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ORIGINAL ARTICLE

Epidermal Growth Factor (EGF)-Like Repeats and Discoidin I-Like Domains 3 (EDIL3): A Potential New Therapeutic Tool for the Treatment of Keloid Scars

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Abstract In keloids, the mechanism underlying the excessive accumulation of extracellular matrix after injury of the skin is unclear, and there is no effective treatment because of the incomplete understanding of their pathogenesis; thus, a high recurrence rate is observed. We studied a new marker of keloids to determine a new treatment strategy. First, the keloid gene expression profile (GSE44270) was analyzed (downloaded from the Gene Expression Omnibus database) and the new keloid marker candidate, epidermal growth factor (EGF)-like repeats and discoidin I-like domains 3 (EDIL3) which were upregulated in keloid samples was identified. Knockdown of EDIL3 is known to suppresses angiogenesis by downregulating relevant inhibitory factors that can limit the supply of survival factors to tumor cells from the circulation via the vascular endothelial cells. In keloids, the mechanism of action of EDIL3 may be similar to that in tumors; the inhibition of apoptosis in tumor cells via a reduction in the apoptosis of blood vessels by upregulating an angiogenic factor. To determine whether EDIL3 is involved in keloid formation, we performed knockdown of EDIL3 in keloid fibroblasts *in vitro* by transfection with anti-EDIL3 small interfering RNA (via microporation). EDIL3 was upregulated in keloid fibroblasts compared with normal fibroblasts in collagen type I, II and III. Our results indicate the control of EDIL3 expression may be a new promising treatment of keloid disease also the molecular targeting of EDIL3 may improve the quality of treatment and reduce the formation of keloids.

Keywords Keloid scar · EDIL3 · Collagen · siRNA · Transfection

Abbreviations

DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
GEO	Gene Expression Omnibus
KFs	Dermal fibroblasts from keloid scar
NFs	Dermal fibroblasts from normal skin
PBS	Phosphate buffered saline
siRNA	Small interfering RNA

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1 Introduction

Wound healing can be roughly subdivided into three stages: inflammatory, growth (proliferation phase), and mature (maturation phase). A keloid is a fibrous tissue that is the result of the irregular excessive accumulation of extracellular matrix components. A keloid can extend around and beyond the size of the site of origin of the original wound and inflammation [1–5]. Pathogenesis of keloid is reported to involve both genetic and environmental factors, but the exact mechanism is unknown [6–8]. Although local tissue factors, particularly tension [7, 9],

wound infection [10], and endocrine factors [11], are known to be involved in keloid formation, the exact mechanism is unknown. As a result of studies on the keloid patients, it became clear that the high incidence of keloids in dark-skinned people is due to genetic factors [8, 12]; among them, the genetic factors are believed to play an important role in keloid formation [13, 14]. Keloids occur mainly in African Americans or Asian people and develop in the damaged skin if a person is genetically predisposed (about 15% of the general population) and the skin is damaged [15]. In these groups, treatments either fail or have a negative impact on the quality of life of the patients: many patients experience physical and psychological discomfort from the treatment, and the recurrence rate is high [14, 16, 17]. Thus, identification of genes involved in keloid formation may provide new insights into the development of treatment for keloids prevention.

Epidermal growth factor-like repeats and discoidin I-like domains 3 (EDIL3) is a glycoprotein that can be connected to the surface of endothelial cells and to the extracellular matrix via an extracellular-matrix protein that is expressed on vascular endothelial cells and macrophage [18]. Leukocytes can be prevented from adhering to the surface of vascular endothelial cells by this anti-inflammatory protein, EDIL3, which acts as an integrin ligand. In addition, in response to inflammatory stimuli, the EDIL3 expression is downregulated and not only involved in cell adhesion and migration but also involved in angiogenesis and cell death. EDIL3 has been reported to be associated with modulation of inflammation [19–21]. Specific expression of EDIL3 has been found in various cancer types, such as colon cancer, hepatocellular carcinoma, bone sarcoma, basal cell carcinoma, and astrocytoma [22–25]. Thus, so far, EDIL3 has been studied primarily in relation to its role in ischemic disease and as a biomarker of cancer [25, 26].

In this study, we aimed to evaluate the association of EDIL3 with keloid formation. To identify a new marker candidate of keloid, we analyzed gene expression using data from the microarray database in Gene Expression Omnibus (GEO) to identify a new marker of keloids [27]. As a result, EDIL3 gene was confirmed, which is upregulated in keloids; it was not yet reported whether there is a fundamental difference in EDIL3 expression between dermal fibroblasts from normal skin (NFs) and dermal fibroblasts from keloid scar (KFs). The exact mechanism of EDIL3 in keloids is not yet fully understood; thus, we hypothesize that EDIL3 promotes proliferation of KFs as well as the production of collagen. After knockdown of EDIL3 gene by small interfering RNA (siRNA), we assessed whether the proliferation of KFs is suppressed and assessed the decrease in collagen production. siRNA can specifically suppress expression of a particular gene, with minimal effects on unrelated factors. It is widely used for the treatment of genetic diseases, cancer, and viral diseases by targeting a specific protein that causes such diseases [28]. For transfection method of siRNA, microporation is performed, which results in high efficiency in delivery without effects on the cells [29–31]. In this study, by regulating EDIL3 expression with siRNA in KFs *in vitro*, we aimed to determine whether this protein can be a new future candidate factor that can be used for inhibition of keloid formation.

2 Materials and methods

2.1 Microarray data and the data source

Gene expression data on nine scars associated with a keloid were downloaded as microarray profiles (GSE44270) from the GEO database (http://www.ncbi.nlm.nih.gov/geo/ accession # GSE44270) [27] (Table 1).

