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Original article

Hyperoxia induces epigenetic changes in newborn mice lungs

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ABSTRACT

Supplemental oxygen exposure is a risk factor for the development of bronchopulmonary dysplasia (BPD). Reactive oxygen species may damage lung tissue, but hyperoxia also has the potential to alter genome activity via changes in DNA methylation. Understanding the epigenetic potential of hyperoxia would enable further improvement of the therapeutic strategies for BPD.

Here we aimed to identify hyperoxia-related alterations in DNA methylation, which could affect the activity of crucial genetic pathways involved in the development of hyperoxic lung injury.

Newborn mice (n = 24) were randomized to hyperoxia (85% O_2) or normoxia groups for 14 days, followed by normoxia for the subsequent 14 days. The mice were sacrificed on day 28, and lung tissue was analyzed using microarrays developed for the assessment of genome methylation and expression profiles.

The mean DNA methylation level was higher in the hyperoxia group than the normoxia group. The analysis of specific DNA fragments revealed hypermethylation of > 1000 gene promoters in the hyperoxia group, confirming the presence of the DNA-hypermethylation effect of hyperoxia.

Further analysis showed significant enrichment of the TGF-β signaling pathway (p = 0.0013). The hypermethylated genes included *Tgfbr1*, *Crebbp,* and *Creb1*, which play central roles in the TGF-β signaling pathway and cell cycle regulation.

Genome expression analysis revealed in the hyperoxia group complementary downregulation of genes that are crucial for cell cycle regulation (*Crebbp, Smad2,* and *Smad3*).

These results suggest the involvement of the methylation of TGF-β pathway genes in lung tissue reaction to hyperoxia. The data also suggest that hyperoxia may be a programming factor in newborn mice.

1. Introduction

Supplemental oxygen administered to premature babies might injure the lungs [1] and, consequently, contribute to the development of bronchopulmonary dysplasia (BPD). However, the pathogenesis of BPD, which is a serious complication of prematurity, is complex and includes a range of external risk factors, as well as genetic susceptibility. Functional alterations of genomic pathways related to several genes encoding important growth factors, such as vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), and insulin-like growth factor (IGF), are among key factors responsible for such susceptibility [2]. VEGF regulates endothelial cell differentiation and angiogenesis and plays a central role in the formation of embryonic vasculature [3]. TGF-β is involved in inhibition of branching morphogenesis and alveolarization in embryonic lung development [4]. IGF is also involved in growth and injury repair processes in many organs, including the lungs [5].

It is well known that oxidative stress due to reactive oxygen species (ROS) and reactive nitrogen species (RNS) can (chemically) damage lung tissue. However, hyperoxia also has the potential to alter the genome activity in lung cells by inducing DNA modifications. ROS/RNS modify cytosine with the oxidative conversion of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine. Peroxides can also modify cytosine to 5-chlorocytosine, which mimics 5-mC [6,7]. The above changes induce

Abbreviations: BPD, bronchopulmonary dysplasia; VEGF, vascular endothelial growth factor; TGF-β, transforming growth factor-β; IGF, insulin-like growth factor; 5-mC, 5-methylcytosine; ROS, reactive oxygen species; RNS, reactive nitrogen species; DAVID, Database for Annotation, Visualization and Integrated Discovery; KEGG, Kyoto Encyclopedia of Genes and Genomes

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improper DNA methylation by inhibiting the binding of DNA methyltransferase 1 (Dnmt1) to DNA [8,9]. The subsequent alteration of the methylation pattern within the CpG sequences can, in turn, result in gene silencing [10]. The above processes might serve as an example of epigenetic regulation of genome activity.

Understanding the epigenetic potential of hyperoxic lung injury might enable further improvement of therapeutic strategies. Therefore, in this study, we aimed at identifying specific, hyperoxia-related alterations of DNA methylation, which could affect the activity of crucial genetic pathways involved in the development of hyperoxic newborn lung injury. To achieve this, we used an established model with newborn mice exposed to long-term hyperoxia [11] and performed whole-genome methylation analysis with complementary expression assessment of specific genes of interest in lung tissue.

