compared to adults [10], but they also have diminished/compromised endogenous antioxidant activity relative to full term infants [9], which is thought to contribute to the critical consequence of hyperoxic insult in BPD pathogenesis. While multiple studies are under investigation [11], overall clinical trials of antioxidant therapies [e.g., superoxide dismutases (SODs), vitamins A and E, N-acetylcysteine, metalloporphyrin] in management of BPD have remained inconclusive although recombinant human SOD has shown marginal efficacy (see review [12]). Nuclear factor, erythroid-derived 2, like 2 (Nfe2l2) or NF-E2-related factor 2 (Nrf2) is an indispensable transcriptional regulator for diverse antioxidant enzyme and host defense protein genes bearing antioxidant response elements (AREs) that bind Nrf2. Critical roles of Nrf2 function have been well defined *in vivo* using adult mice with genetically engineered *Nrf2* and its cytoplasmic inhibitor Kelch-like ECH-associated protein (*Keap1*) in acute lung injury (ALI), emphysema, allergy and asthma, pulmonary fibrosis, lung tumor, and respiratory syncytial virus disease [13]. Roles for Nrf2 in organ development and neonatal disease have also recently been identified in murine experimental BPD. Augmented lethality, lung injury, and arrested saccular-to-alveolar transition after hyperoxia exposure were found in neonatal mice deficient in *Nrf2* ($Nrf2^{-/-}$) compared to wild type controls [14,15]. The surviving juvenile $Nrf2^{-/-}$ mice had decreased surfactant-producing type 2 cells [15], which indicated long term pulmonary outcome and the predisposition potential for oxidative pulmonary disease in adults or adolescents. Data also suggested a therapeutic potential to enhance Nrf2-mediated pulmonary responses in BPD pathogenesis. Supporting the role for Nrf2-ARE responses, increased BPD risk was associated with functional polymorphisms in *NRF2* and in ARE-responsive glutathione-s-transferase (*GSTP1*), NAD(P)H:quinone oxidoreductase 1 (*NQO1*), and UDP glucuronosyltransferase 1 family, polypeptide A1 (*UGT1A1*) in several human cohorts [16–18].

In order to further elucidate the gene transcripts affected by Nrf2 in healthy and diseased lungs we performed genome-wide lung cDNA microarray analyses in the murine model of neonatal ALI. The analyses demonstrated that Nrf2-mediated protection against hyperoxia-induced ALI phentoypes may be through transcriptional regulation of genes associated with DNA replication and cell cycle, various metabolism and small molecular processes, and cell–cell interaction as well as redox homeostasis in the saccular phase lungs of newborn mice [14,19]. We also found altered lung transcriptomes for sustaining lung morphogenesis, cell growth machinery, and lymphocyte immunity during saccular lung maturation in hyperoxia-susceptible $Nrf2^{-/-}$ neonates [14], suggesting their roles in predisposing the immature lung to oxidant-induced disorders. Among the key Nrf2 effectors contributing to the protection against BPD-like disorders are glutathione peroxidase 2 (Gpx2) and macrophage receptor with collagenase structure (Marco) [14].

