Main title: Pulmonary Vascular Disease is evident in gene regulation of
experimental Bronchopulmonary dysplasia

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Abstract:

Objective: To examine the gene expression regarding Pulmonary Vascular Disease in experimental Bronchopulmonary Dysplasia in young mice.

Premature delivery puts babies at risk of severe complications. Bronchopulmonary dysplasia (BPD) is a common complication of premature birth leading to lifelong affection of pulmonary function. BPD is recognized as a disease of arrested alveolar development. The disease process is not fully described and no complete cure or prevention is known. The focus of interest in the search for treatment and prevention of BPD has traditionally been at airspace level; however, the pulmonary vasculature is increasingly acknowledged in the pathology of BPD. The aim of the investigation was to study the gene expression in lungs with BPD with regards to Pulmonary Vascular Disease (PVD).

Methods: We employed a murine model of hyperoxia induced BPD and gene expression microarray technique to determine the mRNA expression in lung tissue from young mice. We combined gene expression pathway analysis and analyzed the biological function of multiple single gene transcripts from lung homogenate to study the PVD relevant gene expression.

Results: There were $n=117$ significantly differentially regulated genes related to PVD via down-regulation of contractile elements, up- and down-regulation of factors involved in vascular tone and tissue specific genes. Several genes also allowed for pinpointing gene expression differences to the pulmonary vasculature. The gene *Nppa* coding for Natriuretic peptide, a potent vasodilator, was significantly down-regulated and there was a significant up-regulation of *Pde1a* (Phosphodiesterase 1A), *Ptger3* (Prostaglandin E receptor 3) and *Ptgs1* (Prostaglandin-Endoperoxide Synthase 1).

Conclusion: The pulmonary vasculature is affected by arrest of secondary alveolarization as seen by differentially regulated genes involved in vascular tone and pulmonary vasculature suggesting BPD is not purely an airspace disease. Clues to prevention and treatment may lie in the pulmonary vascular system.

Rationale:

This paper shows how the gene expression in lung tissue from young mice with Bronchopulmonary dysplasia suggests that the pulmonary vasculature expresses genes differently than controls. Newborn mice pups subjected to chronic hyperoxia from birth mimicks the environment in which premature babies reside after being born prematurely. Literature supports that lung function parameters from prematurely born

humans is permanently reduced and significantly more so if they also develop Bronchopulmonary Dysplasia.

When examining the gene expression in newborn mouse lungs subjected to chronic hyperoxic damage alone, the confounding factors that premature newborns display (infections, hyperbaric ventilation etc) are limited so some parts of the molecular background of disrupted alveolarization can be further explored. The cardiovascular system is closely linked to BPD through increased risk of pulmonary hypertension and Pulmonary Vascular Disease (PVD) and this paper gives a new insight by gathering all PVD-relevant genes from a whole transcriptome analysis performed on lung tissue homogenate from young mice after chronic hyperoxia treatment.

Introduction:

Bronchopulmonary dysplasia (BPD) is a common complication for premature neonates and affects the respiratory system in early childhood and into adulthood [1, 2, 3] and no prevention or treatment is known. Patients with BPD have persistently reduced lung function and lowered exercise tolerance, decreased diffusing capacity and increased bronchial hyperreactivity later in childhood, often misdiagnosed as asthma.[4, 5, 6, 7] There is increasing evidence of BPD affecting the heart and cardiovascular system. [8, 9, 10] BPD is a clinical diagnosis however; the diagnostic criteria include no description of the underlying pathology [11].

Traditionally BPD was considered to be an airspace disease for which modes for prevention and treatment has been sought; however, the vascular pathology in BPD is increasingly acknowledged. [12, 13]. The pathology of BPD is described as an arrest of secondary alveolarization resulting in abnormal and disturbed vascular development with abnormalities at alveolar capillary level in turn leading to up- and down-stream affection of the pulmonary vasculature [14, 15]. The aberrant growth, function and structure in pulmonary vasculature and the consequences of these occurring in lungs with BPD have been termed Pulmonary Vascular Disease (PVD) [16, 17]. PVD is likely to be an emerging problem in the near future as the babies who survive the Neonatal Intensive Care Units are going through childhood with complications from BPD also concerning the pulmonary vasculature.

