Expression profiles of long non-coding RNAs during fetal lung development

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Received February 17, 2019; Accepted April 29, 2020

DOI: 10.3892/etm.2020.9273

Abstract. With advances in neonatology, a greater percentage of premature infants now survive and consequently, diseases of lung development, including bronchopulmonary dysplasia and neonatal respiratory distress syndrome, have become more common. However, few studies have addressed the association between fetal lung development and long non-coding RNA (lncRNA). In the present study, right lung tissue samples of fetuses at different gestational ages were collected within 2 h of the induction of labor in order to observe morphological discrepancies. An Affymetrix Human GeneChip was used to identify differentially expressed lncRNAs. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analyses were performed. A total of 687 lncRNAs were identified to be differentially expressed among three groups of fetal lung tissue samples corresponding to the three embryonic periods. A total of 34 significantly upregulated and 12 significantly downregulated lncRNAs (fold-change, ≥1.5; $P<0.05$) were detected at different time points (embryonic weeks 7-16, 16-25 and 25-28) of fetal lung development and compared with healthy tissues Expression changes in lncRNAs n340848, n387037, n336823 and ENST00000445168 were validated by reverse transcription-quantitative PCR and the results were consistent with the GeneChip results. These novel identified lncRNAs may have roles in fetal lung development and the results of the present study may lay the foundation for subsequent in-depth studies into lncRNAs in fetal lung development and subsequent clarification of the pathogenesis of neonatal pulmonary diseases.

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Key words: fetal lung development, long non-coding RNA, microarray, bioinformatics analysis

Introduction

Long non-coding RNAs (lncRNAs) are characterized by their length (>200 nucleotides), intron/exon structure, the presence of a 3' untranslated region and termination region, and a limited coding potential supported by the absence of open reading frames (1). Biochemically, lncRNAs are thought to mediate local gene expression as cis-regulatory elements, affect transcription of multiple genes as trans-regulatory elements and act as a scaffold for chromatin structure maintenance (2,3). In terms of function, lncRNAs have been reported to participate in numerous biological processes, including X chromosome inactivation, genomic imprinting, cell cycle regulation and the regulation of stem cell pluripotency (4,5). Additionally, the molecular functions of lncRNAs have been highlighted to have roles in various diseases, particularly those relevant to endocrinology, reproduction, metabolism, immunology, neurobiology, muscle biology and cancer (6-9).

Several studies have investigated the association between neonatal lung diseases and lncRNAs. Cheng *et al* (10) reported 9 lncRNAs that were potentially associated with bronchopulmonary dysplasia (BPD) and these data may provide novel insight into the biological roles of lncRNAs in the pathogenesis of BPD. Numerous lncRNAs are significantly differentially expressed in various lung diseases. For instance, metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) has been reported to have an important role in lung cancer progression (11-13). Although the loss of MALAT1 does not affect lung development (14), a previous study indicated that the upregulation of MALAT1 may protect preterm infants with BPD by inhibiting apoptosis (15). Deletions of chromosomes encompassing other lncRNAs may cause lethal lung developmental disorders (16). Szafranski *et al* (17) demonstrated that deletion of a small non-coding methylated region at 16q24.1, included in lncRNA genes, caused the lethal lung developmental disorder alveolar capillary dysplasia with misalignment of pulmonary veins with parent-of-origin effects in a human model. These data indicated that lncRNAs may regulate fetal lung development.

As the survival rate increases among preterm infants, respiratory distress syndrome (RDS) and BPD are becoming more and more common (18). One widely accepted cause of BPD is insufficient fetal lung development during pregnancy (19).

Additionally, a lack of a pulmonary surfactant synthesized by type II alveolar epithelial cells is acknowledged as a major cause of RDS (20). Consequently, there may be a close association between neonatal respiratory diseases and lung development.

Fetal lung development is a complex and continuous process. The development of lung structure includes the embryonic, fetal and postnatal stages (21). Fetal embryonic lung development may be divided into the pseudo-glandular period (7-16 weeks of gestation), canalicular period (16-25 weeks of gestation) and terminal saccular period (25 weeks to full term gestation) (22). Different stages of lung development have different characteristics (23). During the terminal saccular period, with the appearance of alveolar septum, capillaries, elastic fibers and collagen fibers, terminal vesicles become alveolar (21). Alveolar maturation begins at 30 weeks of gestation. Lung potential gas volume and surface area increase from 25 weeks of gestation to full term (24). Increased alveoli, lung volume and surface area provide the anatomical potential for gas exchange and, therefore, provide the basis for fetal survival following birth (25-27). In the present study, three periods of fetal lung development, divided by gestational week, were investigated through the examination of morphological and lncRNA expression changes in three groups of fetal lung tissue samples. An Affymetrix Human GeneChip was employed to assess the differential expression of lncRNAs between three phases of fetal lung development. Bioinformatics methods were also used to analyze the potential functions and pathways associated with the protein-coding genes associated with the differentially expressed lncRNA. These data may provide a theoretical basis for the prevention and treatment of neonatal lung developmental diseases, including RDS and BPD.

Materials and methods

Patients and samples. The present study protocol was approved by the Ethics Committee of Nanjing Maternal and Child Health Care Hospital, Nanjing, China [approval no. (2014) 74]. All of the patients included provided written informed consent to participate in the current study. Abortion was most commonly induced due to personal or social factors, rather than due to congenital problems.

