

Expression Profiles of Long Noncoding RNA in UVA-Induced Human Skin Fibroblasts

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Keywords

Long noncoding RNA · UVA irradiation · Fibroblasts · Skin · Photoaging · Mechanisms

Abstract

Background: Long noncoding RNA (lncRNA) are differentially expressed across stages of differentiation and development, but the role of lncRNA in human skin photoaging mechanisms remains poorly understood. **Objective:** This study aimed to determine lncRNA expression changes in human dermal fibroblasts (HDF) induced by repeated UVA irradiation and to explore correlations between lncRNA and skin photoaging prognosis. **Methods:** In the UVA-HDF group, HDF were subjected to repeated UVA irradiation (10 J/cm² UVA twice daily for 7 days); in the control group, HDF received no irradiation. High-throughput sequencing was used to detect lncRNA expression profiles. Functional annotation analysis and pathway enrichment were performed via Gene Ontology and KEGG. Predicted lncRNA target genes were identified by bioinformatic analysis. **Results:** In the UVA-HDF group, 1,730 lncRNA exhibited over 2-fold expression changes compared with the control group: 1,494 were upregulated, and 236 downregulated. Predicted lncRNA targets were associated with matrix metalloproteinases, ca-

thepsin D, mitogen-activated protein kinase and TGF- β signaling pathways, and collagen fiber metabolism following repeated UVA damaging mechanisms. **Conclusions:** lncRNA profiles were aberrantly expressed in UVA-HDF and might play a key role in skin photoaging. This study provides novel insights into the repeated UVA-damaging pathology and potential targets for treatment of human skin photoaging.

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Introduction

Photoaging is associated with skin diseases such as basal cell carcinoma, squamous cell carcinoma, chronic actinic dermatitis, and actinic reticuloid and light-sensitive dermatoses. Although it has been reported that protein oxidative damage, mitochondrial DNA deletion, accelerated telomere shortening, membrane/nuclear signal alterations, and proteases such as matrix metalloproteinase (MMP) and cathepsins were involved in skin photoaging [1], the mechanism inducing skin photoaging still remains to be elucidated.

Yue Zheng and Qingfang Xu contributed equally to this work.

The Human Genome Project revealed that 97% of the human genome is referred to as noncoding RNA (ncRNA), which is classified into 2 broad categories: small ncRNA (<200 bp) and long ncRNA (lncRNA, >200 bp). Many lncRNA are recognized as important modulators for nuclear functions and exhibit distinct nuclear localization patterns. Others must be exported to the cytoplasm to perform their regulatory roles [2]. Studies have revealed that lncRNA are involved in cell cycle regulation, splicing, mRNA decay, RNA protein regulatory network, genome rearrangement, as well as gene expression at transcriptional and posttranscriptional levels [3, 4].

lncRNA have been identified as tightly linked to the differentiation of various of organs and tissues, including the human skin [5]. Lopez-Pajares et al. [6] demonstrated that lncRNA-MAF:MAFB was linked to transcription factors regulating epidermal differentiation. Liang et al. [7] found that lncRNA groups were aberrantly expressed in keloids indicating that differentially expressed lncRNA may play a key role in keloid formation. Yue et al. [8] suggested that differentially expressed lncRNA might be considered as potential candidate genes for further study of molecular mechanisms of hair follicle morphogenesis.

Recent evidence indicated that several skin diseases were closely associated with lncRNA. Wang et al. [9] found that TSIX, an lncRNA, was overexpressed in systemic sclerosis dermal fibroblasts in vivo and in vitro; the upregulation of TSIX seen in systemic sclerosis fibroblasts might result from activated endogenous TGF- β signaling and play a role in constitutive upregulation of collagen in these cells. Tsoi et al. [10] observed that over 40% of the novel lncRNA were differentially expressed, and the proportions of previously annotated lncRNA were lower in psoriasis lesions than uninvolved or normal skin.

Correlations between lncRNA and the prognosis of skin cancer have also been investigated. Bikle et al. [11] noted increased expression of several well-known oncogenes, including H19, HOTTIP, and Nespas, and reduced expression of tumor suppressor genes, such as Kcnq1ot1, lincRNA-p21, in mouse keratinocytes lacking vitamin D receptor. Lessard et al. [12] reported that lncRNA CASC15 was involved in melanoma progression and phenotype switching. Sand et al. [13] found that expression profiles of lncRNA were changed in cutaneous squamous cell carcinoma (cSCC). Further study noted that LINC00162 (named PICSAR – p38 inhibited cSCC associated lncRNA) is involved in cSCC progression via the activation of the extracellular signal-regulated kinase 1/2 signaling pathway following downregulation of DUSP6 expression [14].