2. Material and methods

2.1. Animal experiment

A total of 24 newborn mice (C57Bl/6Tac) were randomized to hyperoxia (85% O_2; 12 animals) or normoxia (21% O_2; 12 animals) groups for 14 days, followed by normoxia conditions for all animals for the subsequent 14 days. All mice had free access to food and water and were kept under standard conditions in A-Chambers (O_2 – monitor ProOX110, CO_2 – monitor ProCO2 P120, BioSpherix). Lung tissue was harvested on day 28 after euthanasia with a zolazepam/tiletamine/xylazine/fentanyl cocktail. Tissue samples were snap-frozen in liquid nitrogen immediately after cessation of circulation for the subsequent analysis.

The experiments were carried out in Oslo and approved by the Norwegian board of animal research welfare (NARA 50/13-5458).

2.2. Microarray methylation analysis

Genomic DNA was isolated from tissue samples using a MasterPure DNA Purification Kit (Epicentre). DNA concentration and purity were measured using a UV spectrophotometer NanoDrop 1000 (Thermo Scientific). A total of 5 μg of purified genomic DNA was resuspended in 250 μl of PBS and sonicated using an ultrasonic processor UP100H (Hielscher) at 100% amplitude for 5 min, in an ice-water bath. The sonication conditions were optimized to obtain DNA fragments ranging between 200 and 1000 base pairs in size. The degree of DNA fragmentation was determined using an Agilent 4200 TapeStation Instrument. After sonication and fragmentation analysis, the volume of each sample was adjusted to 250 μl with phosphate-buffered saline. Next, 200 μl was used for immunoprecipitation of methylated fragments and the remaining 50 μl as a genomic DNA reference.

The subsequent microarray analysis of the genome methylation pattern was performed using Mouse CpG Island microarrays, enabling assessment of 88,737 probes representing specific genome methylation sites in, or within 95 base pairs from, 15,342 CpG islands. The microarray experiment was performed according to Agilent Microarray Analysis of Methylated DNA Immunoprecipitation protocol (version 2.3.1).

Subsequently, we used the Genomic Workbench software (Agilent) for analysis of methylation data. The above software calculates normalized Z-score from the Gaussian distribution to effectively judge the methylation status of a given probe on the array. The combined Z-score, which is a summation of the left and right Gaussian Z-scores, reflects the location of a probe log-ratio value in relation to the Gaussian distribution of probes with similar temperature melting. A strong positive value of the combined Z-score means that a given probe is methylated, and a strong negative value means that it is unmethylated.

Next, we calculated the mean combined Z-scores for each probe and the average for all mean combined Z-scores for the hyperoxia and normoxia groups. Then, we assessed the differences between groups for each genomic fragment. To decrease the probability of false-positive findings, we focused only on those probes for which the calculated difference exceeded three standard deviations. Eventually, we obtained a final list of probes-of-interest. Subsequently, we assessed the list using the Database for Annotation, Visualization and Integrated Discovery (DAVID) [12] to identify differentially methylated genes and genomic pathways.

2.3. Complementary expression analysis of genes of interest

Total RNA was extracted from the remaining fragments of lung tissue using an RNeasy Mini Kit (QIAGEN). Subsequently, RNA samples were used for expression analysis of previously identified genomic pathways, which revealed significant methylation differences between hyperoxia and normoxia groups. We applied SurePrint G3 Mouse Gene Expression 8 × 60 K microarrays (Agilent Technologies), according to the manufacturer's protocol. The expression profiles of the genes of interest were compared between the normoxia and hyperoxia groups using Partek computer software for microarray data analysis (www.partek.com). Statistically significant ($p < 0.05$) expression differences exceeding 20% (Fold Change > 1.2) were further evaluated.

