Original Article

Identification of Estrogen-associated Intrinsic Aging Genes in Chinese Han Female Skin by cDNA Microarray Technology

YAN Wei[1], ZHAO ZhenMin[1,#], ZHANG LiLi[2], WANG DunMei[2], YAN Li[1], YIN NingBei[1], WU Di[1], and ZHANG Feng[2]

1.Plastic Surgery Hospital, Chinese Academy of Medical Science, Peking Union Medical College, Beijing 100144, China; 2.CAS Key Laboratory of Genome Sciences and Information, Beijing Institute of Genomics, Chinese Academy of Sciences, Beijing 100029, China

Abstract

Objective Estrogens play an important role in intrinsic skin aging. The associated changes in global gene expression are poorly understood.

Methods We used the Illumina microarray platform to obtain comprehensive gene expression profiles in female Chinese Han skin, and confirmed the data by quantitative real-time PCR (Q-RT-PCR).

Results We found 244 genes significantly related to estrogen-associated intrinsic skin aging, and some of these genes were confirmed by Q-RT-PCR. We also performed functional analysis by both Gene Ontology annotation and enrichment of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways database. The functional analysis revealed 11 biological pathways (including the KEGG pathways, the mitogen-activated protein kinase signaling pathway and metabolic pathways), that were associated with multiple cellular functions which may be involved in intrinsic skin aging.

Conclusion This study suggests that estrogen-associated intrinsic skin aging is a complicated biological process involving many genes and pathways.

Key words: Estrogen; Intrinsic aging; Microarray; Metabolism

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INTRODUCTION

China is becoming an aging society, and there is particular interest in improving the quality of life in old age, with prevention of age-associated diseases. Changes in the skin reflect the aging process in humans, so more research on skin aging is being performed. Skin aging can be classified into extrinsic aging (ultraviolet light (UV)-induced) and intrinsic aging (genetic factor-induced or endocrine-induced)[1]. Estrogen, as one of the sex hormones, has a major influence on the endocrine environment, and plays an important role in intrinsic skin aging. Many studies have shown a direct positive correlation between estragon and intrinsic skin aging, and the use of hormone replacement treatment (HRT) has also indirectly demonstrated the beneficial effect of estrogen on skin aging[2-3]. In women, estrogen levels decline rapidly after the menopause as a result of the loss of ovarian follicles, and accelerate skin aging. There is an increase in skin dryness, fine wrinkling, poor healing, and hot flashes, which result from skin atrophy, decreased collagen and water content, decreased sebaceous secretions, loss of elasticity, and manifestations of hyper-androgenism[4-5]. As much as 30% of skin collagen is lost in the first 5 years after the menopause, and the loss increases with the number of years following the menopause[6-7]. Estrogen clearly has an important

[#]Correspondence should be addressed to ZHAO ZhenMin. Tel: 13693500583. E-mail: zhaozhenmin0098@vip.sina.com
Biographical note of the first author: YAN Wei, female, born in 1980, PhD, majoring in plastic surgery.
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function in many components of human skin including the epidermis, dermis, vasculature, hair follicles and sebaceous, eccrine and apocrine glands. Thus, it has significant roles in skin aging, pigmentation, hair growth, sebum production and skin cancer. Molecular mechanisms responsible for the estrogen-associated intrinsic skin aging have been extensively studied. It appears that estrogens may have 2 different mechanisms of actions: 1) a genotropic effect regulating the transcription and expression of genes directly or indirectly; and 2) a cytoplasmic effect activating signaling pathways that affect cell survival and/or modulate other growth factor signaling. Regarding the close relationship between estrogen and intrinsic skin aging, many studies have focused on HRT to reverse the skin aging processes in estrogen-deficient populations, but there are still controversial debates on the mechanism[8]. The dilemma about HRT mainly results from lack of knowledge of the molecular pathways affected by estrogen in humans. Although significant advances have been made in recent years using skin organ culture, little is known in detail about the biological processes and the genes involved, especially in the skin of female Chinese Han. Furthermore, past studies mainly focused on a particular type of skin cell (e.g., fibroblast, keratinocyte, or melanocyte) or a section of skin only (e.g., epidermis, dermis, or connective tissue), ignoring the overall biological network of complete skin tissue. Our study was designed to identify all possible genes and biologic processes involved in estrogen-associated intrinsic skin aging by analysis of Chinese Han female full-thickness skin samples using microarray technology. Our study aimed not only to confirm but also to extend the findings from previous studies about the correlation between estrogen and intrinsic skin aging. This study provides a foundation for future molecular research and prevention of estrogen-associated intrinsic skin aging.

MATERIALS AND METHODS

Subjects

Thirteen healthy Chinese Han women (mean age 50 y; range: 46–55 y) without any specific age-related family history, smoking or alcoholism, were recruited from the Plastic Surgery Hospital of Peking Union Medical College (PUMC), Beijing, China. They were divided into 2 groups according to their menstrual history. Group I included 7 pre-menopausal women (mean age 47.4 y; range: 46–50 y), and group II included 6 post-menopausal women (mean age 53 y; range 50–55 y; mean years of postmenopause 3.3 y; range 1–5 y) (Table 1). All skin samples were obtained from the abandoned tissue of post-auricular skin (UV protected) during rhytidectomy surgery. Fat tissue was removed and full-thickness skin was obtained within 5 min of removal of skin tissue from the body. The sample was immediately snap frozen in liquid nitrogen and stored at –80°C for future RNA isolation. This study followed the Declaration of Helsinki protocols and was approved by the Institutional Review Board at the Plastic Surgery Hospital of PUMC, Beijing, China.