The inclusion criteria were as follows: i) Gestational age of the fetus at the time-point of abortion was 7-28 weeks; ii) fetal abortion was performed due to personal or social factors; iii) pregnant females were aged 20-35 years; and iv) pregnant females provided written informed consent.

The exclusion criteria were as follows: i) Pregnant females with a history of hypertension or diabetes, or kidney, heart, connective tissue or autoimmune diseases; ii) pregnant females with a known history of exposure to radioactivity, toxic substances or drugs; iii) maternal use of glucocorticoids (e.g., dexamethasone, prednisone or beclomethasone) prior to abortion; iv) pregnant females with signs of infection, including positive amniotic fluid culture, increased C-reactive protein or procalcitonin; and v) previously detected chromosomal abnormalities or congenital malformations of the fetus.

Abortion procedure. Procedures took place between August 2014 and February 2015 at Nanjing Maternity and Child Health Care Hospital. Physicians explained the medical method and possible adverse reactions of abortion to the pregnant females who then provided voluntary written informed consent. Females at 8-13 weeks of gestation took mifepristone (200 mg orally), then misoprostol (400 μg sublingually) followed by mifepristone (200 mg orally) after 24-48 h. If the abortion was not complete, repeated misoprostol was taken sublingually every 3 h and up to 4 doses were administered until complete abortion. For females at 14-28 weeks of gestation, amniocentesis was performed prior to the intra-amniotic injection of 0.5% rivanol solution (100 mg) to induce contractions and initiate labor. The pregnant females took mifepristone (25 mg orally), which was used to soften the cervix (28-30). Fetal right-lung tissue samples were collected within 2 h of labor. Fetal lungs were isolated and were divided into three groups according to the fetal gestational age. These groups were termed S1 (embryonic week, 7-16), S2 (embryonic week, 16-25) and S3 (embryonic week, 25-28). A total of 10 samples were collected at S1 (mean age, 27.2±1.56 years; mean fetal gestational age, 13.57±0.73 weeks) 14 samples at S2 (mean age, 25.5±0.84 years; mean fetal gestational age, 20.73±0.72) and 12 samples at S3 (mean age, 25.92±1.13 years; mean fetal gestational age, 26.75±0.23 weeks). The median overall gestational age was 21.35 weeks with a range of 15.58-26.18 weeks.

The right lungs were washed with PBS and cut into several parts (2x4x6 mm), all of which were kept for histological examination. A 5 mg sample of each of the lungs was cut into small pieces in homogenization buffer (Trevigen, Inc.). The lungs were homogenized with a Sonifier (Branson Ultrasonics Corporation) with an amplitude of 14 microns for 10 sec. The cell supernatant was obtained by centrifugation at 12,000 x g for 15 min at 4˚C. Total RNA from the right lungs was isolated from the supernatant using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RNA quality and quantity were measured on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). A total of 1 μg of total RNA was taken from each sample and subjected to 1.5% agarose gel electrophoresis (120 V) for 15 min to determine the integrity of 28 and 18s ribosomal RNA under a gel imager (Bio-Rad Laboratories, Inc.) and to ensure that there were no residual RNA enzymes.

Histology. Lung tissues were fixed with 4% buffered paraformaldehyde at 4˚C overnight, dehydrated [50% ethanol for 2 h, 70% ethanol for 2 h, 85% ethanol for 2 h, 95% ethanol for 2 h, anhydrous ethanol for 1.5 h and anhydrous ethanol (fresh configuration) II for 1.5 h] and embedded in paraffin. Sections with a thickness of 3-4 μm were prepared for H&E staining and immunohistochemistry. Sections were dewaxed with xylene and rehydrated in a graded series of ethanol/water solutions. The sections were then stained with hematoxylin for 5 min, differentiated with 1% ethanol hydrochloride for 3 sec and transferred to eosin solution for 2 min. All procedures were performed at room temperature. The sections were then dehydrated and mounted. A total of three sections were randomly selected from each sample and a total of 108 H&E-stained sections were taken for image analysis, which was performed under a light microscope (BX51; Olympus Corporation) at magnifications of x200 and x400 to observe changes in fetal lung development between groups S1, S2 and S3.

Table I. Reverse transcription-quantitative PCR primers.

Gene	Primer sequence (5'-3')
n387037	Forward TGGAAATTGGAAGAGCACAA
	Reverse TGTATGAGGGTGCATGGAAA
n340848	Forward TTACAAGCTCCATCAGCACAG
	Reverse TCCACCTGTTCATTGGTTCA
ENST00000445168	Forward GGTGGCAGAGCTAGAACTCG
	Reverse GGGTAAGCCTCGTGTACCAA
n336823	Forward TTGTGGGCCTCCTCATATTT
	Reverse GAAGTTTGTCCACCGCAAAG
GAPDH	Forward AACTTTGGCATTGTGGAAGG
	Reverse GGATGCAGGGATGATGTTCT

