Silencing of *ERRα* gene represses cell proliferation and induces apoptosis in human skin fibroblasts

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Abstract. Estrogen-related receptor (ERR) is an orphan nuclear receptor structurally akin to the estrogen receptor. ERR is expressed in tissues with active energy metabolism and regulates intracellular metabolic functions. Additionally, ERRs are known to be strongly expressed in the epidermis of skin tissue, but their functions are unknown. The present study investigated the function of ERRα in human skin fibroblasts. ERRα expressed in human dermal fibroblast TIG113 was knocked down using small interfering (si)RNA and gene expression was comprehensively analyzed using microarrays 48 h later. Pathway analysis was performed using Wikipathways on genes exhibiting expression changes of ≥1.5-fold. Expression of cell cycle-related and apoptosis-related genes was compared using reverse transcription-quantitative PCR. After treating TIG113 cells with siERRα for 72 h, cell proliferation was assessed using the Cell Counting Kit-8 or a scratch wound healing assay and apoptotic cells were measured using the Poly Caspase Assay Kit. Cell cycle analysis was performed using flow cytometry. The expression of the *ERRα* gene was suppressed by siRNA. The expression of genes associated with cell cycle-related pathways were decreased while that of those associated with apoptosis-related pathways increased. Furthermore, the expression of cell cycle-related genes such as cell division cycle 25C, cyclin E and cyclin B1 was decreased and the expression of apoptosis-related genes such as caspase3 and Fas cell surface death receptor was increased. Cell proliferation was suppressed and the number of apoptotic cells increased ~2-fold in ERRα-knockdown TIG113 cells. Cell cycle analysis revealed that the number of cells in the Sub-G_1 phase increased and that in the S and G_2/M phases decreased. The present study suggested that ERRα is an essential for the survival of human skin fibroblasts.

Introduction

Estrogen-related receptor (ERR) has a similar structure to that of estrogen receptor and is an orphan nuclear receptor whose endogenous ligand is unknown (1). ERR has a high homology with the DNA-binding domain of the estrogen receptor and binds to the estrogen-responsive element on the promoter. However, it has low homology with the ligand-binding domain and estrogen does not act as a ligand for ERR (1,2).

ERRα, ERRβ and ERRγ are the three subtypes of ERR. ERRα and ERRγ are expressed in tissues with active energy metabolism, such as the heart, kidneys, skeletal muscle and adipose tissues (3,4). In addition, it has been reported that ERRα and ERRγ regulate intracellular metabolic functions, such as oxidative phosphorylation in mitochondria (5-7). It has further been reported that ERRβ is expressed in the placenta and villous tissue and involved in placenta formation (8).

Estrogen regulates a variety of physiological and disease processes, including reproduction, bone remodeling and breast cancer, among others. It has been revealed that ERR shares target genes and regulatory proteins with estrogen receptor (9). Furthermore, ERR actively influences estrogen responses and it has been suggested that pharmacologically modulating ERR activity may be useful for the prevention and treatment of various symptoms related to women's health (9).

The skin is an estrogen-sensitive organ and skin fibroblasts produce extracellular matrix components, such as collagen, hyaluronic acid and elastin (10-12). These components are also related to skin antiaging and wrinkles and sagging of the skin are likely to occur in menopausal women owing to the decreased secretion of estrogen (10). Furthermore, skin fibroblasts express estrogen receptors α and β and are susceptible to estrogen (13). As ERR interacts with estrogen signaling (14) and is expressed in the skin, it may also play an important role in skin antiaging.

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Abbreviations: CASP3, caspase 3; CDC25C, cell division cycle 25C; ERR, Estrogen-related receptor; FAS, Fas cell surface death receptor; PGC-1α, peroxisome proliferator-activated receptor gamma, coactivator 1 α

Key words: apoptosis, cell proliferation, estrogen-related receptor α, skin fibroblast

In normal human skin, ERRα and ERRβ are expressed in epidermal keratinocytes (15,16) and ERRγ is expressed in keratinocytes and fibroblasts (17). However, their functions remain unknown.

The present study analyzed the function of ERRα in human skin fibroblasts by silencing its gene expression. It performed microarray and pathway analyses and reverse transcription quantitative (RT-q) PCR. Cell proliferation and apoptosis-positive cells were examined and the cell cycle was analyzed using flow cytometry. The present study is the first to report the function of ERRα in human skin fibroblasts, to the best of the authors' knowledge.

Materials and methods

Cell culture. Human normal adult skin fibroblasts (TIG113; JCRB0539) and human neonatal foreskin fibroblasts (NFF; KF-4009, passage 2, https://www.kurabo.co.jp/bio/cell-tissue/skin/03/) were obtained from the Health Science Research Resources Bank (Japan) and KURABO, respectively. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; FUJIFILM Wako Pure Chemical Corporation) with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA), 100 units/ml penicillin and 100 µg/ml streptomycin (FUJIFILM Wako Pure Chemical Corporation). All culture experiments were conducted at 37˚C in a humidified incubator supplemented with 5% CO_2.

Small interfering (si)RNA transfection. The expression of the human ERRα gene was silenced using transient transfection of ERRα siRNA (siERRα; cat. no. sc-44706; Santa Cruz Biotechnology, Inc.), which was performed using DharmaFECT 1 transfection reagent (Horizon Discovery Ltd.), according to the manufacturer's instructions. TIG113 cells were incubated with 50 nM siRNA at 37˚C for 24-72 h before use in subsequent assays. As a negative control, TIG113 cells were transfected with Silencer Negative Control #1 siRNA (siNC; cat. no. 4390843; Thermo Fisher Scientific, Inc.).

Microarray analysis and Wikipathways. TIG113 cells were seeded in a 100-mm cell culture dish, cultured until 80% confluence as described in the *Cell culture* section and then transfected with siERRα or siNC. After 48 h, total RNA was extracted using the RNeasy Mini kit (Qiagen GmbH), according to the manufacturer's instructions.

