Distinct Expression Profiles of IncRNAs Between Regressive and Mature Scars

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Key Words
IncRNAs • Expression profiles • Regressive scar • Mature scar

Abstract
Background: Recent studies suggest that long non-coding RNAs (IncRNAs) play crucial roles in human diseases. The function of IncRNAs in abnormal scar pathogenesis remains poorly understood. Methods: In this study, we examined the IncRNAs expression profiles among regressive and mature scars following caesarean sections. A total of 30,586 IncRNAs and 26,109 mRNAs were analyzed by microarrays (Human LncRNA Array v3.0, Arraystar, Inc.). Results: In total, we identified 1,871 IncRNAs and 817 mRNAs with differential expression between regressive and mature scar individuals (fold change ≥3, p≤0.001). A set of differentially expressed IncRNA transcripts, in particular, IncRNA8975-1, AC097662.2 and RP11-586K2.1, were confirmed using qRT-PCR. Gene ontology and pathway analysis revealed that compared to mature scars, many processes over-represented in regressive scars are related to the immune system. Conclusion: Our results show significantly altered expression profiles of IncRNAs and mRNAs between regressive and mature scars. These transcripts are potential molecular targets for inhibiting abnormal scar formation following caesarean sections.

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Introduction

Female patients receiving caesarean sections (CSs) suffer from abnormal scarring in wound healing [1]. Abnormal scars, including hypertrophic scars and keloids, cause functional and cosmetic issues, are characterized by excessive collagen deposition and exhibit increased extracellular matrix production [2, 3]. Numerous factors have been reported to participate in the wound healing process, such as transforming growth factor beta (TGF-β), interleukin-6, homeobox B13 and platelet-derived growth factor [4]. However, the etiology and mechanism of abnormal scar formation are still poorly understood. In terms of their clinical appearance, hypertrophic scars are classified as early (1 to 2 months), proliferative (3 to 6 months), regressive (approximately 2 years), and mature (at least 4 years) during wound healing [5]. This classification is helpful in revealing the biological changes and mechanisms underlying scar formation and regression.

Emerging evidence shows that noncoding RNAs (ncRNAs) play crucial roles in the development of skin fibrosis [6]. Multiple miRNAs contribute to the scar wound healing etiology [7-10]. LncRNAs (Long non-coding RNAs), are defined as longer than 200 nucleotides, transcribed by RNA polymerase II (RNA PII), but lack an open reading frame [11]. They were found to regulate protein-coding (pc) gene expression at both the transcriptional and post-transcriptional levels [12]. Recently, IncRNAs were found to be associated with fibrosis and TGF-β [13-15], though their roles in abnormal scar wound healing remain largely unknown.

Previously, several studies investigated the differences among patients with abnormal scars and normal individuals [8, 10, 16, 17]. The aim of the present study was to examine distinct expression profiles of IncRNAs between regressive scars and mature scars using third-generation IncRNA microarray techniques. Altogether, 1275 and 596 IncRNAs were upregulated and downregulated (fold-change≥3.0), respectively. We report that IncRNA9875-1, AC097662.2 and RP11-586K2.1 are respectively associated with collagen type I alpha 2 (COL1A2), collagen type IV alpha 3 (COL4A3) and matrix metalloproteinase 16 (MMP16) genes, which may affect collagen synthesis or degradation. This study will be helpful in exploring the differences between regressive scars and mature scars from the perspective of lncRNAs during scar progression.

Materials and Methods

Preparation of tissues

Abdominal skins were obtained from female patients receiving second caesarean sections (CSs) at the Nanjing Maternity and Child Health Care Hospital following informed consent and written permission. The cesarean scar on patients received no drug treatment before the surgical procedure. All specimens were immediately frozen in liquid nitrogen, followed by storage at -80 °C. Diagnosis of a regressive scar or mature scar was confirmed by histology tests. Patients’ information was listed in Table 1.

Ethics statement

This study was approved by the Medical Ethics Committee of Nanjing Maternity and Child Health Care Hospital (No. [2012] 58). Women attending our hospital for caesarean sections read information about the purpose of the study, and written informed consent was obtained from each participant.