Table 1. Basic Characteristics of the Subjects

Subject ID	Age (Years)	Group ID	Beadchip Version
1	46	I	V2.0
2	46	I	V2.0
3	46	I	V3.0
4	47	I	V3.0
5	47	I	V3.0
6	50	I	V3.0
7	50	I	V2.0
8	50	II	V2.0
9	52	II	V3.0
10	53	II	V3.0
11	53	II	V3.0
12	55	II	V2.0
13	55	II	V2.0

Note. Group I: pre-menopausal; Group II: post-menopausal.

RNA Preparation and Microarray Processing

Total RNA was isolated using the TRIzol reagent following the manufacturer's RNA isolation protocol (Invitrogen). RNA integrity and purity were checked by gel electrophoresis and spectrophotometry, respectively. Suitable RNA samples were stored at -80 °C until analysis.

Illumina HUMANWG-6 V2.0 and HUMANWG V3.0 Beadchips (Sentrix Human WG-6; Illumina) were used to generate the global gene expression profiles for 6 and 7 samples, respectively. The 2 versions of Beadchips shared the same procedure. In brief, 500 ng total RNA of each sample was used to generate biotinylated cRNA by *in vitro* transcription using the Illumina R TotalPrep Amplification Kit (Ambion) according to the manufacturer's instructions. Then, 1 500 ng biotinylated cRNA per sample was

hybridized to Beadchips. The hybridization, and subsequent incubation, staining, washing, and drying of the Beadchips were processed according to the standardized operating procedures (Illumina). The Beadchips were scanned on the Beadarray Reader (Illumina). The microarray data have been deposited in the Gene Expression Omnibus database .

Microarray Data Analysis

Genespring GX 10.0 (Agilent) software was used to determine the differentially expressed genes (DEGs) in the pre-menopausal and post-menopausal skin samples. The data was first preprocessed using the default parameters. Then, we selected the quantile algorithm to normalize the preprocessed data because this algorithm showed the best performance for normalization. For the normalized data, we used the method of filtering by flags and selected those probes with Present (P) or Marginal (M) flags (which equates to a detection $P<0.05$) as the expressed probes. Other detailed parameters in this step were: $P=0.99$, $M=0.95$ and at least 60% of samples in one out of the 2 conditions had acceptable values. Among these expressed probes, we determined the DEGs using the *Significance Analysis* function implemented in GeneSpring GX. In this step, the *Unpaired t-test* algorithm and *Asymptotic analysis* method were used for significance analysis and obtaining *P*-values, respectively, and the *P*-value cutoff was set at $P<0.05$. The Gene Ontology and National Center for Biotechnology Information (NCBI) database was used to annotate the observed genes' functions. The Web-based Gene Set Analysis Toolkit (WebGestalt) was used for further functional enrichment analysis.

Quantitative Real-Time Polymerase Chain Reaction

Microarray data were validated by SYBR-green-based quantitative real-time polymerase chain reaction (Q-RT-PCR). The housekeeping gene *ACTβ* was used as the internal control. For each sample, 1000 ng total RNA was reverse transcripted to cDNA using M-MuLV Reverse Transcriptase (NEB) according the manufacturer's instructions. Reactions were performed in triplicate using a DNA Engine Option TM 2 thermal cycler (MJ Research^{TM}). Fold changes were calculated using Livak's 2-ΔΔCT method.

RESULTS

RNA Purity and Integrity

The RNA isolated from 13 skin samples were checked by gel electrophoresis and spectrophotometry, respectively. The ratio of 260/280 was 1.7–2.1. The band of 28S rRNA was nearly twice that of 18S RNA in brightness. All results were good and met the requirements of the Illumina Beadchip (Figure 1).

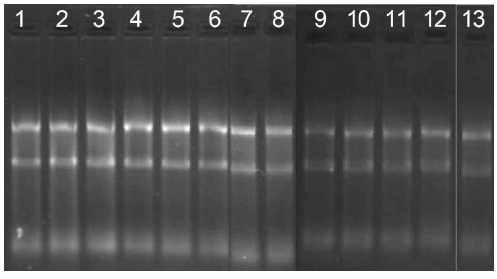

Figure 1. The brightness of 28S rRNA (top strip) was nearly twice that of 18S RNA (bottom strip).

Microarray Data Pre-processing and Differentially Expressed Genes

It took 2 years to collect samples that met both the quantity and quality requirements. During the period, the microarray Beadchips were updated and we had to use 2 different versions of Beadchips during the study. These 2 versions were comparable because the majority (37 352 common probes; 77% overlap) of the Beadchip content were common and we determined these common probes to be the basis of the microarray analysis. The quality of the microarray data was evaluated according to the suggestions of the Illumina microarray platform and all the detected parameters fell within their expected ranges. The background of the Beadchip scan was clean and clear, demonstrating that all signals collected in it had good consistency and sensitivity (Figure 2).

Determination of the expressed genes generated a total of 13 968 (13 968/37 352, 37.4%) distinct genes which passed the following 2 criteria: detection $P<0.05$, more than 60% of the values in any one of the 2 groups had acceptable values. Identification of the DEGs among these expressed genes then yielded 244 genes (131 up-regulated genes, 113 down-regulated genes in the pre-menopausal compared with post-menopausal

samples) (Figure 3) which passed the cut-off of *P*<0.05 using the *Unpaired t-test* algorithm and the *Asymptotic analysis* computation method (Tables 2A, B). These genes underwent further functional enrichment analysis into 11 possible pathways (Table 3).