2.2 Cell origin and cell culture

Keloid skin samples (n = 9) and normal skin samples (n = 5) (Table 2) were acquired after obtaining informed consent from all patients [full ethical approval was obtained from Seoul St Mary's Hospital, Republic of Korea (KCMC06BR067)] prior to surgery. KFs were isolated from discarded keloid tissues of patients who underwent a surgical procedure (n = 9). The resulting skin samples were washed with phosphate-buffered saline (PBS; Wisent Inc., Quebec, Canada) and separated into the dermis and epidermis using Dispase II (Roche, Basel, Switzerland) for 2 h at 37 °C in a humidified atmosphere containing 5% CO_2. The collected dermis was digested with 0.1% type II collagenase (Sigma) in a celltibator for 30 min at 37 °C. Primary KFs and NFs were isolated from the tissue samples by enzymatic digestion. KFs were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Wisent Inc.) and 1% antibiotic–antimycotic solution (Gibco, Carlsbad, CA, USA). When the cells reached 90% confluence, KFs were passaged after trypsinization and used for analysis at passages 3–5.

2.3 Total-RNA extraction and reverse transcription-polymerase chain reaction (RT–PCR)

Total cellular RNA was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and examined for purity and concentration using a photometer (NanoDrop ND). 1 μg RNA

Table 1 Genes differentially expressed fold change in KFs vs. NFs

Symbol	Gene name	KFs/NFs
POSTN	Periostin, osteoblast specific factor	7.96
RGS4	Regulator of G protein signaling 4	5.80
EDIL3	EGF-like repeats and discoidin I-like domains 3	5.77

KFs: dermal fibroblasts from keloid scar, NFs: dermal fibroblasts from normal skin

Table 2 Profile of each sample for primary culture

Patient	Gender	Age (years)	Biopsy site
Normal			
NFs 1	Female	55	Leg
NFs 2	Female	29	Earlobe
NFs 3	Male	22	Earlobe
NFs 4	Male	36	Abdomen
NFs 5	Female	50	Abdomen
Keloid			
KFs 1	Male	26	Occipital
KFs 2	Female	21	Earlobe
KFs 3	Female	28	Earlobe
KFs 4	Male	22	Earlobe
KFs 5	Female	27	Umbilicus
KFs 6	Female	20	Earlobe
KFs 7	Female	21	Earlobe
KFs 8	Female	29	Earlobe
KFs 9	Male	16	Earlobe

NFs: dermal fibroblasts from normal skin, KFs: dermal fibroblasts from keloid scar

and primers (CycleScript RT PreMix, Bioneer, South Korea) were utilized for complimentary DNA (cDNA) synthesis (cDNA synthesis kit, Bioneer). cDNA was obtained by reverse transcription of 1 mg of total RNA from each sample using 200 U of M-MLV Reverse Transcriptase (Invitrogen). The target genes that we analyzed were EDIL3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was an internal control. PCR was performed in a total volume of 20 μl; the reaction mixture contained a 1-μg sample of cDNA, each primer at 10 pμ, distilled water, and the AccuPower PCR PreMix (Bioneer). The amplification conditions were as follows: initial denaturation at 94 °C for 2 min; then 40 cycles of 94 °C for 30 s, 58 °C for 10 s, and 72 °C for 30 s; and termination by a final extension at 72 °C for 7 min. The PCR products were loaded onto 1% agarose gel for electrophoresis, and the mRNA data from each target gene were normalized to GAPDH gene. Expression of each gene was quantified by means of Gel Doc (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and Multi Gauge software (Fujifilm, Tokyo, Japan). The RT–PCR analysis was conducted in triplicate.

2.4 DNA fragment purification and sequence analysis

PCR products were visualized on 1% agarose gel in 1× TAE buffer. After running, the separated DNA was visualized on a UV transilluminator, and the desired bands were excised from the gel. DNA was extracted using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI) protocol according to the manufacturer's instructions. DNA was placed in a 1.5 ml microcentrifuge tube, vortexed, and incubated at 50 °C–65 °C until the gel slice was completely dissolved. The supernatant was discarded, and the pellet was resuspended in the volume of the buffer specified for each protocol. Each extract was brought to a final volume of 40 μl in molecular biology water, and the purity of DNA was measured with NanoDrop ND-1000 spectrophotometer by calculating the ratio of absorbance at 260 and 280 nm. Samples were stored at 4 °C until analysis and then sequenced in both directions using universal forward and reverse primers at Cosmogenetech (Korea).

2.5 Knockdown of EDIL3 by siRNA using microporation transfection

Transfection was performed as described previously [32]. An EGFP expression vector, pEGFP-N1, was purchased from Clontech (BD Bioscience, Palo Alto, CA, USA). For electroporation, cells were either trypsinized and resuspended in PBS (Wisent Inc., Quebec, Canada) with ECM 830 (BTX Inc., San Diego, CA, USA) or in resuspension buffer with the MicroPulser™ and Nucleofector®; 2×10^5 cells were used for each electroporation, and 2 μg of DNA in PBS was mixed with the cell suspension and electroporated into the cells, as described previously. For microporation, DNA was mixed with 10 μl of resuspension buffer. Then, 2×10^5 cells were electroporated under preoptimized square pulse conditions (1300 V, 30 ms, and one pulse). The electroporated cells were incubated in 500 μl of DMEM (Gibco) supplemented with 10% of FBS (Wisent) without antibiotics for 24 h, and then the medium was replaced with the same medium containing antibiotics. The cells were harvested with extraction buffer after transfection for 96 h.

