Original Paper

Overexpression of LncRNA AC067945.2 Down-Regulates Collagen Expression in Skin Fibroblasts and Possibly Correlates with the VEGF and Wnt Signalling Pathways

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Key Words

Long non-coding RNA • AC067945.2 • Collagen • Fibroblasts

Abstract

Background/Aims: Long non-coding RNAs (lncRNAs) are thought to play crucial roles in human diseases. However, the function of lncRNAs in hypertrophic scar formation remains poorly understood. **Methods:** Utilizing qRT-PCR, we explored the expression changes of AC067945.2. Overexpression of AC067945.2 in normal skin fibroblasts was performed by transient plasmid transfection. Western blot was used to check the proteins' expression changes. Cell Counting Kit-8 (CCK-8) assay and Annexin V/7-AAD staining were used to examine cell proliferation and apoptosis, respectively. mRNA-seq was applied to dissect the differentially expressed mRNAs in AC067945.2 overexpressed cells. We also performed ELISA to detect the VEGF secretion. **Results:** AC067945.2 was down-regulated in hypertrophic scar tissues. Overexpression of AC067945.2 did not affect cell proliferation, but it mildly promoted early apoptosis in normal skin fibroblasts. Furthermore, AC067945.2 overexpression inhibited the expression of COL1A1, COL1A2, COL3A1 and α -SMA proteins. Transforming growth factor- β 1 (TGF- β 1) could inhibit the expression of AC067945.2. Based on mRNA-seq data, compared with mRNAs in the control group, 138 mRNAs were differentially expressed, including 14 up-regulated and 124 down-regulated transcripts, in the AC067945.2 overexpression group. Gene ontology and pathway analyses revealed that AC067945.2 overexpression was correlated with developmental processes, binding, extracellular region, and the vascular endothelial

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cell growth factor (VEGF) and Wnt signalling pathways. ELISA confirmed that AC067945.2 overexpression could repress VEGF secretion. **Conclusion:** Taken together, our data uncovered the functions of a novel lncRNA AC067945.2, which might help us understand the mechanisms regulated by AC067945.2 in the pathogenesis of hypertrophic scar formation.

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Introduction

Hypertrophic scar (HS) is a pathologic result of wound healing [1]. It is characterized by erythematous, raised, and inflexible skin tissues that significantly affect the patients' quality of life [2]. This condition can be addressed using various interventions including pressure therapies, skin grafting, steroids, silicone dressings and surgical removal [3]. However, no definitive treatment is available for HS. HS is caused by the excessive accumulation of extracellular matrix (ECM) components including collagen, α -smooth muscle actin (α -SMA) and fibronectin partly due to the recruitment of inflammatory cells and increased numbers of fibroblasts [4]. Emerging evidence indicates that activated fibroblasts with a higher capacity for proliferation and collagen synthesis contribute to the formation and development of hypertrophic scars [5]. Cell apoptosis and apoptosis-related processes also play important roles in hypertrophic scar formation [6]. Although numerous genes involved in hypertrophic scarring have been identified, the molecular mechanisms underlying this process are not well understood.

Long non-coding RNAs (lncRNAs) are a class of transcripts longer than 200 nucleotides that lack protein coding potential [7]. By regulating gene expression, lncRNAs play crucial roles in multiple biological processes such as development, differentiation and carcinogenesis [8-11]. Specific lncRNAs are correlated with fibrotic disorders including hypertrophic scar [12], keloid [13], and lung fibrosis [14], indicating that lncRNAs may be involved in imbalances in ECM deposition. Recently, our group discovered that AC067945.2 (UCSC hg38; also called NONHSAT076109 in NONCODEv4 and HG495060 in GenBank), a lncRNA located upstream of the STAT1 gene on chromosome 2 (UCSC hg38, chr2:191017954-191019079), is dysregulated in regressive scars compared to that in mature scar tissues [15]. However, the functional role of AC067945.2 in hypertrophic scars has not been elucidated.