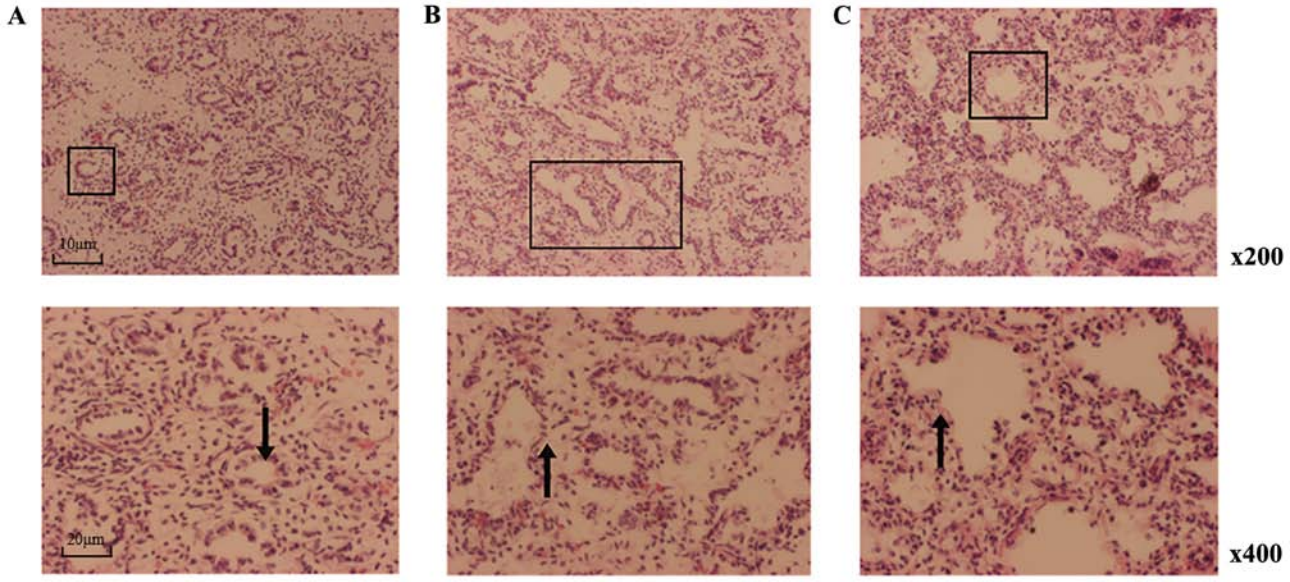

Figure 1. Morphology of lung tissues at different stages of fetal development. (A) Representative images of group S1 (7-16 weeks); the box indicates bronchioles and the arrow points at high columnar epithelial cells, arranged in rings. (B) Representative images of group S2 (E16-E25 weeks); the box indicates respiratory bronchi and the arrow indicates short columnar cells. (C) Representative images of group S3 (25-28 weeks); the box delineates the alveolar sac and the arrow points to squamous epithelial cells. Scale bar, 10 μm (upper panel) or 20 μm (lower panel). Magnification, x200 (upper panel) or x400 (lower panel).

Affymetrix Human GeneChip analysis. The GeneChip® Human Transcriptome Array 2.0 (Affymetrix Inc.) serves as an advanced and comprehensive gene expression profiling tool for whole-transcript coverage available on any microarray platform (31). Probes are distributed across the full length of a gene, including specific probes covering splice junctions, providing a more complete and accurate picture of overall gene expression with additional capacity for transcript isoform analysis. In brief, following the extraction of total RNA, 10 μg of RNA was used to synthesize double-stranded complementary (c)DNA using an Ambion WT expression kit (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The cDNA was then fragmented and labelled with the Affymetrix GeneChip WT terminal labelling kit (Affymetrix Inc.), according to the manufacturer's protocol. The fragmented cDNA was hybridized using the GeneChip hybridization, wash and stain kit (Affymetrix Inc.), according to the manufacturer's protocol. Hybridization was performed at 65˚C with rotation for 16 h in an Affymetrix GeneChip Hybridization Oven 645 (Affymetrix Inc.). The GeneChip arrays were washed and stained on an Affymetrix Fluidics Station 450 (Affymetrix Inc.), followed by scanning on a GeneChip Scanner 3000 (Affymetrix Inc.). The microarray analysis was performed by Genminix Informatics Co., Ltd.

Reverse transcription-quantitative PCR (RT-qPCR). A total of 0.1 g of lung tissue was homogenized in a homogenizer (Kinematica AG). Total RNA was isolated from fetal lungs using TRIzol® reagent. cDNA was synthesized with the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics Co., Ltd.), according to the manufacturer's protocol. An aliquot of 1 μg total RNA was added to each reaction mixture. RT-qPCR was performed on an ABI 7500 thermal cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.) with SYBR Green (Roche Diagnostics Co., Ltd.). The thermocycling conditions were as follows: 95˚C for 5 min,

Table II. Downregulated differentially expressed lncRNAs between the three stages of fetal lung development.

A, S2 vs. S1

lncRNA	Fold-change
n335774	0.06935588
n339745	0.182483003
n334591	0.186404811
ENST00000445168	0.194416962
n342740	0.223988722
n336928	0.225022474
n336102	0.234549609
n335785	0.254695963
n333031	0.272814078
n332815	0.275703789
OTTHUMT00000312398	0.30750654
n409772	0.320872488
n338599	0.34498774
n334031	0.346874815
n406915	0.347614219
n345353	0.365559716
ENST00000559553	0.370347627
NR_024408	0.371285274
n333438	0.376855146
TCONS_l2_00020561-XLOC_l2_010703	0.381374424

B, S3 vs. S2

lncRNA	Fold-change
n337632	0.186974574
n336928	0.221248411
n334074	0.231631923
n335516	0.250004679
TCONS_l2_00009549-XLOC_l2_005089	0.322783111
n336841	0.35573011
n410723	0.39944174
n336683	0.412094182
n333958	0.414088201
TCONS_00000280 XLOC_000357	0.415518323
n336585	0.417651927
n333432	0.424395128
n408121	0.425823065
ENST00000557067	0.428630673
ENST00000448680	0.4427573
n381789	0.457561636
n332792	0.469538382
n333380	0.482586011
n340854	0.486920451
n335620	0.488461581