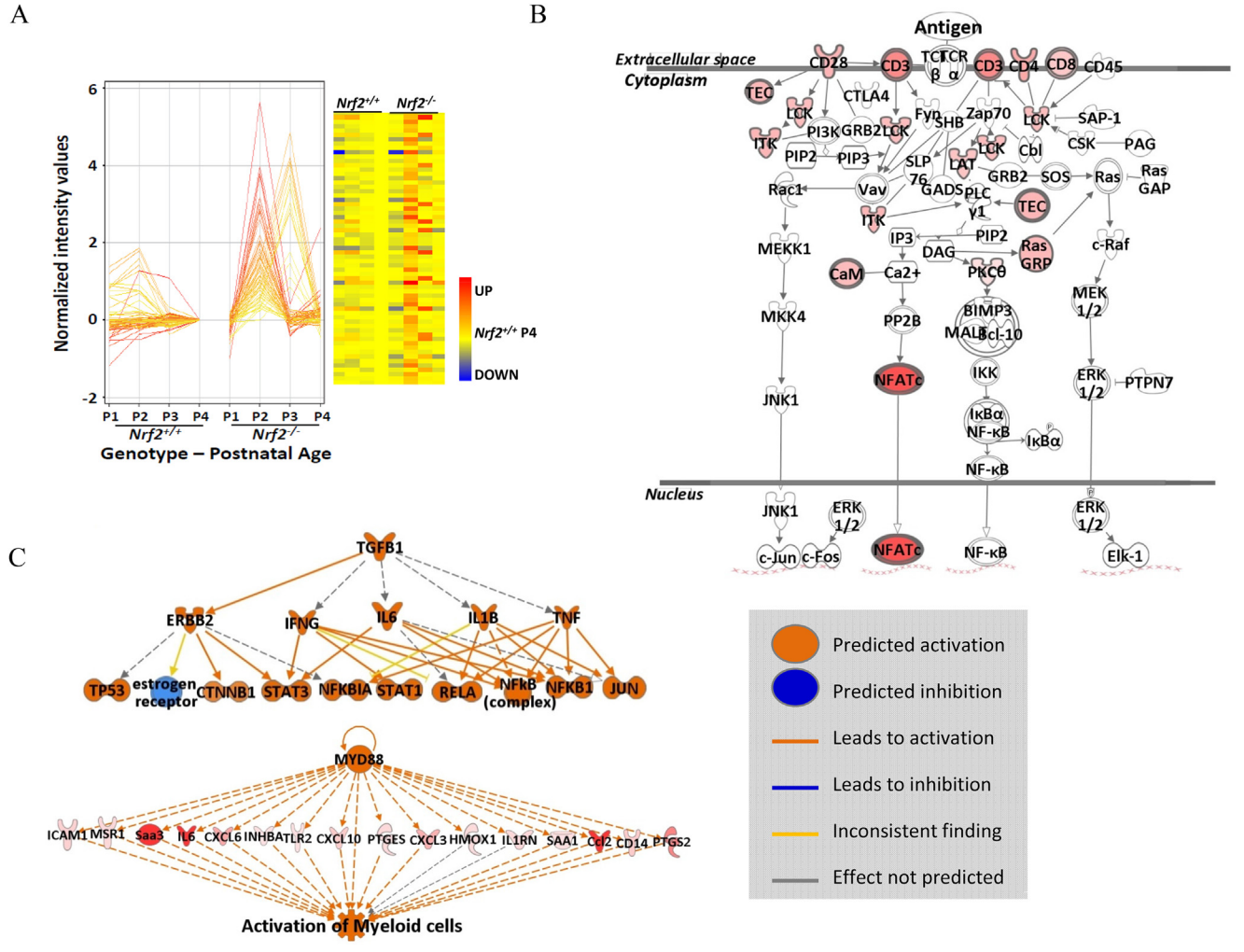
In the current review, we revisited selected sets of our neonatal lung microarray data which were differentially regulated by Nrf2 basally, and during hyperoxia exposure, in order to demonstrate the value of transcriptomics and bioinformatic approaches for identifying novel gene transcripts and gene networks that may contribute to normal lung development and ALI pathogenesis [14]. We elucidated direct Nrf2 downstream target genes by searching genomic sequence near transcription start sites (TSS) for Nrf2/Maf binding sites (or AREs) using a position weight matrix (PWM) model [20] and mapping AREs into peaks of Nrf2 chromatin immunoprecipitation-sequencing (ChIP-seq) [21–24*]. We also determined chemicals and drugs that are predicted to perturb the mode of gene expression (higher or lower in $Nrf2^{-/-}$ compared to $Nrf2^{+/+}$ at baseline or after hyperoxia) by The Library of Integrated Cellular Signatures (LINCS) analysis. The results from these recently developed bioinformatics tools provide informative clues for promising intervention compounds in BPD protection or treatment.

Transcriptome data for bioinformatics analyses

The microarray data were deposited in Gene Expression Omnibus (GEO, accession number GSE29632) and in the National Institute of Environmental Health Sciences (NIEHS) Chemical Effects in Biological Systems data base (CEBS, accession number: 005-00003-0012-000-0). We reanalyzed the previously profiled data sets with a newer version of GeneSpring (12.6, Agilent Technologies, Inc., Santa Clara, CA) to generate gene lists for further analyses. A number of bioinformatics tools have been developed to query transcription profiles for gene networks, canonical pathways, and co-expressed genes [e.g. Ingenuity Pathway Analysis (IPA, Qiagen, Redwood City, CA), Genomic Regions