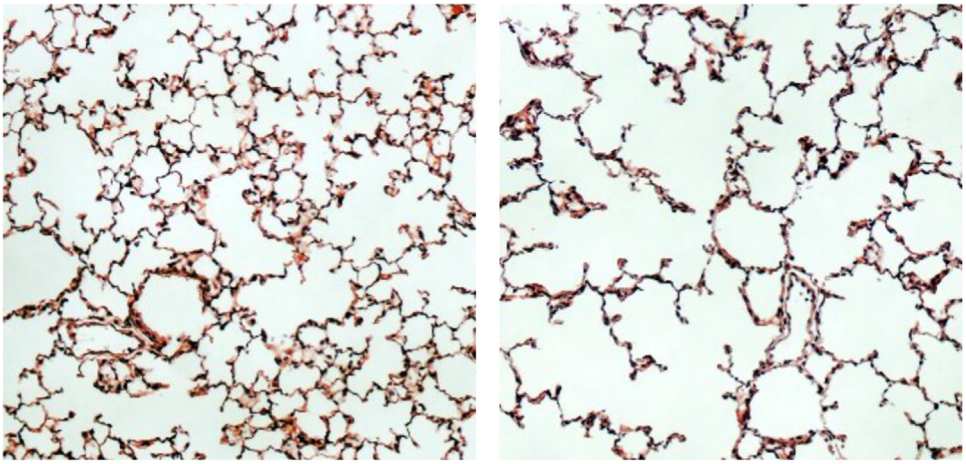

Fig. 1. The observed hyperoxia-related lung damage. Mouse lung tissue at 100× magnification: normal development (normoxia) on the left; arrested alveolarization (hyperoxia) on the right.

3. Results

Histological assessment of lung tissue samples confirmed the presence of oxygen-related lung damage in the hyperoxia group. Fig. 1 shows arrested alveolarization with enlarged alveolar spaces and simplified alveolar structure resulting from prolonged hyperoxia.

The mean methylation level of all microarray probes was increased in the hyperoxia group compared to the normoxia group (average values for mean combined Z-scores 0.57 and − 0.31, respectively). This suggests the presence of an overall DNA-hypermethylation effect of hyperoxia, with a resulting shift of the methylation status of the majority of gene promoters from “unmethylated” in the normoxia group towards “methylated” in the hyperoxia group.

The analysis of specific DNA fragments revealed 1259 probes that were hypermethylated in the hyperoxia group and 252 probes that were hypermethylated in the normoxia group (Supplementary data). The linearity of the Q-Q (quantile-quantile) plots of the mean combined Z-scores in each group suggests the normal distribution of the data (Fig. 2).

Further analysis using DAVID software identified 1011 mouse genes among 1259 probes with increased methylation in the hyperoxia group. Genomic pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway database [13] revealed statistically significant enrichment of five genomic KEGG pathways (Pathways in cancer, TGF-β signaling pathway, Hippo signaling pathway, Melanogenesis, and Prostate cancer pathway) with the greatest gene enrichment ratio for the TGF-β signaling pathway ($p = 0.0013$ with False Discovery Rate correction for multiple comparisons; 18 out of 85 genes in the pathway identified; Fold Enrichment ratio = 4.1).

The hypermethylated genes included the *Tgfbr1* gene, which is pivotal for the pathway, as well as *Crebbp* and *Creb1* genes, which play central roles in cell cycle regulation. The hypermethylated elements of

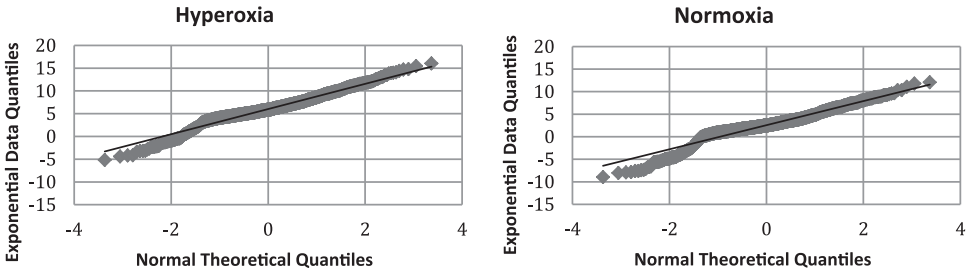

Fig. 2. Q-Q (quantile-quantile) plots of combined Z-scores in the hyperoxia and normoxia groups, suggesting the normal distribution of data.