C, S3 vs. S1

lncRNA	Fold-change
n336928	0.049785865
ENST00000445168	0.108667618
n339745	0.117414297
n335774	0.144621906
n336102	0.179262671
n409772	0.243588174
n338817	0.26669321
n336823	0.27863222
n335785	0.281131886
TCONS_l2_00020561-XLOC_l2_010703	0.283204481
n342740	0.288809679
n345353	0.295015569
n334591	0.295785862
ENST00000557067	0.303030728
n336841	0.322103669
n333432	0.322647779
n341886	0.322920291
n336585	0.333848393
NR_039890	0.343190138
ENST00000500843	0.344062843

Groups: S1, embryonic weeks 7-16; S2, embryonic weeks 16-25; S3, embryonic weeks 25-28. LncRNA, long non-coding RNA.

followed by 40 cycles of 95˚C for 20 sec and 55˚C for 20 sec. At the end of each run, a melting curve analysis was performed at 72˚C to monitor primer dimers and formation of non-specific products. Relative quantification of gene expression in multiple samples was achieved by normalization to the expression of an endogenous control gene, GAPDH. The relative expression levels were calculated by the $2^{-\Delta\Delta Cq}$ method (32). Primer sequences are listed in Table I.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. The GO project offers a controlled vocabulary to label gene and gene product attributes in any organism (geneontology.org; date of access, May 2015). Categories covered by GO analysis include biological process, cellular component and molecular function. GO analysis provides an interpretation of the relevance of genes differentially expressed between the groups by suggesting possible functions of the genes and functions associated with the genes. Fisher's exact test and the χ^2 test were performed to calculate the P-value and false discovery rate of each GO term function. The input used in the bioinformatics analysis was the crossover genes of differentially expressed lncRNA-associated genes and differential mRNA genes co-expressed with lncRNA that were screened in the lncRNA expression profiling results. The criterion for screening differentially expressed genes for statistical significance was $P<0.05$, thus screening out the significant functions exerted by the differentially expressed genes.

KEGG (kegg.jp/kegg/pathway.html) pathway analysis is a functional analysis tool, mapping a set of genes that may be associated with a certain lncRNA to potential pathways they are accumulated in. Fisher's exact test and χ^2 test were used

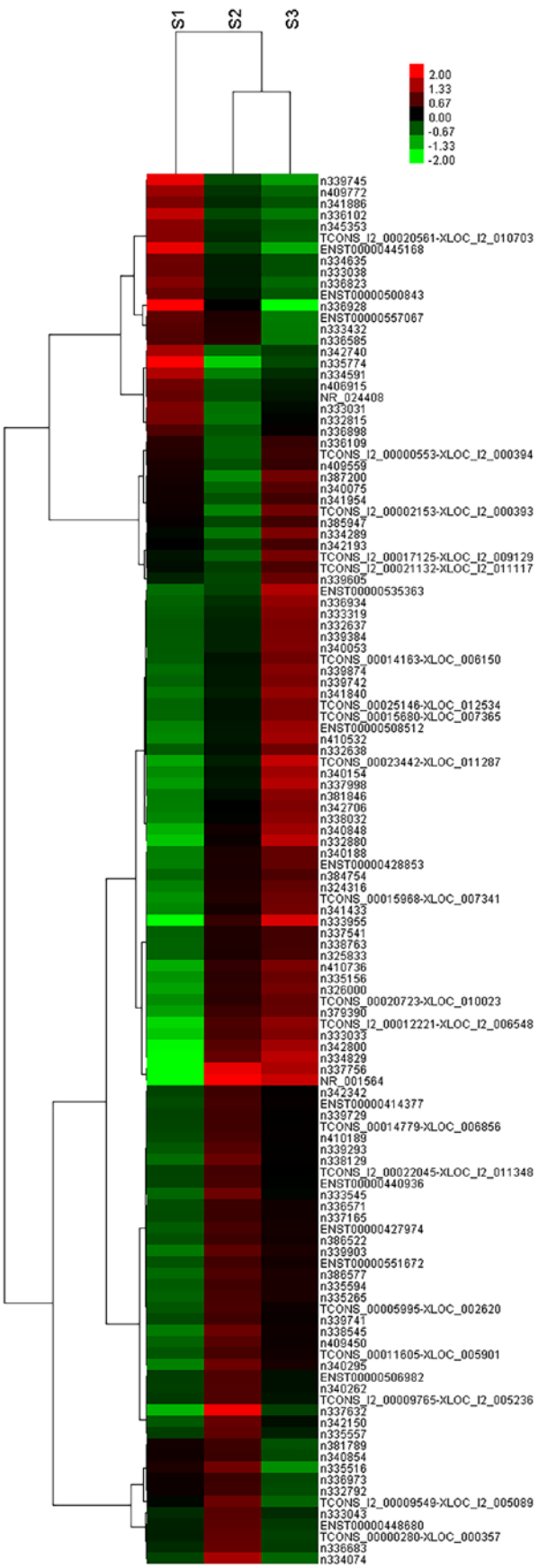

Figure 2. Hierarchical clustering of lncRNA expression in the 3 stages of embryonic development. Distinguishable lncRNA expression profiles were observed. Red indicates significantly increased expression. Green denotes significantly reduced expression and black indicates no difference in expression levels. Groups: S1, embryonic weeks 7-16; S2, embryonic weeks 16-25; S3, embryonic weeks 25-28. lncRNA, long non-coding RNA.