Enrichment of Annotations Tool (GREAT, http://bejerano.stanford.edu/great/public/html/), Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov/), Extracting Patterns and Identifying co-expressed Genes (EPIG, http://www.niehs.nih.gov/research/resources/software/biostatistics/epig/index.cfm)]. In order to provide the most recent updates in gene identity and networks, we focused first on Nrf2-dependently varied gene transcripts (>2-fold at least one time point, n = 396) from the saccular stage (post-natal days P1–P3, characterized by simple, poorly septated saccules) and the more mature late saccular/early alveolar phase (P4, bearing branched septa and multilobular alveoli). While developmental gene variation was highest between P1 and P4 in *Nrf2*$^{+/+}$ mice, the greatest genotype effects were found at days P2–P3. Our previous visual profile analysis also revealed distinct sets of genes which were markedly overexpressed at P2–P3 of *Nrf2*$^{-/-}$ mice [14], so we used analysis by similar entities (GeneSpring, Agilent technologies, Santa Clara, CA) to identify 141 correlated transcripts (Figure 1-A). Using IPA we found that among the canonical pathways affected in this Nrf2-dependent transcript set was T cell receptor signaling (Figure 1-B), which has not been previously implicated in studies of this transcription factor.

Figure 1



Transcriptomics and molecular events in *Nrf2*-deficient murine lungs during saccular-to alveolar transition and bronchopulmonary dysplasia (BPD)-like pathogenesis. (A) cDNA microarray and profile analysis elucidated a set (n = 141) of markedly elevated genes during postnatal days 2 and 3 (P2–P3) in *Nrf2*-deficient (*Nrf2*$^{-/-}$) mice compared to their wild type controls (*Nrf2*$^{+/+}$). Data were normalized to the average expression level of *Nrf2*$^{+/+}$ mice at P4. The expression profiles of these genes are depicted as a heat map. Color bar indicates relative expression intensity. (B) Function analysis determined T cell receptor signaling as one of the most predominant molecular events in *Nrf2*-deficient saccular phase lungs. The intensity of the red highlight for the affected genes in the pathway indicates the level of significance (*p* value). (C) Pathway analysis demonstrated that transforming growth factor beta 1 (TGF-β1) is one of the key upstream regulators for severe BPD-like disease development in *Nrf2*$^{-/-}$ neonates and myeloid differentiation primary response gene 88 (Myd88) and related innate immune signaling pathway genes are essential in the heightened inflammatory responses in *Nrf2*$^{-/-}$ neonates in the current model. Analyses were done using GeneSpring software and Ingenuity Pathway Analysis.

Table 1 Potentially functional antioxidant response elements (ARE) on Nrf2-dependent acute lung injury (ALI) genes determined in a neonate mouse model.