The RNA (1 µg) was used to produce biotin-labeled complementary RNA (cRNA). The labeled and fragmented cRNA was subsequently hybridized to the SurePrint G3 Human Gene Expression microarray (8x60 K ver. 3; Agilent Technologies Inc.). Labeling, hybridization, image scanning and data analysis were performed at Macrogen Japan and the Research Institute of Bio-System Informatics (Iwate, Japan). The TIG113 microarray datasets are available at http://www.ncbi.nlm.nih.gov/geo under accession code GSE245234 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE245234). The ratio of gene expression change in cells treated with siERRα to that in cells treated with siNC was expressed as fold change. Genes with 1.5-fold or greater upregulation or downregulation (n=3) following siRNA transfection in TIG113 cells were subjected to biological pathway enrichment analyses using Wikipathways (version number 20201210; https://www.wikipathways.org/).

Reverse transcription-quantitative (RT-q) PCR. TIG113 cells were seeded in 6-well plates and cultured as described in the *Cell culture* section until they reached 80% confluence and were then transfected with siERRα or siNC. After incubating at 37˚C for 48 h, cells were washed twice with PBS. Total RNA was extracted from the TIG113 cells using the RNeasy mini kit (Qiagen KK) according to the manufacturer's instructions. cDNA was reverse-transcribed from total RNA using the PrimeScript RT Master Mix (Takara Bio, Inc.) according to the manufacturer's instructions. Levels of each mRNAs were quantified by qPCR using TB Green Premix Ex Taq II (Tli RNaseH Plus; Takara Bio, Inc.). The thermocycling conditions were as follows: 30 sec at 95˚C, followed by 40 cycles of 5 sec at 95˚C and 30 sec at 60˚C. Transcription levels were normalized to those of *GAPDH* cDNA. The primer sequences were as follows (5'-3'): *ERRα*, forward GGCCCTTGCCAA TTCAGA and reverse GGCCTCGTGCAGAGCTTCT (18); *ERRβ*, forward GTCTCATACCTACTGGTGGC and reverse AGGTCACAGAGAGTGGTCAG (19); *ERRγ*, forward CAG ACGCCAGTGGGAGCTA and reverse TGGCGAGTCAAG TCCGTTCT (19); *CDKN1C*, forward GCGGCGATCAAG AAGCTGTC and reverse CCGGTTGCTGCTACATGA AC (20); peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (*PGC-1α*), forward AGCCTCTTTGCC CAGATCTT and reverse GGCAATCCGTCTTCATCC AC (21) caspase 3 (*CASP3*), forward GCGGTTGTAGAA GAGTTTCGTG and reverse CTCACGGCCTGGGATTTC AA (22); Fas cell surface death receptor (*FAS*), forward CAA TTCTGCCATAAGCCCTGTC and reverse GTCCTTCAT CACACAATCTACATCTTC (23); cell division cycle 25C (*CDC25C*), forward GCAGAAGTGGCCTATATCGCT and reverse TTCCACCTGCTTCAGTCTTGG (24); cyclin E2 (*CCNE2*), forward TCAAGACGAAGTAGCCGTTTAC and reverse TGACATCCTGGGTAGTTTTCCTC (25); cyclin B1 (*CCNB1*), forward AATAAGGCGAAGATCAACATG GC and reverse TTTGTTACCAATGTCCCCAAGAG (26); and *GAPDH* (NM_001256799.3), forward TGAGAACGG GAAGCTTGTCA and reverse TCTCCATGGTGGTGAAGA CG. The *GAPDH* primers were designed using the Primer 3 Plus interface (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). PCR specificity was assessed using melting curve analysis. All samples were analyzed in duplicate and relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (27). Three independent experiments were performed.

Western blotting. TIG113 cells were seeded in 30-mm plates and cultured as described in the *Cell culture* section until they reached 80% confluence and then transfected with siERRα or siNC. After incubating at 37˚C for 72 h, cells were washed twice with PBS. TIG113 cell lysates were prepared with RIPA lysis buffer (Santa Cruz Biotechnology, Inc.). The protein concentrations were determined using a Takara BCA Protein Assay kit (Takara Bio, Inc.). Total protein (20 µg/lane) was separated by SDS-polyacrylamide gel electrophoresis on 12% (w/v) polyacrylamide gels and was electroblotted onto Hybond nitrocellulose membranes (Cytiva). Subsequently, blocking was performed with 3% non-fat milk powder at room temperature for 2 h. Blots were probed with anti-ERRα antibody (cat. no. 13826; Cell Signaling Technology, Inc.; 1:300) or anti-β-actin antibody (cat. no. 81115-1-RR; Proteintech

Group, Inc.; 1:1,000) at 4˚C overnight, followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (cat. no. ab6721; Abcam; 1:2,000) at room temperature for 1.5 h. The signal was detected using ImmunoStar Zeta (FUJIFILM Wako Pure Chemical Corporation), according to the manufacturer's protocol. Luminescent images were analyzed using a LumiCube (Liponics).

Cell proliferation assay. A total of 4,000 TIG113 cells were seeded in a 96-well plate, cultured as described in the *Cell culture* section for 24 h and then transfected with siRNA. TIG113 cell proliferation was analyzed with a Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Inc.) following the manufacturer's protocol. Briefly, the CCK-8 reagent was added to the cells for 1.5 h at 37˚C. Absorbance was measured on a Benchmark microplate reader (Bio-Rad Laboratories, Inc.) at a wavelength of 450 nm. The number of cells treated with siNC for 0 h was defined as 100% and the increase rate at each time point was expressed as a percentage.