Hematoxylin & eosin staining and masson’s trichrome

Hematoxylin & eosin staining was performed as previously described [5]. Briefly, tissue samples were fixed for 2 hours in 4% paraformaldehyde in phosphate buffered saline (PBS), stored overnight in 10% formalin, and paraffin-embedded. Cross-sections (5 μm) were cut and used for staining with hematoxylin and eosin. Masson’s trichrome method was used to examine collagen deposition as previously reported [15]. Skin tissues were fixed in 4% paraformaldehyde overnight, dehydrated in 70% ethanol, cleared in xylene and embedded in paraffin wax. Sections (4 μm) were prepared and stained with Masson’s trichrome. Images were taken using a Zeiss AX10 microscope equipped with an AxioCam HRc camera and processed with Axiovision software (Zeiss, Oberkochen, Germany).
RNA isolation and quality control

Total RNA was isolated from each tissue using a homogenizer (IKA, Germany) with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and purified with an RNeasy Mini Kit (Qiagen, Hilden, Germany) as per the manufacturer’s protocol. The NanoDrop ND-1000 was used to measure RNA quantity. RNA Quality was tested with the Agilent 2100 Bioanalyzer (Agilent Technologies).

Microarray analysis

Arraystar Human LncRNA Microarray V3.0 is designed for the global profiling of human LncRNAs and protein-coding transcripts (Kancheng, Shanghai, China). Approximately 30,586 LncRNAs and 26,109 coding transcripts can be detected using the third-generation LncRNA microarray. The LncRNAs are carefully constructed using the quality-controlled, public transcriptome databases (Refseq, UCSC knowngenes, Gencode, etc.), as well as landmark publications. Each transcript is represented by a specific exon or splice junction probe, which can identify individual transcript accurately. Positive probes for housekeeping genes and negative probes are also printed onto the array for hybridization quality control.

Sample preparation and microarray hybridization were performed based on the manufacturer’s standard protocols with minor modifications. Briefly, mRNA was purified from total RNA after removal of rRNA (mRNA-ONLY™ Eukaryotic mRNA Isolation Kit, Epicentre). Each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3’ bias utilizing a random priming method (Arraystar Flash RNA Labeling Kit, Arraystar). The labeled cRNAs were hybridized onto the Human LncRNA Array v3.0 (8 x 60K, Arraystar). After washing the slides, the arrays were scanned by the Agilent Scanner G2505C.

The Agilent Feature Extraction software (version 11.0.1.1) was used to analyze acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v11.5.1 software package (Agilent Technologies). After quantile normalization of the raw data, LncRNAs and mRNAs that had flags in at least 1 or 2 samples as “Present” or “Marginal” (“All Targets Value”) were chosen for further data analysis. Differentially expressed LncRNAs and mRNAs between the two samples were identified through fold change filtering. Pathway and GO analyses were applied to determine the roles these differentially expressed mRNAs played in these biological pathways or GO terms. Finally, hierarchical clustering was performed to show the distinguishable LncRNAs and mRNAs expression patterns among samples.

Validation of microarray data and examination of COL1A2 expression by quantitative reverse transcription-polymerase chain reaction

To confirm the microarray data and examine COL1A2 expression, expressions of selected LncRNAs and COL1A2 were tested using quantitative real-time polymerase chain reactions (qRT-PCR) with the SYBR green method on an Applied Biosystems Viia™ 7 Dx (Life Technologies, USA). The sequences of PCR primer sets used for the qRT-PCR are listed in Table 2. COL1A2 expression levels were normalized to internal
control gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), using the 2^(-ΔΔCt) method [18]. lncRNAs expression levels were normalized to internal control gene, 18S rRNA, using the double standard curves method [19].

**Statistical analysis**

Data were analyzed using the SPSS 20.0 software package (SPSS, Chicago, IL, USA) with an independent-samples T test between two groups. All values were represented as mean ± standard deviation (SD) from at least three independent experiments. Statistical significance was defined as P < 0.05.