Table 3 Nucleotide sequences of primer sets used for GAPDH, EDIL3, type I collagen (Col I), type II collagen (Col II) and type III collagen (Col III) in RT–PCR analysis

Gene	Human primer sequence
GAPDH	Sense 5′-GCCAAAGGGTCATCATCTCTG-3′
	Antisense 5′-CATGCCAGTGAGCTTCCCGT-3′
EDIL3	Sense 5′-GTGAACTGTCGGGTTGTTCTGAG-3′
	Antisense 5′-GGTTCCCAAGTGAACATGTCCAT-3′
siEDIL3-1	Sense 5′-ACAUGACUGCCUAUCAGUA-3′
	Antisense 5′-UACUGAUAGGCAGUCAUGU-3′
siEDIL3-2	Sense 5′-CUCAGUAUGUAAGACUCUA-3′
	Antisense 5′-UAGAGUCUUACAUACUGAG-3′
Type I collagen (Col I)	Sense 5′-CGAAGACATCCCACCAATCAC-3′
	Antisense 5′-GATCGCACAACACCTTGCC-3′
Type II collagen (Col II)	Sense 5′-ACCTCTAGGGCCAGAAGGAC-3′
	Antisense 5′-GTGACAAAGGAGAGGCTGGA-3′
Type III collagen (Col III)	Sense 5′-AAAGGGGAGCTGGCTACTTC-3′
	Antisense 5′-GCGAGTAGGAGCAGTTGGAG-3′

GAPDH glyceraldehyde 3-phosphate dehydrogenase, *EDIL3* epidermal growth factor-like repeats and discoidin I-like domains 3, *siEDIL3* small interfering epidermal growth factor-like repeats and discoidin I-like domains 3

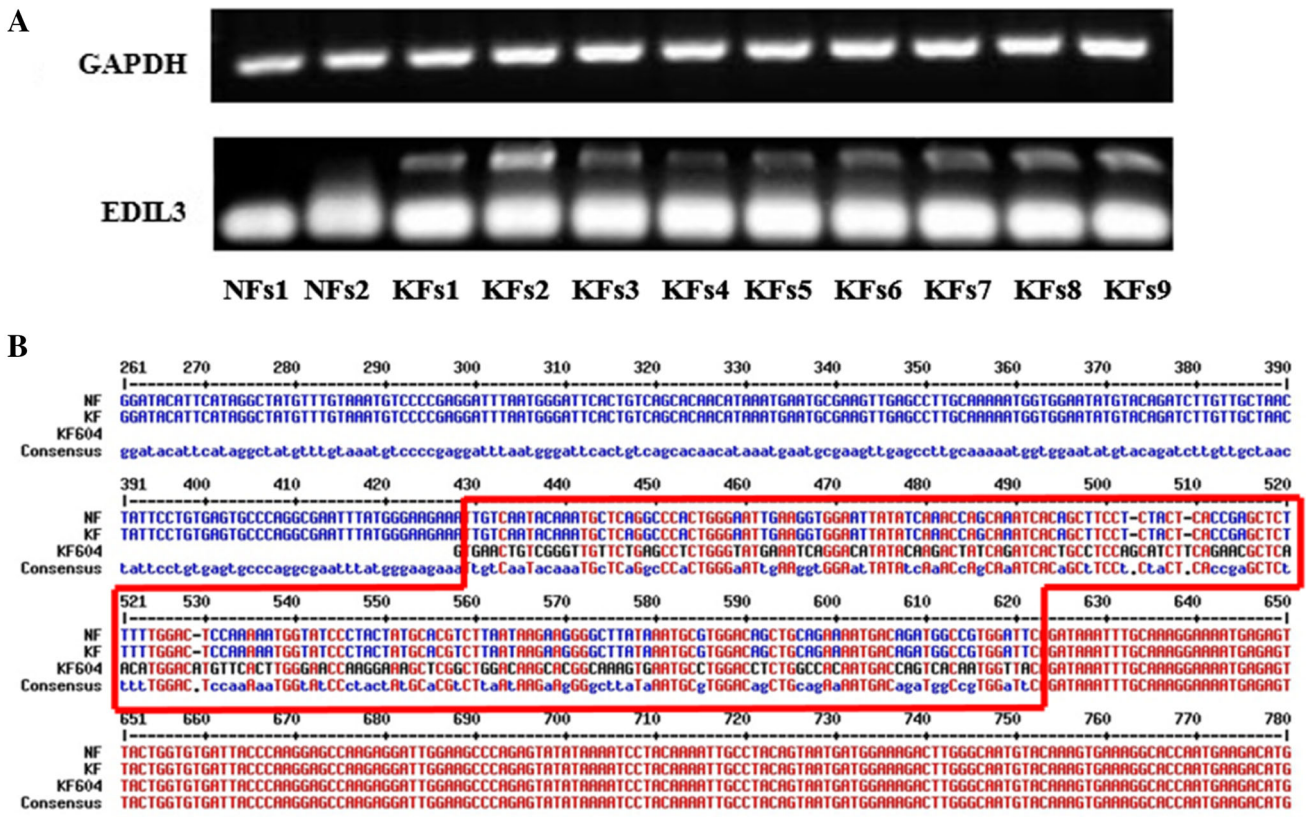

Fig. 1 The difference of EDIL3 variation 2 in NFs and KFs. (**A**) The difference of EDIL3 expression between NFs and KFs according to RT–PCR results; we confirmed the specific band. (**B**) The genomic sequence of the open reading frame region in the EDIL3 gene (Gene Bank accession number NM_001278642). The red box indicates a part of the occurring transcript variant. EDIL3: epidermal growth factor-like repeats and discoidin I-like domains 3, NFs: dermal fibroblasts from normal skin, KFs: dermal fibroblasts from keloid scar