Scratch wound healing assay. TIG113 cells were seeded in triplicate 6-well plates and cultured as described in the *Cell culture* section until they reached 80% confluence and then transfected with siERRα or siNC in DMEM containing 10% fetal bovine serum. After 24 h, the surface of the dishes was scratched linearly with a 200-μl pipette tip and the cells were incubated in serum-free DMEM for 48 h at 37˚C. Images were captured using a phase-contrast and an inverted microscope (CK40; Olympus Corporation; magnification, x40) equipped with an Anyty digital microscope camera (3R-DKMCO4; 3R solution). The wound area for each treatment was calculated by averaging three individual measurements at 0 and 48 h using ImageJ software (ver.1.53; National Institutes of Health). Cell migration was expressed as the percentage of the scratch area filled by migrating cells 48 h post-scratch: migration (%)=(scratch area at 0 h-scratch area at 48 h/scratch area at 0 h) x100.

Apoptosis detection. A total of 4,000 TIG113 cells were seeded in a 96-well plate, cultured as described in the *Cell culture* section for 24 h and then transfected with siERRα or siNC. After 72 h, apoptosis was detected using the Poly Caspase Assay Kit Green FLICA (ImmunoChemistry Technologies, LLC). Relative fluorescent units were measured using a Tecan Infinite 200 Pro Microplate reader (excitation, 530 nm; emission, 590 nm; Tecan Group, Ltd.).

Cell cycle analysis. Cell cycle analysis was performed as in our previous study (28). Briefly, TIG113 cells were seeded in a 100-mm cell culture dish and cultured as described in the *Cell culture* section until they reached 80% confluence. Cells were transfected with siERRα or siNC and cultured for 72 h prior to DNA staining. Cells were washed in PBS, resuspended in propidium iodide (PI)/RNase Staining Buffer (BD Biosciences) and incubated for 15 min at 25˚C. PI fluorescence (FL3) was measured using an FC500 flow cytometer (Beckman Coulter, Inc.). Data were analyzed using the MultiCycle AV software (Phoenix Flow Systems).

Type I collagen and hyaluronan quantification in the medium. TIG113 cells were seeded in a 30-mm cell culture dish, cultured as described in the *Cell culture* section until they reached 80% confluence and were then transfected with siERRα or siNC. After 72 h, the supernatant was collected and filtered through a sterile filter (0.2 μm). Type I collagen and hyaluronan secreted into the medium were quantified using a human collagen type I enzyme-linked immunosorbent assay (ELISA) kit (cat. no. EC1-E105; ACEL, Inc.) and Hyaluronan Quantification Kit (cat. no. HA-KIT; Iwai Chemicals Company, Co., Ltd.), respectively, following the manufacturer's instructions.

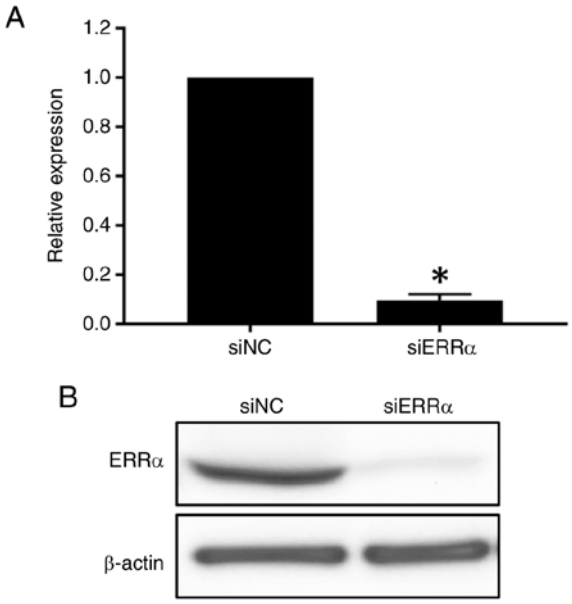

Figure 1. Silencing of ERRα in TIG113 cells. TIG113 cells were transfected with siERRα or a siNC and cultured for 48-72 h. (A) The level of ERRα mRNA expression 48 h after siRNA transfection was evaluated using reverse transcription-quantitative PCR. (B) ERRα protein after 72 h after siERRα transfection was evaluated using western blotting. The data represent the mean ± standard deviation of at least three independent experiments. *P<0.05 vs. siNC. ERRα, estrogen-related receptor α; si, small interfering; NC, negative control.

Statistical analysis. The results are expressed as mean ± standard deviation. Statistically significant differences were determined using Welch's t-test or Kruskal-Wallis analysis with the Steel post-hoc test between two groups and multiple groups, respectively, using Bell Curve for Excel ver. 4.04 (Social Survey Research Information Co., Ltd.). Wikipathways that were significant were determined by Fisher's Exact Test. Furthermore, p.adjust was calculated by performing multiple testing corrections using the Benjamini-Hochberg method. Values with p.adjust <0.05 were considered statistically significant. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of ERRs and ERRα silencing. To investigate the function of ERRα in human skin fibroblasts, ERRα expression in TIG113 cells was suppressed using siRNA targeting *ERRα*. The *ERRα* siRNA (siERRα) significantly decreased *ERRα* expression at the mRNA level compared with a nontargeting control (siNC; P<0.05; Fig. 1A). Furthermore, western blot analysis revealed that the ERRα expression was reduced in