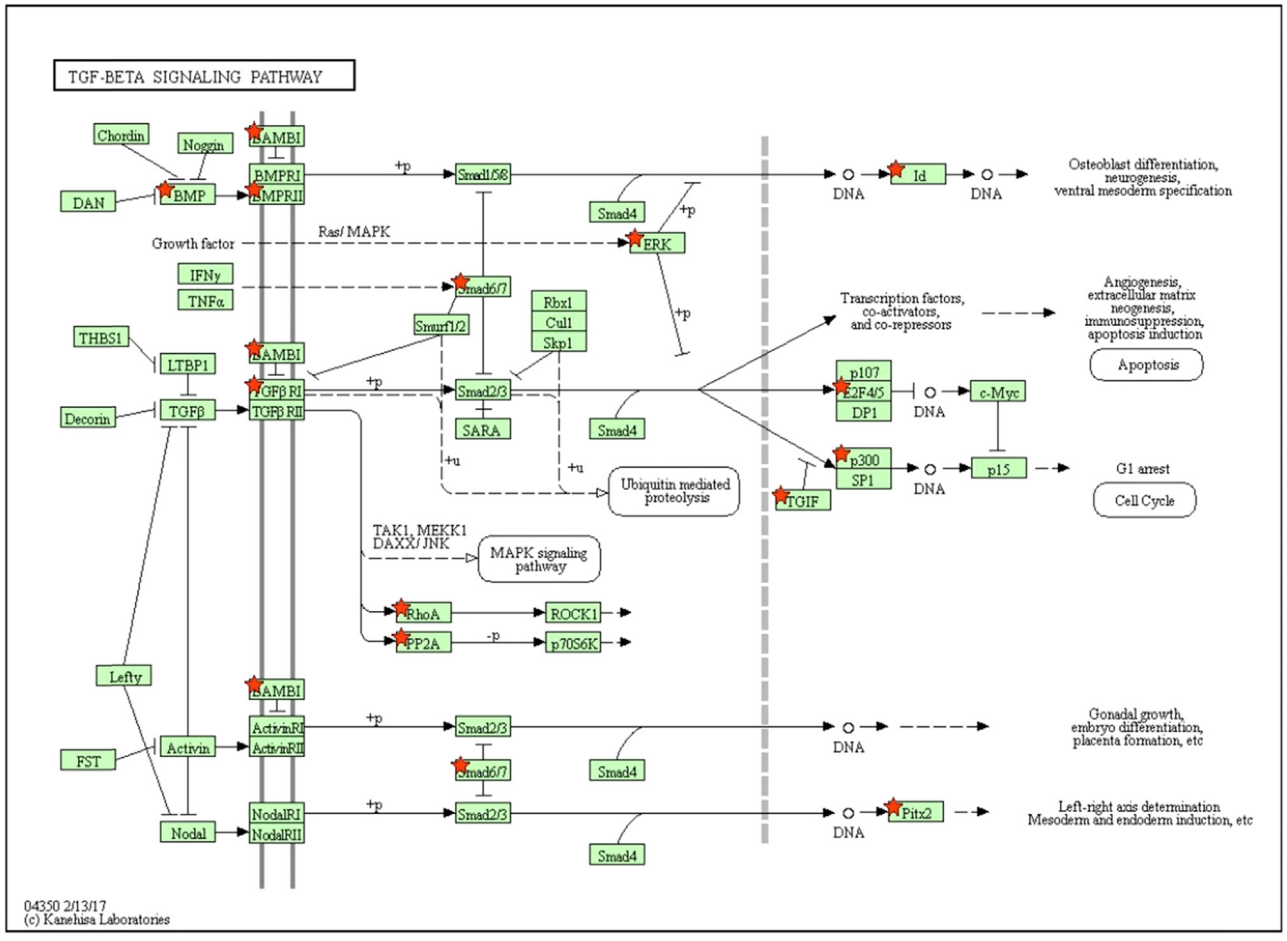

Fig. 3. Methylation pattern in the hyperoxia group; TGF-β pathway scheme is taken from KEGG pathway database.

Table 1
TGF-beta pathway-related genes that exhibited differences between groups in methylation and expression.

Methylation analysis – hypermethylated TGF-beta pathway genes in hyperoxia group					
Gene symbol	Gene name	Genomic location of the methylated DNA fragment (according to NCBI37/mm9)	Mean Z-score in hyperoxia group	Mean Z-score in normoxia group	Mean Z-score difference (SD)
Bambi	BMP and Activin Membrane Bound Inhibitor	chr18:3508199–3508243	3.79	0.63	3.16
Bmp4	Bone Morphogenetic Protein 4	chr14:47007792–47007836	9.73	6.56	3.17
		chr14:47007582–47007626	6.17	2.74	3.43
Bmp6	Bone Morphogenetic Protein 6	chr13:38437991–38438035	9.67	6.52	3.15
Bmpr2	Bone Morphogenetic Protein Receptor Type 2	chr1:59820636–59820681	6.82	2.79	4.03
Crebbp	CREB Binding Protein	chr16:4212906–4212950	6.57	2.97	3.6
		chr16:4213107–4213151	6.97	3.33	3.64
E2f5	E2F Transcription Factor 5	chr3:14611165–14611209	5.5	2.11	3.39
Ep300	E1A Binding Protein P300	chr15:81416218–81416262	7.06	3.9	3.16
Gdf7	Growth Differentiation Factor 7	chr12:8304878–8304922	4.99	1.94	3.05
		chr12:8305295–8305339	7.79	3.99	3.8
