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**Original Paper**

# Microarray Profiling of TGF-β1-Induced Long Non-Coding RNA Expression Patterns in Human Lung Bronchial Epithelial BEAS-2B Cells

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**Key Words**
Long non-coding RNA • Microarray • TGF-β1 • BEAS-2B

**Abstract**
***Background/Aims:*** TGF-β1 mediated radiation-induced bystander effects (RIBE) have been linked with malignant transformation and tumorigenesis. However, the underlying mechanisms are not fully understood. ***Methods:*** To reveal new molecules of regulatory functions in this process, lncRNA microarray was performed to profile both lncRNA and mRNA expression patterns in human lung bronchial epithelial BEAS-2B cells treated with TGF-β1 at a concentration measured in the medium conditioned by directly irradiated BEAS-2B cells. The potential functions of the differentially expressed lncRNAs were predicted by GO and KEGG pathway analyses of their co-expressed mRNAs. Cis- and trans-regulation of the lncRNAs were analyzed and the interaction networks were constructed using Cytoscape. qRT-PCR was conducted to validate the results of microarray profiling. The functions of 4 lncRNAs in TGF-β1-induced EMT were explored by detecting the changes of EMT markers. ***Results:*** 224 lncRNAs were found to be dysregulated, among which 6 lncRNAs were chosen for expression validation by qRT-PCR assay. Pathway analyses showed that differentially expressed lncRNAs are highly correlated with cell proliferation, transformation, migration, *etc*. Trans-regulation analyses showed that the differentially expressed lncRNAs most likely participate in the pathways regulated by four transcriptional factors, FOS, STAT3, RAD21 and E2F1, which have been identified to be involved in the modulation of oncogenic transformation, cell cycle progression, genomic instability, etc. Knock-down of lnc-ABCA12-5, lnc-THEMIS-2 or lnc-HPN-AS1 suppressed the EMT induced by TGF-β1 to varying degrees. ***Conclusion:*** Our findings suggest that the differentially expressed lncRNAs induced by TGF-β1 play crucial roles in the

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oncogenic transformation and tumorigenesis, which provide a better understanding of the underlying mechanisms related to tumorigensis induced by LD/LDR radiations.

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## Introduction

Ionizing radiation (IR) is one kind of well-established human carcinogen, which has been proven to be able to cause various kinds of tumors [1]. Extensive researches have focused on the health effects of IR in the hope of understanding the risk of IR exposure. In contrast to the traditional radiobiological viewpoint that the biological effects of IR are restricted to directly hit cells, the concept of radiation-induced bystander effects (RIBE) has updated our perception of the biological effects of IR [2]. It is realized that the existing radiotherapy models should be reconsidered due to the discovery of radiation-induced secondary carcinogenesis in distant organs after radiotherapy [3], as well as the carcinogenesis induced by low-dose/low-dose-rate (LD/LDR) radiations, for example, the astronauts exposed to LD/LDR high atomic number (Z) and energy (HZE) space radiations [4]. It is generally believed that both gap junctional intercellular communication (GJIC) and soluble factors generated by the irradiated cells are involved in the RIBE [2], of which transforming growth factor-β1 (TGF-β1) is a key soluble factors [5-7]. Although TGF-β1-related signaling pathways were studied extensively, the mechanisms underlying the TGF-β1-induced tumorigenesis remain unclear, especially its role in LD/LDR radiations- or RIBE-induced tumorigenesis.

Long non-coding RNAs (lncRNAs), with a size longer than 200 nt and showing no protein-coding capacity, have been found to regulate gene expression at post-transcriptional level or by chromatin modification and function as key regulators of diverse biological processes, such as development, cell proliferation, differentiation and apoptosis, organogenesis, and diseases [8-11]. Up to now, a great number of researches have shown that lncRNAs are closely related to human cancer [12, 13]. Besides, several studies also linked lncRNAs with epithelial-mesenchymal transition (EMT) and tumorigenesis [14]. For example, hypoxia-induced lncRNANUTF2P3-001 contributes to tumorigenesis of pancreatic cancer [15]. HULC enhances EMT to promote tumorigenesis and metastasis of hepatocellular carcinoma [16]. MALAT1 modulates TGF-β1-induced endothelial-to-mesenchymal transition (EndMT) of endothelial progenitor cells through regulation of TGFBR2 and SMAD3 *via* miR-145 [17]. RMRP promotes carcinogenesis and serves as a novel biomarker for gastric cancer [18]. RP11-708H21.4 is down-regulated in colorectal cancer (CRC) samples, while its up-regulation blocked AKT/mTOR pathway and repressed *in vivo* CRC xenograft tumor growth [19]. However, the relevance of lncRNAs in the tumorigenesis induced by IR or RIBE has not yet been characterized.

Here, we performed lncRNA microarray assays to profile the lncRNA expression patterns in BEAS-2B cells treated with a particular concentration of TGF-β1 (detected in the radiation conditioned medium) and the untreated BEAS-2B cells. Over 300 lncRNAs and mRNAs were found differentially expressed significantly. Several of the differentially expressed lncRNAs were validated by qRT-PCR. The functions of these differentially expressed lncRNAs were predicted according to their co-expressed genes and the GO (gene ontology) analysis. The genes cis- and trans-regulated by the differentially expressed lncRNAs were further analyzed. The present study revealed that the aberrant lncRNA expression pattern may play an important role in the malignant transformation of BEAS-2B cells induced by TGF-β1.

## Materials and Methods

*Cell culture and TGF-β1 treatment*

Human bronchial epithelial cell line BEAS-2B was maintained in DMEM medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 1% penicillin sodium and 100 μg/mL streptomycin, at 37°C in 5% $CO_2$ in a humidified incubator (Thermo Scientific, Asheville, NC, USA). The 80%confluence of BEAS-2B cells in Φ100 mm culture dishes (Corning, NY, USA) was subjected to recombinant Human TGF-β1 (PeproTech, Rocky Hill, NJ, USA) treatment for 2 h with a concentration at 230 pg/mL, which was measured in the medium conditioned by BEAS-2B cells exposed to X-ray irradiation. Briefly, the BEAS-2B cells were exposed to 2 Gy X-rays and the media were collected up to 24 hours post-irradiation. The concentrations of TGF-β1 were measured using Human TGF-β1 ELISA Kit (Multi Sciences, Hangzhou, China). Thus the cells used for microarray profiling were incubated with the media containing TGF-β1 at a concentration of 230 pg/mL for 2 hours before samples collection.