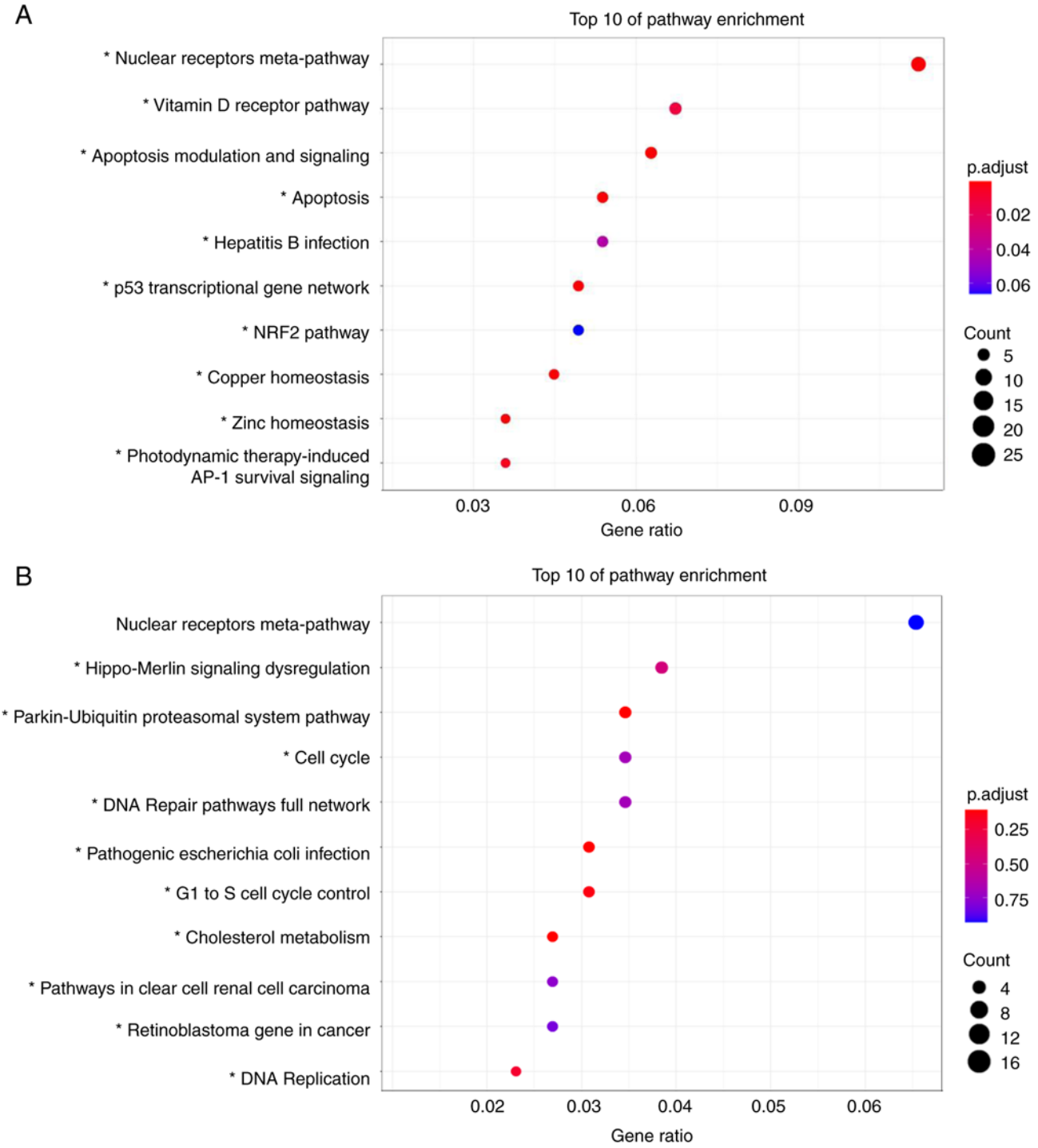

Figure 2. Wikipathways analysis in ERRα-silenced TIG113 cells. (A) Upregulated top 10 enrichment pathways were analyzed using Wikipathways for 48 h siERRα transfection. (B) Downregulated top 10 enrichment pathways were analyzed using Wikipathways for 48 h siERRα transfection. *P<0.05. ERRα, estrogen-related receptor α; si, small interfering.

siERRα-treated TIG113 cells (Fig. 1B). Moreover, microarray analysis revealed that the high expression of *ERRα* compared with that of estrogen receptors α and β suggested the importance of ERRα in skin fibroblasts (Table SI). In addition, when the expression of *ERRα*, *ERRβ* and *ERRγ* in TIG113 cells was analyzed using RT-qPCR, the relative expression level of *ERRβ* was only 1.3% that of *ERRα*, and *ERRγ* was not notably expressed (P<0.05; Fig. S1A). The same experiments using human NFFs yielded similar results (P<0.05; Fig. S1B).

Pathways enrichment analysis. Microarray analysis found 580 upregulated and 738 downregulated genes (Table SII) that had a fold change of 1.5-fold upon *ERRα* knockdown (n=3). Using the genes whose relative expression changed by 1.5-fold or more, biological pathway analyses in TIG113 cells were performed using Wikipathways. The top 10 upregulated or downregulated pathways detected using Wikipathways are shown in Fig. 2A and B, respectively. The 'Nuclear Receptors Meta-Pathway' ranked the highest in upregulated