Gene symbol	Gene title	PWM[a]	MS	Distance to transcription start site	[c]Data source	[b]FD (baseline) or trend/ *p* value (hyperoxia)
Akr1b8	aldo-keto reductase family 1, member B8	19.4, 13.7	0.922, 0.934	–3742, –4012	P2 Days 1–2	–2.0 Low (0.001/0.002)
Aox1	aldehyde oxidase 1	15, 12.6	0.943, 0.836	–323, –225	P1–P4 Day 3	–2.3/–2.7/–2.4/–2.1 Low (0.001)
Atp1b1	ATPase, Na+/K+ transporting, beta 1 polypeptide	15.3, 12.1	0.917, 0.891	–972, –817	P3	2.4
Birc6	baculoviral IAP repeat-containing 6	13.9	0.877	–800	P2	–2.0
Bscl2	Bernardinelli-Seip congenital lipodystrophy 2 homolog	18.7, 11.7	0.947, 0.843	204, –4499	Day 3	Low (0.009)
Cbr3	carbonyl reductase 3	14.1	0.875	–736	Day 3	Low (0.003)
Cdca7	cell division cycle associated 7	11	0.795	–2462	P3	–2.3
Cltc	clathrin, heavy polypeptide (Hc)	15.2–10.9	0.935–0.797	–1984, –1307, –1795, –542	Day 1	Low (0.017)
Cyb5a	cytochrome b-5	18.5–13	0.952–0.895	–2308, –2742, –1836	Day 3	Low (0.007)
Egr1	early growth response 1	11.9	0.839	–3334	P3	–2.7
Ehd1	EH-domain containing 1	11.5	0.89	–3075	Day 2	High (0.001)
Eif4g2	eukaryotic translation initiation factor 4, gamma 2	13.7	0.893	–644	Day 2	High (0.001)
Fgfbp1	fibroblast growth factor binding protein 1	14.5	0.942	–1238	Day 3	Low (0.003)
Fkbp5	FK506 binding protein 5	15	0.931	–4611	P3	2.2
Ftl1	ferritin light chain 1	20.7	0.981	–1087	Day 2	Low (0.002)
Gclc	glutamate-cysteine ligase, catalytic subunit	21.6	0.99	–3795	Days 1–2	Low (0.005/0.004)
Glipr1	GLI pathogenesis-related 1 (glioma)	12.5	0.884	–3418	Day 2	Low (0.003)
Gsta3	glutathione S-transferase, alpha 3	12.9	0.86	–126	P1, P3, P4	–4.9/–4.8/–5.3
Hexa	hexosaminidase A	13, 10.5	0.836, 0.81	–2057, –1779	Day 2	Low (0.01)
Hmox1	heme oxygenase (decycling) 1	19.3–10.8	0.968–0.862	–3873, –3925, –3201	Day 2	Low (0.002)
Hsp90aa1	heat shock protein 90, alpha (cytosolic), class A member 1	16.3, 10.3	0.947, 0.867	–2986, –4958	Day 2	High (0.005)
Hspa8	heat shock protein 8	13	0.904	–1158	Day 1	High (0.027)
Htatip2	HIV-1 tat interactive protein 2, homolog	16.4	0.954	–54	Day 3	Low (0.003)
Ift80	intraflagellar transport 80 homolog	10.8	0.851	–3728	P3 Day 2	–2.6 Low (0.003)
Il6	interleukin 6	18.3	0.965	–256	Day 2	Low (0.007)
Kif5b	kinesin family member 5B	10.6	0.907	–1043	P1–P4	2.6/2.9/2.8/2.7
Malat1	metastasis associated lung adenocarcinoma transcript 1	15.3	0.904	–4261	P3	–2.0
Mcm5	minichromosome maintenance deficient 5, cell division cycle 46	10.2	0.829	–750	P3	–2.4
Me1	malic enzyme 1, NADP(+)-dependent, cytosolic	18	0.944	8	Days 1, 3	Low (0.001/0.003)
Mki67	antigen identified by monoclonal antibody Ki 67	11.8	0.849	–740	P3	–2.3
Nek6	NIMA (never in mitosis gene a)-related expressed kinase 6	18	0.967	–4345	Day 2	High (0.004)
Nfat5	Nuclear factor of activated T-cells 5 (Nfat5), transcript variant a, mRNA	10.3	0.896	–928	P2	–2.2
Nqo1	NAD(P)H dehydrogenase, quinone 1	16.4	0.87	–422	Day 3	Low (0.003)
Oasl2	2′–5′ oligoadenylate synthetase-like 2	10.7	0.883	–61	P2	2.3

Table 1 (*continued*)

Gene symbol	Gene title	PWM[a]	MS	Distance to transcription start site	[c]Data source	[b]FD (baseline) or trend/ *p* value (hyperoxia)
Pgd	phosphogluconate dehydrogenase	18.6, 11.7	0.971, 0.808	−4145, −4177	Day 1	Low (0.03)
Pir	pirin	20, 12.6	0.958, 0.909	118, −219	Day 1	Low (0.007)
Pola1	polymerase (DNA directed), alpha 1	12.5	0.872	−3001	P3	−2.5
Prdx1	peroxiredoxin 1	15.7−14.9	0.923−0.937	−138, −2451, −4889	Day 2	Low (0.001)
Rrm2	ribonucleotide reductase M2	16.2	0.947	−3190	P3	−2.4
Slc7a11	solute carrier family 7 (cationic amino acid transporter, y+ system), member 11	14.1	0.843	−99	Day 3	Low (0.007)
Sord	sorbitol dehydrogenase	11.8	0.899	−4245	P1	−2.0
Srxn1	sulfiredoxin 1 homolog	12.7	0.862	−50	Days 2–3	Low (0.004/0.004)
Timp1	tissue inhibitor of metalloproteinase 1	13	0.877	−1957	Day 1	Low (0.04)
Tlcd2	TLC domain containing 2	13.7	0.877	−4977	P1 Day 3	2.1 High (0.002)
Tomm20	Translocase of outer mitochondrial membrane 20 homolog	10.3	0.915	−131	Day 2	Low (0.002)
Txnl1	thioredoxin-like 1	13.1	0.9	45	Day 2	Low (0.001)
Txnrd1	thioredoxin reductase 1	17.9, 10.6	0.928, 0.84	−97, −403	Days 1–3	Low (0.005/0.001/0)
Ubc	ubiquitin C	12.7, 10.6	0.907, 0.871	−1794, −254	Days 1, 3	Low (0.009/0.003)
Wac	WW domain containing adaptor with coiled-coil	16.8	0.873	−3912	P2	−2.2