In this study, fifteen hypertrophic scar samples and corresponding matched normal skin tissues were used to perform qRT-PCR analysis. The results showed that AC067945.2 was down-regulated in hypertrophic scar tissues. Using plasmid transfection, we found that AC067945.2 affected early apoptosis and the expression of collagen and α -SMA proteins in normal skin fibroblasts. Distinct expression profiles of mRNAs between the AC067945.2 overexpression and control groups were examined using mRNA-seq techniques. Altogether, 14 and 124 mRNAs were up-regulated and down-regulated (fold-change ≥ 2.0 , FDR ≤ 0.5), respectively, in the AC067945.2 overexpression group. Gene ontology and pathway analyses revealed that AC067945.2 overexpression was correlated with the VEGF and Wnt signalling pathways. Taken together, our data uncovered the function of a novel long non-coding RNA AC067945.2 and provided clues for a new regulatory mechanism in the pathogenesis of hypertrophic scars.

Materials and Methods

Ethics statement

This study was approved by the Medical Ethics Committee of The Affiliated Obstetrics and Gynaecology Hospital of Nanjing Medical University (No. [2013]48). Patients admitted to our hospital for scar removal were provided information about the purpose of the study, and written informed consent was obtained from each participant.

Tissue samples

Fifteen hypertrophic scar (HS) tissues and their matched normal skin (NS) tissues were obtained from 15 different patients who were admitted to the The Affiliated Obstetrics and Gynaecology Hospital of

Nanjing Medical University for scar removal. A diagnosis of hypertrophic scarring was confirmed by routine pathological examination. The collected skin samples were divided into two fragments: one was used for the isolation and culture of fibroblasts, and the other was immediately frozen in liquid nitrogen for preparation of total RNA.

Cell culture and TGF- β 1 treatment

Cultures of normal skin fibroblasts (NSFs) were established as previously described [12]. Explants were cultured in DMEM (Invitrogen, USA) supplemented with 10% foetal bovine serum, 100 μ g/mL streptomycin and 100 U/mL penicillin at 37°C in 5% CO₂. The fibroblasts in this study were used between their third and fifth passages. Recombinant human TGF- β 1 was purchased from R&D Systems (Catalogue Number: 240-B, Minneapolis, USA) and dissolved in sterile 4 mM HCl (Sangon, Shanghai, China) containing 1 mg/mL bovine serum albumin (abs49001013a, Absin, Shanghai, China) to a concentration of 20 μ g/mL according to the manufacturer's protocol. Indicated cells were treated with 10 ng/mL TGF- β 1.

Quantitative reverse transcription PCR

Total RNA was isolated from each tissue and corresponding cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as we previously described [12]. Transcript levels for AC067945.2 were normalized to GAPDH cDNA levels using the 2^(-ΔΔCt) method. The primer sequences were as follows: AC067945.2 (150 bp) forward primer, CCTTACACACTCCGCTCTCA and reverse primer, TATCTTGCCTGGGTTCTCTCAT; GAPDH (226 bp) forward primer, GAAGGTGAAGGTCCGAGTC and reverse primer, GAAGATGGTGATGGGATTTC. We used Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) to confirm the specificity of each primer. The data represented the mean for the same sample from three independent experiments. Each value used for the Fig. was normalized to the average value of the respective control.

Plasmid transfection

A 623-nt AC067945.2 coding sequence was cloned into the pcDNA3.1(-) plasmid by Shanghai Sangon Biotech (China). Control (pcDNA3.1(-)) or overexpression (AC067945.2 in pcDNA3.1(-)) vectors were mixed with Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) and added to cells plated at 80% confluency in 6-well plates following the manufacturer's protocol; for each well, 1 μ g plasmid or 2 μ L Lipofectamine 2000 reagent was diluted with 125 μ L Opti-MEM (Thermo Fisher Scientific, USA) for 5 minutes, and then diluted DNA was added to diluted Lipofectamine 2000 reagent. After incubation for 20 minutes, 250 μ L DNA-lipid complex was added to cells in each well. The cell cultures were incubated at 37°C in 5% CO₂. At 72 h after plasmid transfection, the cells were harvested for the evaluation of overexpression efficiency and subsequent analyses via qRT-PCR.