A relationship between BPD and Pulmonary Hypertension (PH) is well known and BPD-associated PH complicates the clinical course, ultimately leading to poor long-term survival [10, 18, 19]. Case reports include right ventricular failure in babies with BPD thought to be a consequence of PH [20]. Pulmonary Vascular Resistance is increased in infants with BPD and is seen to contribute to further PVD in turn

suggesting the relevance of PVD as a contributing component in the pathology in BPD.[11, 21],[17] [22]

There is a demand for knowledge about the molecular biology behind PVD in BPD in the search for possible prevention and treatment options. Gene expression from human lung tissue with BPD is scarce and often obtained postmortem or with co existing pathology and gene expression analyzed from nucleated cells from blood samples will not reflect cellular processes in the lung tissue.

The aim of the study was to explore the gene expression regarding pulmonary vasculature and PVD in lungs with experimental BPD. To achieve this objective we performed a whole transcriptome mRNA analysis examining the gene expression in lungs from young mice with hyperoxia induced experimental BPD and further selected out all PVD relevant gene transcripts.

This is to our knowledge the first microarray study of the whole transcriptome of mouse lungs with hyperoxia induced BPD specifically searching for PVD relevant gene transcripts.

Results:

We found that $n=117$ gene transcripts directly relating to pulmonary vasculature and altered vascular tone, including possible PH, were significantly differentially expressed in lung tissue between normoxia and hyperoxia groups. Eighty-five were down-regulated and thirty two were up-regulated. All genes ($n=117$) involved in PVD are presented in a supplementary table with gene names, description of names, short description of function, mode of regulation and fold change values. The experiment was validated by qRT-PCR and there was a strong positive correlation between microarray expression values and qRT-PCR values, $r=0.905$, $p<0.001$ (figure 1).

There were no differences when accounting for gender.

A total of 311 gene transcripts were significantly differentially expressed in the whole transcriptome study.

Pathway analysis:

Eight KEGG (Kyoto encyclopedia of Genes and Genomes) pathways were recognized after analyzing the gene set with the DAVID Bioinformatics Resources 6.8 National Institute of Allergy and Infectious Diseases resource. Five pathways are directly related to cardiac muscle function these being the pathways for; hypertrophic cardiomyopathy, dilated cardiomyopathy, cardiac muscle contraction, arrhythmogenic right ventricular cardiomyopathy and viral myocarditis. Three pathways involved in muscle activity, contractility and energy production; calcium signaling, focal adhesion and PPAR ((Peroxisome Proliferator-Activated Receptor) signaling.

There were more down-regulated ($n=25$) than up-regulated genes ($n=13$) (table 1).

The genes are overlapping between the pathways and the relationships between the shared genes are visualized in table 1.

Discussion:

Our results consisting of 117 significantly differentially expressed genes all related to pulmonary vasculature and vascular tone add up to suggest in part the molecular basis of Pulmonary Vascular Disease in lungs with experimental BPD. Our findings being that three fourths of the significantly differentially expressed genes are down-regulated, largely genes involved in contractility, supports the theory that the pulmonary vascular tone is altered in lungs with BPD. Out of the 117 genes related to pulmonary vasculature, 35 genes are directly linked to cardiac cells or related to cardiac (cell) development and function. In addition four out of seven pulmonary vascular disease relevant KEGG pathways are cardiac muscle specific ([table 1](#)). Troponin C type 1 (*Tnnc1*), troponin I type 3 (*Tnni3*), troponin I3 interacting kinase (*Tnni3k*) and troponin T type 1 (*Tnnt1*) and 2 (*Tnnt2*) are all significantly down-regulated in our study and are represented in these pathways. Cardiac muscle cells are described in lung vasculature in mice [23, 24]. They are found as sleeves of striated cardiomyocytes covering the wall of inter pulmonary and pulmonary veins. These cells are capable of hypertrophic response comparable to that seen in cardiomyopathies [24] and may function as right ventricular cardiac muscle. This pulmonary venous myocardium is also known to display granules containing natriuretic peptide [24, 25]. It is therefore interesting that Natriuretic Peptide A (*Nppa*), the gene for atrial natriuretic peptide is down-regulated in our gene set. Natriuretic Peptide A is locally released from pulmonary myocardial cells and directly regulates the blood pressure in the pulmonary vascular circuit. Natriuretic Peptide A is closely related to ProBNP, which has shown to correlate with pulmonary vessel pressure, pulmonary vascular resistance and right atrial pressure, additionally,

ProBNP is used clinically in the assessment of PH in babies [18, 21]. The down-regulation of these genes may be a sign of vascular compensation for an increased pulmonary vascular pressure due to dysfunctional vascular development.