Id1	Inhibitor of DNA Binding 1	chr2:152560730–152560774	5.72	2.46	3.26
Id3	Inhibitor of DNA Binding 3	chr4:135700657–135700701	5.06	2.05	3.01
Id4	Inhibitor of DNA Binding 4	chr13:48356696–48356740	5.08	2.08	3.0
Mapk1	Mitogen Activated Protein Kinase 1	chr16:16983547–16983591	7.16	3.07	4.09
Pitx2	Paired Like Homeodomain 2	chr3:128903185–128903231	2.78	− 0.41	3.19
Ppp2cb	Protein Phosphatase 2 Catalytic Subunit Beta	chr8:34710437–34710481	5.25	1.73	3.52
Rhoa	Ras Homolog Family Member A	chr9:108208556–108208600	4.94	1.82	3.12
Smad7	Smad Family Member 7	chr18:75528547–75528591	5.64	2.02	3.62
Tgfbr1	Transforming Growth Factor Beta Receptor 1	chr4:47365606–47365650	6.06	2.63	3.43
Tgif2	TGFB Induced Factor Homeobox 2	chr2:156666261–156666305	5.6	2.5	3.1
Complementary expression assessment – TGF-beta pathway genes with significantly higher expression in normoxia group					
Gene symbol	Gene name	Fold change (Normoxia/Hyperoxia)	Comparison of expression between groups; *t*-test (p value)		
Crebbp	CREB Binding Protein	1.95	0.013		
Smad1	Smad Family Member 1	1.43	0.04		
Smad2	Smad Family Member 2	1.31	0.045		
Smad3	Smad Family Member 3	1.2	0.038		
Acvr1c	Activin A Receptor Type 1C	1.51	0.041		

the TGF-β signaling pathway are shown in Fig. 3.