**Results**

**Clinical characteristics of the patients and histology of skin samples**

Cesarean scar skin samples were collected from 12 patients in the study. According to the hypertrophic scar classification system, in terms of their clinical appearance and duration, a regressive scar was present for approximately 2 years, and the mature scar was present for at least 4 years during wound healing. As shown in Table 1, the mean (SD) age
revealed that more collagen existed in the regressive scar compared to mature scar (Fig. 1C-D). Furthermore, qRT-PCR results demonstrated that COL1A2 expression in the regressive scars was 1.53-fold higher than in the mature scar (Fig. 1E).

To examine the potential biological functions of lncRNAs in scar progression, we collected lncRNAs from quality-controlled databases such as Gencode, RefSeq, UCSC Knowngene and two landmark publications (Fig. 2A). Scatter plot visualization and hierarchical clustering show lncRNA expression variations and patterns between the two groups of regressive and mature scars (Fig. 2B-C). Up to 1871 lncRNAs were differentially expressed in the regressive scar samples compared with the mature scar.

**lncRNA microarray profiling**

To examine the potential biological functions of lncRNAs in scar progression, we determined the lncRNA and mRNA expression profiles between regressive scars and mature scars through microarray analysis (Fig. 2). lncRNAs are collected from quality-controlled databases such as Gencode, RefSeq, UCSC Knowngene and two landmark publications (Fig. 2A). Scatter plot visualization and hierarchical clustering show lncRNA expression variations and patterns between the two groups of regressive and mature scars (Fig. 2B-C). Up to 1871 lncRNAs were differentially expressed in the regressive scar samples compared with the mature scar.
In total, 817 mRNAs were differentially expressed between regressive and mature scar samples as revealed by scatter-plot visualization and hierarchical clustering (Fig. 2D-E). Among these mRNAs, 519 were upregulated, whereas 298 were downregulated in regressive scars compared to mature scars using the fold-change threshold of 3.0.

**Real-time quantitative PCR validation**

To validate the microarray profiling expression data, we selected 8 differentially expressed lncRNAs according to their associated mRNAs (Table 5). Real-time quantitative
PCR (qRT-PCR) analysis was performed on additional independent regressive scar and mature scar samples from women receiving caesarean sections (Table 1). The results...
revealed that similar up-regulation or down-regulation was observed in both microarray and qRT-PCR samples for the 8 lncRNA transcripts (Fig. 3). Therefore, our microarray data were reliable and stable. Among the 8 lncRNA transcripts, lncRNA8975-1 and AC097662.2 expression in regressive scars was 1.55 and 1.95-fold higher, respectively, than in the mature scars. In contrast, RP11-586K2.1 expression in mature scars was 7.29-fold greater than in regressive scars.
Expression signatures of dysregulated lncRNAs between regressive scar and mature scar

To examine the dysregulated lncRNA expression signatures, we analyzed up-regulated or down-regulated lncRNAs according to classification, length distribution and chromosome distribution (Fig. 4). In this study, the majority of lncRNAs with differential expression were intergenic (~47%), within introns of protein coding genes (~19%), natural antisense to protein coding loci (~16%), or bidirectional (~5%), and the remainder representing overlapping transcripts from exons or introns in both sense and antisense directions (Fig. 4A). The lncRNAs are mainly between 400 and 2400 bp in length (Fig. 4B). Up and down regulated lncRNAs were shown in different chromosomes (Fig. 4C-D).

mRNA profiling by microarray

Differentially expressed mRNAs were also detected between regressive and mature scar samples (Fig. 2D; fold change≥2, p≤0.001; lists of differentially expressed mRNAs were constructed (see Table 6-7)). Gene ontology (GO) analysis revealed that numerous biological processes were involved. Many of these processes that are up-regulated in regressive scars are related to immune system processes and cellular developmental processes (Fig. 5A). In contrast, many of these processes that are down-regulated in regressive scars are involved in ion transport and multicellular organismal processes (Fig. 5B). Pathway analysis indicated that the chemokine and TNF signaling pathways, as well as insulin secretion, are mostly found in both regressive scars and mature scars during progression (Fig. 5C-D).

