The transcriptome of nitrofen-induced pulmonary hypoplasia in the rat model of congenital diaphragmatic hernia

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BACKGROUND: We currently do not know how the herbicide nitrofen induces lung hypoplasia and congenital diaphragmatic hernia in rats. Our aim was to compare the differentially expressed transcriptome of nitrofen-induced hypoplastic lungs to control lungs in embryonic day 13 rat embryos before the development of embryonic diaphragmatic defects.

METHODS: Using next-generation sequencing technology, we identified the expression profile of microRNA (miRNA) and mRNA genes. Once the dataset was validated by both RT-qPCR and digital-PCR, we conducted gene ontology, miRNA target analysis, and orthologous miRNA sequence matching for the deregulated miRNAs *in silico*.

RESULTS: Our study identified 186 known mRNA and 100 miRNAs which were differentially expressed in nitrofen-induced hypoplastic lungs. Sixty-four rat miRNAs homologous to known human miRNAs were identified. A subset of these genes may promote lung hypoplasia in rat and/or human, and we discuss their associations. Potential miRNA pathways relevant to nitrofen-induced lung hypoplasia include PI3K, TGF- β , and cell cycle kinases.

CONCLUSION: Nitrofen-induced hypoplastic lungs have an abnormal transcriptome that may lead to impaired development.

MicroRNAs (miRNAs) are small (17–24bp), noncoding RNAs that regulate different biological functions by modulating expression and stability of target genes posttranscriptionally (1,2). The role of miRNAs in lung development is currently poorly defined. Deregulation of miRNAs, and consequently, loss of fine adjustments to target genes can lead to lung diseases (3).

Babies with congenital diaphragmatic hernia (CDH) have a hole in their diaphragm and abnormal lung development resulting in lung hypoplasia and persistent pulmonary hypertension. CDH constitutes about 8% of major congenital anomalies and has the highest mortality rate (4). In rodents, nitrofen

induces CDH and lung hypoplasia similar to the phenotype observed in humans (5). Whether the nitrofen-rat model shares the same pathogenesis with human CDH remains to be determined. Some hypotheses suggest that nitrofen influences diaphragm and lung development in rodent embryos similar to the human condition (6–8). Thus far, the transcriptomic changes associated with nitrofen-modulated developmental signaling have not been characterized in the lung.

In this study, we used next-generation sequencing (NGS) technology to characterize transcriptome differences between control lungs and nitrofen-induced hypoplastic lungs in embryonic lung tissues prior to the development of the diaphragmatic defect. We hypothesize that transcriptome changes contribute to abnormal lung development in the nitrofen rat model of CDH. Exploring such phenotypic mechanisms in rats could help improve our understanding of abnormal lung development in human CDH.

RESULTS

NGS Sequencing Results

A total of 5,045,279 single-end (50bp) MiSeq miRNA sequence reads were generated and 153,253,385 pair-end (50×35) SOLiD sequence mRNA reads were generated from nitrofen-treated and control lung samples. Over 90% of the mRNA reads were mapped on the *Rattus norvegicus* reference genome (rn5) using Lifescope v2.5.1 software (ThermoFisher, Grand Island, NY) with two-mismatch settings.

miRNA and mRNA Profile Induced by Nitrofen

Once the MiSeq sequences were normalized, a large number of unaligned sequences were identified possibly due to the low abundance of embryonic RNA. Due to the nature of working with limited material derived from embryonic animals, a pooled sample collection was analyzed to strengthen our alignment profile. To compensate for the pooled effects of the data, a strict statistical filtering of the raw miRNA dataset provided a focused approach identifying differentially expressed miRNAs

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in the nitrofen-induced hypoplastic lungs. The dataset resulted in 100 significantly induced miRNAs ($P < 0.01$) that were differentially induced by a fold change greater than 1.5 (see **Supplementary Table S1** online). The sequences of other small RNA species including ribosomal (rRNA), small nuclear, small nucleolar and transfer were annotated against the University of Santa Cruz genome browser (9) (see **Supplementary Figure S1** online). Analysis of the mRNA transcriptome identified 186 significantly induced mRNA coding genes ($P < 0.01$) (see **Supplementary Table S2** online). Nitrofen treatment induced 80 mRNA genes by >twofold change while 106 genes were downregulated by <onefold change relative to controls.