ARE, antioxidant response element; PWM, position weight matrix; MS, matrix significance.
[a] Selected bound AREs determined by mouse ChIP-seq analyses [21−24] with PWM greater than 10 in 5′-untranslated region (UTR) and 5 kb promoter of the genes are shown. Expanded data for full gene list and ARE search in Appendices A and B.
[b] Fold difference at baseline (postnatal days P1, P2, P3, or P4) or expression trend and *p* value during hyperoxia exposure (Days 1, 2, or 3) in *Nrf2*$^{-/-}$ neonates compared to *Nrf2*$^{+/+}$ neonates.
[c] Microarray analyses data sources of the Nrf2-dependent genes − baseline (P1−P4) or during hyperoxia (Days 1−3).

Following similar approaches, we also analyzed transcript expression changes induced by postnatal hyperoxia exposure (1−3 days starting from P1) in a Nrf2-dependent manner ($p < 0.01$, >2-fold $n = 437$). Description of these Nrf2-dependently modulated neonatal lung genes and their expression profiles as well as the associated functions and molecular networks have been published previously [14].

Putative ARE search for potential Nrf2 downstream effectors

The putative Nrf2 binding sites (AREs) within 5 kb of TSS were predicted using a PWM model [20], and they were identified as functional AREs only if they were under ChIP-seq peaks [21,25**−27]. About 15% (62 out of 424) or 19% (73 out of 377) of transcripts bearing potential AREs in the Nrf2-dependent neonatal lung genes at baseline or after hyperoxia, respectively (Table 1, Appendices A, B) had nearby putative AREs. For the lung genes basally heightened in *Nrf2*$^{-/-}$ neonates at P2−P3, a motif discovery analysis using MEM-ChIP [28] elucidated two enriched motifs for DNA binding proteins, Ets1 (the significance value, e = 2.3e−9) and Klf4 (e = 1.4e−2) in the TSS +/−1 kb regions and others including Etv6 ($e = 1.1e^{-7}$), Nrf1 ($e = 3.3e^{-5}$), E2f4 ($e = 7.6e^{-5}$), Tcfl5 ($e = 1.4^{e-4}$), and Sp2 ($e = 5.3e^{-4}$) in TSS +/−5 kb regions. Ets family genes are known to associate with cancer progression and studies using gene deficient mice demonstrated that Ets1 regulates hematopoietic cells (T cell, B cell, and natural killer cell) and Etv6 also has a role in hematopoiesis and embryonic angiogenesis [29]. Nrf1, similar to Nrf2, is known to regulate ARE-bearing antioxidants but it also modulates inflammatory responses [30]. Klf4 (Krüppel-like factor 4) is a pluripotent transcription factor known to be involved in carcinogenesis or anti-carcinogenesis by modulating various genes (e.g., p21, cyclins, Bcl2) in cell cycle and survival [31]. It corresponds to the pathway analysis (http://www.ingenuity.com/products/ipa) in which these genes were highly linked to T cell development and T cell receptor signaling (Figure 1-B) with interleukin 7 and TCF3 as predicted upstream regulators. It is therefore postulated that *Nrf2*-deficiency in immature lung activates compensatory mechanism to evoke acute immune responses for host defense. It is also suggested that these compensatory immune responses in *Nrf2*-deficient saccular lung may predispose the hyperoxia-induced

BPD-like disorder in which transforming growth factor beta 1 (TGF-β1) is one of the key upstream regulators and myeloid differentiation primary response gene 88 (Myd88) plays a key role in inflammatory cell infiltration (Figure 1-C)