Figure 2. A random screenshot of the scanned image. The background of the Beadchip is clean and clear, which demonstrates that all signals collected in it have good consistency and sensitivity.

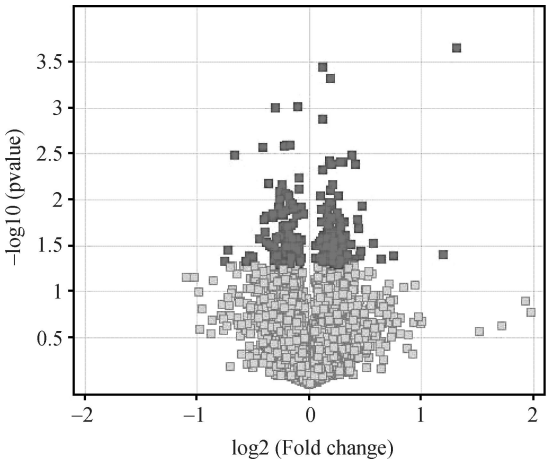

Figure 3. Volcano plots of genes. Each spot represents a detected gene. Red spots represent the significant differentially expressed genes. The grey spots represent other nonsignificant differentially expressed genes.

Table 2A. Up-regulated Differentially Expressed Genes in Skin of Pre-menopausal Women Compared with Post-menopausal Women (*P*<0.05)

Symbol	GenBank Number	*P*-value	FC	Symbol	GenBank Number	*P*-value	FC
PEX6	NM_000287.2	1.02e-4	1.4	*C9orf140*	NM_178448.2	3.00e-2	1.3
PRR4	NM_001098538.1	2.15e-4	2.5	*C1orf198*	NM_032800.1	3.07e-2	1.1
ANKRD13B	NM_152345.4	3.48e-4	1.1	*SELS*	NM_203472.1	3.08e-2	1.0
PPIL2	NM_014337.3	4.70e-4	1.1	*PITRM1*	NM_014889.2	3.08e-2	1.1
MUC4	NM_018406.3	1.28e-3	1.1	*SEMA5B*	NM_001031702.2	3.14e-2	1.1
SULT1A3	NM_003166.3	3.17e-3	1.3	*PRRT1*	NM_030651.3	3.20e-2	1.2
RNF10	NM_014868.3	3.71e-3	1.1	*SLMO1*	NM_006553.2	3.23e-2	1.1
PHF12	NM_001033561.1	3.78e-3	1.2	*ALDH2*	NM_000690.2	3.25e-2	1.2
CHTF18	NM_022092.1	3.84e-3	1.2	*TCP11L2*	NM_152772.1	3.28e-2	1.1
LOC650739	XR_019385.1	3.99e-3	1.3	*COL5A3*	NM_015719.3	3.29e-2	1.2
FBXO46	XM_934743.2	4.09e-3	1.1	*LYG1*	NM_174898.2	3.30e-2	1.1
KLC2	NM_022822.1	4.66e-3	1.1			3.35e-2	1.1
ADC	NM_052998.2	6.70e-3	1.2	*LOC388237*	XM_934263.1	3.37e-2	1.1
HIST1H2AE	NM_021052.2	7.73e-3	1.1	*EPB41L5*	NM_020909.2	3.53e-2	1.2
		8.87e-3	1.1	*TBC1D3C*	NM_001001418.2	3.56e-2	1.4
CLCN6	NM_001286.2	8.93e-3	1.2	*SIN3B*	NM_015260.1	3.59e-2	1.2
LZTS2	NM_032429.1	1.04e-2	1.1	*RIPK4*	NM_020639.2	3.60e-2	1.2
MAN2A2	NM_006122.2	1.10e-2	1.1	*ZC3H7A*	NM_014153.2	3.63e-2	1.2
WDR26	NM_025160.4	1.14e-2	1.1	*TCF7L1*	NM_031283.1	3.64e-2	1.2
TMEM80	NM_174940.2	1.15e-2	1.4	*ARMCX6*	NM_019007.3	3.74e-2	1.1
ELAC2	NM_018127.5	1.17e-2	1.1	*C16orf84*	NM_001012762.1	3.77e-2	1.1
NT5M	NM_020201.3	1.24e-2	1.1	*PRKRIP1*	NM_024653.3	3.80e-2	1.1
VPS41	NM_014396.3	1.35e-2	1.2	*APEH*	NM_001640.3	3.80e-2	1.1
IDH3G	NM_174869.1	1.41e-2	1.1	*MUC7*	NM_152291.1	3.85e-2	2.3
LOC387882	NM_207376.1	1.53e-2	1.1	*TBC1D8*	NM_007063.3	3.86e-2	1.2
ABCA2	NM_212533.2	1.59e-2	1.2	*MCOLN3*	NM_018298.9	3.93e-2	1.3
PRPF8	NM_006445.3	1.61e-2	1.2	*RORC*	NM_001001523.1	3.96e-2	1.2
DDIT3	NM_004083.4	1.61e-2	1.3	*SLC47A2*	NM_152908.3	3.97e-2	1.7
RGMB	NM_173670.2	1.67e-2	1.1	*PEX16*	NM_057174.1	3.97e-2	1.2
CAPN3	NM_024344.1	1.69e-2	1.2	*PIGZ*	NM_025163.2	3.98e-2	1.2
LOC646920	XM_929880.1	1.70e-2	1.1	*OLFML2A*	NM_182487.2	3.99e-2	4
USP5	NM_003481.2	1.71e-2	1.1	*BCL9L*	NM_182557.2	3.99e-2	1.2
ARHGAP10	NM_024605.3	1.75e-2	1.2	*LOC284821*	XR_016232.1	4.04e-2	1.1
NY-SAR-48	NM_033417.1	1.87e-2	1.1	*MLYCD*	NM_012213.2	4.07e-2	1.1
FKBPL	NM_022110.3	1.88e-2	1.1	*PSMG3*	NM_032302.2	4.08e-2	1.2