Functional Enrichment Analysis of mRNA

The differentially expressed transcript datasets were entered into QIAGEN's Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, CA) for mRNA (see **Supplementary Table S2** online) and miRNA pathway analysis (see **Supplementary Table S1** online), PANTHER Gene Ontology classification system (10) for functional gene expression analysis (**Figure 1**), and finally differentially expressed genes were mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) (11). IPA analysis identified several signaling pathways including cell survival mechanisms, lipid metabolism, cellular movement and more tissue-specific mechanisms such as connective tissue development. Using PANTHER transcript classification, processes such as metabolism, protein binding, catalytic activity, WNT signaling pathway and protein functions such as transferase activity and nucleic acid binding were most apparent.

Using a binomial distribution and Log2 conversion of the gene expression data, genes were entered into KEGG (11) and set to a binomial distribution of expression (upregulation, red, downregulation, green). Pathways of significant interest included WNT, transforming growth factor (TGF)- β signaling and retinol metabolism (**Figure 2**).

We grouped the identified miRNAs on the basis of their sequence similarity into 83 families. Sequence comparison revealed 64 human orthologs to previously described rat miRNAs (mirBase release 21 (12)). We identified 10 miRNA families with at least 10 sequence reads at the embryonic stage in human that are potentially relevant to the nitrofen-induced lung pathology in the rat. We identified 19 putative interacting miRNA-mRNA pairs highly relevant to nitrofen-induced lung development (**Table 1**). This linkage between miRNAs and mRNA was also statistically linked using IPA software.

Nitrofen-induced Changes in miRNA Expression Affect Gene Transcription

From the differentially expressed miRNAs and mRNA genes, we identified 78 differentially correlated pairs of miRNAs and mRNAs. The assessment of miRNA and mRNA expression levels revealed significant positive and negative correlations in 8 and 11% of the identified pairs, respectively (**Table 1**). The correlations suggest potential functional relationships that contribute to the pathogenesis of lung hypoplasia upon nitrofen treatment. Of the identified mRNA reads, EPHX3, PSG19,

HNF4a, CAR7 and KCNH7 displayed the highest significant differential expression (fold change >6). Amongst the next 82 mRNAs that had a more moderate yet substantial upregulated expression during embryonic and fetal lung development, (fold change >2) were 14 genes: ATP5F1, CD99, PYGO2, MIP, CCDC126, STXBP6, ATP5I, FAM111A, RPI35A, TNKS2, TPMT, ID4, RPS3A, PSMA6. Also, 34 genes were downregulated (fold change <0.5) during embryonic and fetal lung development.

PCR Validation of the Differential Transcripts and miRNAs

Due to the variable nature of gene expression in developmental tissues along with the significant phenotypic changes induced by nitrofen treatment in embryonic rats, a survey of 32 commonly used endogenous genes was performed (see **Supplementary Figure S2** online). Comparing both nitrofen and control E21 lungs, we identified both ABL and GAPDH as excellent endogenous control genes for RT-qPCR (CV: 0.0769, M value: 0.2221). These two genes were used in tandem through a geometric normalization approach for all functional gene expression analysis (digital PCR, dPCR and RT-qPCR). Two-way ANOVA of results run in Prism (ver 6.0) using a Sidak repeated measures test identified that only PSG19 was significantly upregulated compared to control ($P = 0.0010$). Chromatin licensing and DNA replicating factor 1 (CDT1) was significantly downregulated due to nitrofen treatment (two-tailed unpaired t -test, $P = 0.0420$). Using the same cDNA samples and primer sets from the dPCR, RT-qPCR was also conducted. As expected, a similar trend in gene expression was observed. However, no significant changes were shown for PSG19 when compared to control (two-way unpaired t -test, $P = 0.0558$). Using the calculated fold change in gene expression from both dPCR and RT-qPCR, a linear regression was constructed to determine the correlation between RNA-sequencing and gene expression validation (**Figure 3**). Using these methods, dPCR (slope = 0.592, $R^2 = 0.34$) was shown to be a more accurate method to assess changes in gene expression to that of RT-qPCR (slope = 0.457, $R^2 = 0.11$). Furthermore, large changes in gene expression were more strongly correlated between dPCR and RNA sequencing compared to smaller changes.