Comparing lncRNA expression between early-passage and late-passage fibroblasts, Abdelmohsen et al. [15] assessed numerous transcripts in all lncRNA for skin aging research and detected novel changes with senescence in senescence-associated lncRNA (SAL-RNA). However, lncRNA changes in photoaged human skin remain unknown, and whether lncRNA participate in repeated UVA-caused skin photoaging mechanisms is also unclear. This study investigated lncRNA expression changes in human dermal fibroblasts (HDF) induced by repeated UVA irradiation to assess possible correlation of lncRNA with skin photoaging.

Materials and Methods

Cell Culture

HDF were isolated from the foreskin dermis of children aged between 6 and 8 years with the approval of the Medical Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University and in accordance with the Helsinki Declaration. Informed consent was obtained from all guardians of the donor participants. Cells at passages 2–4 were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Life Technologies) in a 37°C humidified incubator containing 5% CO₂.

UVA Light Exposure

In the UVA irradiation group, HDF were irradiated with 10 J/cm² UVA twice daily for 7 days using a UVA lamp (SUV100; Sigma, St. Louis, MO, USA) emitting a wavelength of 340 ± 5 nm. The emission spectrum of the UVA lamp ranges from 320 to 400 nm. UVA irradiation was measured by a UVX digital radiometer (Sunkun, Guangzhou, China). Control cells were treated under the same conditions but without UVA irradiation.

Senescence-Associated β -Galactosidase Staining

Senescence-associated β -galactosidase (SA- β -Gal) staining, which marks senescent cells, was performed according to previous reports [16]. SA- β -Gal staining solution (CST, Boston, MA, USA) was used according to the manufacturer's instructions. The numbers of positive cells (dark blue cells after staining) per 200 cells were counted.

Flow-Cytometric Analysis

Twenty-four hours after the last UVA irradiation, HDF were fixed in 70% ethanol for 12–16 h at 4–6°C, then incubated with 1 mg/ml RNase R (Sigma-Aldrich, St. Louis, MO, USA) for 0.5 h at 37°C. Propidium iodide (Sigma-Aldrich), at a concentration of 50 mg/ml, was used to label DNA for 0.5 h at 4–6°C. DNA content was assessed by flow cytometry (Beckman Coulter, High Wycombe, UK), and results were analyzed using the Modifit LT v3.2 software.

Western Blot

HDF were collected and protein obtained after the cells were split in BSA and PMSF solution. Protein extracts were prepared

from fibroblasts using protein extraction reagents (Keygen Biotech, Nanjing, China). The Pierce BCA assay (Thermo Fisher Scientific, Rockford, IL, USA) was performed to determine protein concentrations. Protein membranes were incubated overnight with primary mouse monoclonal antibodies (1:1,000 dilution) against p21/WAF-1 (Cell Signaling Technology, Boston, MA, USA) and p16/INK-4a (Abcam, Cambridge, UK), and incubated with secondary antibody (1:3,000 dilution; Cell Signaling Technology) conjugated with horseradish peroxidase for 1 h at 25°C and then assayed by chemiluminescence (ECL advance detection kit; Millipore, Billerica, MA, USA).

RNA Extraction and cDNA Synthesis

Total RNA from HDF of both groups were isolated using TRIzol (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions, followed by a DNase I digestion for 30 min to remove genomic DNA. Extracted total RNA, after purification and checking for quality, were fragmented and reverse transcribed to cDNA using random primers and reverse transcriptase.

lncRNA Differential Expression Test and Clustering Analysis

In both groups, differential expression analysis of lncRNA was performed via Cuffdiff program which used the Cufflinks transcript quantification engine to calculate gene and transcript expression levels and test them for significant differences on the basis of fragments per kilobase per million mapped fragments [17]. The pheatmap R package was then used for unsupervised clusters of differentially expressed genes and experimental conditions. Resulting *p* values were adjusted using the Benjamini and Hochberg approach to control the false discovery rate.

Functional Annotation Analysis

Functional annotation analysis was performed via Gene Ontology (GO) database (<http://www.geneontology.org>), including molecular function, cellular component, and biological process information. *p* values were adjusted using the Benjamini approach.

Pathway Enrichment

KOBAS software was used to identify genes showing statically significant differential expression in KEGG pathways [18]. *p* values were adjusted using the Benjamini and Hochberg approach, and *p* < 0.05 was regarded as statistically significant difference.

Target Gene Analysis

lncRNA and mRNA 3'-UTR sequences were used to find the sRNA binding site by miRanda (<http://www.microrna.org/>). Cytoscape (<http://www.cytoscape.org/>) was applied to build a lncRNA-sRNA-mRNA interaction network.

Quantitative Reverse Transcription Polymerase Chain Reaction

qRT-PCR was performed to detect 2 upregulated lncRNA according to the SYBR method. cDNA was synthesized from RNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). qRT-PCR was performed with the GoTaq® qPCR Master Mix (Promega) and the ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). Average²- $\Delta\Delta C_t$ values were calculated and selected. The primers used for PCR amplification are shown in Table 1.