Table III. Upregulated differentially expressed lncRNAs between the 3 stages of fetal lung development.

A, S2 vs. S1

lncRNA	Fold-change
NR_001564	47.08876564
n337756	32.68860714
n337632	10.19552386
n339163	9.001615862
NR_003349	8.523677449
n334829	8.462693991
NR_003347	8.142292223
NR_003298	7.369409383
NR_003314	7.246287266
NR_003303	6.652354265
NR_003355	6.62124723
NR_003359	6.399445927
n342800	6.291303669
NR_003297	6.236367731
n333955	5.766172512
NR_001291	5.567478185
NR_003308	5.567478185
NR_003348	5.40086692
NR_002974	5.268747348
NR_002581	5.046048245

B, S3 vs. S2

lncRNA	Fold-change
n386326	4.555394507
NR_029493	4.4777873
NR_024065	4.340466177
n382996	4.289896973
NR_036677	4.269902123
NR_026703	3.970979739
n408293	3.920158536
n387200	3.917295604
ENST00000535363	3.798942827
TCONS_l2_00002153-XLOC_l2_000393	3.759703782
n334289	3.748358652
ENST00000459059	3.538294177
n340146	3.474537616
TCONS_00023442-XLOC_011287	3.453784337
ENST00000379816	3.283596831
TCONS_l2_00017125-XLOC_l2_009129	3.263300854
NR_004407	3.159558548
NR_028502	3.103420093
NR_002581	3.063370788
n337998	3.052347039

C, S3 vs. S1

lncRNA	Fold-change

Table III. Continued.

NR_001564	35.47473495
n337756	23.19649621
NR_002581	15.45791679
n333955	14.17277149
n334829	13.39219004
NR_026703	10.89074695
n339163	10.31981355
n342800	9.393546857
NR_004407	8.566180043
n332880	8.210340188
TCONS_00023442-XLOC_011287	7.217168811
TCONS_l2_00012221-XLOC_l2_006548	7.062979945
NR_002974	6.958339161
NR_003349	6.388459125
n340848	6.261155795
n337998	6.218717152
n333033	6.04561874
NR_003347	5.995399957
NR_002977	5.681622584
NR_003314	5.591673021

Groups: S1, embryonic weeks 7-16; S2, embryonic weeks 16-25; S3, embryonic weeks 25-28. lncRNA, long non-coding RNA.

Table IV. Specific fold-changes of ≥1.5 consistently upregulated lncRNAs identified following screening.

Comparison	n34048	n387037
S2 vs. S1	2.905918	1.571261
S3 vs. S2	2.154622	1.509102
S3 vs. S1	6.261156	2.371193

Groups: S1, embryonic weeks 7-16; S2, embryonic weeks 16-25; S3, embryonic weeks 25-28. Fold-change, the ratio of the geometric mean of the same probe signal value between groups.

Table V. Specific fold-changes of ≥1.5 consistently downregulated lncRNAs identified following screening.

Fold-change	n336823	ENST00000445168
S2 vs. S1	0.414761	0.194416
S3 vs. S2	0.471789	0.458941
S3 vs. S1	0.278632	0.108668

Groups: S1, embryonic weeks 7-16; S2, embryonic weeks 16-25; S3, embryonic weeks 25-28. Fold-change, the ratio of the geometric mean of the same probe signal value between groups.

to identify differentially expressed genes. P<0.05 was used to screen and obtain significantly associated pathways. The probability of enrichment of a differentially expressed gene set in a term entry was represented by an enrichment score (EC), with a higher EC indicating a higher significance of the entry. The EC was calculated as the negative base 10 log of the P-value.

Statistical analysis. All quantitative data are expressed as the mean ± standard error of the mean. All experiments were repeated ≥3 times with similar results. Data were analyzed using a SPSS statistical package (version no. 17.0; SPSS, Inc.) and the results of the RT-qPCR were evaluated by one-way ANOVA with the Student-Newman-Keuls post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Histology. In the S1 group, the bronchial tree extended to numerous bronchioles, which were composed of epithelial cells. In a single layer of cubic cells, cells were tall, columnar and arranged in rings. In the S2 group, terminal bronchioles branched out into greater numbers of respiratory bronchioles and cubic epithelial cells appeared short and columnar compared with S1. A dilated alveolar lumen, increased alveolar septa, thinner interstitium and hyperplasia of capillaries were observed in the fetal lungs in the S2 and S3 groups. Alveolar sacs formed in the S3 group and pulmonary alveoli took shape. In S3, a greater number of alveolar septa appeared compared with the S1 and S2 groups, and the interstitium was thinner compared with the S1 and S2 groups. In S3, epithelial cells had a cubic or flat shape (Fig. 1).

lncRNA microarray profiles. Affymetrix Human GeneChip was utilized to determine the expression spectrum of lncRNAs during fetal lung development. As a result, 687 lncRNAs were indicated to be differentially expressed among the three groups (S1, S2 and S3) of fetal lung tissue samples. According to these data, there were 34 upregulated lncRNAs and 12 downregulated lncRNAs that were significantly differentially expressed among all combinations of S1 vs. S2 vs. S3 (fold-change ≥1.5). Among the 687 differentially expressed lncRNAs, 39 downregulated and 202 upregulated lncRNAs were identified in the S2 vs. S1 comparison (fold-change >2). Furthermore, 24 lncRNAs were downregulated and 78 upregulated in the S3 vs. S2 comparison (fold-change >2) and 77 downregulated and 535 upregulated lncRNAs were identified in the S3 vs. S1 comparison (fold-change >2; Tables II and III). Hierarchical clustering was performed in order to display distinguishable lncRNA expression profiles among the groups. Taken together, these data were consistent with the notion that different lncRNAs may be involved in the different phases of lung development (Fig. 2).