DISCUSSION

The purpose of this study was to determine which mRNAs and miRNAs are differentially expressed in the embryonic rat lung after nitrofen treatment and which developmental signaling pathways are affected. Large changes in our gene expression data show a similar trend between NGS and functional analysis, however, smaller changes in gene expression are lost amongst the gene expression variability in this embryonic treatment despite identifying key endogenous control genes that are stable after embryonic nitrofen treatment.

Conservation in Structure and Function of RNA-coding Genes Can Be used to Distinct Rat From Human in Terms of Their Responses to Nitrofen

Some miRNA profiling studies during lung organogenesis demonstrated that the overall expression profile was similar for

Figure 1. Transcript gene functional analysis was conducted using the PANTHER gene ontology database. Gene ontology has been categorized into (a) biological processes, (b) cellular components, (c) molecular function, (d) biological pathways, and (e) protein classification.

mouse, rat, and human lung tissue, suggesting evolutionary conservation (13). MiRNA expression profiling by NGS technology has the advantage of both sensitivity, distinguishing between miRNAs that differ in sequences by as little as a single nucleotide and high throughput. Using NGS sequencing technology,

we obtained reads from both the 5' and 3' arms of the pre-miRNAs. The identified rat miRNAs showed strong conservation with known human miRNAs in mirBase with only three unique miRNA seeds missing from known vertebrate homologs. Although these are unique and represent rat-specific sequences,

Existing evidence based on the hairpin fold structure suggests phylogenetic conservation of our rno-miRNA data (15). For example, the orthologous hsa-miR-200c-3p that was enriched in the rat lung tissues and upregulated upon nitrofen treatment exhibited seed conservation with its host gene. Our findings, with regard to lung development, are consistent with increasing evidence from human studies on the effects of teratogenic and toxic triggers on miRNA profiling, and reconcile with orthologous studies matching the nitrofen-mediated upregulation of rat miR-101a-5p, -3p and human miR-144-5p *in vivo* in human airway epithelial cells upon cigarette smoke (16). Both of these miRNAs are also known to target genes associated with lung diseases such as the cystic fibrosis transmembrane conductor regulator gene in human (16). We also reported the upregulation of rat miR-193b after nitrofen exposure. Others attributed cystic fibrosis transmembrane conductor regulator replenishment and cancer regression to the upregulation of miR-193b in human (17). Thus, miR-193b may rescue cystic fibrosis transmembrane conductor regulator downstream of nitrofen in rat lung hypoplasia—a finding that warrants further investigation. We report a 4.04-fold increase in rno-miR-193-3p after nitrofen treatment and a high level of conservation with its human ortholog, demonstrating hsa-miR-193-3p to be a putative antiproliferative molecule that may play a role in CDH progression by a similar mechanism in human.

Table 1. Correlations of the microRNA (miRNA)–mRNA pairs

Pearson correlation coefficient	miRNA annotation (mirBase)	Fold change (nitrofen/control)	P value	Gene
1	rno-miR-3585-5p	2.90	0.01	TPD52L1
1	rno-miR-142-5p	0.63	0.00	ABHD2 , BAZ2A , CYP26B1 , EIF4E3, HIPK2, IKZF2, LRAT , MAML1, ROBO1, SRGAP1
1	rno-miR-504	0.63	0.00	RNF44
1	rno-miR-325-3p	0.55	0.00	B3GNT7, CFTR, CNN1 , FAM189B, FAM189B, FOXN3, GPR155 , IKZF2, INHA, LRRN1, MAML1, MUSTN1, NR5A2 , TRAK1
1	rno-miR-615	0.43	0.00	IKZF2
1	rno-miR-384-5p	0.32	0.00	GCNT2, HIPK2, IKZF2, ITGB3, MAML1, MYH11 , NR5A2 , PCDH20, R3HDM1, RNF44 , SBK1, SCN8A
1	rno-miR-216b-3p	0.19	0.00	FOXN3, IKZF2, SPRED2
1	rno-miR-291a-5p	0.19	0.00	BAZ2A , PARM1, PRKAA2, TGFB3
–1	rno-miR-592	4.50	0.00	CYP26B1
–1	rno-miR-877	3.99	0.00	FOXN3
–1	rno-miR-215	3.43	0.00	ABHD2
–1	rno-miR-3585-5p	2.90	0.01	BAZ2A , IKZF2, ITGB3, SIRPA , SPRY4, SRGAP1, TGFB3
–1	rno-miR-484	2.38	0.00	FOXN3, PCDHGA8 , TGFB3
–1	rno-miR-369-3p	2.12	0.00	ADCY5 , AHDC1, CYP26B1, FOXN3, GREM2, IKZF2
–1	rno-miR-142-5p	0.63	0.00	DNAL1
–1	rno-miR-504	0.63	0.00	PDE1B
–1	rno-miR-325-3p	0.55	0.00	FIBIN, GADD45G, MASP1, MRPS25, MYO7B, PDE1B, PPCS, TMEM205
–1	rno-miR-384-5p	0.32	0.00	ADO, ATP2B2, DCUN1D2
–1	rno-miR-216b-3p	0.19	0.00	ID4