LINCS analysis for chemical targets

LINCS analysis was performed on the Nrf2-dependently modulated neonatal lung gene sets using the L1000CDS^2 search engine (http://amp.pharm.mssm.edu/L1000CDS2/#/index). We used gene transcripts significantly higher or lower (>2-fold) in *Nrf2*$^{-/-}$ mice compared to the same exposure condition or postnatal age, chose "reverse" mode for small molecule signatures that reversed our input, and allowed the small molecule combination [32]. For the gene sets markedly high only in *Nrf2*$^{-/-}$ at baseline, we used the signatures and their relative expression ratio between *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ as input for the analysis. For the top 50 search results ranked by the "search score" obtained from the L1000CDS^2 analysis (when up and down lists were available) or the largest cosine distance (*Nrf2*$^{-/-}$ baseline), we performed additional statistical tests for significance. To do so, we downloaded the original L1000 gene expression signatures computed using the characteristic direction signature method [33*] for each chemical compound perturbation and the respective cell line landmark gene signatures and all ~22,000 L1000 genes. We then implemented the hypergeometric test that produced a *p* value for each enrichment result. All gene signatures were annotated with Entrez symbols and duplicate gene entries were filtered prior to the test.

We next used LINCS to identify via signature overlap assessment, transcript profiles from cell-based drug perturbation experiments that overlap with transcript profiles generated for hyperoxia exposure responses (Table 2, Appendix C). At baseline, significant overlaps with perturbation-induced profiles were determined only at P4 for the Nrf2-dependent transcripts and they included anti-inflammatory (e.g., Zolantidine, Meclocycline, Fenbufen) and chemotherapeutic (e.g., Tipifarnib) agents (Table S3). Significantly high overlaps were also found between our differential transcript lists at all three time points. The minimum overlap ratios (the input differential transcripts and the signature differential transcripts divided by the effective input) ranged from 0.0349 to 0.0581 across the time points.

Among many perturbing agents (Table 2, Appendix C), L-sulforaphane and 15-delta prostaglandin J2 (15d-PGJ2) were predicted to reverse the Nrf2-dependent transcriptome changes during the BPD-like

Table 2 Representative list of drugs identified using the L1000CDS^2 search engine of the Library of Integrated Cellular Signature (LINCS) analysis to be predicted to act on Nrf2-dependent lung transcriptome during bronchopulmonary dysplasia (BPD) pathogenesis.