Discussion

Hypertrophic scar formation is an abnormal sign of wound healing following caesarean section. However, after long periods, scar hyperplasia automatically regresses without

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between regressive and mature scars. Using a third-generation lncRNA microarray, we detected 1275 upregulated and 596 downregulated lncRNAs in regressive scars compared to mature scars. In addition, we confirmed 8 differentially expressed transcripts by qRT-PCR. Our study provides a comprehensive profile of the lncRNAs and coding transcripts expression between regressive and mature scars.

During scar regression, most cells undergo apoptosis, and collagen gradually degrades. Type I, IV collagen is important in wound healing [20, 21]. Type I collagenolysis is mediated by secreted, as well as membrane-anchored members of the matrix metalloproteinase (MMP) gene family during fibroblast-mediated matrix remodeling [22]. MMP16 is part of

| Gene Symbol | Fold Change (RS/MS) | RNA length | chrom | strand | txStart |
|-------------|---------------------|------------|-------|--------|---------|
| CHRDL2      | 7.42174534          | 1699       | chr11 | -      | 74407473|
| COQ6        | 24.3273959          | 1615       | chr14 | +      | 74416954|
| HLA-DRB5    | 23.02169073         | 1171       | chr6  | +      | 32485153|
| SERPINB4    | 21.28794013         | 1736       | chr18 | -      | 61304429|
| LAMP5       | 19.52651955         | 2059       | chr20 | +      | 94950044|
| NOX4        | 18.68373485         | 4270       | chr11 | -      | 89057521|
| MBLN1       | 18.27651316         | 5390       | chr3  | +      | 152017193|
| IFNG        | 16.41087364         | 1240       | chr12 | -      | 68548549|
| COL12A1     | 16.31602217         | 1157       | chr9  | -      | 75794041|
| POS         | 15.50995782         | 2158       | chr14 | +      | 75745480|
| LOC839831   | 14.87226313         | 2430       | chr7, g000195, random | - | 49237 |
| PLCH1       | 13.82471138         | 6128       | chr3  | -      | 155197670|
| TIMD4       | 13.74991164         | 1290       | chr5  | -      | 156346292|
| CCL3 L1     | 12.44581843         | 804        | chr17 | -      | 34628384|
| KRT13       | 12.31187118         | 1693       | chr1  | -      | 39657232|
| VHL         | 12.10775881         | 1014       | chr1  | -      | 156268414|
| JAK1M1      | 11.96346603         | 2593       | chr4  | -      | 6055481|
| AKR1B14     | 11.86314878         | 1625       | chr7  | +      | 134233484|
| FOXE1       | 11.56048016         | 3473       | chr9  | +      | 100615336|
| IFT12       | 10.43020245         | 3505       | chr10 | +      | 91061705|
| DPT         | 10.38908733         | 1749       | chr4  | -      | 168664964|
| PRT5S23     | 10.34651872         | 3806       | chr11 | +      | 86514490|
| DHC1H14     | 10.12034241         | 1997       | chr1  | +      | 225117355|
| RGRIP1L1    | 10.06476758         | 5959       | chr16 | -      | 53633817|
| KMO         | 8.9243389832        | 5266       | chr1  | -      | 24195458|
| LAX1        | 9.047495262         | 3027       | chr14 | -      | 23503288|
| MKK         | 9.717478372         | 3658       | chr10 | -      | 27961802|
| MATN3       | 9.525754666         | 2600       | chr2  | +      | 20191812|
| CCL4        | 9.349831099         | 667        | chr17 | +      | 34431291|
| REIL1       | 9.28531872          | 3622       | chr4  | +      | 37612255|
| KRT13       | 9.275451271         | 1719       | chr17 | -      | 39657232|
| JUN         | 9.247801066         | 3348       | chr11 | -      | 59246642|
| CHRNA3      | 9.16398848          | 3318       | chr15 | -      | 78887664|
| SPANKXN1    | 8.789518579         | 613        | chrX  | +      | 144329910|
| EDN3        | 8.78372317          | 2617       | chr20 | +      | 57675498|
| MAPRE2      | 8.646008655         | 4337       | chr18 | +      | 32558207|
| CYR61       | 8.47787234          | 2295       | chr11 | +      | 86046444|
| SCT         | 8.42308996          | 514        | chr11 | -      | 626312|
| COL6A3      | 8.38543952          | 4088       | chr2  | -      | 238290259|
| SULTE1      | 8.204452802         | 1780       | chr4  | -      | 70706929|
| PCSK5       | 8.18542943          | 3369       | chr9  | -      | 78905559|
| CD69        | 8.028874099         | 1762       | chr12 | -      | 9905081|
| FCGR3A      | 7.987724643         | 2406       | chr1  | -      | 161511590|
| ASPN        | 7.941867342         | 2541       | chr9  | -      | 95218488|
| PRMT7       | 7.708755371         | 2665       | chr18 | +      | 107599266|
| CHRNA5      | 7.65348355          | 2040       | chr15 | -      | 78885394|
| LYSMD4      | 7.509854151         | 2929       | chr15 | -      | 100267611|
| NLRP5       | 7.44690745          | 3805       | chr19 | +      | 56511091|
| ASPN        | 7.43259779          | 2308       | chr19 | +      | 95218488|
| TCN1        | 7.426527358         | 1577       | chr11 | -      | 59620280|
Table 7. The top 50 down-regulated mRNAs in the RS compared with the MS filtered by a fold-change >3.0.