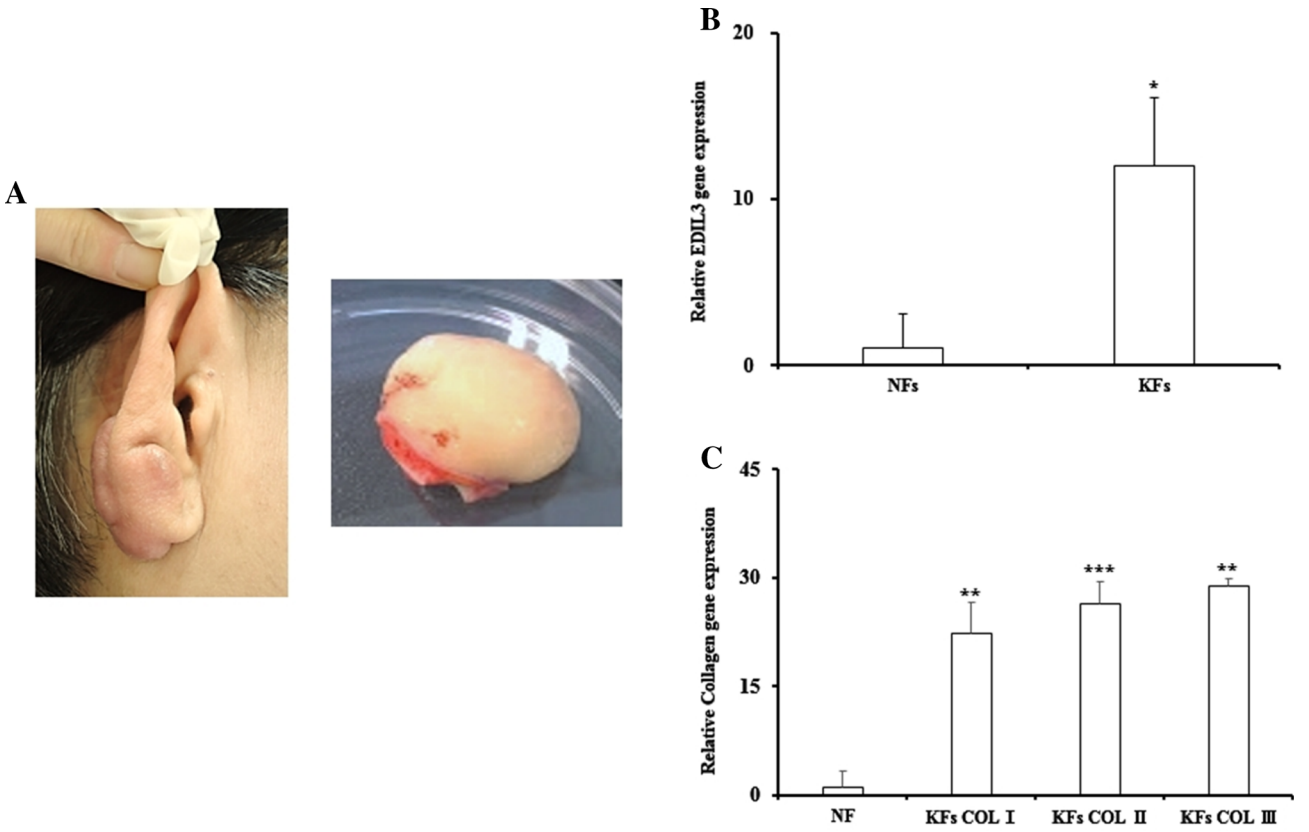

Fig. 2 Clinical appearance of the keloid of patient 3 and the mRNA expression of EDIL3 and type I, type II and type III collagens in NFs and KFs. **A** The keloid tissue on the earlobe of a 28 years old female was firm and hard like accumulation of cartilaginous tissue (patient 3 in Table 2). **B** EDIL3 mRNA levels were measured by real-time quantitative RT–PCR; there was an 11-fold difference in mRNA expression between NFs and KFs. **C** Differences between NFs and KFs in terms of mRNA expression of type I and type II collagens according to the real-time quantitative RT–PCR. These data show statistically significant differences according to Student's t-test (means ± SD, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). EDIL3: epidermal growth factor-like repeats and discoidin I-like domains 3, NFs: dermal fibroblasts from normal skin (n = 5), KFs: dermal fibroblasts from keloid scar (n = 9)

2.6 Quantitative real-time RT–PCR

Total RNA was prepared using the TRIzol® reagent (Invitrogen) according to the manufacturer's instructions, and purity of the extracted RNA was calculated from the data on optical density at 260 and 280 nm. cDNA was prepared by reverse transcription of 1 mg of total RNA from each sample using M-MLV Reverse Transcriptase (Invitrogen). Real-time PCR was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA). The final PCR mixture contained 0.5 µl each of the forward primer and reverse primer (final concentration of each: 500 nM) (Table 3), 25 µl of the Power SYBR Green PCR Master Mix (Applied Biosystems), and 1µl of the sample (equivalent to 50 ng of RNA). Real-time PCR was performed on an ABI Prism 7900 HT instrument (Applied Biosystems), and universal cycling conditions were used (2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C). Threshold cycle values were determined by automated threshold analysis in the ABI Prism software, version 1.0 (Applied Biosystems). Transcription of the target gene was analyzed by the $^{\Delta\Delta}$ threshold cycle method ($2^{-\Delta\Delta}$CT) to determine the fold changes.

2.7 Western blotting

After incubation, cells were treated under the appropriate conditions, washed with ice-cold PBS, and lysed in lysis buffer. Total protein was extracted by means of T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Rockford, IL, USA) containing 1% of a protease inhibitor cocktail (Sigma-Aldrich). Protein concentration was measured with a Quick Start Bradford protein assay kit (Bio-Rad Laboratories, Inc.). The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 10% gel and transferred onto nitrocellulose blotting membranes (Amersham, Buckinghamshire, United Kingdom). After blocking with 5% skim milk in Tris-buffered saline containing 0.1% of Tween 20 for 1 h, we incubated the membranes with primary antibodies against