Also, a total of 227 (mouse) genes were identified among the 252 probes with increased methylation in the normoxia group. However, no significant methylation differences were observed for specific genomic pathways.

Microarray methylation data have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-6489.

The complementary expression analysis of TGF-β pathway genes revealed significant downregulation of five genes in the hyperoxia group. Three of these genes (*Crebbp, Smad2,* and *Smad3*) are crucial for cell cycle regulation. Higher expression of the above genes in the normoxia group functionally corresponds with the previously described hypermethylation of genes in the hyperoxia group. The genes with different methylation patterns and differential expression levels are listed in Table 1 and in Fig. 4.

In addition, we observed high percentage of genes involved in apoptosis regulation among all genes exhibiting statistically significant expression differences between hyperoxia and normoxia groups (45 out of 470 genes). The majority of them were apoptosis inhibitors with decreased activity in the hyperoxia group. We also identified 10 genes involved in reaction to oxidative stress. The lists of the above genes are available in the Supplementary Table.

4. Discussion

Hyperoxia-related alterations of cell signaling and genome expression have been thoroughly described and reviewed in the literature [14–17]. It was shown that the immaturity of the antioxidant defense system, especially in extremely preterm infants [18,19], may be a predisposing factor to BPD, as the use of high oxygen concentrations, and positive pressure ventilation upon resuscitation and in the neonatal intensive care unit. However, less is known about the influence of hyperoxia on DNA methylation. In a recent study, widespread hypermethylation of DNA was demonstrated in a rat model of BPD [20]. Other studies have reported increased global DNA methylation levels in human cells in case of short-term treatment with oxygen, whereas long-term treatment had the opposite effect [21]. However, epigenetic mechanisms are complex, and the DNA methylation level might depend not only on external factors, such as the concentration of oxygen and duration of hyperoxia, but also from tissue-specific responses to oxygen.

An important obstacle in studying methylation-related epigenetic mechanisms using next-generation sequencing is the lack of established algorithms for identification of functionally important alterations of DNA methylation among very many hypermethylated DNA regions [21]. Therefore, in our study, we focused on assessment of the methylation of gene promoters, which is a classical regulatory mechanism of gene expression [22]. We applied microarrays developed specifically for methylation studies and validated our findings with complementary gene expression analysis.

The present study is, to our knowledge, the first to assess lung-specific DNA methylation and genome expression in the context of BPD pathogenesis. Based on these data, we suggest that hyperoxia is a programming factor in the newborn lung with potentially lifelong consequences. One weakness of this study is that we have not separated the different cells in the lung and, consequently, do not know which cell types are affected.

Lung injury and repair processes involve many cellular activities, including cell growth, differentiation, and remodeling of extracellular matrix components. TGF-β belongs to a group of pivotal growth factors