*The lncRNA and mRNA microarray expression profiling*

Total RNA extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol was quantified by NanoDrop™ ND-2000 (Thermo Fisher Scientific, Sunnyvale, CA, USA) and the RNA integrity was assessed using the Agilent Bio-analyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The microarray profiling was carried out in the lab of Shanghai OE Biotech Company. Total RNA were transcribed to double strand cDNA, then synthesized into cRNA and labeled with Cyanine-3-CTP. The labeled cRNAs were hybridized onto the microarray. After washing, the arrays were scanned by the Agilent Scanner G2505C (Agilent Technologies, Santa Clara, CA, USA). The raw data was acquired by analyzing the array images using Feature Extraction software (version 10.7.1.1, Agilent Technologies). Genespring (version 13.1, Agilent Technologies) were then employed for quantile normalization and basic analysis of the raw data. Differentially expressed genes or lncRNAs were identified through fold change as well as *P* value calculated with t-test. The threshold set for up- and down-regulated genes was a fold change>2.0 and a *P* value<0.05. Finally, Hierarchical Clustering was performed to display the distinguishable genes' expression pattern among samples. The false discovery rate (FDR)was used to evaluate the significance of the *P* value and a FDR<0.05 was considered to be significant.

*The construction of the co-expression network of lncRNAs with mRNAs*

Since the most of the lncRNAs in the current databases have not yet been functionally annotated, the prediction of their functions is based on the functional annotations of their co-expressed mRNAs. For each differentially expressed lncRNA, the Pearson correlation coefficient (PCC) of its expression value was calculated with that of each dysregulated mRNA. The co-expressed mRNAs with PCC>0.8 or <–0.8 and a correlation *P* value<0.05 were identified as statistically significant. The functional enrichment analyses of the co-expressed mRNAs were performed using the hypergeometric cumulative distribution function. The GO/KEGG pathway annotations were assigned to the lncRNAs as the predicted functions (http://david.abcc.ncifcrf.gov, http://www.genome.jp/kegg/).

*Cis-regulation of lncRNA and the construction of the TF-lncRNA network*

Firstly, the gene locations for different lncRNAs on the chromosome were determined, then the co-expressed genes (*P* value of correlation<0.05) located within the 300 kbp windows upstream and downstream of the differentially expressed lncRNAs were identified as the potential "cis-regulated mRNAs". For the TF-lncRNA network construction, hypergeometric cumulative distribution was adopted to calculate the enrichment significance of the co-expressed genes of the differentially expressed lncRNAs in every transcription factor (TF) term. The co-expressed genes with a FDR<0.01 were deemed as enrichment in the TF term. The networking sketch of TF and lncRNA relationship was generated using Cytoscape software (http://www.cytoscape.org).

*Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)*

qRT-PCR was carried out in a two step procedure. Reverse transcription (RT) of total RNA was conducted using an All-in-One™ First-Strand cDNA Synthesis Kit (GeneCopoeia, Rockville, MD, USA). The real-time PCR analysis was carried out using a SYBR Premix Ex Taq II Kit (Takara, Japan) in a 20 μL system. The PCR amplification was performed using an ABI ViiA™ 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and initiated by 10 min at 95°C before 40 thermal cycles, each at 95°C for 15 s, 55°C for 15 s and with an extension at 72 °C for 10 s. Melting curve analysis was performed at the end of the PCR cycles to validate the specific generation of the expected PCR product. The expression levels of lncRNAs or mRNAs were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and were analyzed by C(t) value comparison method. All experiments were done in triplicate. The primers were synthesized by Shanghai Sangon Biotech and all sequences are listed in Table 1.

**Table 1.** Primer pairs used for qRT-PCR

| Genes | Primer pairs |
|---|---|
| GAPDH | Forward: GCACCGTCAAGGCTGAGAAC<br>Reverse: TGGTGAAGACGCCAGTGGA |
| lnc-ERVFRD-1-1 | Forward: TACAAGCTCCATCAGCACAGG<br>Reverse: TGGTTCACTGGGCTGCTATC |
| lnc-HPN-AS1 | Forward: TCAAAGACCATGAGCCGAGC<br>Reverse: CACGTCTACACCACATGGCT |
| lnc-THEMIS-2 | Forward: CGTGTACGTGCGTTAGAGGT<br>Reverse: CGCCTGTAGCACAAAAGCAA |
| lnc-ABCA12-5 | Forward: AACCTACGGATGACTCGTGC<br>Reverse: ACACGGAAGTGGATGGACAAA |
| lnc-WDR12-4 | Forward: GCGATGGAGCAGCTATCAGA<br>Reverse: TGGTGACTTCAAATCCTCAACCA |
| lnc-SBDS-25 | Forward: ACTTCGACTCCTTGAAGCTGG<br>Reverse: CGGTGACGGGATAGTTTGTGA |
| lnc-ITGB6-4 | Forward: CAGCTCAGCAAAACGAACCT<br>Reverse: ACACTGAGAGTCCACAGATACTTC |
| E-cadherin | Forward: TGCCCAGAAAATGAAAAAGG<br>Reverse: GTGTATGTGGCAATGCGTTC |
| N-cadherin | Forward: ACAGTGGCCACCTACAAAGG<br>Reverse: CCGAGATGGGGTTGATAATG |
| Fibronectin 1 | Forward: CAGTGGGAGACCTCGAGAAG<br>Reverse: TCCCTCGGAACATCAGAAAC |
| Vimentin | Forward: GAGAACTTTGCCGTTGAAGC<br>Reverse: GCTTCCTGTAGGTGGCAATC |

*Statistical Analysis*

All data are presented as means ± SE. Statistical significance was determined by Student's *t*-test between the indicated groups. $P<0.05$ was considered to be statistically significant.

## Results

*Expression profiles of lncRNA and mRNA in BEAS-2B cells treated with TGF-β1*

224 lncRNAs were identified with significantly differential expression in BEAS-2B cells treated with TGF-β1 at a concentration of 230 pg/mL compared with untreated BEAS-2B cells (fold change>2; FDR<0.05), among which 161 were significantly upregulated while 63 downregulated. Unsupervised hierarchical clustering analysis of the expression of 224 lncRNAs and 99 mRNAs was shown in heat map (Fig. 1). Among the dysregulated transcripts of lncRNA, lnc-ERVFRD-1-1 was the most upregulated, with a FC (fold change) of 8.29, while lnc-WDR12-4 was the most downregulated, with a FC of –4.98. The top 20 differentially expressed lncRNAs identified using microarray analyses are listed in Table 2. 99 mRNA transcripts were found to be dysregulated by the same criteria as lncRNAs (fold change>2; FDR<0.05), with 82 upregulated and 17 downregulated, among which KCNJ12 and OR51B2 were most upregulated and downregulated, respectively, with FCs of 17.43 and –29.34.