Table 1. qRT-PCR Primers

Primer name	Sequence (5'-3')
β-Actin-F	CATGTACGTTGCTATCCAGGC
β-Actin-R	CTCCTTAATGTCACGCACGAT
lnc-KRTAP5-6-3-F	GGACCAAGACCCTGCAATGA
lnc-KRTAP5-6-3-R	CCTCCCTGATATGCCCCGA
lnc-POFUT2-5-F	ACAGGAGGGGCCATGACTAT
lnc-POFUT2-5-R	GTCTCCTCACAGGGTTCAGC
lnc-AC010336.2-1-F	GCTGGTGTGTCAGCTCCACATCT
lnc-AC010336.2-1-R	TCTCGTTTTCTCCGTCAGCG
lnc-AHNAK2-7-F	AAGTTGCCACGCCCCACTCA
lnc-AHNAK2-7-R	GGCTGGACACCCACAAAGG
lnc-AC118344.1-1-F	CAGTGCCCAGGGCTTATTCT
lnc-AC118344.1-1-R	ACTGTCAGCATTGTGGCTCT
lnc-MAP2K6-4-F	GACAAGATGCTCTGGTCAAACC
lnc-MAP2K6-4-R	CAGATACCCAACCTTGCTGC
lnc-DCAF4L2-3-F	CTGCGGCAACCTTTAACCAC
lnc-DCAF4L2-3-R	CAGCAGACAAAACTGCCCC
lnc-LUM-1-F	AGCATCTTGCCTGGATGAGC
lnc-LUM-1-R	ACAGTCCCTGTGCTGTATGTC
lnc-FCN2-6-F	CTATGAAGGGATTGGAGGACC
lnc-FCN2-6-R	GTCCTCACCGGCTCGATAAT
lnc-GLI3-4-F	GGTACCGGAACCTGCAGAAA
lnc-GLI3-4-R	TGGCCTTGACCATCTTTGCT

Statistical Analysis

All experiments were done in triplicate. Statistical analysis was performed with SPSS 19.0 statistical software. Data are presented as means ± SD. Analysis of variance or *t* test analysis was performed to compare differences between groups. *p* < 0.05 was considered statistically significant.

Results

Phototoxicity of Repeated UVA Irradiation

HDF subjected to consecutive UVA treatment (10 J/cm², twice daily for 7 days) presented increased SA-β-gal expression in >90% of cells, suggesting that aging was successfully induced, whereas in the control group the rate of positively staining fibroblasts was <20% (Fig. 1).

Flow-cytometric results showed that compared with the nonirradiated HDF (control), the number of UVA-irradiated HDF increased from 53 ± 2.4 to 77 ± 4.1% (*p* < 0.05) in G0/G1 phase and decreased from 37 ± 5.3 to 20 ± 3.9% (*p* < 0.05) in S phase and from 10 ± 1.1 to 2 ± 0.8% (*p* < 0.05) in G2/M phase. This G1 phase arrest was induced by consecutive UVA treatments.

Western blotting analysis showed increased expression of cell cycle- and senescence-associated proteins