As certain lncRNAs exhibited more significant fold-changes among the three groups, 4 lncRNAs were selected based on these data, including two downregulated lncRNAs (n336823 and ENST00000445168; Table IV) and two upregulated lncRNAs (n3408848 and n387037; Table V).

RT-qPCR. The expression levels of the selected lncRNAs were confirmed by RT-qPCR. Among these differentially

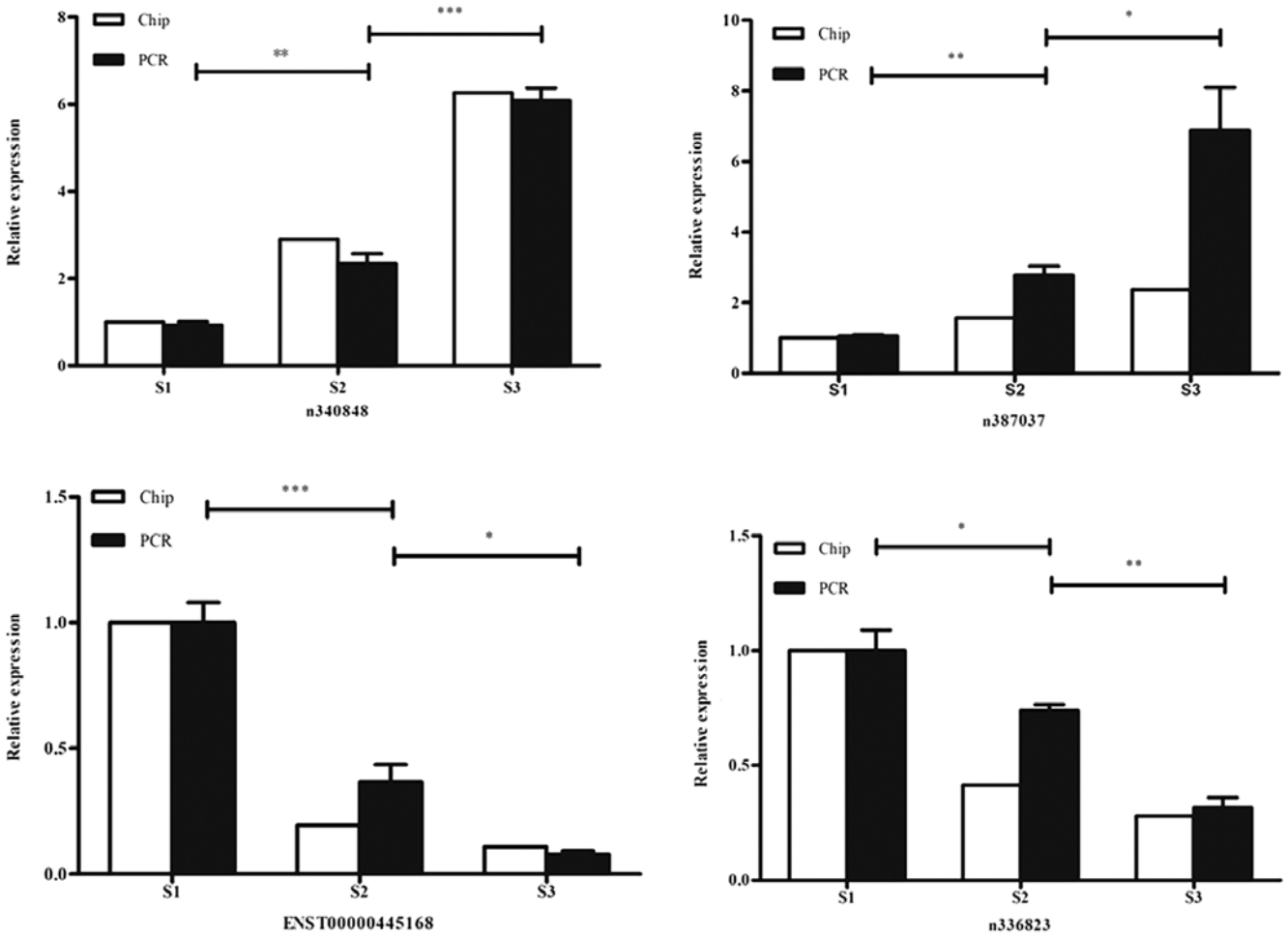

Figure 3. Validation of GeneChip results by RT-qPCR. n340848 and n387037 progressively increased in expression as the gestational age increased. ENST00000445168 and n336823 expression was reduced with the gestational age. Groups: S1, embryonic weeks 7-16; S2, embryonic weeks 16-25; S3, embryonic weeks 25-28. Statistical analysis was performed on RT-qPCR data. ***P<0.001, **P<0.01 and *P<0.05 vs. indicated groups. RT-qPCR, reverse transcription-quantitative PCR.

expressed lncRNAs, n340848 and n387037 were indicated to be continuously increased in expression with progression through the three phases of lung development, while the expression levels of ENST00000445168 and n336823 were reduced with progression. These results were consistent with the GeneChip data obtained. The relative trends in expression of these lncRNAs are presented in Fig. 3.