Cell Counting Kit-8 (CCK-8) assay

Cell proliferation was analysed using the CCK-8 kit (Vazyme, Nanjing, China). In brief, 24 h after plasmid transfection, NSFs were seeded at 5×10³ cells per well in 96-well plates with five replicates per condition. At 0, 24, 48, 72 and 96 h, 10 μ L of CCK-8 solution was added to each well. After 1.5 h of incubation, the absorbance was measured at 450 nm on a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Cell apoptosis assay

72 h after plasmid transfection in NSFs, the cells were subjected to cell apoptosis assay. Apoptosis was detected by Annexin V/7-AAD staining with an apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, 10⁶ treated cells were incubated with Annexin V/7-AAD for 20 min at room temperature. Apoptosis were then analyzed by Accuri-C6 flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). The assays were performed according to the manufacturer's instructions.

Western blot

The protocol was the same as we previously reported [12]. Briefly, proteins (15 μ g) were separated by SDS-PAGE and transferred to nitrocellulose membranes (Amersham, Chalfont, UK). The membranes were blocked in 5% non-fat dry milk followed by incubation with primary antibodies. Then, the membranes were incubated with either goat anti-rabbit IgG or goat anti-mouse IgG horseradish peroxidase (HRP)-

conjugated secondary antibodies (1:2000, Abcam) and developed using chemiluminescence reagents (ECL; Amersham). The primary antibodies used were as follows: anti-COL1A1 rabbit monoclonal antibody (1:1000; ab138492, Abcam), anti-COL1A2 rabbit polyclonal antibody (1:1000; ab96723, Abcam), anti-COL3A1 mouse monoclonal antibody (1:1000; ab6310, Abcam), anti-alpha-smooth muscle actin rabbit monoclonal antibody (1:1000; ab32575, Abcam), and anti-GAPDH mouse monoclonal antibody (1:2000; ab8245, Abcam). GAPDH was used as a loading control. Band intensity was quantified by densitometry of three independent experiments using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The values used for the histogram were normalized to GAPDH.

Measurement of VEGF concentration in cell culture supernatant and patient serum

The concentration of VEGF from cell culture supernatant or sera from 12 patients was assessed using a human VEGF ELISA kit (R&D Systems, Minneapolis, USA). ELISA was performed following the manufacturer's protocol. Briefly, 50 μ L of standards or samples (including cell culture supernatant and sera from 12 patients) was added to antibody pre-coated microtiter plates and incubated for 45 minutes at 37°C. After washing, biotinylated anti-IgG was added to the plates and incubated. Then, streptavidin-HRP was added to the plates and incubated. Chromogen Solution A and B were subsequently added. The reaction was stopped with the Stop Solution. Then, absorbance was measured at 450 nm on a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The manufacturer-provided range for VEGF levels was 15.6-2000 pg/mL.

Detection of the differentially expressed mRNAs in the AC067945.2 overexpression group using an mRNA-seq technique

Seventy-two hours after plasmid transfection in NSFs in 6-well plates, the cells from 2 wells of the AC067945.2 overexpression group and 2 wells of the control group were dissolved in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and sent to Vazyme (Vazyme Biotech Co., Ltd, Nanjing, China) for the following treatment.

RNA purity was checked using the Nano Photometer® spectrophotometer (IMPLEN, CA, USA), and RNA concentration was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit with the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

The transcriptome library for sequencing was generated using the VAHTSTM mRNA-seq v2 Library Prep Kit from Illumina® (Vazyme Biotech Co., Ltd, Nanjing, China) following the manufacturer's recommendations. Clustering of the index-coded samples was performed using VAHTS RNA Adapters set1/set2 for Illumina® (Vazyme Biotech Co., Ltd, Nanjing, China) according to the manufacturer's instructions.