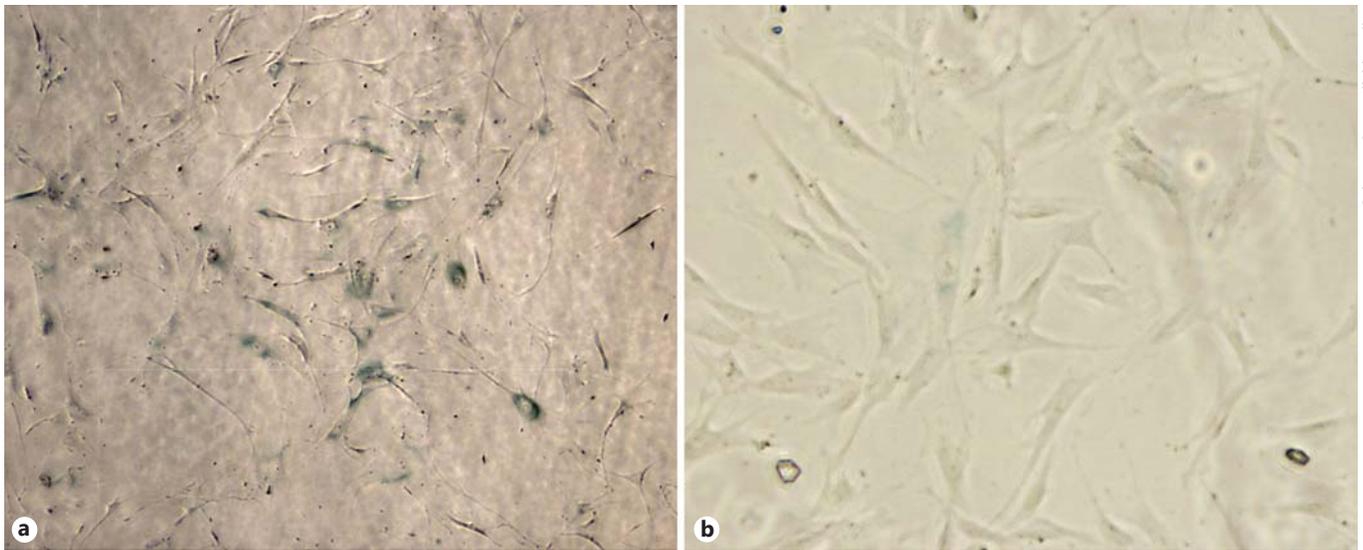


Fig. 1. After 7 days of UVA irradiation, 90% human dermal fibroblasts (HDF) presented increased expression of senescence-associated β -galactosidase. In the control group, however, the rate of positively staining fibroblasts was <20%. $\times 40$. **a** HDF irradiated with UVA for 7 days. **b** HDF without irradiation (control group).

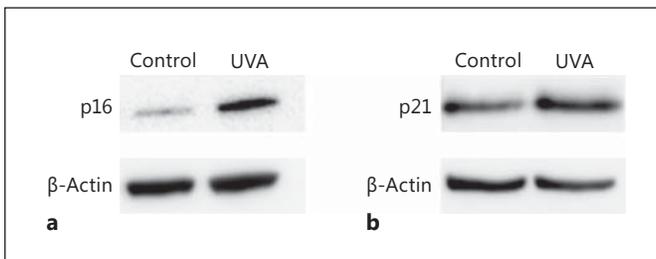


Fig. 2. After 7 days of UVA irradiation, the expression of p16/INK-4a and p21/WAF-1 proteins was increased in Western blot analysis. The protein level was normalized to β -actin. **a** p16/INK-4a. **b** p21/WAF-1.

p16/INK-4a and p21/WAF-1 in HDF subjected to repeated UVA irradiation (Fig. 2).

lncRNA Detection by High-Throughput Sequencing

In the group treated with repeated UVA irradiation, 1,730 lncRNA exhibited >2-fold expression changes compared with the healthy control group: 1,494 were upregulated and 236 were downregulated (Fig. 3).

Characterization of Differentially Expressed lncRNA and Molecular Functional Analysis

GO significant enrichment analysis showed that these differentially expressed lncRNA were mainly involved in

cell division, molecular functions, and biological processes, which have been associated with UVA-induced damaging mechanisms (Fig. 4). Molecular pathway analysis revealed that repeated UVA-induced changes in lncRNA mainly affected molecular signaling, cytokine-cytokine receptor interactions, focal adhesion, signaling pathways regulating the pluripotency of stem cells, and pathways involved in cancer development/progression (Fig. 5).

lncRNA-Targeted sRNA-mRNA Network Prediction

To validate results of sequencing, we selected 10 lncRNA with >2-fold changes with the highest signal value of probes after UVA irradiation (Table 2).

GO analysis of photoaging-associated proteinases and lncRNA showed that lnc-POFUT2-5 and lnc-FCN2-6, produced from the gene loci COL18A1 and COL5A1, respectively, were >2-fold increased after repeated UVA irradiation. Regarding the interaction between lncRNA and signal transduction pathway analysis, we found that lnc-AC010336.2-1, lnc-AHNAK2-7, lnc-AC118344.1-1, and lnc-MAP2K6-4 were most probably associated with MAPK signaling pathway changes while lnc-GLI3-4 and lnc-LUM-1 might be associated with changes in the TGF- β signaling pathway, which play an important role in skin photoaging mechanisms. lnc-DCAF4L2-3 was probably associated with MMPs while lnc-KRTAP5-6-3 was probably associated with cathepsin D (Table 3).