It has been shown that long term survivors of BPD have increased airway bronchial hyper reactivity [26]. This involves the bronchial smooth muscle cells and they could theoretically be candidates for differential regulation in genes involving muscle contraction and hypertrophy. However; bronchial smooth muscle does not contain striated or myocardial type muscle, at least not described so far, but this will be true for troponins (Troponin C, Troponin I and Troponin T), which are cardiac specific. There was no significant differential regulation in troponins in the blood samples taken at the same time in the same animals (unpublished data) and no heart tissue added to our lung homogenate.

Our findings regarding the cardiac specific/cardiac muscle cell related down-regulated mRNA for genes relevant for pulmonary vascular disease are novel in that they allow for pinpointing the pulmonary vasculature as a site for differential gene regulation of muscle activity and elements of contractility.

These findings are supported by additional pathway hits, one being the KEGG pathway for Ca^{2+} regulation. The role of Ca^{2+} channels in pulmonary arterial hypertension at a molecular level has been discussed and astringent control of Ca^{2+} regulation in smooth muscle cells (SMC) is a part of blood pressure control in pulmonary vessels [27]. Calcium and the Ca^{2+} signaling pathway participate in the development of the lung [28, 29, 30].

Another relevant pathway discovered by our study is the focal adhesion pathway. Focal adhesion is involved in actin skeleton remodeling and in increased cell movement as in stress. It is likely that response to stress could occur in vessels with disturbed muscle tone possibly working to control a situation resembling persistent hypertension caused by abnormal development of lung microvasculature. The gene Actinin 2 (*Actn2*) is down-regulated and represented in both the focal adhesion pathway and arrhythmogenic right ventricular cardiomyopathy pathway, suggesting a relationship between these pathways.

We found significantly differentially expressed genes from the PPAR α pathway in our gene set. PPARs are transcription factors and are involved in energy production and expressed in smooth muscle cells (SMC). PPARs are also involved in inflammation and in release of some pro-inflammatory and pro-angiogenic factors that are relevant to BPD as oxygen supplementation is known to cause inflammation [31].

From the 117 genes that are related to PVD and vascular tone some gene transcripts are particularly relevant and are presented in the following paragraphs. There was significant up-regulation of Phosphodiesterase 1A (*Pde1a*), Prostaglandin E receptor 3 (*Ptger3*) and Prostaglandin-Endoperoxide Synthase 1 (*Ptgs1*) in addition to down-regulation of Phosphodiesterase 4D Interacting Protein (*Pde4dip*) and Microsomal glutathione S-transferase (*Mgst2*). Phosphodiesterases cause vascular relaxation and prostacyclins are pulmonary vasodilators and are both used in the management of PH in BPD in babies [21]. This could suggest that the BPD inflicted lung produces phosphodiesterase and prostaglandin E for the functional purpose of vascular relaxation. These findings are consistent with previous studies [32].

It has been postulated that there is a permanent vascular hypertension in BPD [14, 17]. Down-regulation of genes of contractile elements and of vascular muscle tissue could indicate that the lung attempts to respond to a higher pressure in the pulmonary vascular system by chronic functional vascular relaxation [21]. Mourani and Abman [16] have discussed how PVD in BPD could result in functional PH through impaired and suboptimal gas exchange and altered pulmonary blood flow distribution even if it is not severe enough to be clinically recognized as frank PH and our findings seem to support this.