(Continued)

Symbol	GenBank Number	*P*-value	FC	Symbol	GenBank Number	*P*-value	FC
BTNL9	NM_152547.3	1.89e-2	1.2	*LOC113230*	XM_053966.11	4.17e-2	1.2
TNPO2	NM_013433.3	1.92e-2	1.2	*SERGEF*	NM_012139.2	4.17e-2	1.1
SREBF1	NM_001005291.1	2.02e-2	1.4	*ELMO3*	NM_024712.3	4.19e-2	1.2
CNTNAP3	NM_033655.3	2.17e-2	1.2	*FAM158A*	NM_016049.3	4.19e-2	1.2
PCSK1N	NM_013271.2	2.22e-2	1.1	*NXPH4*	XM_938935.2	4.19e-2	1.2
MKNK1	NM_003684.3	2.27e-2	1.2	*KIAA1737*	NM_033426.2	4.21e-2	1.1
SLC25A12	NM_003705.2	2.30e-2	1.1	*ATP5B*	NM_001686.3	4.22e-2	1.1
		2.34e-2	1.2			4.23e-2	1.1
BCS1L	NM_001079866.1	2.37e-2	1.1	*LOC652889*	XM_942615.1	4.25e-2	1.0
AKAP1	NM_003488.3	2.41e-2	1.2	*PGA3*	NM_001079807.1	4.28e-2	1.2
FLYWCH1	NM_020912.1	2.42e-2	1.1	*NSUN7*	NM_024677.3	4.32e-2	1.2
LOC645100	XM_939316.1	2.46e-2	1.1	*DFNB59*	NM_001042702.2	4.33e-2	1.3
AP3D1	NM_003938.5	2.46e-2	1.1	*JPH1*	NM_020647.2	4.36e-2	1.1
KIAA0284	NM_015005.1	2.46e-2	1.2	*HES1*	NM_005524.2	4.40e-2	1.6
OIP5	NM_007280.1	2.51e-2	1.1			4.45e-2	1.1
EZH2	NM_152998.1	2.55e-2	1.2	*TMEM80*	NM_174940.2	4.53e-2	1.2
IDH3B	NM_174856.1	2.58e-2	1.1	*IGSF9*	NM_020789.2	4.56e-2	1.2
MAP3K10	NM_002446.2	2.59e-2	1.2			4.57e-2	1.3
C16orf74	NM_206967.1	2.61e-2	1.1	*SAMD10*	NM_080621.4	4.61e-2	1.1
PDE1B	NM_000924.2	2.69e-2	1.1	*C2orf24*	NM_015680.3	4.61e-2	1.1
CYP4F3	NM_000896.1	2.70e-2	1.2	*DKFZp434N035*	NM_032262.1	4.62e-2	1.1
C3orf21	NM_152531.3	2.76e-2	1.2	*SETD8*	NM_020382.3	4.64e-2	1.2
GTPBP3	NM_133644.1	2.78e-2	1.1	*ZNF512B*	NM_020713.1	4.66e-2	1.1
ST7L	NM_138729.2	2.79e-2	1.2	*C6orf136*	NM_145029.1	4.74e-2	1.2
AHCYL2	NM_015328.1	2.86e-2	1.1	*H2AFZ*	NM_002106.3	4.80e-2	1.1
CA13	NM_198584.1	2.88e-2	1.2	*RPS6*	NM_001010.2	4.80e-2	1.2
PLEKHG4	NM_015432.2	2.89e-2	1.2	*PRKCSH*	NM_001001329.1	4.90e-2	1.2
PLEKHH1	NM_020715.2	2.91e-2	1.5	*SNORD38A*	NR_001456.1	4.94e-2	1.2
GCUD2	NM_207418.2	2.93e-2	1.1	*ETHE1*	NM_014297.3	4.96e-2	1.2
TTLL7	NM_024686.4	2.94e-2	1.1	*PMS2L2*	NR_003614.1	4.98e-2	1.1
GSTA2	NM_000846.3	3.00e-2	1.1				

Note. The blank space on the form represent the genes not annotated in detail in the Gene Ontology database.

Table 2B. Down-regulated Differentially Expressed Genes in Skin of Pre-menopausal Women Compared with Post-menopausal Women (*P*<0.05)