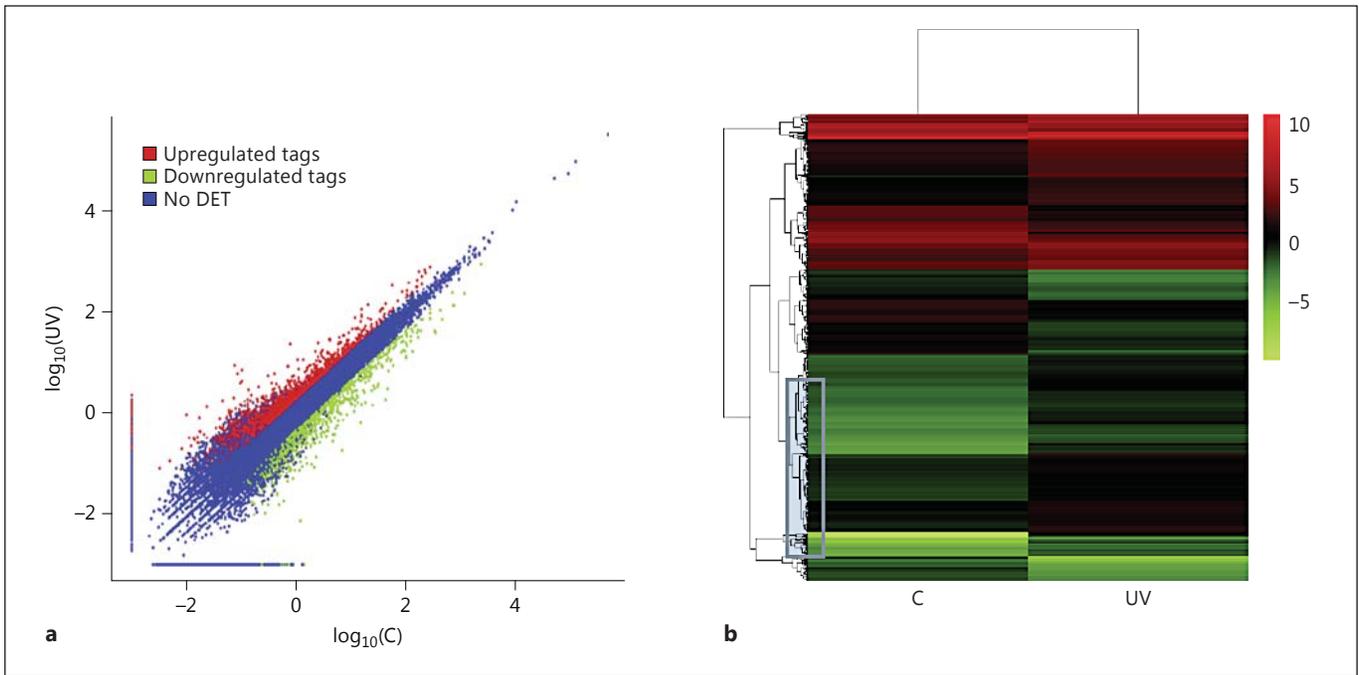


Fig. 3. Differentially expressed lncRNA profile and unsupervised cluster. **a** Gene expression: control vs. UV irradiation. $FDR \leq 0.001$ AND \log_2 ratio ≥ 1 . High-throughput sequencing showed that in the HDF group with repeated UVA irradiation, 1,730 lncRNA exhibited over 2-fold expression changes compared to the control group. Among these 1,494 lncRNA were upregulated, while 236 were downregulated after 7 days of UVA irradiation (reads per thousand base pairs per million reads). DET, differentially expressed transcripts. **b** Unsupervised cluster of differentially expressed lncRNA.

Table 2. Changes in lncRNA expression (UV vs. control) induced by repeated UVA irradiation

lncRNA changes	Gene ID	x-fold change	Up-/down-regulation
lnc-POFUT2-5	COL18A1	6.36	Upregulation
lnc-FCN2-6	COL5A1	3.12	Upregulation
lnc-AC010336.2-1	MAP2K7	3.54	Upregulation
lnc-AHNAK2-7	AKT1	3.31	Upregulation
lnc-AC118344.1-1	AKT2	2.92	Upregulation
lnc-MAP2K6-4	MAP2K6	0.41	Downregulation
lnc-GLI3-4	INHBA	2.24	Upregulation
lnc-LUM-1	DCN	0.22	Downregulation
lnc-DCAF4L2-3	MMP16	2.51	Upregulation
lnc-KRTAP5-6-3	CTSD	0.52	Downregulation

Discussion

lncRNA were found to have various functions in skin fibroblasts [7, 10]. Recent studies disclosed that UV stimulation could alter lncRNA expression. Zeng et al. [19] found that following UVB irradiation (20 mJ/cm²) the ex-

Table 3. lncRNA target prediction by Gene Ontology

lncRNA changes	Targeted gene	Targeted network
lnc-POFUT2-5	COL18A1	Collagen
lnc-FCN2-6	COL5A1	Collagen
lnc-AC010336.2-1	MAP2K7	MAPK signaling pathway
lnc-AHNAK2-7	AKT1	MAPK signaling pathway
lnc-AC118344.1-1	AKT2	MAPK signaling pathway
lnc-MAP2K6-4	MAP2K6	MAPK signaling pathway
lnc-GLI3-4	INHBA	TGF- β signaling pathway
lnc-LUM-1	DCN	TGF- β signaling pathway
lnc-DCAF4L2-3	MMP16	Matrix metalloproteinase
lnc-KRTAP5-6-3	CTSD	Cathepsin D

pression of 807 lncRNA and 69 stress response-related genes had changed >2-fold. Expression levels of lnc-GKN2-1:1, lnc-CD1D-2:1, and lnc-SGCG-5:4 and ROS content were significantly increased after UVB irradiation [19]. Hall et al. [20] reported that lincRNA-p21 was highly inducible by UVB and had a key role in triggering UVB-