*Validation of the microarray data using qRT-PCR*

The most upregulated lncRNA ERVFRD-1-1 and downregulated lncRNA WDR12-4 were chosen for expression validation using qRT-PCR. Besides, 4 lncRNAs (HPN-AS1, THEMIS-2, ABCA12-5, and SBDS-25) were screened out for expression validation of the microarray data using qRT-PCR on the basis of lncRNA function predictions, which were the core regulatory lncRNAs in the TF-lncRNA network. These lncRNAs were speculated to be involved in the oncogenic transformation. Consistent with microarray data, it is indicated by the qRT-PCR results that lncRNAs ERVFRD-1-1, HPN-AS1, THEMIS-2 and ABCA12-5 were upregulated significantly and that WDR12-4 and SBDS-25 were downregulated significantly in the BEAS-2B cells treated with TGF-β1 of 230 pg/mL compared with control cells (Fig. 2).

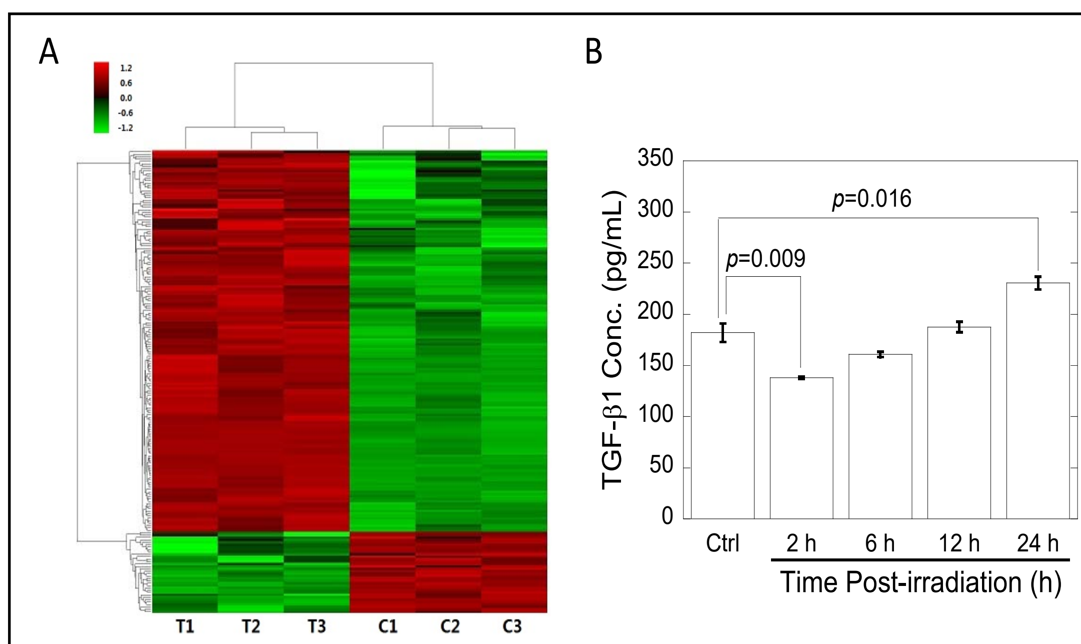


**Fig. 1.** Heat map of the differentially expressed lncRNAs in BEAS-2B cells treated with TGF-β1 at a concentration of 230 pg/mL. (A) Each row represents one lncRNA, while each column represents one sample. C1-C3 represent independent control samples, while T1-T3 represent three independent TGF-β1-treated samples. The relative lncRNA expression level is depicted according to the color scale. Red indicates up-regulation; green indicates down-regulation. (B) The concentrations of the TGF-β1 in the medium of BEAS-2B cells exposed to 2 Gy X-ray irradiation were assayed by ELISA.

**Table 2.** Top 10 up-regulated and down-regulated lncRNAs and mRNAs in BEAS-2B cells treated with TGF-β1 compared with untreated BEAS-2B cells

| Up-regulated lncRNAs | | Down-regulated lncRNAs | | Up-regulated mRNAs | | Up-regulatedmRNAs | |
|---|---|---|---|---|---|---|---|
| lncRNA | FC | lncRNA | FC | mRNA | FC | mRNA | FC |
| ERVFRD-1-1 | 8.29 | WDR12-4 | 4.98 | KCNJ12 | 17.43 | OR51B2 | 29.34 |
| SMAD5-7 | 7.99 | ADCK3-3 | 4.71 | ANGPTL4 | 8.09 | MAF1 | 8.73 |
| MRPS18B-2 | 5.31 | IFT74-7 | 4.42 | COL27A1 | 7.74 | TRIOBP | 7.20 |
| GPR27-10 | 5.12 | FILIP1L-6 | 3.83 | LOC645427 | 7.24 | ATPIF1 | 7.03 |
| NIPSNAP3B-4 | 4.93 | DCD-1 | 3.54 | ROGDI | 7.16 | MKRN1 | 6.71 |
| CISTR | 4.73 | TET3-1 | 3.44 | EDN1 | 6.72 | STX10 | 6.60 |
| EIF2AK4-7:1 | 4.12 | EXOC3-5 | 3.33 | KCNJ18 | 6.22 | CD274 | 5.96 |
| COL4A2-AS2-2 | 4.06 | ENST00000440802 | 3.21 | BMF | 5.14 | DRAM1 | 5.45 |
| TRPA1-2 | 3.99 | HFE2-2 | 3.19 | NEDD9 | 4.66 | MPRIP | 5.19 |
| ABCA12-5 | 3.89 | COL8A1-3 | 3.19 | PTPRE | 4.56 | CBX1 | 4.66 |

*Co-expression profiles of lncRNA and mRNA and the prediction of lncRNA function*

