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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, arrest of saccular-to-alveolar transition, and lung injury, 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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Keywords

Bronchopulmonary dysplasia; acute lung injury; prematurity; mRNA expression; pathway analysis; canonical; LINCS1000CDS² search engine; inflammation; antioxidant

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 of 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 (< 1,000 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 these stem cells after hyperoxia exposure did not provide convincing results (see review [8]).

In utero expression of airway antioxidant enzymes are known to increase toward term gestation to prepare for birth into an oxygen (O₂)-rich (from 3% to 21% O₂) 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*^{-/-}) mice 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 phenotypes 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 NIEHS Chemical Effects in Biological Systems (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. Following similar approaches, we also analyzed gene transcript expression changes induced by postnatal hyperoxia exposure (1–3 days starting from P1) and were Nrf2-dependent (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 Ets6 ($e = 1.1e^{-7}$), Nrf1 ($e = 3.3e^{-5}$), Ets4 ($e = 7.6e^{-5}$), Tcf15 ($e = 1.4e^{-4}$), 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 regulate hematopoietic cell (T cell, B cell, and natural killer cell) and Ets6 also play roles 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 the 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 response for host defense. It is also suggested that these compensatory immune responses in *Nrf2*-deficient sacculus lung may predispose the hyperoxia-induced BPD-like disorder in which transforming growth factor beta 1 (TGF- β 1) is one of the key upstream regulator 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 gene signatures obtained from our microarray gene expression experiment on the *Nrf2*-dependently modulated neonatal lung gene sets using the L1000CDS² search engine (<http://amp.pharm.mssm.edu/L1000CDS2/#/index>). We took 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 molecular 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 L1000CDS² 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 pathogenesis. These findings 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- κ B) inhibition, it is known as a key regulator of 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 sulforaphane, 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 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 pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Transcriptome analysis provided molecular aspects of BPD pathogenesis.
- Pathway analysis indicated roles for Nrf2 in neonatal lung disease.
- Bioinformatic analyses suggested potential drug targets and therapeutic compounds for BPD.

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 were 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 of the current model. Analyses were done using GeneSpring software and Ingenuity Pathway Analysis.

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 bioinformatics 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 (E2f4), or colony stimulating factor 2 (Csf2) may participate in the Nrf2-ARE pathway during normal saccular-to-alveolar transition. Hyperoxia exposure to 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. *Nrf2* deficiency in saccular phase lung suppressed these cellular processes and aberrantly

activated upstream molecules including transforming growth factor beta 1 (TGF- β 1) and myeloid differentiation primary response gene 88 (Myd88) 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 *Nrf2*^{-/-} neonate lung may predispose the 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 play roles in the 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.

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Potentially functional antioxidant response elements (ARE) on NrF2-dependent acute lung injury (ALI) genes determined in a neonate mouse model.