Genes expressed in the lung are in bold. The *P* value indicates the likelihood of the focus genes in a network being found together by random chance. The same computation was used for GO analysis of the initial gene list. The columns are sorted in descending order of *P* value.

Please refer to Supplementary Table S2 online for full names of the gene symbols.

We report a >twofold increase in the expression of another highly conserved miRNA; let-7d-3p in nitrofen-induced hypoplastic lungs. This result is in contrast to Pandit and colleagues (18), who demonstrated that a lack of let-7d promotes alveolar thickening of alveolar compartments exclusively. The latter study demonstrated that a lack of let-7d causes epithelial-to-mesenchymal transition in lung epithelial cells from idiopathic pulmonary fibrosis patients, mediated by TGF- β by a SMAD-dependent mechanism. Nitrofen induced rno-let-7b-5p by 3.89-fold and -7d-3p by 2.27-fold, that target the Ras transcripts downstream of the fibroblast growth factor receptors (19), thus downregulating lung branching and epithelial cell proliferation. Mir-145 consists of a specific miRNA signature associated with pulmonary hypertension and regulates airway smooth muscle cell differentiation according to previous studies (20). Our results indicate that miRNA rno-miR-145-3p and -5p is a putative component in CDH abnormal lung development in both rat and humans, corroborating the results from others (20). Consequently, future studies should target miR-145 to investigate if it plays a role in persistent pulmonary hypertension in CDH.

miRNAs Mediate Nitrofen-induced Cell Cycle Arrest and Proliferation Inhibition

Most differentially expressed genes from the nitrofen lungs are clustered, demonstrating a strong expression profile in the

developing lung. Nitrofen persists in the lung at elevated levels for 3 days and hence a single dose is enough for inducing intense irreversible damage to the cells (5). Nitrofen induces genotoxicity that modulates several miRNAs, and thereby alters critical cell cycle control signaling networks. We and others have previously reported in both the nitrofen rat and mouse model that cell cycle-associated proteins are downregulated in nitrofen-induced lung hypoplasia (8,21,22). Nitrofen has been demonstrated to suppress cell proliferation by inhibiting regulators of DNA replication (8), activation of the mitochondrial apoptosis axis Bcl2, Bax, and Bak, inhibition of Bcl-xl, and executing the activation of p38-mitogen-activated protein kinase P-38 MAPK in cultured type II pneumocytes (23). ABHD2 plays an important role in maintaining lung structure integrity and is expressed in alveolar type II cells in mice lungs. In mouse pulmonary smooth muscle cells, ABHD2 triggers the differentiation of monocytes to macrophages. Its deficiency often presents emphysema-like symptoms including increased macrophage infiltration, increased inflammatory cytokines, a protease/anti-protease imbalance and enhanced apoptosis in a strikingly similar fashion to human pathology (24). Here, we identified a nitrofen-perturbed immune response pattern in the lung and implicated a role for ABHD2. Similarly to nitrofen, the lung carcinogen benzo(a)pyrene downregulates miR-142-5p significantly (25).