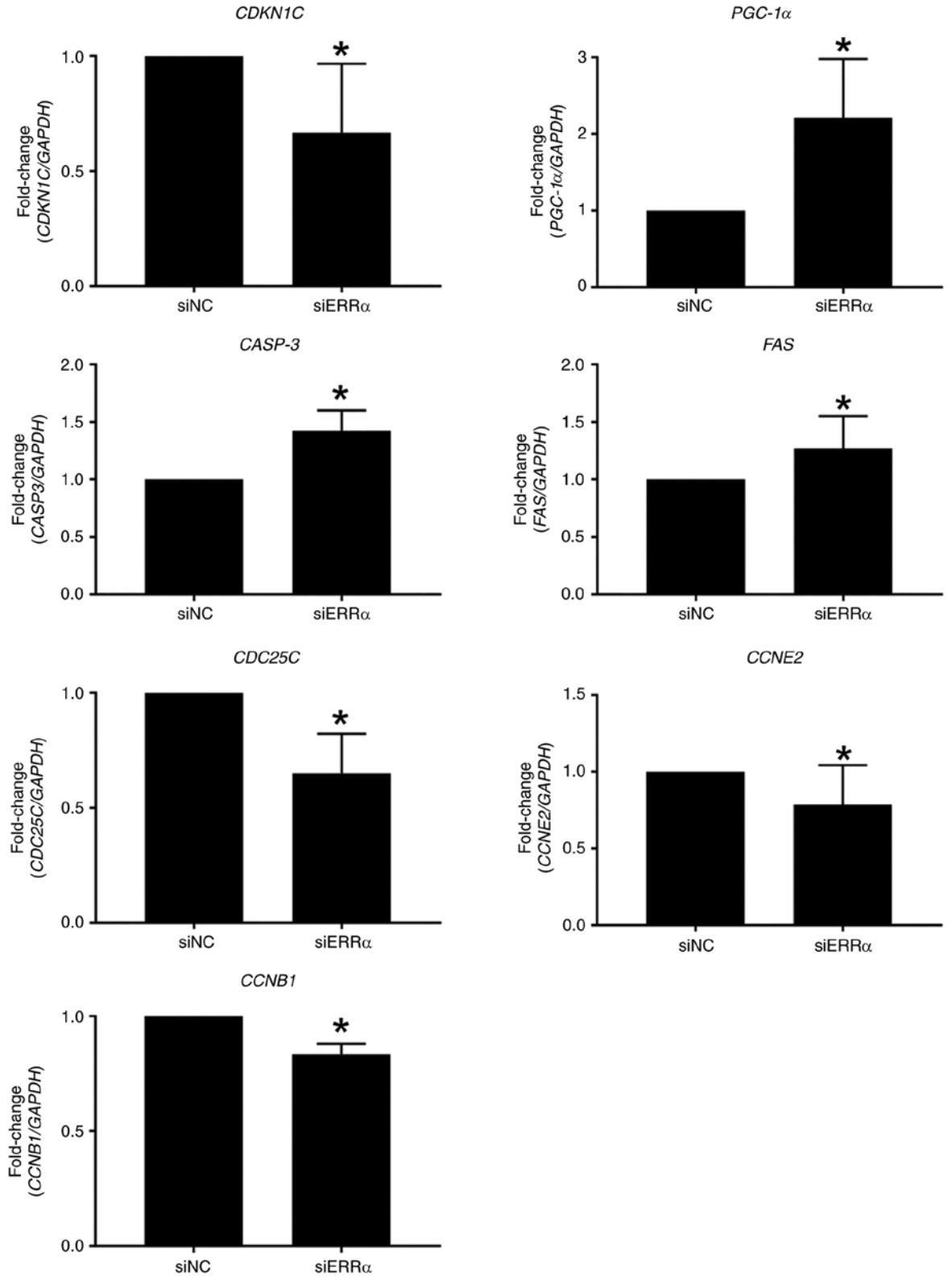

Figure 3. Validation of gene expression in TIG113 cells. TIG113 cells were transfected with siERRα or siNC and cultured for 48 h. The mRNA levels of each gene were quantified using reverse transcription-quantitative PCR. Relative expression was normalized to that of *GAPDH*. Data represent the mean ± standard deviation of three independent experiments. *P<0.05 vs. siNC. *ERRα*, estrogen-related receptor α; si, small interfering; NC, negative control; *CDKN1C*, cyclin-dependent kinase inhibitor 1C; *PGC-1α*, peroxisome proliferator-activated receptor gamma, coactivator 1 α; *CASP3*, caspase 3; *FAS*, Fas cell surface death receptor; *CDC25C*, cell division cycle 25C; *CCNE2*, cyclin E2; *CCNB1*, cyclin B1.

and downregulated pathways, but no significant difference was observed in downregulated pathways. The upregulated pathways were mainly apoptosis-related pathways, such as 'Apoptosis Modulation and Signaling' and 'p53 transcriptional gene network' (Fig. 2A). Furthermore, the downregulated pathways were related to cell cycles such as 'Cell Cycle',

Table I. Fold change of selected gene expression by ERRα silencing.

A, Nuclear receptors meta-pathway (upregulation)

Gene symbol	Gene name	Fold change
TGFB2	Transforming growth factor, β 2	3.65±0.45
SLC2A14	Solute carrier family 2 (facilitated glucose transporter), member 14	3.05±0.31
SLC2A3	Solute carrier family 2 (facilitated glucose transporter), member 3	2.53±0.22
ABCC3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	2.37±0.30
RGS2	Regulator of G-protein signaling 2	2.77±0.58
CDKN1C	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	2.21±0.19
PGC-1α	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	2.39±0.22
CYP3A5	Cytochrome P450, family 3, subfamily A, polypeptide 5	2.30±0.22
ESR1	Estrogen receptor 1	2.05±0.19
EPHA2	EPH receptor A2	2.10±0.20
CYP3A7	Cytochrome P450, family 3, subfamily A, polypeptide 7	2.41±0.41
SLC7A11	Solute carrier family 7 (anionic amino acid transporter light chain, xc-system), member 11	1.88±0.16
SLC6A6	Solute carrier family 6 (neurotransmitter transporter), member 6	1.94±0.07
CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1	1.73±0.09
SPRY1	Sprouty homolog 1, antagonist of FGF signaling (Drosophila)	1.86±0.40
PDK4	Pyruvate dehydrogenase kinase, isozyme 4	2.01±0.34
GCLC	Glutamate-cysteine ligase, catalytic subunit	1.71±0.06
JUNB	Jun B proto-oncogene	1.92±0.27
HBEGF	Heparin-binding EGF-like growth factor	1.61±0.07
SLC7A5	Solute carrier family 7 (amino acid transporter light chain, l system), member 5	1.75±0.06
SLC39A8	Solute carrier family 39 (zinc transporter), member 8	1.76±0.12
PPARA	Peroxisome proliferator-activated receptor alpha	1.71±0.12
LRRC8A	leucine rich repeat containing 8 family, member A	1.56±0.06
PPP1R14C	Protein phosphatase 1, regulatory (inhibitor) subunit 14C	1.70±0.10
ABCC2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	2.11±0.78