Other gene transcripts from our study represent proteins involved in muscle contraction and constituents of muscle contractile elements and in being significantly differentially expressed they can suggest a functional affection of muscle cells in the lung inflicted with experimental BPD. The contractile elements are anchored to the cytoskeleton by focal adhesion which allows for communication between cells and extracellular matrix allowing cross talk that aids synchronized function (e.g. keeping vascular tone) in the vessels [33]. Our findings include transcripts from genes that are involved in contractile elements that are general for smooth muscle cells, not just cardiac muscle cell specifically. There are several possible origins for genes relating to muscle cells and contractility. Myofibroblasts, fibrocytes and smooth muscle cells (SMC) are all contractile and it is possible that circulating progenitor cells that differentiate into these contractile cells, and they may be recruited due to abnormal vascular tone (e.g. PH) and inhabit the pulmonary vascular system in the BPD lung [27, 34]. Fibroblast growth factor/Myocyte-Activating Factor (*Figf12*) and Bone morphogenetic protein 7 (*Bmp7*) are down-regulated in our samples and Transforming Growth Factor β 3 (*TGF β 3*) is up-regulated, all three are known to be related to PH

through BMP/TGF β signaling [27, 35]. The effect of PH on cells in the extracellular matrix in the pulmonary vessels may account for some of the differences in gene regulation that we have seen. These cells may express genes for contractile elements and might be programmed to “loosen” the vascular tone resulting from abnormal lung development (figure 2).

Finally, the gene VEGF-D (*Figf*) is up-regulated in our findings and this is consistent with literature [36]. The vascular-endothelial growth factors (VEGFs) are known to be important in angio- and vasculogenesis in the lung [14, 36]. The gene *Flt-3* is up-regulated in our findings, and is thought to have relation to angiogenesis, [36, 37]. The pulmonary vascular development precedes the alveolar development and the relationship of angiogenesis (formation of new blood vessels from pre-existing ones) and vasculogenesis (de novo formation of blood vessels) is extensively scrutinized in the context of BPD [14, 33, 36]. The exact mechanism of angiogenesis and vasculogenesis in lungs with BPD is unknown but may be involved in repair and remodeling of the pulmonary vasculature in lungs with BPD [27]. Current research supports the importance of angiogenesis in BPD, however results regarding angiogenesis-relevant factors are divergent [15, 38].

In summary, the results from our gene expression study in mouse lungs with BPD enlighten the background of PVD in BPD. The vessels are different in function; hormones and other factors exert regulatory effects on the pulmonary vasculature in lungs with BPD that leads to chronic PVD (figure 3).

Future studies regarding prevention or therapies for BPD should look further into the pulmonary vascular system. The future scope of our group is to study the gene expression in hearts from the subjects whose lungs are examined in this paper.

Limitations

The transcriptome analysis has limitations with regards to predicting the actual proteomics and metabolomics in the tissues or cells being studied. Interpreting significance in gene expression profiles is sensitive to cut-off values. Additional time points could have been beneficial in order to compare possible fluctuations in gene expression. The BPD hyperoxia model is limited compared to clinical BPD as the pathogenesis is multifactorial. Due to ethical challenges and scarcity of representative human material, current knowledge of the development and pathology of BPD is largely derived from animal models and the limited examinations of lung tissue from BPD non-survivors and severely ill patients are often not representative for the majority of patients with BPD [14]. Mice are born immature with lung development in the sacular stage equivalent to human gestation from around 26-36 weeks of pregnancy. When a mouse is 28 days old as in this study, they are loosely “comparable” to humans in early primary school age, 5-10 yrs of age and with lung development in the alveolar stage. However the mechanisms of cell cycle, inflammation/repair and re-adjustments are relative to the kinetics of molecular biology making absolute comparisons imprecise. The number of mice in each group may affect the results as may the choice of mouse strain.