After clustering, the libraries were sequenced on an Illumina HiSeq XTen platform using a (2×150 bp) paired-end module.

After initial quality control, clean reads were mapped to the reference sequence by using TopHat2 software (v2.1.1). The alignment files generated by TopHat2 were input into the Cufflinks software (v2.2.1), which is a program for the comparative assembly of transcripts and the estimation of their abundance in a transcriptome sequencing experiment by using the measurement unit fragments per kilobase of transcript per million mapped reads (FPKM). After using the Cuffmerge program to merge transcripts of each sample in different materials and stages into a single gtf file that was used to identify differentially expressed genes, we used the Cuffdiff program to find differentially expressed genes (DEGs). The differentially expressed genes were identified with q values ≤ 0.05 and a fold-change of ≥ 2 between two samples.

GO and pathway enrichment analyses

GO enrichment and KEGG pathway enrichment analyses were performed by the Vazyme (Vazyme Biotech Co., Ltd, Nanjing, China), which is the same as we previously reported [16] that uses clusterProfiler data from R/bioconductor software (<http://www.r-project.org> and <http://www.bioconductor.org/>) with public databases that include NCBI Entrez Gene (<http://www.ncbi.nlm.nih.gov/gene>), GO (<http://www.geneontology.org>), KEGG (<http://www.genome.jp/kegg>), and Biocarta (<http://www.biocarta.com>). The enrichment P-values of both the GO and pathway enrichment analyses were calculated using the Fisher's exact test, which was corrected using enrichment q-values (the false discovery rate) that were calculated using John Storey's method.

Statistical analysis

Differences in the data between two groups were analysed using SPSS 20.0 software (SPSS, Chicago, IL, USA) with independent samples *t* tests. The data are presented as the means of results from three experiments with each experiment performed in triplicate. Statistical significance was defined as *P*<0.05. Pearson's correlation analysis was performed using SPSS 20.0 software (SPSS, Chicago, IL, USA). Significant correlation was defined as *P*<0.05.

Results

AC067945.2 is down-regulated in hypertrophic scar tissues

We first examined AC067945.2 expression in 15 hypertrophic scar tissues compared to matched normal skin samples. The results revealed that AC067945.2 mRNA level was significantly reduced in hypertrophic scar tissues compared to that in normal skin tissues (Fig. 1).

Based on bioinformatics analyses, we found that AC067945.2 is a transcript of 623 nucleotides in length and comprises 2 exons (NONHSAT076109 in NONCODEv4). AC067945.2 contains no overlapping sequence with the transcripts from its neighbouring gene STAT1 and is a natural antisense lncRNA (Fig. 2A, 2B). Consistent with AC067945.2 being a non-coding RNA based on the UCSC database, the Coding Potential Calculator (CPC) computational algorithm (http://cpc.cbi.pku.edu.cn/programs/run_cpc.jsp) predicted that AC067945.2 has very low coding potential (Fig. 2C) similar to that of MEG9, a well-documented bovine lncRNA with a coding potential score of -0.570863 [12, 17].

AC067945.2 alters early apoptosis and collagen and α -SMA protein expression in normal skin fibroblasts

To further determine the role of AC067945.2, we selected normal skin fibroblasts for overexpression experiments. Overexpression of AC067945.2 was performed using plasmid transfection with the 623 nt AC067945.2 coding sequence. As shown in Fig. 3A, plasmid transfection led to the overexpression of AC067945.2 in NSFs. Subsequently, the CCK-8 assay was used to measure cell proliferation. It was observed that the overexpression of AC067945.2 did not affect cell proliferation (Fig. 3B). We also examined cell apoptosis in human NSFs overexpressing AC067945.2. The results showed that the overexpression of AC067945.2 significantly promoted early apoptosis in NSFs (Fig. 3C).