GO analysis and KEGG pathway analysis. Upregulated transcripts were indicated to be associated with the GO terms cell adhesion, hydrogen peroxide decomposition and protein kinase C of a G protein-coupled receptor signaling pathway (Fig. 4A). The top three biological process terms associated with the downregulated transcripts were G protein-coupled receptor signaling pathway coupled to the cyclic guanosine monophosphate nucleotide second messenger, brain development and cerebral cortex development (Fig. 4A).

Additionally, KEGG enrichment analysis was performed to investigate the possible roles of the lncRNA-associated protein-coding genes. The most significant pathways enriched in the set of upregulated protein-coding genes included cell adhesion molecules, as well as adherens junction and glyceride metabolism (Fig. 5A). Biosynthesis of an amino acid, basal cell carcinoma and glycolysis/gluconeogenesis were the most important pathways enriched in the set of down-regulated genes (Fig. 5B).

Discussion

In the present study, 687 lncRNAs were identified to be differentially expressed among three groups (embryonic periods S1, S2 and S3) of human fetal lung tissue samples. The results revealed 34 upregulated lncRNAs and 12 downregulated lncRNAs, which were significantly differentially expressed among all combinations of S1 vs. S2 vs. S3 (fold-change ≥1.5; P<0.05). Among these differentially expressed lncRNAs, n340848, n387037, n336823 and ENST00000445168 were then validated by RT-qPCR. These results were consistent with the GeneChip results. GO enrichment analysis revealed that the majority of the GO terms associated with these genes belonged to the biological process category. The fact that one lncRNA is able to target numerous genes suggests that lncRNAs may be involved in a series of biological processes.

Among the four lncRNAs selected, lncRNA n340848 is located on chromosome 6, overlapping with a gene called neural precursor cell expressed developmentally

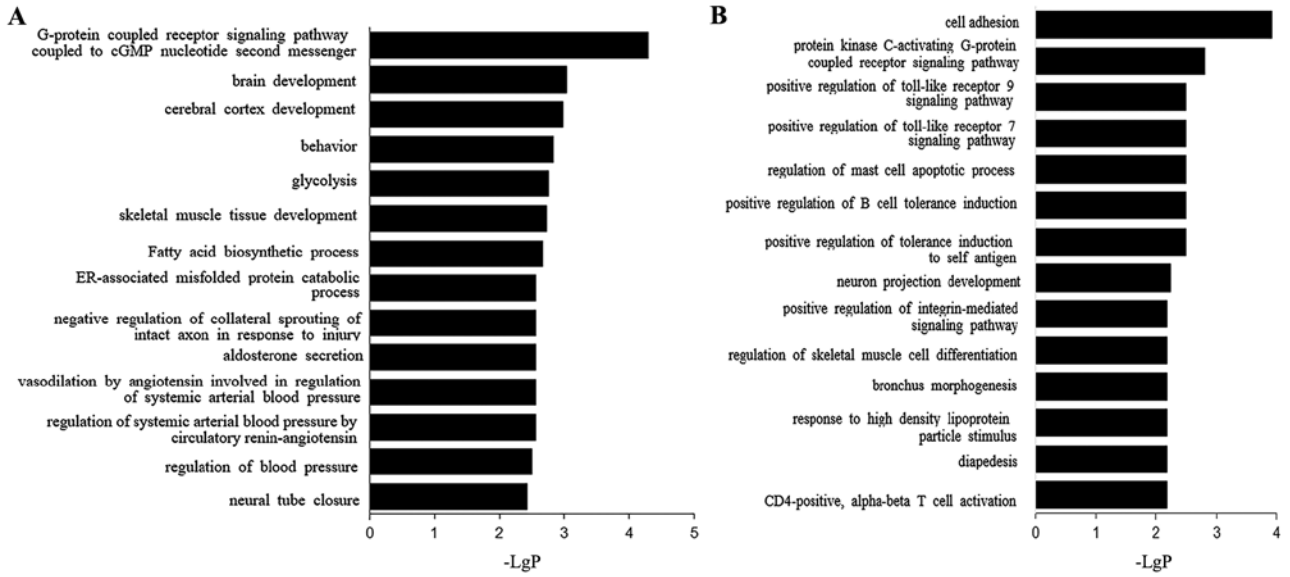

Figure 4. Gene Ontology terms associated with the crossover genes of differentially expressed lncRNA-associated genes and differential mRNA genes co-expressed with lncRNA. (A) Terms associated with upregulated genes. (B) Terms associated with downregulated genes. ER, estrogen receptor; cGMP, cyclic guanosine monophosphate.