Figure 3. Next-generation sequencing (NGS) validation by digital PCR and RT-qPCR. Validation of the expression of a subsample of genes by dPCR. Two-way ANOVA ($P < 0.001$) (a, b). RT-qPCR of the selected NGS transcripts with no significant changes were shown (PSG19 two-tailed t -test ($P = 0.0558$)) (c). (d) Linear regression correlation of RNA-seq (y axis) with dPCR and RT-qPCR data (x axis) using a log2 mean-fold change measure of the genes differentially expressed across the two gene-expression platforms under correlation analysis. Outliers (Ephx3) were identified and removed as having 1.5 SD or larger. Grey indicates control, black indicates nitrofen-treated samples, circles indicates biological replicates, triangles represent dPCR results, and open squares indicate RT-qPCR results.

In the current study, rno-miR-325-3p positively correlated with nitrofen-induced GPR155 and NR5A2. GPR155 is an integral membrane protein related to G-protein-coupled receptors. The mRNAs for GPR155 are widely expressed in adult mouse tissues and during the development of the lung and brain. Rno-miR-384-5p and NR5A2 expressions correlate positively upon nitrofen exposure. MiR-384-5p is a marker of neurotoxicity and regulates apoptotic cell death in myocardial ischemia by modulating PIK3CD pathway (26). Myosin heavy chain 11 is a component of the contractile apparatus of aortic smooth muscle cells and is expressed in neonatal lung smooth muscle cells (27). RNF44 is a poorly understood protein that is encoded by a gene and contains a RING finger motif, a motif often present on proteins involved in protein-protein and protein-DNA interactions. In the current study, nitrofen induced both myosin heavy chain 11 and RNF44 in embryonic lungs, and their expression

positively correlated with miR-384-5P expression. These data suggest a potential role for this pathway in the vascular remodeling observed in CDH-associated pulmonary hypertension.

miRNAs Regulate Nitrofen-induced Airway Smooth Muscle Cell Dysfunction

Airway smooth muscle cells (ASMC) progenitors in the embryonic lung mesenchyme produce fibroblast growth factor-10 required for epithelial cell morphogenesis. Fibroblast growth factor-10 expression is crucial for fine tuning the spatiotemporal ASMC activity early in human development. The relationship between lung hypoplasia, fibroblast growth factor-10 deficiency, and dysfunctional ASMC from birth onward is linked (28), and as a perturbation of this relationship by nitrofen results in abnormal smooth muscle relaxation near term. Calponin (CNN1), a multifunctional

orthologous protein in both rats and humans in ASMC, is tightly restricted to differentiated ASMC lineages including those in the lung during embryonic and postnatal life. In the current study, nitrofen downregulates CNN1, the latter correlates with the miR-504 upregulated expression.

E-cadherin and integrin are major components of the extracellular matrix. Loss of E-cadherin is a hallmark of epithelial-to-mesenchymal transition and synergizes the dedifferentiation capacity of the cell. From our study, we identified a nitrofen-induced upregulation of rno-miR-296-3p. MiR-192/215 has been shown to mediate changes in E-cadherin expression via targeting the ZEB2 family of transcriptional repressors downstream of TGF- β (29). In addition, our study showed downregulation of members of the miR-200 family (rno-miR-200c-3p, rno-miR-200c-5p, and rno-miR-200b-5p) upon nitrofen treatment by >twofold relative to the control. Expression of miR-200 and ZEB1/2 is linked to a negative feedback loop (30) that may involve rno-miR-200c-3p, -200b-5p in a crosstalk with TGF- β . A previous study from Xu *et al.* (31) using immunohistochemistry in the nitrofen rat model demonstrated that TGF- β 1 protein was stronger in CDH lungs.