Hypertrophic scars are related to abundant alpha-smooth muscle actin (α -SMA)-producing myofibroblasts in conjunction with increased levels of type III and type I collagen [18]. Thus, we also determined whether the expression of COL1A1, COL1A2, COL3A1 and α -SMA was changed in fibroblasts overexpressing AC067945.2. Interestingly, western blot analysis showed that the protein expression of COL1A1, COL1A2, COL3A1 and α -SMA was down-regulated in the AC067945.2 overexpression group compared to that in the control group of normal skin fibroblasts (Fig. 3D, 3E). Altogether, these data suggest that the overexpression of AC067945.2 promotes early apoptosis and reduces collagen and α -SMA protein expression in fibroblasts.

TGF- β 1 is a key and critical component in the pathogenesis of hypertrophic scars. It is known that TGF- β 1 signalling is activated in hypertrophic scar tissues. We determined whether AC067945.2 could be regulated by TGF- β 1 in normal skin fi-

Fig. 1. Expression of AC067945.2 in hypertrophic scar tissues. Expression of AC067945.2 was validated in HS tissues (n=15) and matched NS tissues (n=15) by qRT-PCR. **Statistically significant difference at *P*<0.01.

Fig. 2. Characteristics of AC067945.2. (A) Chromosomal location of AC067945.2 and information of the nearest gene based on the UCSC hg38 database. Arrow indicates the lncRNA AC067945.2. (B) Transcript of AC067945.2 from the UCSC hg38 database. (C) Based on the Coding Potential Calculator, AC067945.2 is a non-coding RNA.

Fig. 3. Overexpression of AC067945.2 promotes early apoptosis and reduces collagen and α -SMA protein expression in NSFs. (A) Expression of AC067945.2 was detected by qRT-PCR after plasmid transfection. (B) Cell proliferation was measured using the CCK-8 assay. (C) Apoptosis was assessed by flow cytometry using the Annexin V-PE/7AAD kit. (D) Protein levels of COL1A1, COL1A2, COL3A1, and α -SMA were detected using western blotting. (E) Densitometric analysis of western blots. (F) Expression of AC067945.2 was inhibited by 10 ng/mL TGF- β 1. * $P < 0.05$, ** $P < 0.01$, versus the Control in A-F. Control in A-F: cells overexpressing an empty negative control vector (pcDNA3.1). AC ov in A-F: cells overexpressing the 623 bp AC067945.2 transcript. Blank in F: cells treated with nothing. Control in F: cells treated with vehicle. TGF- β 1 in F: cells treated with 10 ng/mL TGF- β 1.

broblasts. Interestingly, AC067945.2 expression could be significantly repressed by TGF- β 1 treatment (Fig. 3F). These results were consistent with the observation that AC067945.2 was down-regulated in hypertrophic scar tissues (Fig. 1).

Differentially expressed mRNAs were detected by mRNA-seq after AC067945.2 overexpression

To examine the potential biological functions of AC067945.2, we determined mRNA expression profiles through mRNA-seq analysis after overexpressing AC067945.2. Hierarchical clustering and boxplots showed variations and patterns in mRNA expression between the AC067945.2 overexpression and control groups (Fig. 4A, 4B). Scatter plots were used to visualize the differentially expressed mRNAs (Fig. 4C). Up to 138 mRNAs were differentially ex-

Fig. 4. mRNA-seq analysis in the AC067945.2 overexpression group versus the control group. (A) Hierarchical clustering shows variations and patterns in mRNA expression between the AC067945.2 overexpression and control groups. (B) Boxplot shows variations and patterns in mRNA expression between the two groups. The horizontal axis represents the sample name. The vertical axis indicates the value of $\log_{10}(\text{FPKM}+1)$. Each box represents five statistics, including the maximum, the upper quartile, the median, the lower quartile and the minimum value from top to bottom. (C) Scatter plots showing differentially expressed mRNAs including the 14 up-regulated and 124 down-regulated transcripts. Red dots represent the up-regulated mRNAs in the AC067945.2 overexpression group compared to the control group. Green dot represent the down-regulated mRNAs between the two groups.