Symbol	GenBank Number	*P*-value	FC	Symbol	GenBank Number	*P*-value	FC
PRR11	NM_018304.2	9.38e-4	1.1	*ABCB1*	NM_000927.3	2.98e-2	1.3
EVI1	NM_005241.1	9.79e-4	1.2	*DISC1*	NM_001012959.1	2.99e-2	1.1
TMEM34	NM_018241.1	2.46e-3	1.1	*NSL1*	NM_001042549.1	3.08e-2	1.3
PPP5C	NM_006247.2	2.50e-3	1.1		BY797688	3.10e-2	1.2
CDH11	NM_001797.2	2.57e-3	1.2	*RBM12B*	NM_203390.2	3.12e-2	1.3
LOC644544	XM_927666.1	2.67e-3	1.3	*WBSCR18*	NM_032317.2	3.17e-2	1.2
GVIN1	XM_495863.3	3.22e-3	1.6	*COP1*	NM_052889.2	3.20e-2	1.1
	BF507424	5.70e-3	1.1	*C6orf190*	NM_001010923.1	3.23e-2	1.2
ENTPD7	NM_020354.2	6.56e-3	1.3	*PAQR8*	NM_133367.3	3.23e-2	1.3
	BX101207	6.68e-3	1.2	*IFT88*	NM_175605.3	3.28e-2	1.1
KLF3	NM_016531.4	7.52e-3	1.1	*TBX22*	NM_016954.2	3.34e-2	1.1
SUB1	NM_006713.2	7.91e-3	1.2	*LOC442535*	NM_001013738.1	3.38e-2	1.2
FBXO17	NM_024907.5	8.32e-3	1.2	*TMC5*	NM_024780.3	3.45e-2	1.2
HIGD1B	NM_016438.2	8.76e-3	1.1		BI481473	3.47e-2	1.1
	BM666794	8.83e-3	1.1		CB157495	3.50e-2	1.1
BOAT	XM_292512.4	9.63e-3	1.2	*RPS26L*	XR_017804.1	3.50e-2	1.7
NSD1	NM_022455.3	1.06e-2	1.1	*KRT1*	NM_006121.3	3.55e-2	1.2
LOC387841	XM_932678.1	1.09e-2	1.2	*LOC728127*	XR_015281.1	3.58e-2	1.2
	BC038747	1.09e-2	1.1	*LOC653497*	XM_927733.1	3.68e-2	1.1
CACNB4	NM_001005747.1	1.18e-2	1.1	*EEF1A1*	NM_001402.5	3.73e-2	1.1
	CB963631	1.20e-2	1.2	*GALK2*	NM_001001556.1	3.73e-2	1.2
KIF26B	NM_018012.3	1.20e-2	1.1	*LOC283932*	NM_175901.3	3.76e-2	1.1
GABPB2	NM_144618.1	1.22e-2	1.1	*STARD13*	NM_178008.1	3.82e-2	1.2

(Continued)

Symbol	GenBank Number	*P*-value	FC	Symbol	GenBank Number	*P*-value	FC
KIAA0889	NM_152257.1	1.24e-2	1.1	*40609*	NM_022826.2	3.83e-2	1.2
TIE1	NM_005424.2	1.27e-2	1.2	*ZNF549*	NM_153263.1	3.85e-2	1.2
KPTN	NM_007059.1	1.42e-2	1.2	*CCDC149*	NM_173463.2	3.92e-2	1.1
IGSF1	NM_205833.1	1.42e-2	1.0	*IKZF2*	NM_016260.2	3.99e-2	1.1
ZC3HAV1	NM_020119.3	1.42e-2	1.3		AK097979	4.01e-2	1.5
FLJ33360	NM_001001702.1	1.43e-2	1.1	*CAPZA2*	NM_006136.2	4.02e-2	1.2
FOXN2	NM_002158.3	1.46e-2	1.2	*MICB*	NM_005931.2	4.03e-2	1.2
LOC646038	XM_928999.1	1.46e-2	1.2	*MARK1*	NM_018650.3	4.03e-2	1.1
TMEM25	NM_032780.2	1.48e-2	1.3	*ZNF365*	NM_014951.2	4.10e-2	1.4
PHCA	NM_018367.4	1.52e-2	1.3	*LOC732425*	XM_001133480.1	4.16e-2	1.1
CLCA4	NM_012128.2	1.62e-2	1.3	*BTBD9*	NM_052893.1	4.21e-2	1.1
POLDIP2	NM_015584.3	1.77e-2	1.1	*IL33*	NM_033439.2	4.42e-2	1.2
	AL137257	1.85e-2	1.2	*RPS29*	NM_001032.3	4.52e-2	1.1
EIF4E3	NM_173359.3	1.89e-2	1.1		BU624523	4.54e-2	1.2
CCDC88A	NM_018084.3	1.90e-2	1.2	*RPS11*	NM_001015.3	4.55e-2	1.1
RG9MTD3	NM_144964.2	1.91e-2	1.1		BX099724	4.57e-2	1.5
TLR3	NM_003265.2	2.04e-2	1.2	*PARN*	NM_002582.1	4.61e-2	1.1
SP140	NM_007237.3	2.08e-2	1.2		DB113199	4.61e-2	1.1
C6orf204	NM_206921.2	2.09e-2	1.2	*LOC441377*	XM_938599.2	4.6e-2	1.7
DOCK10	NM_014689.2	2.20e-2	1.3	*CD44*	NM_001001391.1	4.70e-2	1.1
CXADR	NM_001338.3	2.36e-2	1.1	*ZNF696*	NM_030895.1	4.76e-2	1.1
CYFIP2	NM_014376.2	2.51e-2	1.2	*C14orf106*	NM_018353.3	4.77e-2	1.2
RNF121	NM_018320.3	2.54e-2	1.1	*UTP6*	NM_018428.2	4.80e-2	1.2
EBF3	NM_001005463.1	2.56e-2	1.2	*ZNF187*	NM_007151.1	4.83e-2	1.1
SDCCAG3L	XM_371118.2	2.58e-2	1.1	*PAPOLA*	NM_032632.3	4.83e-2	1.1
IGSF9B	NM_014987.1	2.61e-2	1.2	*THAP1*	NM_018105.2	4.84e-2	1.1
TMEM66	NM_016127.4	2.62e-2	1.1	*UBE2Q2*	NM_173469.1	4.85e-2	1.2
HCP5	NM_006674.2	2.65e-2	1.4	*CDC42EP1*	NM_152243.1	4.91e-2	1.1
KIAA1958	NM_133465.2	2.66e-2	1.1	*C11orf46*	NM_152316.1	4.91e-2	1.2
MPFL	NM_001025190.1	2.67e-2	1.1	*SLC6A1*	NM_003042.2	4.94e-2	1.1
FOXI2	NM_207426.1	2.75e-2	1.2	*CHST10*	NM_004854.3	4.95e-2	1.1
IFIT1	NM_001548.2	2.78e-2	1.1	*MYOZ1*	NM_021245.2	4.97e-2	1.1
TNPO1	NM_153188.2	2.84e-2	1.3	*LOC284757*	NM_001004305.1	4.98e-2	1.2
KLF12	NM_016285.2	2.95e-2	1.1				