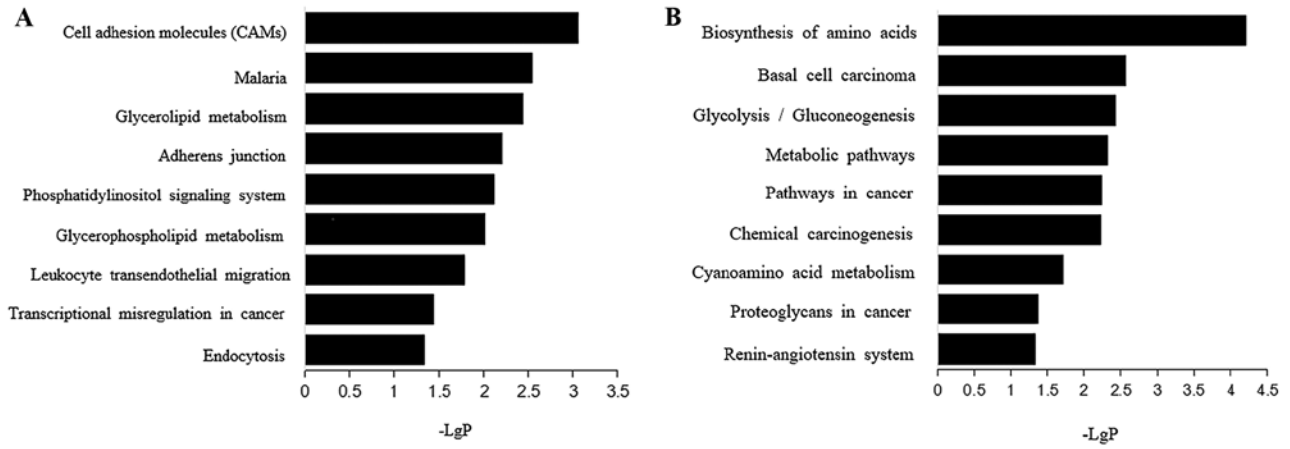

Figure 5. Kyoto Encyclopedia of Genes and Genomes pathways associated with the crossover genes of differentially expressed lncRNA-associated genes and differential mRNA genes co-expressed with lncRNA. (A) Terms associated with upregulated genes. (B) Terms associated with downregulated genes.

down-regulated 9 (NEDD9). The highest levels of NEDD9 mRNA and protein have been detected in lungs and kidneys (33). NEDD9 has been reported to act as a scaffold protein and is part of the Crk-associated substrate family, which regulates protein complex control of cell invasion and differentiation (34). n387037 is a 1,342-bp lncRNA with a genomic overlap with the platelet and endothelial cell adhesion molecule 1 (PECAM1) gene (5). PECAM1 expression has been reported in almost all tissues and is expressed at the highest levels in the placenta, lungs and fat tissues (35). It has been indicated as a novel therapeutic target in neonatal respiratory distress syndrome and ventilator-induced lung injury (36). LncRNA n336823 is 26,063 bp long and overlaps with patched 1, which encodes a member of the patched family of proteins and a component of the hedgehog (HH) signaling pathway (37). HH signaling is crucial for embryonic development and tumorigenesis (38). Numerous studies have indicated that activation of the HH signaling pathway is associated with the progression of multiple solid tumors, including lung cancer (1,39).

lncRNA ENST0000445168 (also known as 02038-202), encoded on chromosome 3, is an intergenic lncRNA, whose transcription occurs entirely within the genomic interval between two adjacent protein-coding genes (40,41). These results indicated that the four lncRNAs mentioned above may take part in the etiology and pathogenesis of disorders of neonatal lung development; however, further research is required for confirmation.

Numerous studies have indicated that various lncRNAs are involved in different biological events (38,42,43). lncRNA NR_033925, also known as forkhead box (FOX)F1-AS1 or FOXF1-adjacent non-coding developmental regulatory RNA, is encoded upstream of FOXF1 (44). It is highly expressed in human lungs and has an important effect on the development of the heart and gastrointestinal tract (45). lncRNA n409380, associated with the FOXP2 gene, promotes embryonic

development and the proliferation of lung epithelial cells (46). lncRNA n335087 and lncRNA n339275 overlap with gene TGF-β receptor 2 and are involved in lung development-associated tracheal morphogenesis and leukocytic protein phosphorylation (17).

Certain studies have addressed the specific expression patterns or function of lncRNAs in lung development. Herriges *et al* (47) screened 363 lncRNAs in the lung and foregut endoderm and indicated that they were spatially associated with transcription factors across the genome. It has been reported that lncRNAs in the lungs are located near genes of transcription factors, including NK2 homeobox 1 (NKX2.1), GATA binding protein 6, FOXA2 and FOXF1, which are essential in foregut and lung development (48,49). One of these lncRNAs, NKX2.1-associated non-coding intergenic RNA (NANCI), performs an important function in lung development by acting upstream of the critical transcription factor NKX2.1 and downstream of Wnt/β-catenin signaling to regulate lung endoderm gene expression and morphogenesis (16). Furthermore, a previous study identified a feedback loop within the NANCI-NKX2.1 gene duplex to explain how this subset acts as a rheostat to buffer the expression of neighboring transcription factor genes, to maintain tissue-specific cellular identity during development and postnatal homeostasis (47).

In conclusion, the results of the present study indicated that the lncRNA expression profile varies among different phases of fetal lung development. These results provided certain indications of the roles of lncRNAs in human fetal lung development. In the future, functional verification, target gene prediction and validation of the identified lncRNAs will be performed *in vitro* and *in vivo* to provide novel insight into the pathogenesis of neonatal pulmonary diseases.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant no. 781270725).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JS, ZB, YY, XGZ and XYZ designed the experiments. JS, ZB, WZ, CM, YS and QK performed the experiments, collected data, generated the figures and interpreted the results. JS, ZB, XGZ and XYZ wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures were approved by the Ethics Committee of Nanjing Maternal and Child Health Hospital, Nanjing, China [approval no. (2014) 74] and all patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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