Fig. 5. GO analyses showing the top 10 enriched ontologies of the 138 differentially expressed mRNAs. The term/gene ontologies on the vertical axis were drawn according to the first letter of the ontology name in descending order. The horizontal axis represents the gene number. The different colours from green to red represent the three ontologies, namely, biological process, cellular component and molecular function.

Fig. 6. KEGG pathway analyses showing the top 20 pathways associated with the 138 differentially expressed mRNAs. The enrichment Q values were calculated using Fisher's exact tests. The term/pathway on the vertical axis was drawn according to the first letter of the pathway name in descending order. The horizontal axis represents the enrichment factor, i.e., (the number of dys-regulated genes in a pathway/ the total number of dysregulated genes)/(the number of genes in a

pathway in the database/the total number of genes in the database). The top 20 enriched pathways were selected according to the enrichment factor value. The selection standards were the number of genes in a pathway ≥ 4 and $q < 0.05$. The different colours from green to red represent q-values. The different sizes of the circles represent the gene number in a pathway.

pressed in the AC067945.2 overexpression group compared with those in the control group (fold-change ≥ 2 , $q \leq 0.05$), among which 14 were up-regulated, and 124 were down-regulated.

Gene ontology (GO) analysis revealed that the 138 differentially expressed mRNAs were involved in numerous biological processes. Many of these processes are related to single organism developmental processes, developmental processes, protein binding, binding, extracellular region and extracellular matrix (Fig. 5). In addition, KEGG pathway analysis indicated that the VEGF, Wnt, p53, MAPK signalling pathways and retinol metabolism were enhanced after overexpressing AC067945.2 in normal skin fibroblasts (Fig. 6).

We then performed ELISA to test whether VEGF could be regulated by AC067945.2. The results showed that the secretion of VEGF in cell culture supernatant was significantly inhibited in the AC067945.2 overexpression group (Fig. 7A). Then, we used the same ELISA kit to examine VEGF levels in sera from 12 patients. Hypertrophic scar (HS) and matched normal skin (NS) tissues were obtained from the patients for detecting the expression of AC067945.2. Pearson's correlation analysis was performed using SPSS 20.0 software (SPSS, Chicago, IL, USA). The data showed that the serum level of VEGF was not correlated with lncRNA AC067945.2 expression in either normal skin or hypertrophic scar tissues from patients (Fig. 7B).

Fig. 7. Overexpression of AC067945.2 represses VEGF secretion in NSF and a correlation graph between serum level of VEGF and lncRNA AC067945.2 expression. (A) ELISA was performed to detect human VEGF levels in NSF culture supernatant 72 h after plasmid transfection. Blank: cells treated with nothing. Control: cells overexpressing an empty negative control vector (pcDNA3.1). AC ov in A-F: cells overexpressing the 623 bp AC067945.2 transcript. * $P < 0.05$, versus the Control group. (B) Pearson's correlation analysis revealed that the serum level of VEGF was not correlated with lncRNA AC067945.2 expression in either normal skin ($P = 0.936$) or hypertrophic scar tissues ($P = 0.236$) from patients.

Discussion

Hypertrophic scar formation is an abnormal sign of wound healing with complicated aetiology [19]. Roles of lncRNAs in hypertrophic scar formation have been rarely documented. Our group previously reported that lncRNA8975-1 could control collagen expression in hypertrophic scar fibroblasts [12]. In this study, we found that AC067945.2 was significantly down-regulated in hypertrophic scar tissues. Further plasmid overexpression experiments demonstrated that AC067945.2 promoted early apoptosis and reduced collagen and α -SMA protein expression. Using mRNA-seq, we detected 14 up-regulated and 124 down-regulated lncRNAs in the AC067945.2 overexpression group compared to the control groups of normal skin fibroblasts. Further KEGG pathway analysis indicated that the VEGF, Wnt, p53, MAPK signalling pathways and retinol metabolism were related to AC067945.2 overexpression.