Note. The blank space on the form represent the genes not annotated in detail in the Gene Ontology database.

Table 3. Common Enriched KEGG Pathways by the Three Analysis Tools: Webgestalt

Pathway Name	Webgestalt			
	O	E	R	*P*
Lysine Degradation	3	0.21	14.45	$1.2e^{-3}$
β-Alanine Metabolism	2	0.10	19.26	$4.8e^{-3}$
Ribosome	3	0.42	7.22	$8.5e^{-3}$
MAPK Signaling Pathway	5	1.27	3.94	$9.3e^{-3}$
Citrate Cycle(TCA cycle)	2	0.15	13.24	$1.0e^{-2}$
Propanoate Metabolism	2	0.16	12.84	$1.06e^{-2}$
Metabolic Pathways	11	5.21	2.11	$1.64e^{-2}$
ABC Transporters	2	0.21	9.63	$1.84e^{-2}$
Arginine and Proline Metabolism	2	0.25	7.85	$2.7e^{-2}$
Insulin Signaling Pathway	3	0.65	4.64	$2.7e^{-2}$
RNA Degradation	2	0.28	7.18	$3.18e^{-2}$

Note. (O): The number of genes in the gene set and also in each category; (E): excepted number in the category; (R): Ratio of enrichment; (*P*): *P*-value hypergeometric test.

Q-RT-PCR

We selected 4 genes: *CAPN3, RORC, MPFL, TLR3*, to validate the microarray expression data by SYBR-green-based Q-RT-PCR. Results of the Q-RT-PCR demonstrated concordance in the direction of change between the 2 different platforms. Sequences of primers, reaction systems, and cycling conditions are presented in Table 4.

DISCUSSION

Previous Studies on Estrogen and Intrinsic Skin Aging

Estrogen exerts its actions through estrogen receptors (ERα and ERβ)[5]. The expression of ERs is variable depending on the location and tissue type. Skin on the face has been shown to express much higher concentrations of ERs than breast and thigh skin[9]. ERβ is more widely distributed within the skin and skin structures than ERα[10]. The variation in

distribution of receptors within the skin suggests that each has a different, cell-specific role[11]. These individual roles, to date, have not been elucidated. ERs have 60% homology and nearly equal binding affinity for a large number of ligands[12]. The effects of endogenous estrogen on the epidermis have indirectly been shown through studies with HRT, which have demonstrated changes in the lipid layer of the epidermis in relation to estrogen status. Increased skin surface lipids and water-holding capacity of the epidermis with the use of HRT suggests that postmenopausal estrogen supplementation enhances the skin barrier function and may prevent dryness. An insight into the effects of estrogen on the dermis has been gained by studies focused on skin moisture, skin thickness, and skin aging. One human study reported that elevated endogenous estrogen improved dermal hydroscopic quality[13]. Another study in postmenopausal women revealed an enhancement of the amount of collagen in facial skin with HRT[14]. These findings suggested a correlation between the dermal composition and estrogen. Estradiol binds keratinocytes with high affinity, and keratinocytes express both ERα and ERβ. At physiologic concentrations, estradiol up-regulates the level of ERβ receptors in keratinocytes and induces keratinocyte proliferation[15]. Estrogens also target human skin fibroblasts. 17-β-Estradiol has been shown to increase fibroblast proliferation in the human pubocervical fascia and to a lesser degree in human skin[16]. In addition, estrogen has been shown to decrease tissue-degrading matrix metalloproteinases in fibroblasts derived from pelvic floor connective tissue[17].

Table 4. Primer Sets and Reaction Conditions of Q-RT-PCR Experiments

Gene	Primer Sequences (Forward, Reverse)	Cycles	Annealing Tm (℃)	Product Size (bp)	Regulation
CAPN3	F 5′GGATTCCACCTCAACAAC 3′ R 5′GCCTAACGAAGCAGCAGA 3′	40	50	103	up
RORC	F 5′ACGGGCATTGATGAGAAC 3′ R 5′GAAGGCAAATACGGTGGC3′	40	50	153	up
MPFL	F 5′ACCCTCTGGCGATTGTCC 3′ R 5′CCGCTTTCCCTCCACTCTA 3′	40	61	203	down
TLR3	F 5′ACCCTCTGGCGATTGTCC 3′ R 5′CGCTTTCCCTCCACTCTAC 3′	40	61	262	down
ACTβ	F 5′AGCGAGCATCCCCCAAAGTT 3′ R 5′GGGCACGAAGGCTCATCATT 3′	40	X*	285	

Note. X* indicates that the annealing Tm of the gene *ACTβ* could be 53, 56, or 58 according to the target genes. Regulation change is in the pre-menopausal samples compared with the post-menopausal samples.