Addendum:**Materials and Methods:****Animal experiment:**

All animal experiments were approved by the Norwegian board of animal research welfare. (NARA 50/13-5458). Newborn mice (C57Bl/6Tac) were randomized [ENREF 22](#) to hyperoxia (85% O₂) or normoxia (21% O₂) within 24 hrs after birth and kept accordingly for 14 days. All animals were kept under normoxic conditions during the subsequent 14 days ([figure 4](#)) [39]. Mothers were switched every 24 hrs to avoid oxygen toxicity. All animals had free access to food and water and kept under standard conditions in A-Chambers, BioSpherix Ltd, Parish, NY, USA, O₂- monitor, ProOX110 BioSpherix Ltd, Parish, NY, USA, CO₂- monitor, ProCO₂ P120, BioSpherix Ltd, Parish, NY, USA). Lung tissue was harvested on day P28 after euthanasia with i.p. ZRF cocktail (Zolazepam/Tiletamine/Xylazine/Fentanyl). Thirty six samples were randomized for histological examination ([figure 5](#)). Lungs were trans-tracheally instilled with paraformaldehyde [40], paraffin embedded, sectioned and stained with Hematoxylin and Eosin and Mean Linear Intercepts (MLI) determined as previously described [40] ([figure 5](#)) (three were excluded due to inadequate inflation). Mothers were switched every 24 hours to prevent oxygen toxicity and to ensure equality in maternal care and nutrition between pups.

Microarray expression analysis:

Total RNA was extracted from lung tissue ($n=33$ hyperoxia and $n=31$ normoxia) by RNeasy Micro Kit – (QIAGEN, Hilden, Germany) then analysed by SurePrint G3 Mouse Gene Expression 8x60K Microarray (Agilent Technologies, CA, USA) from 100 ng RNA pr sample, according to manufacturer's protocol "Two-Color Microarray-

Based Gene Expression Analysis (Low Input Quick Amp Labeling) Protocol v 6.5”.

The universal mouse reference RNA (Agilent Technologies, CA, USA) was used as an internal control. Microarrays were run for each individual.

Quantitative Real-Time PCR (qRT-PCR) validation/analysis:

Microarray results were validated by qRT-PCR method using TaqMan probes. (Figure 3) Nine randomly selected genes (tables 2 and 3) and two endogenous controls were used. cDNA was synthesized by reverse transcription, (800 ng of total RNA) with SuperScript III First-Strand Synthesis SuperMix (Invitrogen, ThermoFisher Scientific, MA, USA). Each qRT-PCR reaction was carried out in duplicates applying specific assays of primers and probes for each gene target (table 3 and 4), using Universal Master Mix II, no UNG (LifeTechnologies, ThermoFisher Scientific, MA, USA). To analyse the qRT-PCR experiments data we used the relative quantification and $2^{-\Delta\Delta Ct}$ algorithm (table 3).

Gene identification

Information regarding genes (descriptions, names, functions and locations of the genes) was searched for in the following databases; Gene Cards

(<http://www.genecards.org/>) , HUGO Gene Nomenclature Committee

(<http://www.genenames.org/>), Ensembl (http://www.ensembl.org/Mus_musculus/),

EMBL-EBI (<http://www.ebi.ac.uk/>), The UniProt Knowledgebase (UniProtKB)

<http://www.uniprot.org/>, Mouse genome informatics <http://www.informatics.jax.org/>,

and NCBI gene (<http://www.ncbi.nlm.nih.gov/gene/>).

The gene set was analyzed in DAVID Bioinformatics Resources 6.8 (<https://david-d.ncifcrf.gov/>)

Statistics:

Microarray data were normalized before a moderated t -test was applied to compare the gene expression of single genes between the groups, using the limma package [41] in R. The results were corrected for multiple testing by applying the Benjamini-Hochberg procedure. A 0.05 significance level of the adjusted p -values was used.

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Figure legends:

Figure 1: Validation of microarray results by qRT-PCR

a $2^{-\Delta\Delta Ct}$ versus microarray fold change expression values for the nine genes selected for validation.

b Fold change values and qRT-PCR values for the nine genes selected for validation. Values above 1 indicate up-regulated genes, values below 1 indicate down-regulated genes.

Figure 2: Combined effects of genes involving muscle contraction, function and composition leads to altered pulmonary vascular tone.

Figure 3: Multiple factors combine to give the condition of Pulmonary Vascular Disease seen in Bronchopulmonary Dysplasia.

Figure 4:

Study design

Figure 5:

Verification of the BPD hyperoxia model by histological examination.

a Mouse lung tissue at equal inflation and magnification.

b Mean Linear Intercepts, groups compared with independent *t*-test.