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

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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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Introduction

Long non-coding RNAs (IncRNAs) 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, IncRNAs 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, IncRNAs 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 IncRNAs 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 IncRNAs. Cheng et al (10) reported 9 IncRNAs that were potentially associated with bronchopulmonary dysplasia (BPD) and these data may provide novel insight into the biological roles of IncRNAs in the pathogenesis of BPD. Numerous IncRNAs 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 IncRNAs 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 IncRNA 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 IncRNAs 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-glândular 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% rivanal 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 (2×4×6 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. 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 dehydrated 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.
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,
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 \(2^{-\Delta\Delta CT} \) 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 \( \chi^2 \) 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 \( \chi^2 \) test were used.

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

| IncRNA         | Fold-change |
|----------------|-------------|
| n335774        | 0.06935588  |
| n339745        | 0.182483003 |
| n334591        | 0.186404811 |
| ENST00000445168| 0.194416962 |
| n342740        | 0.223988722 |
| n336928        | 0.225022474 |
| n336102        | 0.23459609  |
| n335785        | 0.254695963 |
| n333031        | 0.272814078 |
| n332815        | 0.275703789 |
| OTTHUMT00000312398 | 0.30750654 |
| n409772        | 0.320872488 |
| n338599        | 0.34498774  |
| n334031        | 0.34687415  |
| n406915        | 0.347614219 |
| n345533        | 0.36559716  |
| ENST00000559553 | 0.370347627 |
| NR_024408      | 0.371285274 |
| n333438        | 0.376855146 |
| TCONS_00020561-XLOC_0010703 | 0.381374424 |

B, S3 vs. S2

| IncRNA         | Fold-change |
|----------------|-------------|
| n337632        | 0.186974574 |
| n336928        | 0.221248411 |
| n334074        | 0.231631923 |
| n335516        | 0.250004679 |
| TCONS_0009549-XLOC_005089 | 0.322783111 |
| n336841        | 0.355730111 |
| n410723        | 0.39944174  |
| n336683        | 0.412094182 |
| n333958        | 0.414088201 |
| TCONS_0000280-XLOC_000357 | 0.415518323 |
| n336855        | 0.417651927 |
| n333432        | 0.424395128 |
| n408121        | 0.425823065 |
| ENST00000557067 | 0.428630673 |
| ENST00000448680 | 0.4427573  |
| n381789        | 0.457561363 |
| n332792        | 0.469538382 |
| n333380        | 0.482586011 |
| n340854        | 0.486920451 |
| n335620        | 0.488461581 |

C, S3 vs. S1

| IncRNA         | Fold-change |
|----------------|-------------|
| n335774        | 0.049785865 |
| ENST00000445168| 0.10867618  |
| n339745        | 0.11741297 |
| n335774        | 0.144621906|
| n336102        | 0.179262761|
| n409772        | 0.243588174 |
| n338817        | 0.26669321 |
| n336823        | 0.27863222 |
| n335785        | 0.281131886 |
| TCONS_00020561-XLOC_0010703 | 0.283204481 |
| n342740        | 0.288809679 |
| n345533        | 0.295015569 |
| n334591        | 0.295785862 |
| ENST00000557067 | 0.303030728 |
| n336841        | 0.322103669 |
| n333432        | 0.322647779 |
| n341886        | 0.322920291 |
| n336855        | 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.
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 | IncRNA | 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    | 5.26874348 |
| NR_002581    | 5.046048245 |

| B, S3 vs. S2 | IncRNA | Fold-change |
|--------------|--------|-------------|
| n386326      | 4.555394507 |
| NR_029493    | 4.4777873 |
| NR_024065    | 4.340466177 |
| n382996      | 4.28986973 |
| NR_036677    | 4.269902123 |
| NR_026703    | 3.970979739 |
| n408293      | 3.920158536 |
| n387200      | 3.917295604 |
| ENST00000535363 | 3.798942827 |
| TCONS_12_00001215-XLOC_12_000393 | 3.759703782 |
| n334289      | 3.748358652 |
| ENST00000459059 | 3.538294177 |
| n340146      | 3.474537616 |
| TCONS_12_00002344-XLOC_12_0011287 | 3.453784337 |
| ENST00000379816 | 3.283596831 |
| TCONS_12_000017125-XLOC_12_0009129 | 3.263300854 |
| NR_004407    | 3.159558548 |
| NR_0028502   | 3.103420093 |
| NR_002581    | 3.063370788 |
| n337998      | 3.052347039 |

| C, S3 vs. S1 | IncRNA | Fold-change |
|--------------|--------|-------------|
| n386326      | 4.555394507 |
| NR_029493    | 4.4777873 |
| NR_024065    | 4.340466177 |
| n382996      | 4.28986973 |
| NR_036677    | 4.269902123 |
| NR_026703    | 3.970979739 |
| n408293      | 3.920158536 |
| n387200      | 3.917295604 |
| ENST00000535363 | 3.798942827 |
| TCONS_12_00001215-XLOC_12_000393 | 3.759703782 |
| n334289      | 3.748358652 |
| ENST00000459059 | 3.538294177 |
| n340146      | 3.474537616 |
| TCONS_12_00002344-XLOC_12_0011287 | 3.453784337 |
| ENST00000379816 | 3.283596831 |
| TCONS_12_000017125-XLOC_12_0009129 | 3.263300854 |
| NR_004407    | 3.159558548 |
| NR_0028502   | 3.103420093 |
| NR_002581    | 3.063370788 |
| n337998      | 3.052347039 |
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_12_00012221-XLOC_12_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 12 upregulated 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 S1 vs. S2 comparison (fold-change >2). Furthermore, 24 lncRNAs were downregulated and 78 upregulated in the S3 vs. S2 comparison (fold-change >2) and 24 lncRNAs were 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 (n340848 and n387037; Table V).

RT-qPCR. The expression levels of the selected lncRNAs were confirmed by RT-qPCR. Among these differentially
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
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 IncRNAs in lung development. Herriges et al (47) screened 363 IncRNAs in the lung and foregut endoderm and indicated that they were spatially associated with transcription factors across the genome. It has been reported that IncRNAs 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 IncRNAs, 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 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 IncRNA expression profile varies among different phases of fetal lung development. These results provided certain indications of the roles of IncRNAs in human fetal lung development. In the future, functional verification, target gene prediction and validation of the identified IncRNAs will be performed in vitro and in vivo to provide novel insight into the pathogenesis of neonatal pulmonary diseases.

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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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