A method originally demonstrated in Guttman's paper was adopted to predict the functions of the differentially expressed lncRNAs [20]. Simply speaking, the co-expressed mRNAs for each differentially expressed lncRNA was calculated, and then a functional enrichment analysis of this set of co-expressed mRNAs was conducted. The enriched functional terms were used as the predicted functional terms of given lncRNA. The co-expressed mRNAs of lncRNAs were identified by calculating Pearson correlation coefficient with a correlation $P$ value<0.05. Each aberrantly expressed lncRNA was found to be related to hundreds of co-expressed mRNAs. Heat maps were produced by unsupervised hierarchical clustering analysis of the expression values of the co-expressed mRNAs for each differentially expressed lncRNA (Fig. 3). Hypergeometric cumulative distribution function was then used to calculate

the enrichment of functional terms of the co-expressed mRNAs in GO/KEGG pathway annotations. In the above-mentioned correspondence between the differentially expressed lncRNAs and the predicted functional terms, top 200 and top 500 predictive relationships of the predicted confidence level (with the lowest *P* values) were selected and subjected to frequency counting statistics to reflect the overall functional distributions of the differentially expressed lncRNAs. Among all predicted functional terms, top 200 (according to frequency counting) were selected to draw a bar chart.

GO analysis indicated that several functional pathways were enriched. The most frequently predicted functions of differentially expressed lncRNAs are protein binding, transcription co-repressor activity, platelet-derived growth factor receptor binding and 15-hydroxyprostaglandin dehydrogenase (NAD+) activity (Fig. 4A). Of these, 69 were clustered into negative regulation of transcription from RNA polymerase II promoter, 57 into BMP signaling pathway,49 into negative regulation of transforming growth factor beta receptor signaling pathway, and 40 into negative regulation of sequence-specific DNA binding transcription factor activity. By using the same criteria as the GO analysis, KEGG pathway analysis showed that some pathways are highly correlated with the aberrantly expressed lncRNAs, including TNF signaling pathway, transcriptional misregulation in cancer, Rap1 signaling pathway, FoxO signaling pathway, TGF-β signaling pathway, Hippo signaling pathway, small lung cancer signaling pathway, *etc*. (Fig. 4B).

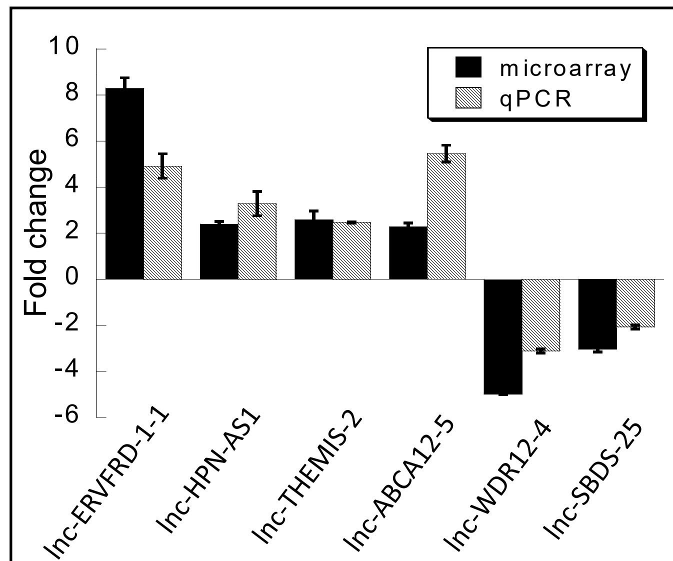


**Fig. 2.** qRT-PCR validation of six differentially expressed lncRNAs. The results were consistent with the microarray data. Bars represent SE.

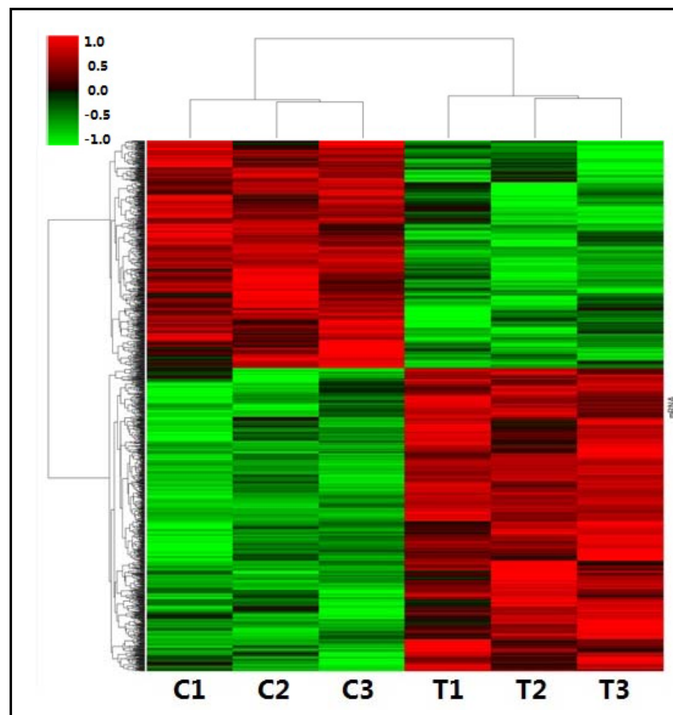


**Fig. 3.** Heat map of the co-expressed mRNAs of the differentially expressed lncRNAs in BEAS-2B cells treated with TGF-β1. Each row represents one mRNA, while each column represents one sample. C1-C3 represent independent control samples, while T1-T3 represent three independent TGF-β1-treated samples. This heat map shows the co-expressed mRNAs of lncRNA HPN-AS1.