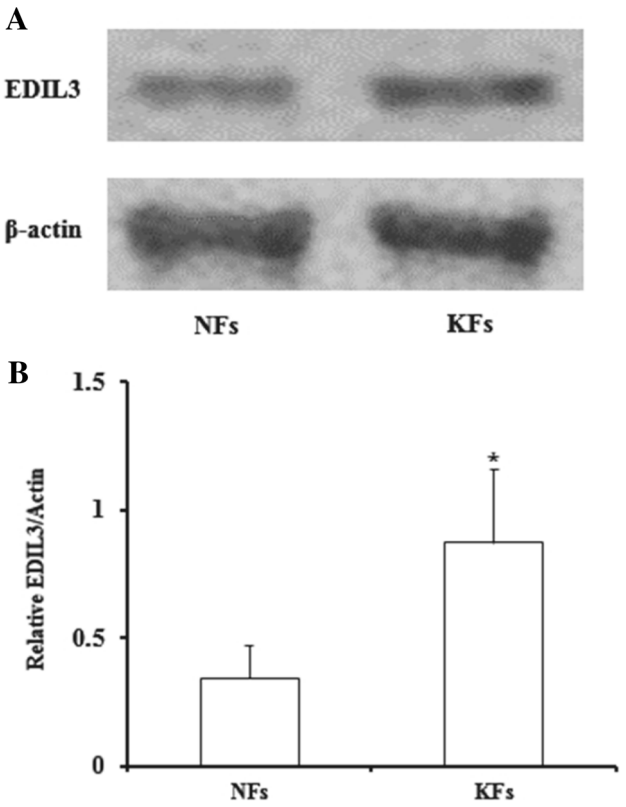

Fig. 3 The differences in EDIL3 protein expression between NFs and KFs. **A** EDIL3 protein expression detected in NFs and KFs by Western blot. **B** Quantitative analysis indicates the EDIL3 protein expression in NFs and KFs. We confirmed an approximately 2.5-fold difference in the protein expression (*$p < 0.05$). All data were normalized to the β-actin. EDIL3: epidermal growth factor-like repeats and discoidin I-like domains 3, NFs: dermal fibroblasts from normal skin (n = 6), KFs: dermal fibroblasts from keloid scar (n = 6)

EDIL3 (1:500, Abcam) and β-actin (1:5000, Sigma-Aldrich). The membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody for 2 h. After washing, we analyzed the membranes using the horseradish peroxidase substrate-enhanced chemiluminescence detection kit (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom) and quantified the signals using LAS 4000 (Fujifilm) and Multi Gauge software (Fujifilm). The Western blot analysis was performed in triplicate.

2.8 Proliferation assays

The proliferation of KFs and NFs was measured with the solution consisting 10% Cell Counting Kit-8 (Dojindo, Kumamoto, Japan), and the cells were incubated in DMEM containing 10% FBS and 1% antibiotic–antimycotic solution. KFs and NFs culture media were replaced with the Cell Counting Kit-8 solution and incubated for 2 h at 37 °C and 5% CO_2, and the supernatant was analyzed by an enzyme-linked immunosorbent assay on the plate reader at 450 nm (Spectramax PLUS384, Molecular Devices, Sunnyvale, CA, USA). The optical density of each well was calculated by means of the relative KFs standard curve. KFs proliferation was documented by photographing the cells under an inverted microscope (Olympus). The proliferation analysis was conducted in triplicate.

2.9 Statistics

Each experiment was independently performed three times. The data were expressed as a mean ± standard error of the mean. Statistical analysis was performed by Student's t-test to determine differences among the groups. Differences with $p < 0.05$ were considered statistically significant.

3 Results

3.1 Microarray analysis of the genes differentially expressed in KFs and NFs; identification of possible target genes

We analyzed the data on the microarray profiles of cellular gene expression from nine keloid samples and three normal skin samples [27] RNA expression of EDIL3 in keloid tissue was upregulated 5.7-fold compared to the healthy skin samples; thus, EDIL3 was selected as a valid candidate gene (Table 1).

3.2 Characteristics of EDIL3 compared between KFs and NFs

In order to verify the validity of the microarray data, we assessed EDIL3 expression in NFs and KFs by RT–PCR. NFs, as shown in Fig. 1A, showed an EDIL3 band, but EDIL3 mRNA was strongly expressed only in KFs samples; only two bands were selected for confirmation. We performed cloning and sequencing after genomic DNA extraction of the band. As a result of testing the similarity of the sequences using MultAlign and NCBI BLAST of specifically detected bands from NFs and KFs, we confirmed that the EDIL3 expressed in keloids was EDIL3 variation 2 (Gene Bank accession number: NM_001278642). These results suggest that EDIL3 variation 2 expressions were different between NFs and KFs (Fig. 1B).

3.3 EDIL3 expression and collagen expression were increased in KFs according to real-time RT–PCR

Results of RT–PCR were once again confirmed by real-time RT–PCR (to more accurately quantify differences in bands) and showed higher EDIL3 expression in cultured KFs than in NFs. Samples of mRNA were extracted from