B, Apoptosis (upregulation)

Gene symbol	Gene name	Fold change
IRF7	Interferon regulatory factor 7	2.97±0.64
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	2.40±0.88
PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1	2.28±0.20
BCL2L11	Bcl2-like 11 (apoptosis facilitator)	2.24±0.28
APAF1	Apoptotic peptidase activating factor 1	2.15±0.13
BBC3	Bcl2 binding component 3	2.10±0.11
CASP1	Caspase 1, apoptosis-related cysteine peptidase	2.02±0.13
CASP3	Caspase 3, apoptosis-related cysteine peptidase	1.81±0.15
CASP4	Caspase 4, apoptosis-related cysteine peptidase	1.72±0.07
TNFRSF21	Tumor necrosis factor receptor superfamily, member 21	1.68±0.16
HRK	Harakiri, Bcl2interacting protein	1.59±0.08
FAS	Fas cell surface death receptor	1.53±0.04

C, Cell cycle (downregulation)

Gene symbol	Gene name	Fold change
CDC25C	Cell division cycle 25C	-2.21±0.55
E2F1	E2F transcription factor 1	-1.79±0.27
PKMYT1	Protein kinase, membrane associated tyrosine/threonine 1	-1.76±0.20

Table I. Continued.

C, Cell cycle (downregulation)		
Gene symbol	Gene name	Fold change
RBL1	Retinoblastoma-like 1	-1.75±0.17
ORC5	Origin recognition complex, subunit 5	-1.72±0.02
MCM6	Minichromosome maintenance complex component 6	-1.72±0.10
CCNE2	Cyclin E2	-1.63±0.10
CCNB1	Cyclin B1	-1.58±0.09
PCNA	Proliferating cell nuclear antigen	-1.56±0.05

Data are presented as the mean of fold change ± standard deviation.

'G_1 to S cell cycle control', and 'DNA Replication' (Fig. 2B). Microarray analysis revealed that the expression of 25 genes belonging to the 'Nuclear Receptors Meta-Pathway', such as *CDKN1C* and *PGC-1α* and 12 apoptosis-related genes, such as *CASP3* and *FAS*, were upregulated. A total of nine cell cycle-related genes, including *CDC25C*, *CCNE2* and *CCNB1* were downregulated (Table I). RT-qPCR validated the aforementioned findings (Fig. 3).

Reduction of cell proliferation and induction of TIG113 apoptosis cells by ERRα silencing. As silencing of *ERRα* downregulates cell cycle-related genes and upregulates apoptosis-related genes in fibroblasts, cell proliferation and apoptosis analyses were performed. *ERRα* was silenced in TIG113 cells and cell proliferation was evaluated every 24 h. Cells continued to proliferate for up to 72 h in siNC. By contrast, in *ERRα*-silenced TIG113 cells, cell proliferation was significantly reduced after 24 h and the difference in cell proliferation increased after 48 and 72 h, suggesting that cell proliferation was suppressed in *ERRα*-silenced TIG113 (Fig. 4A). Furthermore, in the scratch wound healing assay, the migration percentage of TIG113 cells treated with siNC was 68.9%, whereas it decreased to 32.8% with siERRa (Fig. 4B and C).

As silencing of ERRα increased the expression of apoptosis-related genes (Table I and Fig. 3), whether apoptosis was induced was examined. The activity of poly caspase, an apoptosis induction-related enzyme, increased ~2-fold 72 h after transfection with siERRα (Fig. 4D). These results suggest that apoptosis was induced in *ERRα*-silenced TIG113 cells.

Silencing ERRα causes cell cycle arrest in TIG113 cells. TIG113 cells were treated with siERRα for 72 h prior to cell cycle analysis. The siERRα treatment significantly increased the proportion of Sub-G_1 phase cells and decreased the proportion of S and G_2/M phase cells (Fig. 5A and B).

Quantification of type I collagen and hyaluronan. ELISA revealed that the amount of type I collagen produced by TIG113 cells was significantly decreased after transfection with siERRα for 72 h (Fig. S2A). Similarly, the amount of hyaluronic acid was significantly decreased after transfection with siERRα (Fig. S2B).