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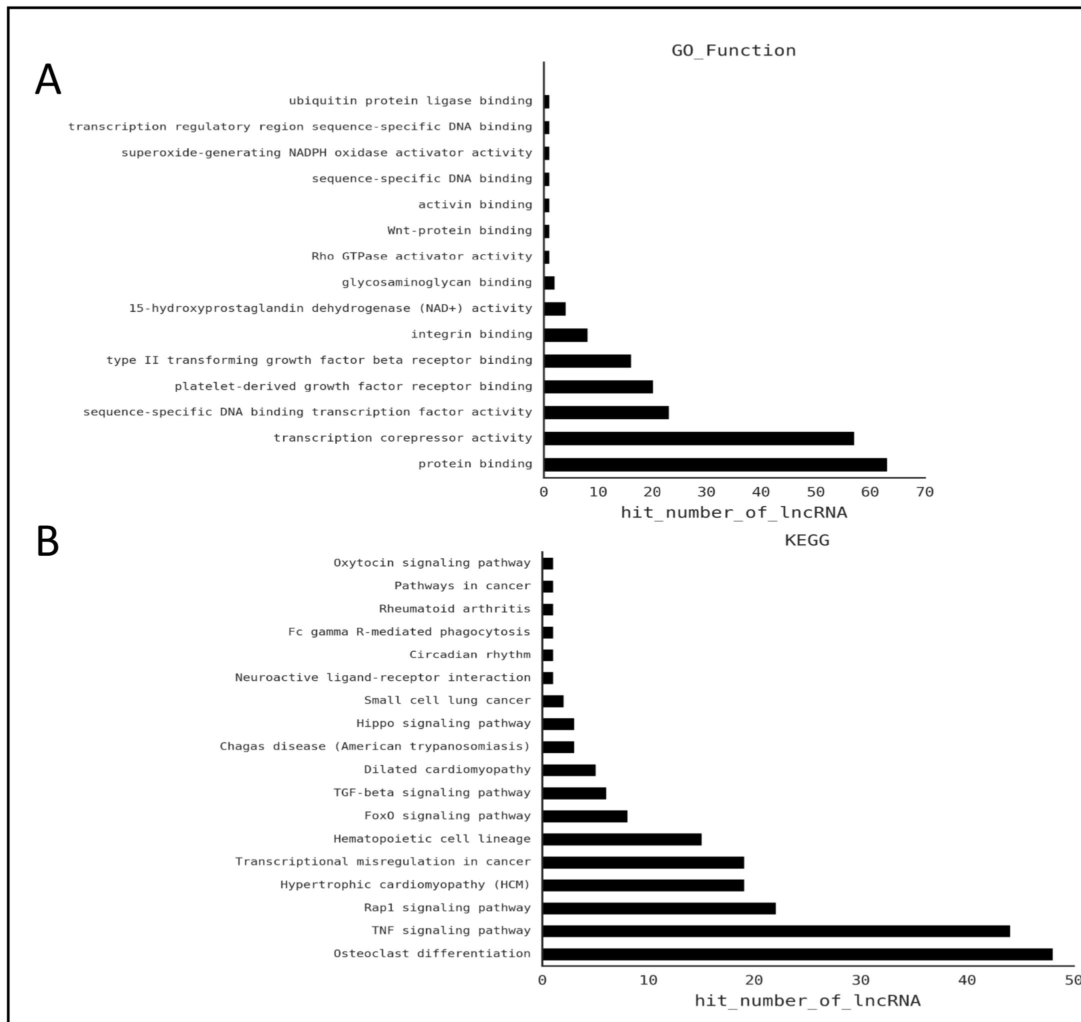


**Fig. 4.** The top 200 GO function terms (A) and KEGG pathways (B) for the co-expression genes of differentially expressed lncRNAs between BEAS-2B cells treated with 230 pg/mL TGF-β1 and the control cells .

*Cis- and Trans-regulation of lncRNAs and the construction of the TF-lncRNA-target gene network*

It is reported that some lncRNAs exert their cis-regulatory functions on the expression of both themselves and the nearby genes located on the same chromosome [8]. The co-expressed genes (*P* value of correlation<0.05) located within the 300 kbp windows upstream or downstream of the differentially expressed lncRNAs were identified as the potential "cis-regulated mRNAs". As for the TGF-β1-treated BEAS-2B cells, 8 lncRNAs with9 cis-regulatory genes were identified. Among these, lnc-ABCA12-5, one of the most up-regulated lncRNAs in the TGF-β1-treated BEAS-2B cells, has a positive cis-regulatory role on the fibronectin1, an important gene functioning in EMT, cell adhesion and migration processes. lnc-DPM2-2 has 2 positive cis-regulatory genes, FAM102A and PIP5KL1, of which PIP5KL1 participates in cell proliferation and migration. The representative chromosomal location of the lncRNA ABCA12-5 and its cis-regulatory gene were showed in Fig. 5.

Besides the cis-regulation analysis, the tran-regulation of the differentially expressed lncRNAs was also analyzed. Based on the analysis of co-expressed genes of the differentially expressed lncRNAs, the hypergeometric cumulative distribution was further employed to calculate the enrichment significance of the co-expressed genes in every transcription factor (TF) term. The co-expressed genes with a FDR<0.01 were deemed as enrichment in the TF

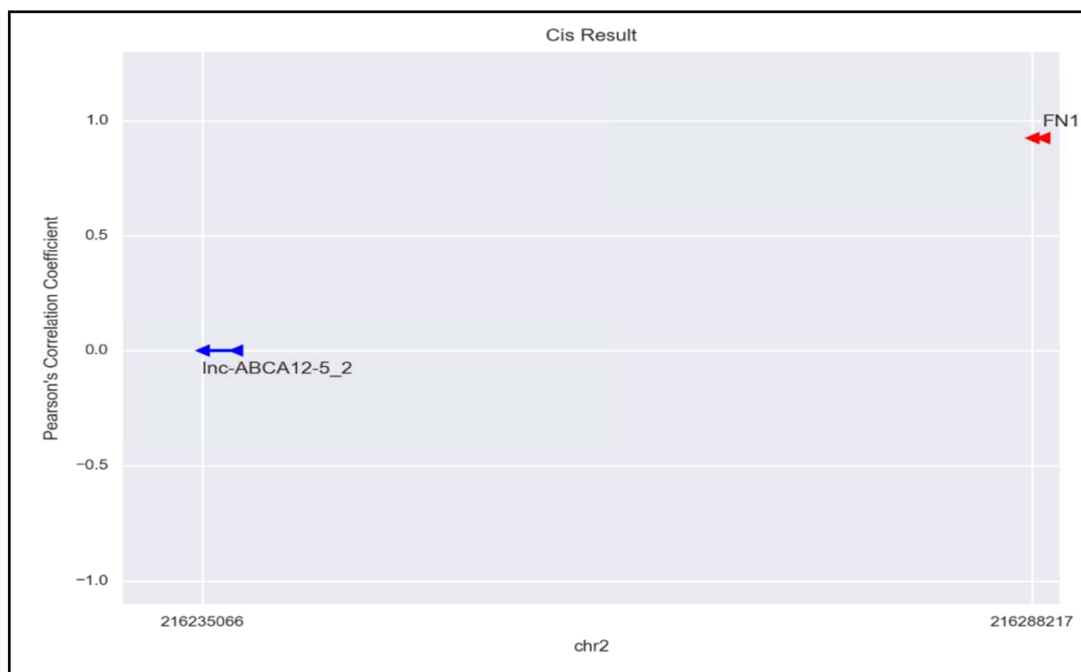


**Fig. 5.** Representative chromosomal locations of lncRNA ABCA12-5 and its cis-regulatory gene. The abscissa is the genomic position while the ordinate is the correlation coefficient. The red line represents mRNA involved in positive regulation relationship with lncRNA while the green line represents negative regulation, the blue line represents lncRNA.