Table 1

Gene Symbol	Gene Title	PWM	MS	Distance to Transcription Start Site	*Data Source	† FD (Baseline) or Trend/p value (Hyperoxia)
<i>Akr1b8</i>	aldo-keto reductase family 1, member B8	19.4, 13.7	0.922, 0.934	-3742, -4012	P2	-2.0 Low (0.001/0.002)
<i>Aox1</i>	aldehyde oxidase 1	15, 12.6	0.943, 0.836	-323, -225	Days 1-2 P1-P4	-2.3/-2.7/-2.4/-2.1 Low (0.001)
<i>Ap1b1</i>	ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide	15.3, 12.1	0.917, 0.891	-972, -817	Day 3 P3	2.4 Low (0.001)
<i>Birc6</i>	baculoviral IAP repeat-containing 6	13.9	0.877	-800	P2	-2.0
<i>Bsc12</i>	Bernardinelli-Seip congenital lipodystrophy 2 homolog	18.7, 11.7	0.947, 0.843	204, -4499	Day 3	Low (0.009)
<i>Chr3</i>	carbonyl reductase 3	14.1	0.875	-736	Day 3	Low (0.003)
<i>Cdca7</i>	cell division cycle associated 7	11	0.795	-2462	P3	-2.3
<i>Cltc</i>	clathrin, heavy polypeptide (Hc)	15.2-10.9	0.935-0.797	-1984, -1307, -1795, -542	Day 1	Low (0.017)
<i>Cyb5a</i>	cytochrome b-5	18.5-13	0.952-0.895	-2308, -2742, -1836	Day 3	Low (0.007)
<i>Egr1</i>	early growth response 1	11.9	0.839	-3334	P3	-2.7
<i>Ehd1</i>	EH-domain containing 1	11.5	0.89	-3075	Day 2	High (0.001)
<i>Eif4g2</i>	eukaryotic translation initiation factor 4, gamma 2	13.7	0.893	-644	Day 2	High (0.001)
<i>Fgf1p1</i>	fibroblast growth factor binding protein 1	14.5	0.942	-1238	Day 3	Low (0.003)
<i>Fkbp5</i>	FK506 binding protein 5	15	0.931	-4611	P3	2.2
<i>Ftl1</i>	ferritin light chain 1	20.7	0.981	-1087	Day 2	Low (0.002)
<i>Gclc</i>	glutamate-cysteine ligase, catalytic	21.6	0.99	-3795	Days 1-2	Low (0.005/0.004)
<i>Gliplr1</i>	subunit GLI pathogenesis-related 1 (glioma)	12.5	0.884	-3418	Day 2	Low (0.003)
<i>Gsta3</i>	glutathione S-transferase, alpha 3	12.9	0.86	-126	P1, P3, P4	-4.9/-4.8/-5.3 Low (0.01)
<i>Hexa</i>	hexosaminidase A	13, 10.5	0.836, 0.81	-2057, -1779	Day 2	Low (0.001)
<i>Hmx1</i>	heme oxygenase (decycling) 1	19.3-10.8	0.968-0.862	-3873, -3925, -3201	Day 2	Low (0.002)
<i>Hsp90aa1</i>	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)
<i>Hspa8</i>	heat shock protein 8	13	0.904	-1158	Day 1	High (0.027)

Gene Symbol	Gene Title	PWM	MS	Distance to Transcription Start Site	*Data Source	† FD (Baseline) or Trend/p value (Hyperoxia)
<i>Htatip2</i>	HIV-1 tat interactive protein 2, homolog	16.4	0.954	-54	Day 3	Low (0.003)
<i>Ifi80</i>	intraflagellar transport 80 homolog	10.8	0.851	-3728	P3	-2.6
					Day 2	Low (0.003)
<i>Il6</i>	interleukin 6	18.3	0.965	-256	Day 2	Low (0.007)
<i>Kif5b</i>	kinesin family member 5B	10.6	0.907	-1043	P1-P4	2.6/2.9/2.8/2.7
<i>Malat1</i>	metastasis associated lung	15.3	0.904	-4261	P3	-2.0
<i>Mems5</i>	minichromosome maintenance deficient 5, cell division cycle 46	10.2 18	0.829	-750	P3	-2.4
<i>Me1</i>	malic enzyme 1, NADP(+)-dependent, cytosolic	18	0.944	8	Days1, 3	Low (0.001/0.003)
<i>Mki67</i>	antigen identified by monoclonal	11.8	0.849	-740	P3	-2.3
<i>Nek6</i>	NIMA (never in mitosis gene a)-related expressed kinase 6	18	0.967	-4345	Day 2	High (0.004)
<i>Nfat5</i>	Nuclear factor of activated T-cells 5 (Nfat5), transcript variant a, mRNA	10.3	0.896	-928	P2	-2.2
<i>Nqo1</i>	NAD(P)H dehydrogenase, quinone 1	16.4	0.87	-422	Day 3	Low (0.003)
<i>Oas12</i>	2'-5' oligoadenylate synthetase-like 2	10.7	0.883	-61	P2	2.3
<i>Pgd</i>	phosphogluconate dehydrogenase	18.6, 11.7	0.971, 0.808	-4145, -4177	Day 1	Low (0.03)
<i>Pir</i>	pirin	20, 12.6	0.958, 0.909	118, -219	Day 1	Low (0.007)
<i>Polr1</i>	polymerase (DNA directed), alpha 1	12.5	0.872	-3001	P3	-2.5
<i>Prdx1</i>	peroxiredoxin 1	15.7-14.9	0.923-0.937	-138, -2451, -4889	Day 2	Low (0.001)
<i>Rrm2</i>	ribonucleotide reductase M2	16.2	0.947	-3190	P3	-2.4
<i>Slc7a11</i>	solute carrier family 7 (cationic amino acid transporter, y+ system), member 11	14.1	0.843	-99	Day 3	Low (0.007)
<i>Sord</i>	sorbitol dehydrogenase	11.8	0.899	-4245	P1	-2.0
<i>Srxn1</i>	sulfiredoxin 1 homolog	12.7	0.862	-50	Days 2-3	Low (0.004/0.004)
<i>Timp1</i>	tissue inhibitor of metalloproteinase 1	13	0.877	-1957	Day 1	Low (0.04)
<i>Tlcd2</i>	TLC domain containing 2	13.7	0.877	-4977	P1	2.1
					Day 3	High (0.002)
<i>Tomm20</i>	Translocase of outer mitochondrial membrane 20 homolog	10.3	0.915	-131	Day 2	Low (0.002)