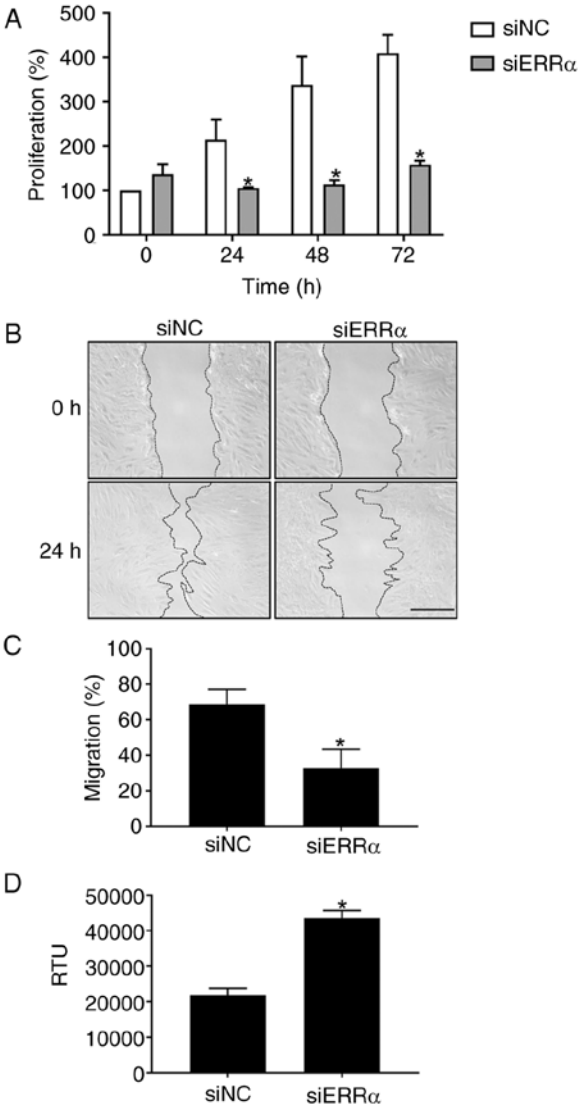

Figure 4. Effect of ERRα knockdown on proliferation and apoptosis in TIG113 cells. TIG113 cells were transfected with siERRα or siNC and cultured for 24-72 h. (A) Proliferation of TIG113 cells was determined every 24 h following siERRα transfection. (B) Following transfection of siRNA for 24 h, it was scratch-wounded and cultured for 48 h. Scale bar, 500 μm. (C) Migration area was calculated using the ImageJ software. (D) Apoptosis of TIG113 cells was determined 72 h after siERRα transfection. The data represent the mean ± standard deviation of at least three independent experiments. *P<0.05 vs. siNC. ERRα, estrogen-related receptor α; si, small interfering; NC, negative control; RFU, relative fluorescent unit.

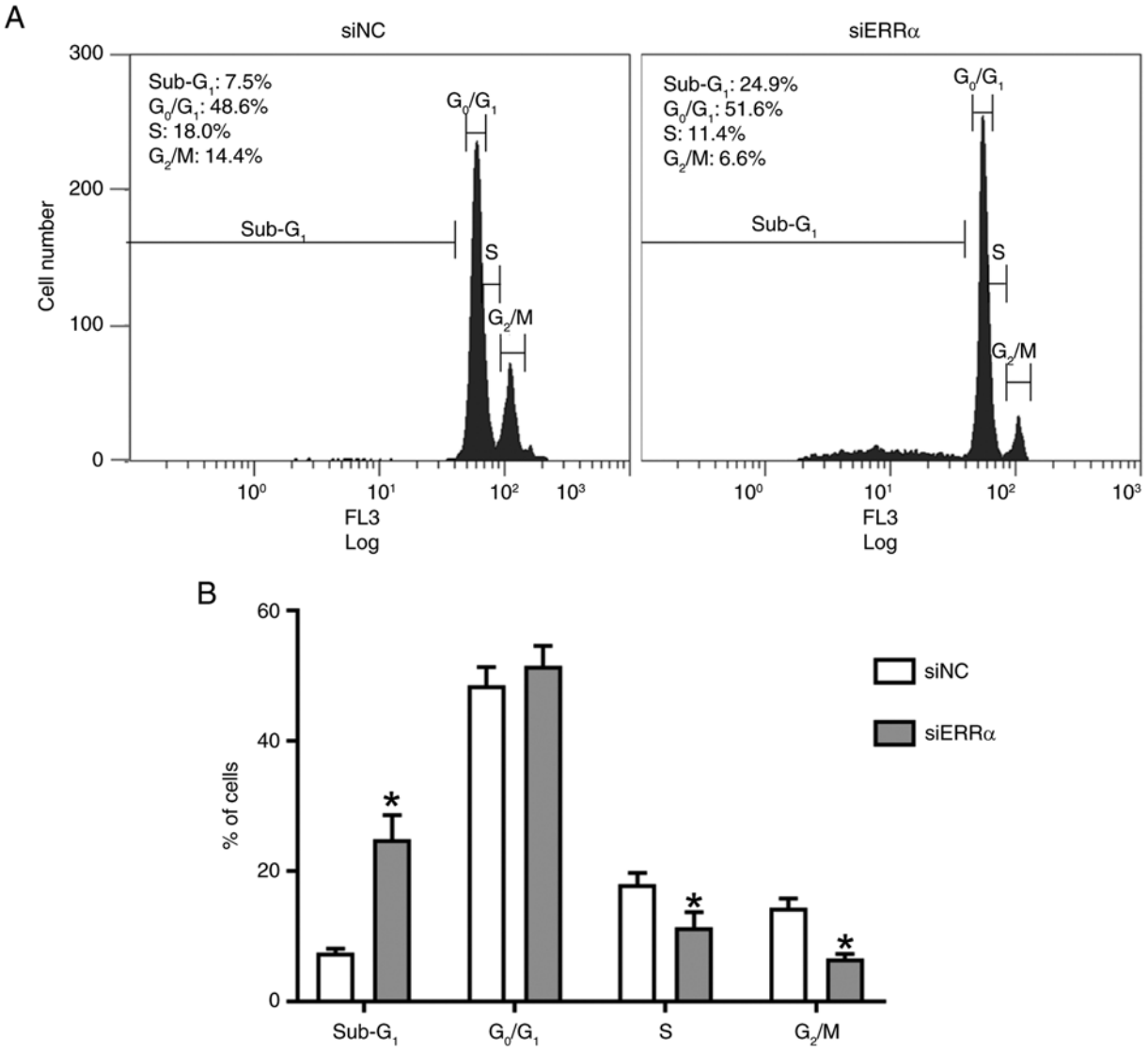

Figure 5. Effect of ERRα silencing on the cell cycle in TIG113 cells. Cell cycle analysis in TIG113 cells treated with siERRα or siNC for 72 h prior to DNA staining and fluorescence-activated cell sorting. (A) Cell cycle plots. (B) Percentages of cells in the Sub-G_1, G_0/G_1, S and G_2/M phases. Data were analyzed using the MultiCycle AV software and represent the mean ± standard deviation of the mean of three independent experiments. *P<0.05 vs. siNC. ERRα, estrogen-related receptor α; si, small interfering; NC, negative control.