Retinoic acid (RA) signaling is essential for fetal lung development and postnatal alveolar septation (32). Nitrofen suppresses the retinoid response element by a mechanism yet to be identified (33) and this suppression is reversible by RA supplementation. CYP26B1 gene encodes cytochrome P450, an RA metabolizing enzyme that is expressed in embryonic lung tissues undergoing morphogenesis. CYP26B1 fine tunes the activity of RA. The primary function of lecithin retinol acyltransferase is to convert retinol to the more functional retinyl esters. We report that nitrofen inhibits CYP26B1 and lecithin retinol acyltransferase. Studies have shown that inhibiting CYP26 augments RA function in human epidermal keratinocytes (34). The above data corroborates with previous findings that RA is diminished upon nitrofen exposure. We find that CYP26B1 and lecithin retinol acyltransferase correlate positively with miR-142-5p expression, whereas miR-592 negatively correlates with CYP26B1 expression. These results confirm the previous evidence from clinical studies that showed retinol levels to be lower in babies born with CDH (35). MiR-10a is an RA target (36) and is localized in the vicinity of the HOXB cluster of chromosome 17 in humans. The HOXB locus is involved in lung branching morphogenesis and has a putative role in determining cell fate decision in the lung (37). Our study identified nitrofen-mediated upregulation of rno-miR-10b. MiR-10a (a close homolog of MiR-10b) is a known mediator of RHOC, and functions through the upstream axis TWIST-miR-10b-HOXD10-RHOC (38). PI3K/mTOR signaling is required for miR-10 activity and depends on their interactions with 5' TOP mRNAs and cell type (39). Doi *et al.* (40) previously reported that PI3K mRNA is decreased in E21 nitrofen-induced hypoplastic lungs. They also showed with immunohistochemistry that PI3K protein was diminished in the distal epithelium of E18 CDH lungs and that PI3K overall intensity was decreased in E21 CDH lungs (40).

Our NGS analysis demonstrated that rno-miR-142-5p and -291a-5p are differentially expressed and correlate positively with BAZ2A, a chromatin-dependent transcriptional regulator—an integral component of chromatin re-modeling complexes. We report that rno-miR-3585-5p negatively alters two genes; BAZ2A and SIRPA; however, the exact mechanism is poorly understood. It has been shown that SIRPA, a member of the signal-regulatory-protein family, can interact with surfactant proteins A and D to suppress alveolar phagocytosis without triggering an inflammatory response (41). This protein can be phosphorylated by tyrosine kinases and is found to participate in signal transduction triggered by CD47 and mediated by various growth factor receptors.

Limitations and Future Research

One of the limitations of our study is the use of the teratogen nitrofen to induce lung hypoplasia and CDH. Even though this model has been widely used and accepted to study the pathogenesis of abnormal lung development and CDH (reviewed by us in (5)), a direct link between nitrofen use and abnormal lung development and CDH in humans has never been established.

When performing a transcriptomic analysis, there are limitations that are inherent to the design of such a study. We have shown that several genes and miRNAs are involved in nitrofen-induced abnormal lung development and CDH. How these transcript differences translate to changes in protein structure and function remains unknown. A high-throughput proteomic survey combined with immunoblotting candidate proteins would be ideal to assess these differences. It is possible that the sampling differences between the NGS study and that conducted by the biological replicates could be the strongest effect being shown. The RNA used for the experiment was pooled from multiple dams whereas RNA used for the functional gene expression analysis was true biological replicates derived from multiple dams. These biological replicates are essential as nitrofen induces a CDH-like lung phenotype in up to 100% of E13.5 rats. Due to this uncertainty in the developmental phenotype, the variability that would be induced at the genetic level due to nitrofen would be more pronounced than shown in the phenotypic presentation of nitrofen-induced CDH. This biological bias combined with the technical variability in NGS complicates the functional assessment of the NGS results. Further extrapolations into the transcriptomic effects of nitrofen warrant a stronger presence of biological diversity amongst samples. An important follow-up study would be a combined miRNA and mRNA sequencing experiment across consecutive stages of lung development. In our study, the age-matched embryonic tissues and the nature of the collected samples limited our primary material and thus we had to pool multiple lungs for analysis. This approach did not permit the proper assessment of biological variation. Additionally, using a mixed sample approach studying a highly heterogeneous organ such as the lung does not permit focusing on functionally distinct regions or substructures, making the establishment of expression profiles less informative. We could not confirm tissue-specific, strain-specific or cell-specific differential miRNA or gene expression pattern for all reads analyzed. This would have

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Total RNAseq Library Preparation and Sequencing

Using 5 µg of ribosomal depleted total RNA (Epicenter Ribo-Zero, Illumina), the total RNA library was created using the SOLiD total RNA-Seq kit (ThermoFisher). Briefly, the RNA was fragmented to 100–200 bp using RNase III before being hybridized and ligated to SOLiD adapters. The library was reverse transcribed to cDNA before being size selected using AmPure XP beads (Beckman Coulter, Mississauga, Canada). Once cDNA libraries were barcoded (SOLiD primers, ThermoFisher) and assessed (Agilent Technologies), libraries were pooled and run for next-generation sequencing on the SOLiD 5500xl instrument (ThermoFisher).