Gene Symbol	Gene Title	PWM	MS	Distance to Transcription Start Site	*Data Source	† FD (Baseline) or Trend/ <i>p</i> value (Hyperoxia)
<i>Txn1l</i>	thioredoxin-like 1	13.1	0.9	45	Day 2	Low (0.001)
<i>Txnrd1</i>	thioredoxin reductase 1	17.9, 10.6	0.928, 0.84	-97, -403	Days 1-3	Low (0.005/0.001/0)
<i>Ubc</i>	ubiquitin C	12.7, 10.6	0.907, 0.871	-1794, -254	Days 1, 3	Low (0.009/0.003)
<i>Wac</i>	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.

¶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. †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.

* Microarray analyses data sources of the Nrf2-dependent genes - baseline (P1-P4) or during hyperoxia (Days 1-3).

Table 2

Representative list of drugs identified using the L1000CDS² search engine of the Library of Integrated Cellular Signature (LINCS) analysis to be predicted to act on Nrf2-dependent lung transcriptome during the bronchopulmonary dysplasia pathogenesis.

Hyperoxia exposure day	Drug name	Activity of compound*	Disease treatment	Minimum overlap ratio	Corresponding <i>p</i> value
day 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	6.280×10^{-6} $/9.929 \times 10^{-4}$
1/3	L-sulforaphane	Induction of Nrf2 and prevent NF- κ B binding	Induce detoxification and xenobiotic enzymes	0.1765/0.2051	3.100×10^{-10} $/7.32 \times 10^{-5}$
1/3	Arachidonyl trifluoromethyl ketone	Inhibits PLA2	Neuroprotective and treatment of multiple	0.1765/0.2564	6.280×10^{-6} 7.650×10^{-4}
1/3	Parthenolide	Binds directly to and inhibit IKK β	Anti-inflammatory and anti-hyperalgesic activities	0.2059/0.2051	6.28×10^{-6} $/9.929 \times 10^{-4}$
3	Celastrol	Inhibits NF- κ B	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 J ₂	PPAR- γ agonist	Antioxidant and anti-inflammatory activities	0.2051	1.210×10^{-10}

Drugs and chemicals were identified using the L1000CDS² 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 L1000CDS² analyses are described elsewhere [41]. Full list of the drugs and chemicals in Appendix 3.

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