term. In the correspondence between the differentially expressed lncRNAs and TF terms, top 200 and top 500 predictive relationships of the predicted confidence level (with the lowest *P* values) were selected and subjected to frequency counting statistics to reflect the overall functional distributions of the differentially expressed lncRNAs. The top 200 (according to frequency counting) were chosen to draw a bar chart, in which 13 TFs were implicated. Most of the potential trans-regulatory lncRNAs were found to participate in pathways regulated by several TFs: FOS, TFAP2A, STAT3, RAD21, and E2F1 (Fig. 6).

The intersection of the set of co-expresed genes of the differentially expressed lncRNAs and the set of target genes of the transcription factors was calculated. Then TFs significantly associated with lncRNAs were obtained by enrichment degree calculation of the intersection using hypergeometric cumulative distribution, so as to identify potential TFs jointly play regulatory roles with lncRNAs. Through the hypergeometric distribution calculation, each lncRNA can obtain multiple lncRNA-TF pairs while each lncRNA-TF pair is the result of multiple genes enrichment. According to the *P* value, the TF-lncRNA network consisting of the top 100 pairs of lncRNA and TF with the highest credibility (Fig. S1 - for all supplemental material see www.karger.com/10.1159/000495052) was produced using the Cytoscape software, while the TF-lncRNA-target gene network was generated based on the top 10 pairs of lncRNA and TF with the highest credibility (Fig. S2). In the TF-lncRNA-target gene network, there are 10 differentially expressed lncRNAs with 268 target genes and 4 core TFs (FOS, STAT3, RAD21, and E2F1) (Fig. S2). For example, ITGA5 and ING1, co-expressed genes for HPN-AS1, are also target genes of FOS. FOS is speculated to regulate the expression of both lncRNA HPN-AS1 and its target genes like ITGA5 and ING1. Thus it is speculated that lncRNA HPN-AS1 plays roles similar to FOS on the regulation of gene expressions.

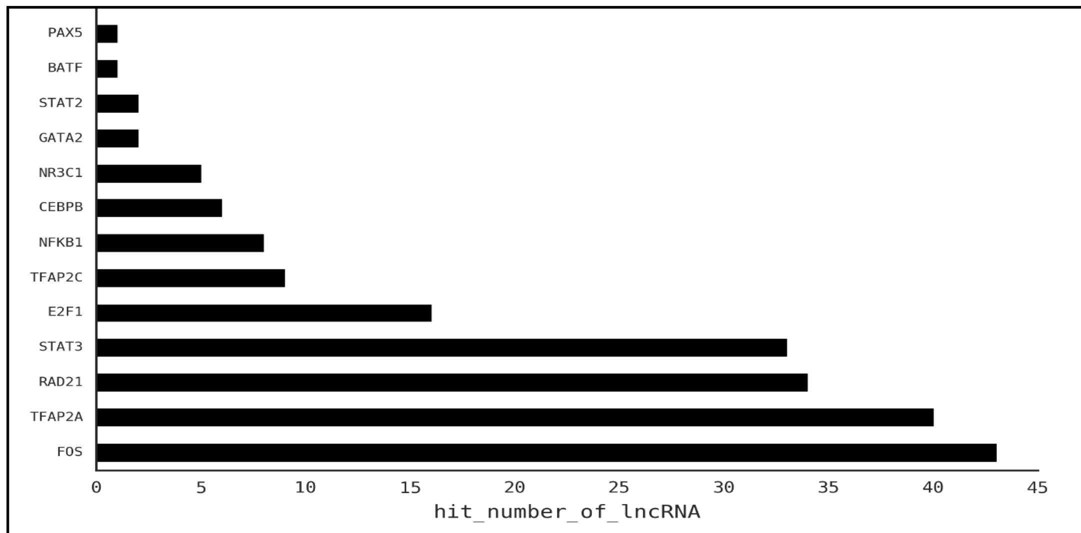


**Fig. 6.** Top 200 pairs of lncRNA and TF of the highest correlation. Most of the differentially expressed lncRNAs are predicted to participate in the pathways regulated by FOS, TFAP2A, RAD21, STAT3 and E2F1.

*Knock-down of lnc-ABCA12-5, lnc-THEMIS-2 or lnc-HPN-AS1 suppressed the EMT induced by TGF-β1 to varying degrees*

To verify the reliability of bioinformatics analysis, we selected 4 lncRNAs for functional assay. siRNAs targeting lnc-ABCA12-5, lnc-THEMIS-2, lnc-HPN-AS1, and lnc-ITGB6-4 were designed and synthesized. As shown in Fig. 7, knock-down of lnc-ABCA12-5, lnc-THEMIS-2 or lnc-HPN-AS1 suppressed the changes of EMT markers (E-cadherin, N-cadherin, fibronectin 1 and vimentin) induced by TGF-β1 to varying degrees, indicating their significant roles in the regulation of TGF-β1-triggered EMT process in BEAS-2B cells. However, knock-down of lnc-ITGB6-4 didn't affect the expressions of the 4 EMT markers. As shown in Fig. S2, lnc-THEMIS-2 is predicted to be regulated by STAT3 while lnc-HPN-AS1 is predicted to be regulated by FOS. Both STAT3 and FOS have been identified as important regulators in EMT and oncogenic transformation. In addition, fibronectin 1 (FN1), an EMT marker correlated with acquisition of tumorigenicity, was identified as a potential cis-regulated mRNA of lnc-ABCA12-5 (Fig. 5). Our findings for these 3 lncRNAs coincide with the known functions of STAT3, FOS and FN1 in the regulation of EMT and oncogenic transformation.