Bioinformatics and Statistical Analysis

SOLiD RNAseq data were mapped to the *R. norvegicus* reference genome (rn5) using Lifescope v2.5.1 software (ThermoFisher) with the two-mismatch settings. The mapped reads were quantified against the University of Santa Cruz-annotated refGenes and normalized gene expression profiles, reads per kilobase per million reads, were generated. The bioconductor package edgeR (42) based on a negative binomial model was used to infer the differentially expressed genes between the treated and control samples. MiSeq sequence reads were used for miRNA profiling. After adaptor removal and quality check, the differentially expressed mature miRNAs were analyzed using the mirTools 2.0 pipeline (43). The rno mirBase v16 with “best match” setting was used. We detected 100 miRNAs by a cutoff of $P \leq 0.01$ and fold change (FC) ≥ 1.5 and their mature sequences were further identified using mirBase. The miRNA and mRNA expression data files of the nitrofen-treated and control lung tissue samples were loaded into the Partek Genomics Suite v6.6 (Partek Inc, St Louis, MO). The Pearson correlations of the miRNAs and their target mRNAs were analyzed against the rno-miR database using the miRNA integration module. For differentially expressed miRNAs, all sequence reads in rat and mRNA targets overlapping with differentially expressed genes were used to perform IPA pathway analysis to obtain a list of enriched pathways (Ingenuity Systems, Redwood City, CA). We identified human ortholog sequences by mapping the short (rno) reads to a known miRNA database (mirBase) using BLASTN and other tools from the mirOrtho database (44) (see **Supplementary Table S3** online). We identified homolog sequences of selected miRNAs among different species using the stem loop structure. Consequently, the aligned sequence conservation of miRNAs was compared across species (see **Supplementary Table S4** online and **Figure 4**). Differential expression of miRNA between paired samples was measured using a Chi-squared statistic. P values were calculated for the null hypothesis that no differential expression existed between the two samples. Final P values are corrected using the Bonferroni correction for multiple hypothesis testing.

Endogenous Control TaqMan Assay

Embryonic rat lungs (E21) were isolated from three different dams, and total RNA was extracted and reverse transcribed as described previously. A rat endogenous control plate (ThermoFisher) was seeded with cDNA (25 ng RNA input per reaction), TaqMan Fast Advanced Master Mix (ThermoFisher), and water to 10 µL. The plates were run on a CFX96 thermocycler (Bio-Rad, Mississauga, Canada) using the recommended cycling parameters and Raw C_t values were reported along with m-value analysis provided by the accompanied software (CFX Manager 3.1; Bio-Rad).

Primer Design and Optimization

Briefly, primers were designed using Primer Quest (Integrated DNA Technologies, Coralville, IA) and verified for specificity using UNAFold (IDT), and Primer-Blast (45) software (see **Supplementary Table S5** online). Primer specificity was verified using conventional molecular techniques before being optimized for SYBR Green annealing temperature and melting curve specificity using gradient RT-qPCR and also determined with digital-PCR (dPCR).

Digital PCR

Samples were diluted to an optimal concentration (1.6 copies/µL in water) using an Early Access Digital PCR Experiment Design application (ThermoFisher). Each sample was combined with QuantStudio 3D Master Mix (ThermoFisher), SYBR I (ThermoFisher), primers (500 nM each) and built up to 14.5 µL with water. Reactions were

loaded, filled, and sealed on separate QuantStudio 3D Digital PCR 20k Chips (ThermoFisher) and run on a QuantStudio 3D chip reader (ThermoFisher) with data analysis completed using the associated online software.

RT-qPCR

Using SsoAdvanced SYBR green supermix (Bio-Rad), each reaction well was loaded with primers (500 nM each), along with 10 ng of template and built up to 20 µL with water. Samples run using a CFX 96 thermocycler with the following cycling parameters: 95 °C 30 s, 40 cycles of 95 °C for 15 s and annealing at 59 °C for 30 s after which a melting curve was performed. Two endogenous controls as determined by the TaqMan assay were used to calculate changes in expression (CFX Manager 3.1, Bio-Rad).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/pr>

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