| Gene Symbol | Fold Change (RS/MS) | RNA length | chrom | strand | txStart |
|-------------|---------------------|------------|-------|--------|---------|
| ILLRN       | -666.433249         | 1865       | chr2  | +      | 113975469 |
| ITHH        | -424.5070825        | 3198       | chr10 | +      | 7742535  |
| MYOCD       | -156.6695757        | 6806       | chr17 | -      | 12569206 |
| GABARAPL1    | -133.6320038        | 1905       | chr12 | +      | 103755400 |
| F8          | -97.62918625        | 9048       | chrX  | -      | 154064063 |
| AGR3        | -86.35827264        | 763        | chr7  | -      | 16899029 |
| FMN2        | -52.23887089        | 6440       | chr1  | +      | 240255184 |
| CRABP1      | -44.2791443         | 772        | chr15 | +      | 78632665 |
| TP53        | -14.1312515         | 1703       | chr4  | -      | 65444403 |
| WIF1        | -44.03518153        | 2240       | chr5  | +      | 240255184 |
| SYNE1       | -43.21885832        | 27439      | chr6  | -      | 152442818 |
| FSG4        | -38.81927839        | 1780       | chr9  | -      | 43696853 |
| HBG1        | -36.37460294        | 584        | chr11 | -      | 52695018 |
| SLC26A6     | -35.46589134        | 2629       | chr3  | -      | 48663155 |
| NOS1        | -35.42739423        | 12189      | chr12 | -      | 117645946 |
| HBG2        | -28.75656973        | 583        | chr11 | -      | 5274420  |
| DNA2        | -25.22823226        | 4287       | chr10 | +      | 70173282 |
| KIAA15499L  | -24.90704411        | 11640      | chr11 | +      | 33563876 |
| ZNF668      | -21.53918151        | 2510       | chr16 | -      | 31072163 |
| PITPNM2     | -21.12472002        | 6736       | chr12 | -      | 124368025 |
| CTAG2       | -20.29942803        | 773        | chrX  | +      | 153808245 |
| ENPP6       | -19.0462539         | 3936       | chr4  | -      | 180598958 |
| PIP         | -18.23022574        | 591        | chr7  | -      | 142829173 |
| OSBPL1A     | -18.19652782        | 4224       | chr18 | -      | 21742109 |
| HBB         | -17.6202494         | 6526       | chr11 | -      | 52466956 |
| HBA1        | -16.26035538        | 576        | chr16 | -      | 2266787 |
| FSG7        | -15.44929233        | 2046       | chr19 | -      | 43428283 |
| HRB         | -15.0091926         | 774        | chr11 | -      | 5254058 |
| CTG2        | -14.24363942        | 993        | chrX  | -      | 153989045 |
| LEPR        | -14.22318831        | 5135       | chr1  | -      | 65886363 |
| ECEL1       | -13.96150591        | 2872       | chr12 | -      | 23343456 |
| SPRR2E      | -13.48079771        | 678        | chr7  | -      | 153065510 |
| PAPPA       | -13.40979995        | 11205      | chr9  | -      | 116916070 |
| FSG1        | -12.96023223        | 2071       | chr19 | -      | 43370612 |
| SPRR2B      | -12.72390612        | 662        | chr1  | -      | 153042717 |
| TPS3BP2     | -12.44566684        | 4670       | chr1  | -      | 223967594 |
| SCEL        | -11.02071406        | 3114       | chr13 | -      | 78109908 |
| GPR132       | -10.74009223        | 3652       | chr14 | -      | 10551570 |
| ODZ2        | -10.65268073        | 9645       | chr5  | -      | 16671842 |
| AHS2        | -10.55450166        | 518        | chr16 | -      | 31592020 |
| MBN1L3      | -10.00951095        | 11502      | chrX  | -      | 131503342 |
| PRKRA       | -9.552698767         | 587        | chr12 | -      | 10998645 |
| FOXC2       | -9.30216853         | 1683       | chr16 | -      | 86600856 |
| ADAM22      | -8.266182666        | 9226       | chr7  | -      | 87565365 |
| FADS2       | -8.084434808        | 3149       | chr19 | -      | 61595712 |
| TDRD12      | -7.934117093        | 1617       | chr19 | -      | 33216678 |
| KRT7        | -7.98266294         | 1752       | chr1  | -      | 22626953 |
| SH3GL2      | -7.772251977        | 2745       | chr6  | -      | 17578952 |
| KCNQ5       | -7.69697757         | 6625       | chr6  | -      | 7331570 |
| FOXD2       | -7.62304203         | 4675       | chr1  | -      | 47901668 |