lncRNAs can modulate gene expression at almost every level [20, 21]. VEGF is an essential factor in the angiogenetic response in hypertrophic scar management. The expression of VEGF increases in early scars, peaks in proliferative scars and decreases in regressive scars [22]. Anti-Vascular Endothelial Growth Factor (Bevacizumab) therapy could reduce hypertrophic scar formation in a rabbit ear wounding model [23]. The mitogen-activated protein kinase (MAPK) pathways are important mediators of inflammatory signalling, and inhibition of p38 MAPK decreases fibroblast contractility *in vitro* and attenuates wound contraction *in vivo* [24]. By analysing our mRNA-seq data using KEGG pathways, we found that the down-regulated mRNAs in AC067945.2 overexpression group were enriched in the VEGF and MAPK signalling pathways. Additionally, VEGF expression was down-regulated in the AC067945.2 overexpression group (Fig. 7A). These results are consistent with the notion that AC067945.2 can reduce collagen expression through the VEGF or MAPK signalling pathway.

TGF- β signalling is a key regulator in the pathogenesis of hypertrophic scars. We found that lncRNA AC067945.2 was down-regulated after TGF- β 1 stimulation (Fig. 3F), consistent with the down-regulation of AC067945.2 in hypertrophic scar tissues (Fig. 1). Up-regulation of the Wnt/ β -catenin pathway has been shown to be induced by transforming growth factor-beta in hypertrophic scars and keloids [25]. Specifically, in normal skin fibroblasts, β -catenin might be involved in myofibroblast transition and in negatively regulating the TGF- β 1-induced myofibroblast transition [26]. Our mRNA-seq data revealed that the down-regulated mRNAs in the AC067945.2 overexpression group were enriched in the Wnt signalling pathway. It is possible that AC067945.2 may reduce collagen expression by affecting the negative regulator of the Wnt signalling pathway.

P53 activates mesenchymal stem cells to suppress hypertrophic scarring [27]. The p53-related apoptotic pathway is important in treating hypertrophic scar patients [28]. Our

mRNA-seq data revealed that the down-regulated mRNAs in AC067945.2 overexpression group were enriched in the p53 signalling pathway. It is possible that AC067945.2 promotes cell apoptosis and reduces collagen expression by affecting the p53 signalling pathway.

In humans, imbalanced apoptosis and proliferation in HSFs can cause hypertrophic scar formation. The proliferation or apoptosis of hypertrophic scar fibroblasts has been reported to be affected by the non-coding RNAs miR-200b, miR-21 and miR-143-3p [29]. We found that AC067945.2 overexpression slightly promoted early apoptosis in normal skin fibroblasts. The functional interactions of lncRNAs, miRNAs and mRNAs might lead to new insights into the pathogenesis of diseases [30, 31]. Therefore, according to our previously reported strategies [12], we used DIANA-LncBase v2, LNCipedia v4.0 and NPInter v3.0 to determine whether any miRNA was associated with AC067945.2. Unfortunately, we could not identify any relevant miRNAs. Whether AC067945.2 interacts with microRNAs to regulate early apoptosis needs further exploration.

Further elucidating of whether AC067945.2 can function through the VEGF, MAPK or Wnt signalling pathway during hypertrophic scarring would be helpful in revealing the underlying biological aetiology and potentially providing useful information for scar evaluation and treatments.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (81501672, 81602774, 81701910); the Jiangsu Maternal and Child Health Research Project (F201608); the Nanjing Medical Science and Technique Development Foundation (YKK15159) and Jiangsu Provincial Medical Youth Talent.

Jun Li projected the experiment. Ling Chen and Jingyun Li performed the sample preparation, cell transfection and qRT-PCR experiments. Qian Li, Xue Li and Xiangdong Hua performed the bioinformatics analysis. Bei Zhou and Yanli Gao performed the statistical analysis. Jun Li wrote and edited the manuscript.

Disclosure Statement

The authors declare that they have no conflict of interests.

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