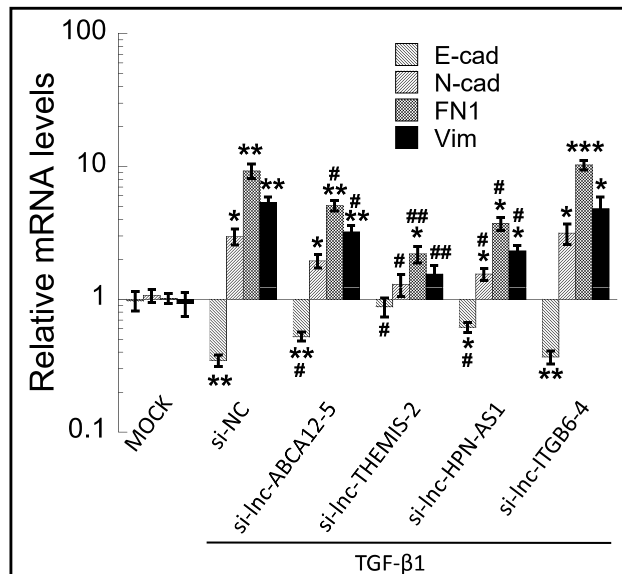


**Fig. 7.** Relative mRNA levels of E-cadherin, N-cadherin, Fibronectin 1 and Vimentin in BEAS-2B cells. BEAS-2B cells were transfected with siRNAs targeting lnc-ABCA12-5, lnc-THEMIS-2, lnc-HPN-AS1, and lnc-ITGB6-4. 24 hours post transfection, TGF-β1 (230 pg/ml) was added to the cells for an additional 48 hours. Asterisks indicate statistical significance relative to control group. Bars represent SE. *P<0.05, **P<0.01 and ***P<0.001. Hash signs indicate statistical significance relative to si-NC group. #P<0.05, ##P<0.01.

## Discussion

The RIBE are observed in many kinds of cells, which happen in a much larger proportion of cells than are traversed by radiation. It has been suggested that irradiated cells are capable of signaling to the neighboring unirradiated cells and manifesting a wide spectrum of biological effects, including micronuclei formation, sister-chromatid exchange (SCE), chromosomal aberrations, malignant transformation, cell death, proliferation and differentiation, *etc.* [21].

Since the discovery of the phenomenon dating back to 1990s [22], the underlying mechanisms have been extensively studied over 20 years. It has been identified that both GJIC [23] and/or soluble factors such as reactive oxygen species (ROS) [24], nitric oxide (NO) [25], calcium fluxes [26], and a series of cytokines such as interleukin-8 (IL-8) [27], tumor necrosis factor-α (TNF-α) [28], and TGF-β1 [5] generated by directly hit cells play roles in the RIBE. RIBE-induced cell malignant transformation most likely contributes to the tumorigenesis induced by LD/LDR radiations [4], such as the lung tumorigenesis caused by radon progeny inhalation in high-level natural radiation areas or lung tumorigenesis in astronauts induced by space radiation [29, 30]. For the LD/LDR radiations, the tumorigenesis is speculated to be induced by RIBE to a large extent as there are few cells traversed by radiation. Thus, elucidating the molecular mechanisms underlying the RIBE-induced malignant transformation is of important significance for the protection from tumorigensis caused by LD/LDR radiations.

TGF-β signaling consists of TGF-β ligands, TGF-β receptors as well as the downstream components. There are more than 30 members in human TGF-β family, including activin, nodal, lefty, myostatin, TGF-β, bone morphogenetic proteins (BMPs), growth and differentiation factor (GDF) and Mullerian inhibitory substance (MIS), which have been found to regulate a wide variety of biological processes including cell growth, apoptosis, differentiation, migration, extracellular matrix (ECM) production, angiogenesis, immunity, and development [31]. TGF-β is synthesized as a precursor protein of 390 amino acid, which is then cleaved by enzymes such as Furin to yield a N-terminal peptide to form a 75 kDa latency associated peptide (LAP) and a C-terminal peptide that dimerizes to form a 25 kDa bioactive cytokine. Then the bioactive TGF-β ligands interact with the type I and type II receptors located in cell surface and transduce the signals by phosphorylating Smad (mothers against decapentaplegic homolog) transcription factors that could translocate into the nucleus to regulate gene expression with the cooperation with other factors [32]. Among the 3 TGF-β isoforms with high degree sequence homology and activities overlapping, TGF-β1 is the most prominent and important.

TGF-β1 was originally identified and named for its ability to induce the anchorage–independent growth of rat kidney fibroblasts [33]. After years of research, it has been found that TGF-β1 functions in a variety of biological processes, such as differentiation [34], growth arrest and pro-apoptotic response [35], EMT [36], migration and invasion [37] and the immunosurveillance [38]. Besides, it has been widely reported that TGF-β1functions in both direct and bystander radiation effects and is a key extracellular sensor and signal of stress responses in irradiated tissues [39]. Studies showing that TGF-β1 mediates radiation-induced EMT in epithelial cells elicit concerns of tumorigenesis induced by RIBE [40-42]. However, the concrete molecular mechanisms remain elusive.

As a kind of important regulatory non-coding molecules, lncRNAs play important roles in many biological processes, and their functions in tumorigenesis, development and tumor metastasis are more striking. However, studies related to TGF-β1-induced lncRNA are rare. Richards *et al.* found that TGF-β1-induced lncRNA-HIT promoted the migration, invasion and EMT of mouse mammary epithelial NMuMG cells [43]. However, the role of TGF-β1-induced lncRNA in oncogenic transformation of lung epithelial cells related to lung tumorigenesis induced by RIBE has not been demonstrated yet. Thus, in the present study, we treated human lung bronchial epithelial BEAS-2B cells with TGF-β1 at a concentration in medium conditioned by directly irradiated cells and conducted lncRNA microarray to explore the

underlying molecular mechanisms related to RIBE-induced cell transformation, in the hope of revealing the correlation between lncRNA expression and malignant transformation of lung epithelial cells induced by RIBE. As a result, the expressions of 224 lncRNAs and 99 mRNAs transcripts were found to be significantly changed compared with the control cells. The verification of expressions of several selected lncRNAs by qRT-PCR suggested that the microarray data are credible. Then the potential functions of the differentially expressed lncRNAs basing on their correlated mRNAs were speculated.