The relatively small number of genes induced by estrogen include *c-fos* (a gene that encodes a protein that is part of the AP-1 transcription factor), epidermal growth factor, epidermal growth factor receptor, and the cell cycle-associated cyclin D1. Apart from *c-fos*, the latter 3 genes lack estrogen response elements[18]. It was recently suggested that estrogen may affect cells through mechanisms that do not involve gene transcription/expression, but through activation of cytoplasmic signaling pathways such as Src/Shc/extracellular signal-regulated kinase (ERK)[19]. This pathway was activated by many transmembrane tyrosine kinase growth factor receptors, which suggested that estrogen may augment growth factor receptor activation. Cross-talk between estrogen and other growth factors, such as insulin-like growth factor 1 (IGF-1) and transforming growth factor-α, was found to protect fibroblasts and HeLa cells from apoptosis[20].

Estrogen-related Genes and Pathways

Our study showed that 244 differentially expressed genes (131 up-regulated genes, 113 down-regulated genes), and 11 biological pathways of the pre-menopausal samples compared with the post-menopausal samples were involved in the estrogen-associated intrinsic skin aging in Chinese Han females, including lysine degradation (*NSD1, ALDH2, SETD8*), RNA degradation (*PARN, PAPOLA*), various metabolic pathways (*MAN2A2, ALDH2, IDH3G, MLYCD, ATP5B, GALK2, IDH3B, NT5M, CYP4F3, AHCYL2, ADC*), β-alanine metabolism (*MLYCD, ALDH2*), arginine and proline metabolism (*ALDH2, ADC*), propanoate metabolism (*MLYCD, ALDH2*), the mitogen-activated protein kinase (MAPK) signaling pathway (*MKNK1, PPP5C, CACNB4, DDIT3, EVI1*), the insulin signaling pathway (*MKNK1, SREBF1,*

RPS6), the citrate cycle (*TCA cycle*) (*IDH3B, IDH3G*), ribosomes (*RPS11, PRS29, RPS6*) and ABC transporters (*ABCB1, ABCA2*). Some genes and biological processes were consistent with previous observations. However, we were unable to match others with those found in the earlier studies. The roles of many novel genes differentially expressed in our study are still unknown and were not subject to particular attention in past reports.

Lysine Degradation Pathway Three genes *NSD1*, *ALDH2*, and *SETD8* are enriched in the pathway of lysine degradation. They mainly are related to the biological pathway from lysine to carnitine. Carnitine, a quaternary ammonium amino acid, occurs in the microbial, plant and animal kingdoms, and is involved in energy metabolism, hormonal action and adaptation to stress. In mammals, carnitine is known for its involvement in fatty acid metabolism, for peroxisomal to mitochondrial trafficking of activated fatty acids destined for β-oxidation[21]. The carnitine shuttle system involves carnitine acyltransferase, which allows the reversible exchange of coenzyme A and carnitine onto fatty acids, and carnitine/acylcarnitine translocase, which allows transport of carnitine esters across intracellular membranes. Carnitine has been ascribed additional roles in animals such as in coenzyme A homeostasis, energy storage in the form of acetylcarnitine, secretion of poorly metabolized acyl residues and regulation of hormonal action[22]. There are many studies of L-carnitine and fatty acid metabolism in skin aging. L-carnitine, an amino acid and a component of a typical human diet, functions as a biocatalyst to facilitate the transfer of fatty acids into mitochondria for metabolism to produce ATP. Trookman et al. explored the possibility of incorporating L-carnitine into an anti-aging hydrating cream for energy enhancement. They found a significant increase in ATP levels and enhanced oxygen uptake in cultured human fibroblasts compared with a placebo formulation. By restoring skin energy reserve levels these results demonstrated that the formulation containing encapsulated L-carnitine improved the appearance and the feel of the skin[23]. The aging of skin has been associated with an increase in size of the adipocytes located within the subcutaneous tissue. Fat mass and tissue distribution change dramatically throughout life. Fat deposits reach a peak by middle or early old age, followed by a substantial decline, together with fat tissue dysfunction and redistribution in advanced old age. Fat tissue growth occurs through increases in the size and number of fat cells. Baldassarri's study on the size of subcutaneous adipocytes of rats revealed that L-acetyl-carnitine re-equilibrates the catabolic deficit of fats in the skin of the elderly[24].

Signaling Pathways Previous studies revealed that signaling pathways play an important role in estrogen-associated intrinsic skin aging. Two relevant signaling pathways, the MAPK signaling pathway and the insulin signaling pathway were identified in our study.