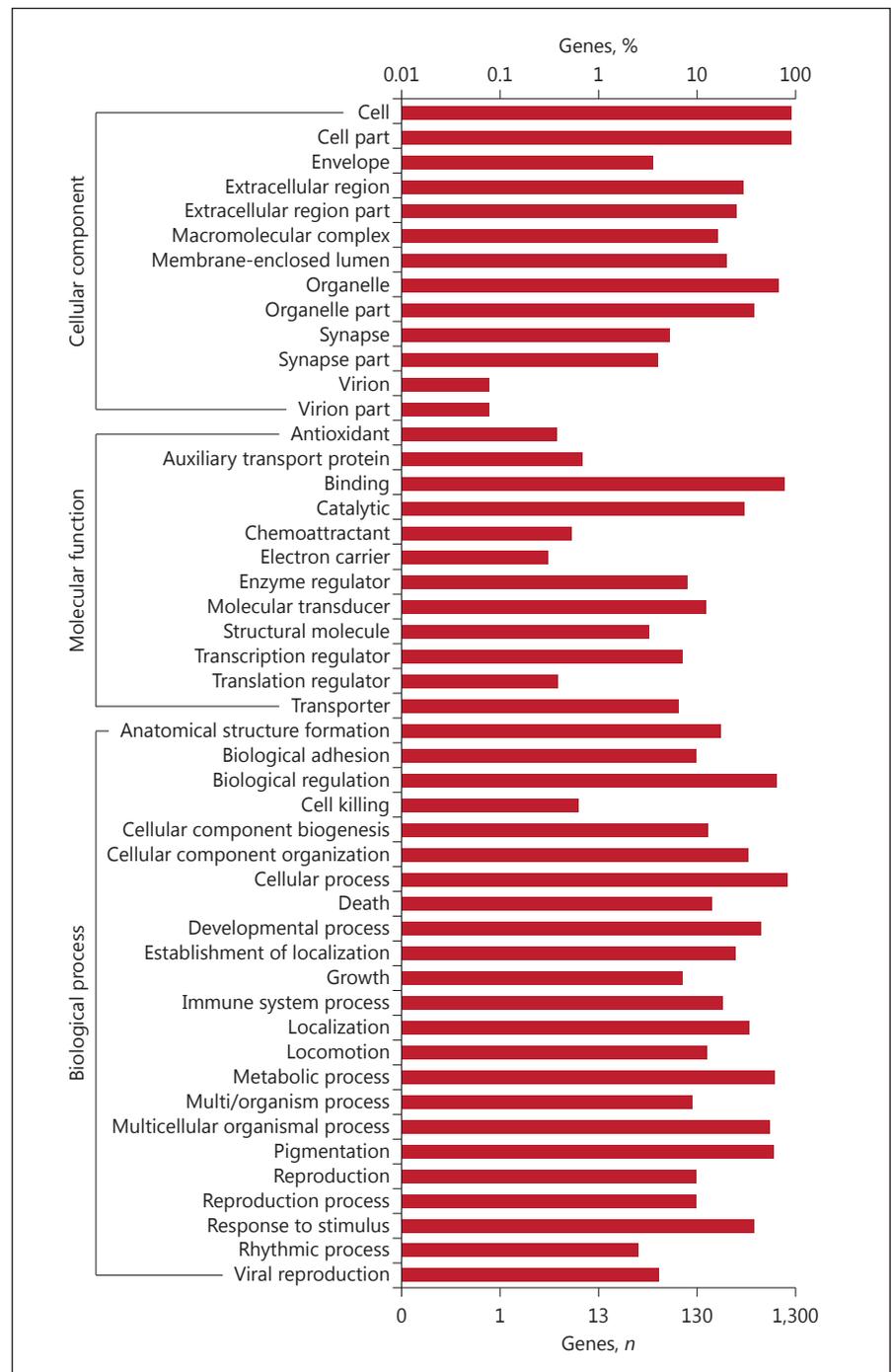


Fig. 4. Differentially expressed lncRNA-related protein coding genes in chronic UVA-treated human dermal fibroblasts were analyzed using Gene Ontology (GO) database (<http://www.geneontology.org>). GO significant enrichment analysis showed these differently expressed lncRNA are mainly involved in cell division, molecular function, and biological process, which are related with chronic UVA damaging mechanisms.

induced apoptotic death. Here, repeated UVA irradiation changed lncRNA expression in human skin fibroblasts. In 1,730 lncRNA, expression was changed >2 folds. Regarding the pathogenesis of skin photoaging, changes mainly affected intracellular signal transduction pathways, DNA damage, immunosuppression, abnormal remodeling of

extracellular matrix (ECM)/angiogenesis, as well as changes in protease expression and activity [21]. Therefore, we biologically analyzed changes in lncRNA which might take part in skin photoaging mechanisms and characterized interactions of lncRNA with MMP, cathepsins D, collagen fiber metabolism, and MAPK and TGF- β signaling.