Since only a small number of known lncRNAs have functional annotations, a method called "guilt by association" is adopted to predict their functions by figuring out their correlated genes and the corresponding signaling pathways [20]. As to the differentially expressed lncRNAs identified in our work, thousands of co-expressed mRNA transcripts were found to be correlated. The functions of the lncRNAs were predicted by functional enrichment analysis of the co-expressed mRNAs. As shown in Fig. 4, the top predicted functions of the differentially expressed lncRNAs contain protein binding, transcription co-repressor activity, platelet-derived growth factor receptor binding and 15-hydroxyprostaglandin dehydrogenase (NAD+) activity, *etc*. Among hundreds of predicted KEGG pathways, highly correlated ones include TNF signaling pathway, transcriptional mis-regulation in cancer, Rap1 signaling pathway, FoxO signaling pathway, TGF-β signaling pathway, Hippo signaling pathway, small lung cancer signaling pathway, *etc*. Almost all predicted pathways participate in the tumorigenesis and cancer progression, implying that these differentially expressed lncRNAs may play potential important roles in the malignant transformation of BEAS-2B cells and lung tumorigenesis induced by RIBE.

We also predicted the cis-regulation relationships of the differentially expressed lncRNAs and their nearby protein coding genes. In our research, 8 lncRNAs with9 cis-regulatory genes were identified. lnc-ABCA12-5, which is up-regulated in the TGF-β1-treated BEAS-2B cells, is predicted to play a potential cis-regulatory role on the FN1 that presents at cell surface and in extracellular matrix and functions as an important gene in EMT, cell adhesion and migration processes. ING-1, functioning as a tumor suppressor protein that can induce cell growth arrest and apoptosis, was down-regulated in the TGF-β1-treated BEAS-2B cells, which has a potential cis-regulatory relationship with the up-regulated lnc-CARKD-2. PIP5KL1, cis-regulated by lnc-DPM2-2, is related to cell proliferation and migration. UACA and VMP1 cis-regulated by lnc-LARP6-4 and lnc-VMP1-3 respectively are linked to regulation of apoptosis and metastasis. The above-mentioned findings suggest that the differentially expressed lncRNAs of potential cis-regulatory functions play significant roles in the EMT, transformation, cell death and migration.

Besides the cis-regulation relationships, the majority of the differentially expressed lncRNAs were found to function in a trans-regulation manner. In the core network of lncRNA-TF pairs, top 200 lncRNA-TF relationships were related to 13 TFs (Fig. 6). Most of the potential trans-regulatory lncRNAs participate in pathways regulated by several TFs, including FOS, TFAP2A, TFAP2C, STAT3, NFKB1, STAT2, RAD21, E2F1, CEBPB, *etc*. The target genes were further added into the TF-lncRNA network to determine the 3-element network correlation, in which 4 core TFs (FOS, STAT3, RAD21, and E2F1) were identified (Fig. S2). FOS is part of the activator protein-1 (AP-1) transcription factor and has been shown to participate in the molecular mechanisms of cell proliferation, differentiation, apoptosis, and transformation. Moreover, the deregulation of FOS could be linked with a variety of pathological processes, such as immunological, skeletal and neurological defects, as well as oncogenic transformation and tumor progression [44]. As to oncogenic transformation, N-terminal phosphorylation of the nuclear phosphoprotein c-Jun plays a key role downstream of the oncogenic transactivation functions of Fos [45]. Interestingly, in our microarray data, FOS was also found to be up-regulated significantly, suggesting that it may play a role in the oncogenic transformation of BEAS-2B cells. STAT3 is a member of the STAT protein family, which is phosphorylated by the receptor-associated kinases in response to cytokines and growth factors, and then forms heterodimers that translocate to the cell nucleus where they function as transcription activators. It has been widely reported that STAT3 play significant

roles in the cell growth, EMT, oncogenic transformation, tumorigenesis, progression and immune evasion of lung cancer [46-48]. The E2F1 transcription factor is realized as a regulator of the cell cycle as well as a potent mediator of DNA damage-induced apoptosis and the checkpoint response. The growth factor stimulation of cells causes phosphorylation of Rb and hyperphosphorylated Rb is released from E2F1 or E2F1/DP complex, and free E2F1 can bind DNA and recruit other transcription factors and co-activators to target gene promoters and induce transcription of a variety of genes to exert diverse functional roles [49]. Since the functions of a large number of E2F1 target genes are related to promoting cell cycle progression, E2F1 is considered to be tumor promoting with its deregulation leading to unrestrained cell cycle progression. However, mutations in E2F1 itself are rare [50]. Thus it is predicted that the epigenetic regulation, such as interactions with histones, noncoding RNAs or post-translational modifications may play crucial roles in the E2F1 relevant tumorigenesis. RAD21, a nuclear phosphoprotein involved in the repair of DNA double-strand breaks and in chromatid cohesion during mitosis, becomes hyperphosphorylated in cell cycle M phase and plays a role in sister chromatid cohesion. Besides, some studies have showed that its dysregulation correlated with human cancer [51, 52]. Since TGF-β causes both DDR signaling activation and DNA damages in bystander cells, its effects on the genomic instability of bystander cells mediated by DNA damages are complicated and worth further studying [7, 53]. Our data showed that among 224 differentially expressed lncRNAs, 28 were found to participate in pathways regulated by FOS while 20 in pathways regulated by STAT3 and 7 in E2F1 regulated pathways, of which3 lncRNAs (HPN-AS1, NR_120367, C15orf54-4) were potentially regulated by FOS with highest credibility, 4 lncRNAs (SBDS-25, FAM155B-3, LINC00200, THEMIS-2) by STAT3, and 1 (lnc-ITGB6-4) by E2F1, implying that these ones most likely participate in the oncogenic transformation of BEAS-2B cells. Furthermore, 2 lncRNAs (AMDHD1-5, FOXP1-IT1) were predicted to be regulated by RAD21 with highest credibility, which may be involved in the DNA repair response and genomic instability induce by TGF-β1.

## Conclusion

In conclusion, our work for the first time studied the genome-wide lncRNA expression patterns in human lung bronchial epithelial cells treated with TGF-β1 for 2 h at a concentration detected in radiation conditioned medium. 224 lncRNAs were found differentially expressed while some were predicted to be involved in the malignant transformation of BEAS-2B cells and tumorigenesis of lung cancer. Since most of the discovered lncRNAs have not yet been functionally annotated [54], the functions of the differentially expressed lncRNAs were predicted by the "guilt by association" approach. In addition, lnc-ABCA12-5, lnc-THEMIS-2 and lnc-HPN-AS1 were found to be involved in the regulation of EMT induced by TGF-β1. The results are of crucial significance for the understanding of molecular mechanisms of lung tumorigenesis induced by LD/LDR radiations as well as for the development of molecule-targeted drugs for radiation protection.

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## Disclosure Statement

The authors declare that they have no conflicts of interest.

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