the matrix metalloproteinase (MMP) gene family that is directly relevant to fibrosis [23]. Previous reports demonstrated that severe hypoxia existed in regressive scars, and oxygen levels return to normal in mature scars, findings that are consistent with dynamic changes in microvessel density [5]. Our results revealed that IncRNA8975-1 and AC097662.2 expression were decreased, whereas RP11-586K2.1 expression was increased during regression from a regressive scar to mature scar. Those IncRNAs were associated with collagen synthesis or degradation genes (COL1A2, COL4A3 and MMP16, Table 5).

IncRNAs play crucial roles in multiple developmental processes and diseases at almost every gene expression regulation level [24-27]. Antisense RNAs usually regulate their counterpart sense mRNA in cis by bridging epigenetic effectors and regulatory complexes at specific genomic loci [28]. A recent study reported that IncRNAs contributed to reduced expression of collagen and increased expression of matrix-degrading enzymes [29]. The functional interactions of IncRNAs, miRNAs and mRNAs could lead to a new explanation for...
the pathogenesis and treatment of pulmonary fibrosis [30]. In our study, the relationship between lncRNAs (lncRNA8975-1, AC097662.2) and mRNAs (COL1A2, COL4A3) was natural antisense; furthermore, RP11-586K2.1 was intronic antisense with MMP16. These results suggest that AC097662.2 may correlate directly with COL4A3 expression and RP11-586K2.1 may correlate directly with MMP16 expression through epigenetic regulations, consistent with decreased collagen synthesis and increased collagen degradation during scar regression. In addition, lncRNA8975-1 expression correlates directly with the mRNA level of type I collagen (COL1A2) (Fig. 1E and Fig. 3).

The immune system is active during cutaneous wound repair [31]. Additional damage caused by the immune system can lead to delayed healing and excessive scar formation [31]. Here, we used a Gene Ontology analysis to prove that the main biological process involved was the immune system process, emphasizing the crucial roles of the immune system during scar regression.

Significant molecular networks, including chemokines and the TNF signaling pathway, participate in cutaneous scar formation and growth [32, 33]. In this study, pathway analysis showed that associated genes of dysregulated lncRNAs are primarily involved with chemokines and the TNF signaling pathway.

For the first time, we report the differentially expressed lncRNA profiles between regressive and mature scars. Studying differential lncRNA expression between regressive and mature scars is helpful in revealing the biological changes underlying scar regression, and potentially providing useful information for scar evaluation.

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