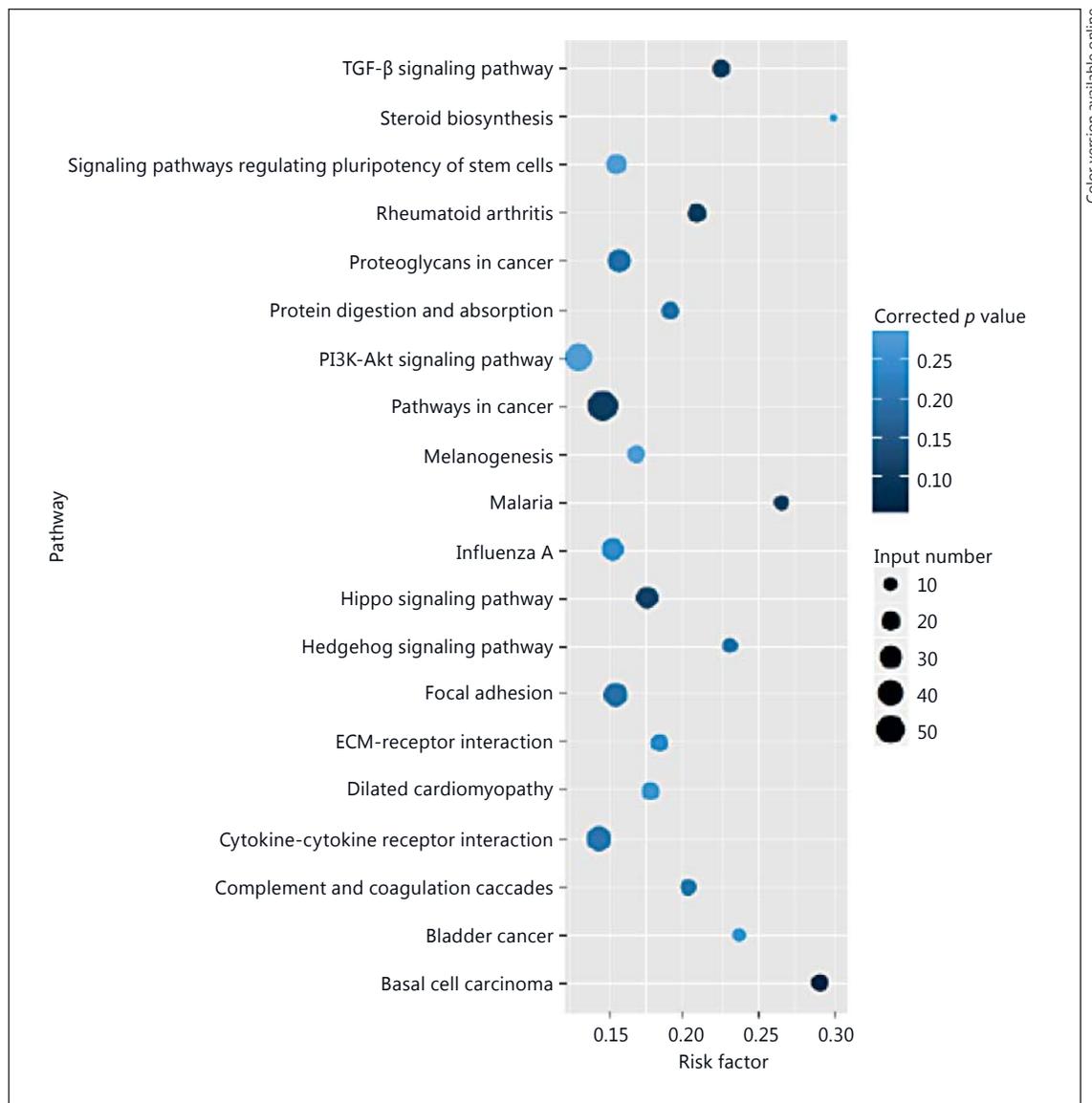


Fig. 5. Molecular pathway analysis revealed that chronic UVA-induced changes in lncRNA were mainly involved in the PI3K-Akt signaling pathway, cytokine-cytokine receptor interaction pathway, focal adhesion, signaling pathways regulating pluripotency of stem cells, and pathways in cancer. ECM, extracellular matrix.