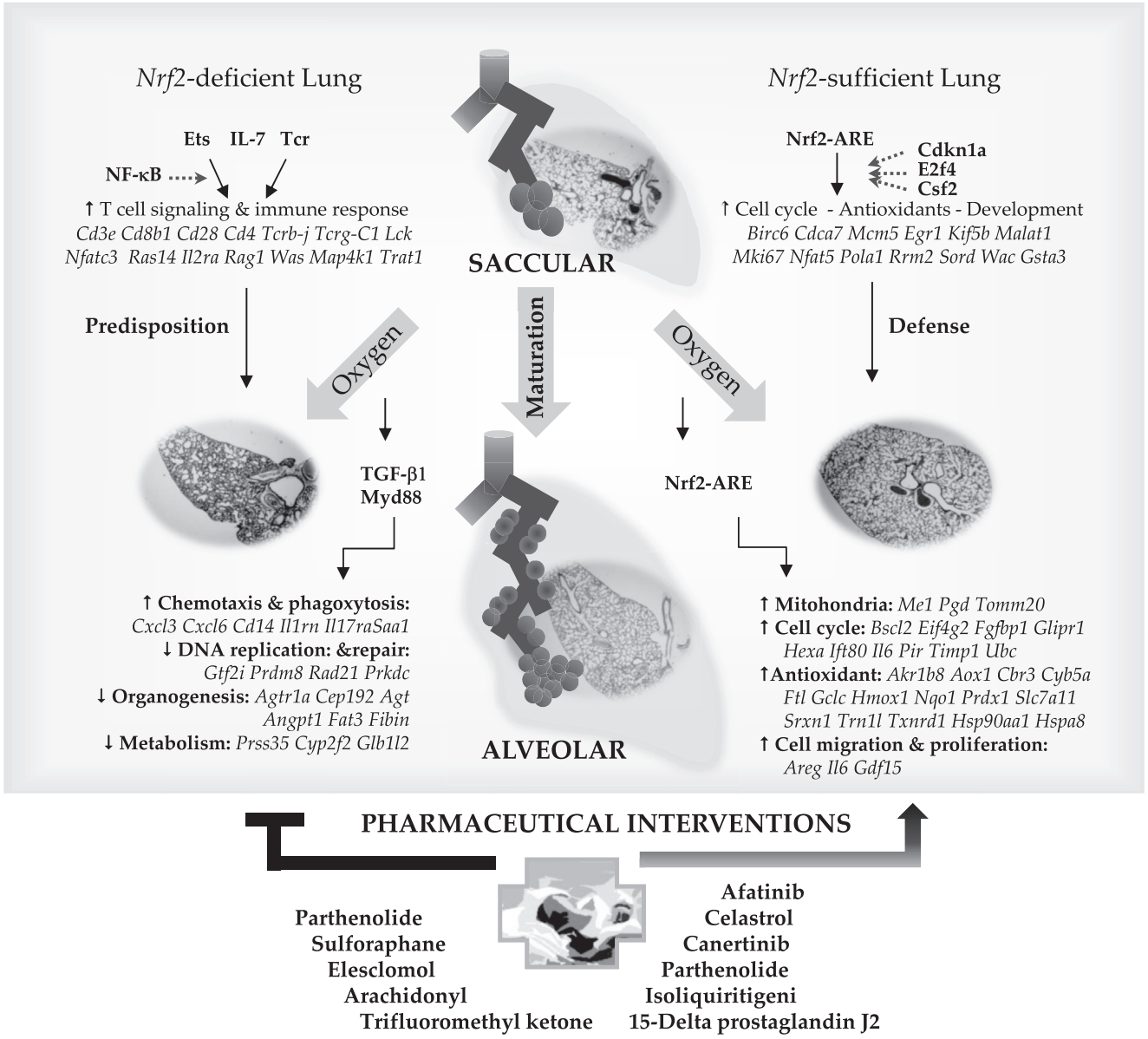
Hyperoxia exposure day	Drug name	Activity of compound[a]	Disease treatment	Minimum overlap ratio	Corresponding *p* value
1	Elesclomol	Induction of cancer cell death by oxidative stress	Anti-cancer activity	0.1765	3.060×10^{-6}
1	Isoliquiritigenin	Inhibits NLRP3 inflammasome	Treatment of inflammatory diseases	0.1765	7.170×10^{-7}
1/3	Parthenolide	Suppression of apoptotic genes	Anti-cancer and anti-inflammatory activities	0.2059/0.2051	$6.280 \times 10^{-6}/9.929 \times 10^{-4}$
1/3	L-sulforaphane	Induction of Nrf2 and prevent NF-κB binding	Induce detoxification and xenobiotic enzymes	0.1765/0.2051	$3.100 \times 10^{-10}/7.32 \times 10^{-5}$
1/3	Arachidonyl trifluoro-methyl ketone	Inhibits PLA2	Neuroprotective and treatment of multiple sclerosis	0.1765/0.2564	$6.280 \times 10^{-6}/7.650 \times 10^{-4}$
1/3	Parthenolide	Binds directly to and inhibit IKKβ	Anti-inflammatory and anti-hyperalgesic activities	0.2059/0.2051	$6.28 \times 10^{-6}/9.929 \times 10^{-4}$
3	Celastrol	Inhibits NF-κB	Antioxidant, anti-inflammatory, and anti-cancer activities	0.2308	4.390×10^{-5}
3	Canertinib	Tyrosine kinase activity	Anti-cancer activity	0.2051	9.010×10^{-6}
3	Afatinib	Inhibits EGFR and HER2	Anti-cancer (NSCLC) activity	0.2051	3.042×10^{-4}
3	15-delta prostaglandin J2	PPAR-γ agonist	Antioxidant and anti-inflammatory activities	0.2051	1.210×10^{-10}

Drugs and chemicals were identified using the L1000CDS^2 search engine for which there are statistically significant overlap ratios of transcript profiles from cell-based perturbation experiments and Nrf2-dependent lung transcriptome analysis in neonatal mice exposed to hyperoxia. Details for L1000CDS^2 analyses are described elsewhere [41*]. Full list of the drugs and chemicals in Appendix C.