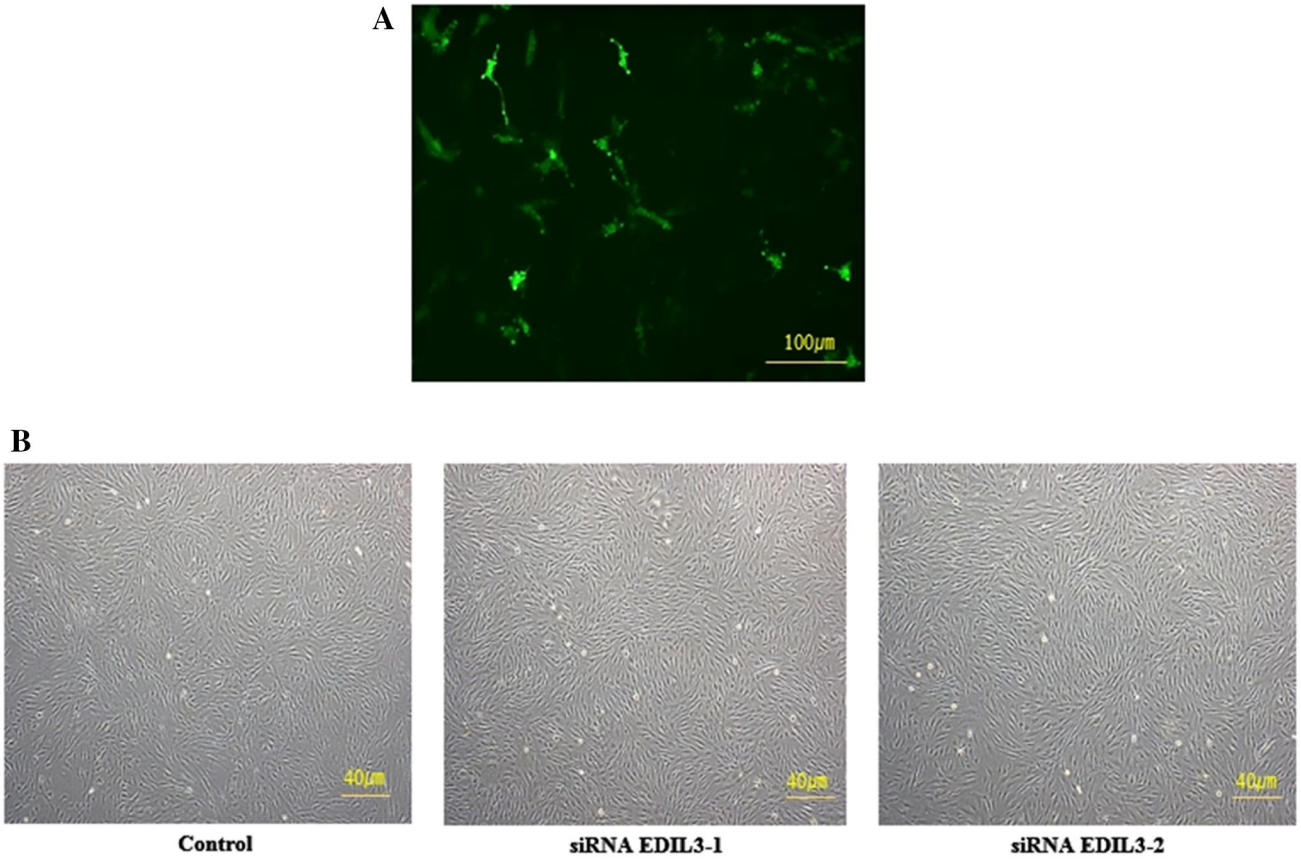

Fig. 4 Transfection efficiency in KFs. **A** pEGFP-vector was transfected in KFs by microporator. The transfection efficiency was verified by quantifying the expression of EGFP by fluorescence microscopy (×100 magnification). **B** The KFs morphology was confirmed after transfection of siRNA EDIL3 by microporation (×40 magnification). EDIL3: epidermal growth factor-like repeats and discoidin I-like domains 3, siRNA: small interfering RNA, KFs: dermal fibroblasts from keloid scar

KFs and NFs from the same patient (patient 3 in Table 2) with a clinically typical keloid on the earlobe (Fig. 2A). Keloid is different from typical scar tissue as it is harder and composed of many different kind of tissue including cartilaginous tissue which is made of type II collagens. The normal tissue was obtained from the normal skin, and the results were confirmed using real-time quantitative RT–PCR. EDIL3 was overexpressed in KFs by 11-fold compared with NFs (Fig. 2B). In addition, type I, type II and type III collagens, which are typically overexpressed in keloids, showed 22-fold, 26-fold and 28-fold overexpression, respectively, in KFs compared with NFs (Fig. 2C). All cells were used at passages 2–4, and the data were analyzed statistically for three independent experiments at the time of measurement. These differences were statistically significant ($*p < 0.05$).

3.4 Western blot analysis of protein expression in NFs and KFs

To quantify the protein level of EDIL3 in KFs, we used Western blotting after extracting total protein from five of the six samples of NFs and KFs. As expected, overexpression of EDIL3 was confirmed in KFs compared with NFs (Fig. 3A). The expression difference was approximately 2.5-fold (Fig. 3B) and was statistically significant ($*p < 0.05$).

3.5 Viability and KFs function were not impaired after transfection

To evaluate the efficiency of EDIL3 siRNA by microporator, we measured EGFP expression by fluorescence microscopy. The EDIL3 siRNA knockdown was performed by means of a microporaton in the conditions of 1300 V, 30 ms, and one pulse. Figure 4 shows that siRNA transfection using microporation methods was efficient to knockdown EDIL3 (no. 8, 1300 V, 30 pulse width, and one pulse). As a result of examination of the cell state after the transfection of KFs, we did not observe any differences in morphology, viability, and confluence between the transfected and control cells. Thus, it was confirmed under a microscope that transfection by microporation does not exert any visibly negative effects on KFs.