Discussion

ERRα is expressed in skin tissue, but its function is unknown. In the present study, *ERRα* was silenced by siRNA in human skin fibroblasts and its function was analyzed. ERRα, ERRβ and ERRγ are expressed in keratinocytes of the skin epidermis and it has been reported that only ERRγ is expressed in fibroblasts (15-17). However, in the present study, the expression levels of *ERRα* in siNC-treated TIG113 cells in microarrays was higher than that of *ERRβ* and γ. As the present study mainly aimed to clarify the function of ERRα in TIG113 cells, the comparison of the expression of ERRα, β and γ, as well as the estrogen receptor, was only a supplementary analysis and thus absolute quantitative expression analysis was not performed. However, detailed analysis using absolute quantification is required to compare the expression levels of ERRs and estrogen receptors α and β in the future. Furthermore, because only a few studies reported expression of ERRs in skin tissues and cells, further analysis with more specimens is required.

Silencing of *ERRα* decreased the expression of cell cycle-related genes such as *CDC25C*, *CCNE2* and *CCNB1*. CDC25C is known to control the transition from the G_1 phase to the S phase and the transition from the G_2 phase to the M phase (29). In addition, cyclin E binds to cyclin-dependent kinase 2 in the G_1 phase to form a complex that is required for the cell cycle transition from the G_1 phase to the S phase where DNA replication is initiated (30) and CCNB1 is a regulatory protein involved in mitosis (31). Furthermore, silencing of ERRα increases the expression of *CDKN1C*, a known cell cycle inhibitor (32). Expression of these genes related to the cell cycle was decreased and cell proliferation was suppressed in ERRα-silenced TIG113, suggesting that a normal cell cycle did not occur. Cell cycle analysis showed that siERRα knockdown decreased the number of cells in the S and G_2/M phases. ERRα regulates *CDC25C* and *CCNB1* in gastric cancer cells, suggesting that it also regulates these genes in fibroblasts (33).

Silencing of *ERRα* enhanced apoptosis and the expression of apoptosis induction-related genes such as *CASP3*

and *FAS*. Furthermore, Sub-G_1 phase cells were increased in siERRα-treated TIG113 cells. An increase in the Sub-G_1 phase was observed in apoptotic cells (34), suggesting that apoptosis was induced by siERR treatment. Caspases are a family of proteases that play central roles in numerous processes, including cell death and inflammation and *CASP3* is an important mediator of apoptosis (35). *FAS* is a type I transmembrane protein and apoptosis is induced upon binding of the Fas ligand (36,37). The results of the present study suggested that increased expression of these apoptosis-related genes induce cell death in ERRα-silenced TIG113 cells. The *p53* gene encodes a protein that has the function of regulating suppression of the cell growth cycle such as DNA repair, cell growth arrest and apoptosis (38). It has recently been reported that ERRα and p53 protein directly bind to regulate colon cancer growth through regulation of mitochondrial biogenesis and that knockdown of ERRα suppresses *p53* gene expression and impairs mitochondrial biogenesis (39). Although no change was observed in the expression level of *p53* in this study (data not shown), it is possible that silencing of *ERRα* abolished its interaction with p53 and reduced mitochondrial biogenesis. ERRα contributes to the proliferation of some cancer cells and knockdown of ERRα reduces cell proliferation and induces apoptosis (40-42), consistent with the results of the present study. This suggested that ERRα also contributes to cell proliferation in normal skin fibroblasts.

The PGC-1 family includes PGC-1α, PGC-1β and PGC-1-related coactivators, which regulate mitochondrial biogenesis (43). PGC-1α induces ERRα expression and interacts with ERRα (44) and the ERRα/PGC-1α axis is known to decrease with aging, accelerating osteoporosis, kidney dysfunction, sarcopenia and neurodegeneration (7). Furthermore, the expression of PGC-1α is enhanced in the myocardium of ERRα-null mice (45), consistent with the findings of the present study. Although the mechanism is not clear, it is possible that the silencing of ERRα in fibroblasts compensates for the enhancement of PGC-1α expression, or that ERRα regulates the expression of PGC-1α.

Thus, knocking down ERRα altered various genes, leading to cell cycle modifications and the induction of apoptosis. However, the present study was unable to identify any genes directly regulated by ERRα. Future research should focus on identifying the direct targets of ERRα.

In the skin, fibroblasts secrete components that contribute to skin antiaging, such as type I collagen and hyaluronan. It was hypothesized that the decrease in cell proliferation was due to a decrease in these components. Furthermore, when TIG113 cells were treated with siERRα, the amount of type I collagen and hyaluronan secreted into the culture supernatant decreased. These results suggested that ERRα may also be an important factor for skin antiaging.

ERRα is an orphan nuclear receptor that can be activated by exogenous agonists such as phytoestrogens such as genistein and daidzein (46), which exhibit estrogenic activity and are found in plants. These compounds share target genes with estrogen receptors and phytoestrogens may activate the ERR pathway, potentially contributing to skin fibroblast proliferation. As estrogen is not an ERR ligand and does not activate ERR, the present study did not investigate the activation of ERRα by estrogen treatment. However, given that various phytoestrogens may act as ligands for ERRα, future research should explore these possibilities to uncover new activators of ERRα.

ERR is expressed in skin tissue, but its function is unknown. The present study found that suppression of ERRα expression using siRNA suppresses cell proliferation and induces apoptosis. As a reduction in skin fibroblasts accelerates skin aging, the discovery of new exogenous ligands for ERRα and activation of ERRα may lead to the development of new skin antiaging treatments.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author. The TIG113 microarray datasets generated in the present study may be found in the Gene Expression Omnibus under accession number GSE245234 or at the following URL: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE245234.

Authors' contributions

NN designed the study. NN, TN, MN, CH and KH performed the experiments and analyzed the data. NN, MN and KH confirm the authenticity of all the raw data. NN and CH wrote the original manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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