Five genes, *MKNK1, PPP5C, CACNB4, DDIT3, EVI1,* are involved in the MAPK signaling pathway, mediating the biological process of proliferation, differentiation, inflammation and apoptosis. The MAPK/ERK (originally called ERK) pathway is a chain of proteins in the cell that communicates a signal from a receptor on the surface of the cell to the DNA in the nucleus of the cell. The signal is activated when a growth factor binds to the receptor on the cell surface and inactivated when the DNA in the nucleus expresses a protein and produces a change in the cell, such as cell division. MAPK regulates the activities of several transcription factors. MAPK phosphorylates and activates *MNK*, which, in turn, phosphorylates *CREB*. MAPK also regulates the transcription of the *c-fos* gene. By altering the levels and activities of transcription factors, MAPK leads to altered transcription of genes that are important for the cell cycle. The MAPK pathway also plays an important role in signal transduction in the biological processes of estrogen-associated intrinsic aging. A recent study reported that estradiol activates *CREB* and *Elk-1* via MAPKs in C2C12 cells, and modulates *c-fos* expression through MAPK activation[25]. The study also established a protective effect of the hormone in C2C12 cells exposed to an apoptotic stimulus, and MAPKs had a role in this estrogen effect[26]. Three genes, *MKNK1, SREBF1, RPS6,* were involved in the insulin signaling pathway. The gene *MKNK1* is mainly associated with glucose and lipid biosynthesis, while *SREBF and RPS6* are involved in protein homeostasis. Insulin activates both MAPK and phosphatidylinositol 3-kinase intracellular pathways. The role of insulin alone as well as its interactions in the regulation of genes and steroid biosynthesis has been examined. The insulin-mediated increase in ovarian steroidogenesis is achieved through enhanced expression of genes, enzymes and proteins that are crucial for the steroidogenic machinery[27]. The insulin/IGF-1 pathway has been shown to affect the lifespan of yeast, worms, flies and mice. Recent studies in humans also suggested an association but this is not yet conclusive. Additional studies of common variants in larger samples will be required to detect modest

effects. In addition, new sequencing technologies may help detect rare variants in this pathway associated with longevity in humans[28].

Metabolic Pathways Four pathways found in our study are related to metabolism, including the metabolic pathway (*MAN2A2, ALDH2, IDH3G, MLYCD, ATP5B, GALK2, IDH3B, NT5M, CYP4F3, AHCYL2, ADC*), propanoate metabolism (*MLYCD, ALDH2*), arginine and proline metabolism (*ALDH2, ADC*), and β-alanine metabolism (*MLYCD, ALDH2)*. The latter 2 pathways are related to amino acids metabolism. The amino acids are essential for proteins which are important in cell signaling, immune responses, cell adhesion, active transport across membranes, and the cell cycle[29].

β-Alanine is a naturally occurring β amino acid, but is not used in the biosynthesis of any major protein or enzyme. It is formed *in vivo* by the degradation of dihydrouracil and carnosine. It is a component of the naturally occurring peptides carnosine and anserine and also of pantothenic acid (vitamin B5) which itself is a component of coenzyme A. Under normal conditions, β-alanine is metabolized into acetic acid. Arginine is classified as an essential amino acid for young mammalian species and as an essential amino acid for adults. In fact, arginine is essential for the growing animal and for the adult organism under pathological circumstances characterized by tissue regeneration such as in the course of inflammatory processes and wound healing, with new collagen synthesis and proliferation of lymphoid cells[30]. L-arginine was reported to have a positive anti-aging effect. Its greater anti-aging potential than any pharmaceutical or nutraceutical agent previously investigated was demonstrated in one study[31].

Previous studies found that there was a link between collagen synthesis and prolidase activity in cultured skin fibroblasts treated with anti-inflammatory drugs[32]. Collagen biosynthesis and prolidase activity are inter-regulated in *MCF-7* and *MDA-MB-231* cells. Prolidase catalyzes the hydrolysis of imidodipeptides to release proline, which is used for collagen resynthesis and cell growth[33-34]. In Kumar's study in roses, exogenous proline was found to suppress the oxidative stress and enhanced the vase life of 'Grand Gala' roses[35].

Do the Results Really Reflect the Association between Estrogen and Intrinsic Skin Aging?

Apart from the changes in the endocrine environment, intrinsic skin aging is attributed to many other factors, such as ethnicity (genetic background) and anatomical variation[36]. In addition, UV exposure and smoking are the main extrinsic factors that influence the process of skin aging[37-38]. Study design, to exclude confounding factors except estrogen, is very important and necessary. In this study, we aimed to match the subjects characteristics at recruitment. All subjects chosen in our study were of the same racial background (Chinese Han female). The mean ages of the 2 study groups were 47.4 years and 53 years, with no significant difference between the 2 groups. All samples were collected from the same anatomical post auricular position without UV exposure. All subjects were healthy without any relevant aging-related family history, smoking or alcoholism. Although it is impossible to exclude all interfering factors, the strict recruitment criteria ensured that the effect of other factors except estrogen in intrinsic skin aging could be minimized.

There was a marked difference in results between the present and previous studies. Most of the related genes and biological pathways involved in this field have not been reported until now. We carried out this study based on full-thickness skin, focusing more on the biological processes network in the whole skin tissue, which provided us with more reliable and comprehensive information about estrogen-associated intrinsic skin aging in the Chinese Han female. Although we could only analyze the involved genes and hypothesize the biological pathways from previous research, it is a major step forward to reveal the correlation between the estrogen and intrinsic skin aging.

Study Limitations

The present study has 2 limitations. First, the number of women enrolled was relatively small. Second, we only identified the genes and pathways involved in estrogen-associated intrinsic skin aging. Further research about those genes and biological pathways are needed in the future.

In summary, our results demonstrate that gene expression is significantly altered in Chinese Han female skin aging coincident with the decline in estrogen levels. Analysis of the identified genes suggests that a wide range of biological processes are involved in estrogen-associated intrinsic aging of skin. The results provide an insight into the effect of estrogen in skin aging and estrogen-related skin diseases. Further study is needed to investigate the biological functions of genes and related biological pathways in estrogen-associated intrinsic skin aging.

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