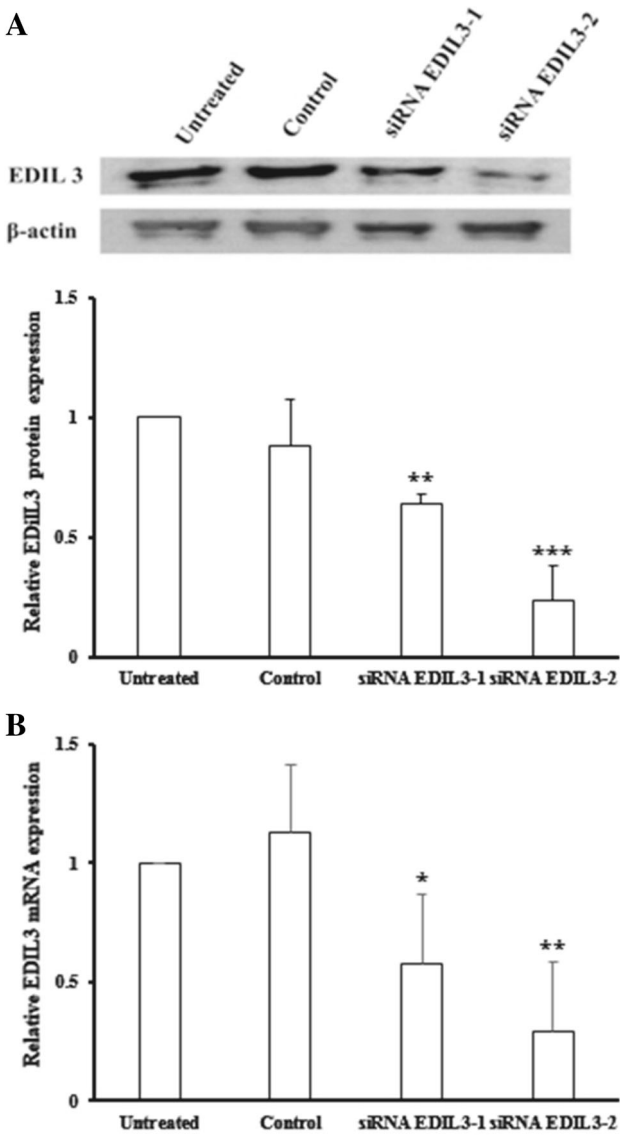

Fig. 5 The small interfering RNA (siRNA) significantly reduces EDIL3 expression of both mRNA and protein in KFs. **A** Western blot analysis of EDIL3 in KFs transfected with siRNA. **B** Results of a real-time quantitative RT–PCR confirmed the knockdown. Expression analysis of EDIL3 mRNA in KFs. The small interfering RNA (siRNA) siEDIL3-1 (or siEDIL3-2) was transfected into the cells by microporation. 96 hours after the transfection, total protein and total mRNA were extracted and probed for EDIL3 expression to confirm that the knockdown was effective (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). KFs: dermal fibroblasts from keloid scar (n = 5)

3.6 Suppression of EDIL3 expression by siRNA

We analyzed EDIL3 expression after the siRNA knockdown in KFs. The anti-EDIL3 siRNA named siEDIL3-1 (or siEDIL3-2) was transfected into the cells by microporation. For 24 h after the transfection, the cells were cultured in antibiotic–antimycotic-free DMEM without FBS; then, 10% FBS and 1% antibiotic–antimycotic solution were added to DMEM (the whole medium was replaced). 96 h after the transfection with the anti-EDIL3 siRNA, it was confirmed that the knockdown was effective both at the protein and mRNA levels. Compared with the results in controls, the Western blot analysis showed that siEDIL3-1 and siEDIL3-2 decreased EDIL3 expression (separately); thus, we confirmed a reliable knockdown of the EDIL3 protein (Fig. 5A). After transfection of siRNA, the Western blot for EDIL3 showed a reduction in the protein level by 27% (for siEDIL3-1) compared with the control, and siEDIL3-2 caused a 72% reduction. Analysis of the EDIL3 mRNA level by real-time quantitative RT–PCR showed that siEDIL3-1 caused a 66% reduction, whereas the siRNA EDIL3-2 resulted in approximately 74% downregulation (Fig. 5B). The data from three independent experiments were statistically analyzed at the time of measurement, and the differences were statistically significant (*$p < 0.05$).

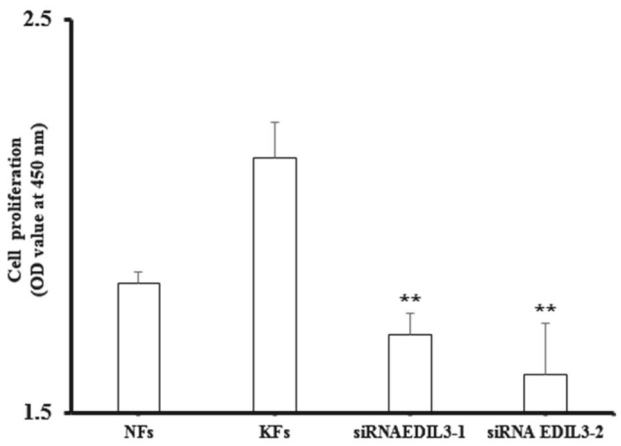

Fig. 6 Inhibition of EDIL3 decreases cell proliferation. The NFs, KFs, and knockdown siEDIL3-1 and siEDIL3-2 cell proliferation was analyzed using CCK-8 assay (**$p < 0.01$) after 7 days. The data are presented as a mean ± standard error of the mean of the ratio of light absorbance at 450 nm. NFs: dermal fibroblasts from normal skin (n = 5), KFs: dermal fibroblasts from keloid scar (n = 5)

3.7 Suppression of cell proliferation in KFs transfected with siEDIL3-1 or siEDIL3-2

We harvested the cells 96 h after the transfection in 96-well plates (in triplicate) and seeded the cells for a 7-day proliferation assay (CCK-8 assay). We confirmed that the EDIL3 knockdown with siRNA produced a 1.1-fold change in the growth rate of NFs; in contrast, siRNA siEDIL3-1 reduced the proliferation rate by 52% and siEDIL3-2 by 74% in KFs (Fig. 6). The data from three independent experiments were statistically analyzed at the time of measurement, and the differences were statistically significant (*$p < 0.05$).