Probe name/Gene name	1 hyperoxia	2 hyperoxia	3 hyperoxia	4 hyperoxia	5 hyperoxia	6 hyperoxia	7 hyperoxia	8 hyperoxia	9 hyperoxia	10 hyperoxia	11 hyperoxia	12 hyperoxia	13 normoxia	14 normoxia	15 normoxia	16 normoxia	17 normoxia	18 normoxia	19 normoxia	20 normoxia	21 normoxia	22 normoxia	23 normoxia	24 normoxia
A_68_P31458878/Bambi	3,8	4,2	3,6	3,6	2,8	3,4	3,8	2,5	3,9	3,8	5,9	4	0,9	-2	-1	0,7	1,5	0,7	0,3	0,7	0,9	1,9	1,7	1
A_68_P29575957/Bmp4	9,2	10	11	9,7	6,5	10	9,9	9,4	11	11	8,4	10	7,6	4,3	5,8	7,1	6,9	7,1	5,2	7,6	6,5	7,6	7,3	5,6
A_68_P29575955/Bmp4	6,4	7	6,2	6,7	4,5	6,8	6,4	5,4	6,4	7	4,2	6,9	2,7	1,4	3,2	3	2,9	2,8	1,8	3,2	2,9	3,4	3,3	2,3
A_68_P28959949/Bmp6	9,9	6,9	9,8	11	7,1	11	11	10	11	12	10	6,6	6,8	4,9	6,1	8	6,5	7	7,5	8,1	6,7	6,3	6,1	4,3
A_68_P20265715/Bmpr2	7,7	3,9	6,7	7,7	5,2	6,7	7,8	7,2	7,5	7,7	8,6	5,1	1,7	-2	1,5	4,4	2,4	4	4	4,4	4,7	2,8	3,3	1,6
A_68_P30508953/Crebbp	6,9	5,7	6,8	6,7	5,5	7,4	7,3	6	7,3	7,3	4,8	7,1	3,4	0	1,2	4	3,7	3,3	2,6	3,8	3,6	4	3,6	2,2
A_68_P30508955/Crebbp	7,6	5,8	6,9	8,1	5,9	7,5	7,8	6,4	7,5	8	5,9	6,3	3,4	0,2	3,1	4,1	3,9	3,6	3	3,7	3,9	4	4,5	2,6
A_68_P21957615/E2f5	5,2	6,2	5,6	5,9	4,5	6,5	6,2	4,9	5,4	5,4	4,4	5,8	1,9	-0	1,9	2,4	2,9	2,5	1,6	2,5	2,5	2,8	2,6	2,1
A_68_P30378502/Ep300	8,4	5	8,6	8,1	2,2	8,3	8,5	8,3	8,5	7,9	6,3	4,6	4,8	2	3,6	6	4,5	4,6	4,5	5,3	4,5	2,2	2,6	2,2
A_68_P28221291/Gdf7	4,7	2,9	5,2	6,1	5,4	6,4	5,4	4,6	5,5	5,5	4,6	3,6	2,1	-1	1,7	2	3,1	2,6	2,2	2,4	2,4	2,7	2,7	0,5
A_68_P28221294/Gdf7	8,7	3,7	8,2	9,6	6	8,6	9	8,7	9,1	9	7,8	5,3	3,6	3	4,6	5,9	3,2	5	4,7	4,9	4,6	3,7	3,7	0,9
A_68_P21750889/Id1	6,5	6,8	6,5	6	4,1	5,2	6,3	4,3	5,9	6,9	4,5	5,7	2,4	0,9	2,9	2,3	2,4	2,4	1,8	2,6	2,3	3,1	3,7	2,6
A_68_P23332311/Id3	4,7	6,8	5,6	4,6	3,4	5,6	5,4	5,4	4,4	5,3	4	5,5	3,7	1,2	1,9	1,3	2,3	1,5	2,1	1,9	1,3	2,3	2,4	2,6
A_68_P29018473/Id4	5,8	3	4,4	5,7	5,7	5,1	5,9	5,4	5,6	5,7	5,3	3,3	2,3	-0	1,7	3	2,7	2,2	2,8	2,3	2,6	2,5	2,4	0,7
A_68_P30571310/Mapk1	8,5	5,7	8	8,2	3,1	7,7	8,1	9,1	7,7	8,1	6,5	5,3	2,1	1,7	2,9	4,7	2,4	4,2	3,5	4,5	4	2,5	2,3	2
A_68_P22518623/Pitx2	2,7	3,8	3	3,2	2,3	3,7	3,7	2,7	2,3	1,7	1,6	2,8	0,1	-6	-2	0,4	1,3	0,5	-1	1	0,3	-0	0,6	-0
A_68_P25769753/Ppp2cb	4,9	4,3	5,5	5,4	5,8	4,4	6	4,5	6	5,6	4,7	5,8	2,2	-2	0,6	1,7	1,6	2,1	2,4	1,6	2,7	3,4	2,9	1,4
A_68_P26814198/Rhoa	5,9	2,5	5,8	5,8	4,7	6	5,3	5,7	5,4	5,5	4,9	1,8	1,3	1,8	2,1	2,3	2,6	1,7	2,1	2,3	2,4	1,8	1,7	-0
A_68_P31836541/Smad7	5,7	2,3	5,9	6,5	5,1	5,9	7	5,1	5,9	6,8	8,6	2,8	2	-3	0,9	3,9	2	3,7	2,6	3,9	3,7	1,9	2,4	0,1
A_68_P22894259/Tgfbr1	6,2	5,8	6,5	6,2	4,9	6,5	7	5,7	6,4	6,6	5,5	5,4	2	1,5	1,1	3,3	2,7	2,9	2,2	3,5	3,2	3,5	3,6	2
A_68_P21773633/Tgif2	6,2	6,1	5,9	6,7	3,7	4,6	6,2	4,7	5,3	5,4	6,9	5,4	2,9	2,9	2,8	3	2,3	1,8	1,8	2,3	1,5	2,9	3,4	2,5