[a] EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; IKKβ, I kappa B kinase beta; NLRP3, NLR Family, Pyrin Domain Containing 3; NSCLC, non-small cell lung cancer; NF-κB; nuclear factor kappa-light-chain-enhancer of activated B cells PLA2, phospholipase 2; PPAR-γ, peroxisome proliferator-activated receptor gamma.

pathogenesis. These finding are consistent with the current concepts of Nrf2 regulation. 15d PGJ2 is not only an Nrf2 activator acting through Keap1 binding [34**] but is also a ligand for peroxisome proliferator-activated receptor gamma (PPAR-γ) which bears functional AREs for Nrf2 modulation [35]. Through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) inhibition, it is known as a key regulator of

Figure 2



Proposed Nrf2-mediated regulatory mechanisms of saccular-to-alveolar transition and bronchopulmonary dysplasia (BPD)-like pathogenesis and potential therapeutic intervention. Comparative microarray analysis of *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ neonates followed by bioinformatic analyses suggested downstream Nrf2 target genes bearing functional AREs in the lung undergoing saccular-to-alveolar transition and BPD-like pathogenesis. Molecules such as cell cycle dependent kinase 1a (Cdkn1a), E2F transcription factor 4 (F2f4), or colony stimulating factor 2 (Csf2) may participate in the Nrf2-ARE pathway during normal saccular-to-alveolar transition. Hyperoxia exposure of the premature lung at saccular phase with simple, poorly septated saccules causes lung injury and interrupts normal alveolar formation. Nrf2-mediated protection is predicted to be through transcriptional activation of antioxidant, cell cycle and DNA repair, and mitochondria-related metabolism genes. *Nrf2* deficiency in saccular phase lung suppressed these cellular processes and aberrantly activated upstream molecules including transforming growth factor beta 1 (TGF-β1) and myeloid differentiation primary response gene 88 (Myd88) that may drive inflammatory cell infiltration and attenuate DNA repair and cell cycle, metabolism, and morphogenesis signaling. Augmented transcriptome of T lymphocyte signaling and related acquired immunity during the saccular-to-alveolar transition in *Nrf2*$^{-/-}$ neonate lung may predispose BPD-like phenotypes. Ets family transcription factor, interleukin 7 (IL–7), or T cell receptor (Tcr) in association with nuclear factor kappa-light-chain enhancer of activated B cells (NF-kB) may have roles upstream of this process. Drugs or chemicals acting on redox balance (e.g., sulforaphane, Celastrol), anti-inflammation (e.g., 15-delta prostaglandin J2, Isoliquiritigenin, Pathenolide, Celastrol), or anti-cancer (e.g., Elesclomol, Pathenolide, Canertinib, Afatinib) mechanisms are suggested as pharmaceutical interventions for BPD pathogenesis.

inflammation [36,37]. We also demonstrated Nrf2-dependent protection against ALI by 15d-PGJ2 in mice [35]. Sulforaphane is a powerful phytochemical Nrf2 agonist, and its role in anti-carcinogenesis and pulmonary protection against bacterial infection following emphysema, respiratory syncytial virus infection, and inhaled arsenic has been demonstrated in rodent studies (see review [13]). Moreover, controlled human studies recently reported the therapeutic potential of sulforaphane in reducing adverse effects of airway toxicants [38–40**]. This LINCS-based approach to investigation of transcript profiles has the potential to identify other drugs or chemicals that behave similar to 15d-PGJ2 and sulphoraphane, and provide additional means to upregulate anti-oxidant and other lung cellular defense mechanisms.

Conclusions

We have described new and emerging bioinformatics-based approaches that may be used to better understand direct Nrf2 downstream effector genes and drugs/chemicals that may act on the Nrf2-ARE pathways for BPD prevention or treatment. Nrf2 effectors bearing AREs determined by functional bioinformatics in the upstream region as well as in the vicinity of the genes (Table 1, Appendices A, B) have a broad spectrum of activity including lung morphogenesis, cell growth machinery, and lymphocyte immunity (Summary in Figure 2). We believe that continued development of analytical tools to investigate global transcriptome changes in the developing lung will provide new insight to potential therapeutic strategies for lung diseases of prematurity, as well as other diseases where Nrf2 has an important role in protecting against pathogenesis.

Author disclosure statement

The authors declare that no competing financial interests exist.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cotox.2016.10.006.

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