MMPs are able to degrade ECM protein components. UVA irradiation could increase activator protein-1 activity and expression of MMPs, including MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-10, MMP-12, and MMP-13 [22]. Studies have revealed that MMP-16, a membrane-type MMP, could degrade fibrillar collagen type I. However, changes in MMP-16 induced by UVA irradiation were not reported, and the underlying mechanisms remain unclear. In our study, lnc-DCAF4L2-3, which is adjacent to the MMP-16 gene coding sequence,

was >2-fold increased. With respect to lncRNA, which may affect the promoter region and inhibit proteinase mRNA transcription, the hypothesis that lncRNA might downregulate some subtype MMP expression induced by UV irradiation was set up and needs to be confirmed by further research.

Cathepsin D, a protein related to epidermal differentiation, plays an important role in skin damage and skin barrier changes following repeated UV exposure. Previous studies found that cathepsin D expression was de-

creased in photodamaged skin [16], and cathepsin D gel could increase the cathepsin D content in the stratum corneum and repair the epidermal barrier in repeatedly photodamaged skin [1]. Keratin-associated proteins (KRTAPs), the major structural hair shaft proteins, are thought to be involved in keratin bundle assembly. lnc-KRTAP5-6-3 was the lncRNA coded by KRTAP genes. Here, we found that lnc-KRTAP5-6-3, which is closely located to the cathepsin D gene coding sequence, was decreased to 52% of the control. This indicated that lnc-KRTAP5-6-3 might act as an upstream regulator of cathepsin D expression changes in UVA-damaged skin.

Interaction between lncRNA and MAPK signaling has been confirmed. Zhang et al. [23] noted that lncRNA-NEAT1 is involved in the TLR4-mediated inflammatory process via activation of the late MAPK signaling pathway. Zou et al. [24] observed that knockdown of lncRNA-MALAT1 could inhibit MEK1, ERK1, p38, and JNK1. They suggested that MAPK pathways might be involved in the mechanisms regulating lncRNA-MALAT1 [24]. Peng and Fan [25] found that the ERK/MAPK pathway was inactivated after lncRNA-CCHE1 knockdown. Chen et al. [26] reported that lncRNA-MALAT1 promoted neurite outgrowth through activation of the ERK/MAPK signaling pathway in N2a cells. Our results indicate that lnc-AC010336.2-1, lnc-AHNAK2-7, lnc-AC118344.1-1, and lnc-MAP2K6-4, which changed in HDF following UVA exposure, are probably involved in the skin photoaging process by interacting with the MAPK signaling pathway.

Studies investigating cross talk between the TGF- β signaling pathway and lncRNA in cells found that several members of the TGF- β pathway are targeted by lncRNA [27]. Wang et al. [28] found that the upregulation of lncRNA-TSIX in systemic sclerosis fibroblasts might activate endogenous TGF- β signaling and play an important role in controlling collagen mRNA stabilization. Fu et al. [29] observed that TGF- β R2 and SMAD2 could be upregulated by ectopic expression of lncRNA-ATB in hepatic stellate cells. Huang et al. [30] found that lncRNA participate in the TGF- β pathway to modulate the expression of ECM genes and myofibroblast differentiation. Zhao et al. [31] suggested that TGF- β /Smad signaling is inhibited by lncRNA-ANRL, which then resulted in reduced p15INK4B expression. We found abnormally increased lnc-GLI3-4 expression but lnc-LUM-1 was remarkably decreased in photo-damaged skin fibroblasts.

Accumulating evidence indicates that lncRNA play critical roles in the regulation of collagen expression in various tissues and organs [32]. Zhou et al. [33] found

that a subset of lncRNA linked to collagen genes and numerous proteins regulate the ECM during formation of fibrotic scars. Wang et al. [34] noted that lncRNA-TSIX played a role in controlling collagen mRNA stabilization in scleroderma dermal fibroblasts. Our research revealed that lnc-POFUT2-5 and lnc-FCN2-6 (produced from the gene loci COL18A1 and COL5A1, respectively) were predicted to be associated with collagen synthesis. This indicated the association between changes in lncRNA by repeated UVA irradiation and collagen-coding genes which might cause collagen abnormalities in photoaged skin.

Our results show that lncRNA profiles were aberrantly expressed following repeated UVA irradiation of skin fibroblasts compared with unirradiated cells, which indicates that differentially expressed lncRNA may play a key role in skin photoaging. The present study provides novel insights into the repeated UVA-damaging pathology and potential targets for treatment of photoaged human skin.

Statement of Ethics

HDF were isolated from the foreskin dermis of children aged between 6 and 8 years with the approval of the Medical Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University, and research was conducted in accordance with the Helsinki declaration. Informed consent was obtained from all guardians of the donor participants.

Disclosure Statement

There are no conflicts of interest associated with this study.

Funding Sources

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