Fig. 4. TGF-β pathway genes exhibiting different methylation patterns.

regulating these cellular activities [23,24]. Type I and type II receptors for TGF-β are serine/threonine kinases that signal through the SMAD family of transcriptional regulators. Temporal fluctuations in expression of both receptors were demonstrated in rats undergoing prolonged exposure to 100% oxygen [24].

Crebbp and *Creb1* genes play critical roles in embryonic lung development [25], growth control, and homeostasis by coupling chromatin remodeling to transcription factor recognition. Crebbp mediates cAMP-gene regulation by binding specifically to phosphorylated CREB1 protein, which is an important regulator of apoptosis in oxidant-mediated responses of lung epithelial cells [26]. cAMP response element binding protein 1 (Creb1) activity is crucial for the development and differentiation of the conducting and distal lung epithelium. The Creb1 transcription factor regulates cellular gene expression in response to elevated levels of intracellular cAMP. It is noteworthy that TGF-β represses the *cyclin A* gene through a cyclic AMP (cAMP) response element, which complexes with the cAMP response element binding protein (Creb1) [27].

We detected fluctuations in the methylation of the genes mentioned above in response to hyperoxia. The findings indicate a role for these genes in the pathogenesis of lung injury, such as in BPD. We also demonstrated complementary expression changes of some important genes from the TGF-β pathway and observed altered expression of several genes involved in apoptosis regulation and in the cellular response to oxidative stress. However, DNA methylation is probably counter-balanced by other epigenetic processes, which might behave dynamically and in a cell-specific manner. Therefore, the observed differences in expression of selected genes might only partially reflect the hyperoxia-related methylation pattern.

An important limitation of the study lies in the fact that the presence or absence of long-term alteration in methylation and protein synthesis may depend on the duration of hyperoxia. We exposed the animals to hyperoxia for a relatively long time. We induced alveolar development arrest and could observe morphological abnormalities in lung tissue, which resemble those seen in BPD. However, we were not able to assess potential short-term fluctuations of the DNA methylation with resulting alterations of genome expression. Detailed investigation of the dynamics of TGF-β pathway expression alterations would probably require repeated assessment of expression changes in lung tissue in a larger study. Also, we propose that the different cell types of the lung should be studied separately.

Some limitations of the mouse model in studies on oxygen exposure in neonates should also be mentioned. These include the relatively high resistance of rodents to oxygen as compared to preterm infants [28], as well as problems to reflect in the animal model the gestational age differences or the effects of prolonged mechanical ventilation.

In summary, our results suggest the involvement of methylation of TGF-β pathway genes in the reaction of lung tissue to hyperoxia. It seems that the excess of oxygen can trigger hypermethylation of the pathway, with subsequent decrease of activity of pivotal genes and stimulation of apoptosis. This, in turn can result in alteration of lung morphogenesis with disturbed branching and alveolarization. However, additional experiments are necessary to elucidate the precise mechanisms of epigenetic influence of hyperoxia on lung tissue and their contribution to development of BPD.

Declaration of interest

None.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2018.04.566.

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