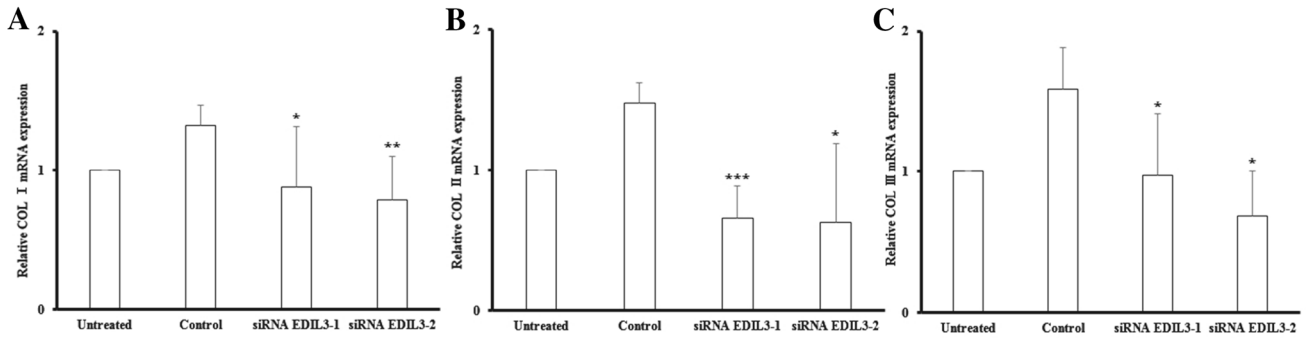

Fig. 7 The mRNA expression of type I, type II and type III collagens under the influence of anti-EDIL3 siRNA according to real-time quantitative RT–PCR. Type I, type II and type III collagens mRNA expressions were calculated as type I, type II and type III collagens mRNA/GAPDH mRNA. KFs after the knockdown of EDIL3 were cultured in 6-well plates 96 h after transfection of siRNA. This knockdown downregulated both type I, type II and type III collagens. **A** Type I collagen mRNA levels were measured by real-time quantitative RT–PCR. **B** Type II collagen mRNA levels were measured by real-time quantitative RT–PCR. **C** Type III collagen mRNA levels were measured by real-time quantitative RT–PCR (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). KFs: dermal fibroblasts from keloid scar (n = 5)

3.8 Suppression of EDIL3 by siRNA downregulates type I and type II collagens deposited by KFs

The collagen expression in KFs which is the result of EDIL3 knockdown by siEDIL3-2 (during 96 h after transfection) was assessed at different time points; we extracted mRNA and analyzed it using quantitative RT–PCR. In several studies, excessive accumulation of type I and type III collagen has been reported in keloids. Because type I, type II and type III collagen has not yet been studied in keloids with different EDIL3 levels (EDIL3 interacts with the αvβ3 integrin), and because EDIL3 expression regulation or influence on type I, type II and type III collagen in KFs has not been studied either, we explored these relations. The mRNA expression of type I collagen was reduced by siEDIL3-1 by 32%; siEDIL3-2 produced a more significant reduction of 40% (Fig. 7A). The mRNA expression of type II collagen was reduced by siEDIL3-1 by 55%, whereas siEDIL3-2 reduced it by 57% (Fig. 7B). The mRNA expression of type III collagen was reduced by siEDIL3-1 by 40%; siEDIL3-2 produced a more significant reduction of 57% (Fig. 7C). The data from three independent experiments were statistically analyzed at the time of measurement, and the differences were statistically significant (*$p < 0.05$).

4 Discussion

In this study, we identified genes, whose mRNA expression levels are upregulated in keloids, on the basis of our analysis of microarray data, allowing researchers to simultaneously measure the expression of thousands of genes [27]. Gene expression in keloids has been studied previously, but the association studies did not provide any strong proof of EDIL3 being differentially expressed. We demonstrated this phenomenon *in vitro*, showing that this characteristic (upregulation of EDIL3) is consistent among KFs of different origin. We observed a significant increase in both mRNA and protein expression of EDIL3 in KFs compared with that in NFs samples.

EDIL3 is a unique ligand of αvβ3 integrin, which is generated by endothelial cells, and this integrin is a part of a secretion signaling pathway of the cell [21, 23]. In one study, it was reported that EDIL3 is present in embryonic blood vessels; by binding to αvβ3, EDIL3 can modulate apoptosis to facilitate the attachment of endothelial cells [21, 33]. Recently, changes in EDIL3 expression were detected in colon and breast cancer as well as in melanoma [23, 24, 34]. EDIL3 participates in the inhibition of apoptosis in tumor cells *in vivo*, and there is a report showing that EDIL3 promotes tumor growth via a reduction in apoptosis of tumor cell and increase number of blood vessels by upregulating an angiogenic factor that is necessary for tumor formation [23]. By down-regulating EDIL3, suppression of angiogenesis is reported in the process of downregulating relevant inhibitory factors that can limit the supply of survival factors to tumor cells [24].

In the present study, when we reduced EDIL3 expression in keloids, we found that the proliferation of KFs decreased. In keloids, the mechanism of action of EDIL3 may be similar to that in tumors because overexpression of EDIL3 is expected to affect angiogenesis [35].

Therefore, we tested whether regulation of expression of EDIL3 has any effect on type I, type II and type III collagen in KFs. Type I and III collagens are already well-known in its role in keloid formation [36], but also additionally other studies revealed up-regulation of collagen II related chondrogenic gene COMP in keloid [37]. Our study also showed that cell proliferation and type I, type II and III

collagens are downregulated when EDIL3 is downregulated. We reviewed the differences in EDIL3 expression between NFs and KFs in multiple studies and did not find any similar reports. Furthermore, the difference in the expression of type II collagen between KFs and NFs was not reported previously either. As type II collagen is expected to play an important role in the progression of keloids; therefore, it would be important to study type II collagen in the future as a therapeutic target, particularly using a microporator as a newly designed electroporator [38].

The transfection method involving microporation is such an efficient approach that transfection can be performed under optimal conditions with minimal damage and toxicity to the cells [29–31, 39]. A viral vector can ensure stable gene expression and high transfection efficiency compared with nonviral vectors. On the other hand, viral vectors have a risk of toxicity to the recipient and also can induce an inflammatory response [38, 40, 41]. Microporation, which uses a nonviral vector, protected the keloid-specific cellular characteristics and established highly efficient transformation protocol that ensured optimal conditions for KFs.

In summary, the effects of siRNA on EDIL3 expression in KFs and NFs were clearly distinguished in this study. siRNA proved effective in downregulating EDIL3 in KFs. Our results show that the EDIL3 knockdown downregulates collagen, which is a characteristic of KFs; furthermore, the proliferation of KFs was found to be decreased. According to our results, EDIL3 has therapeutic potential as an antifibrotic therapy in keloid. We believe that the most important treatment in keloid is antifibrotic therapy because keloid lesions are caused by excessive deposition of the extracellular matrix.

In the future, we are planning to study EDIL3 as a new keloid biomarker; we will further explore the mechanism of knockdown of EDIL3 that causes the reduction in cellular proliferation and collagen synthesis in a keloid.

In conclusion, we confirmed that it is possible to downregulate EDIL3 by means of siRNA to reduce the cellular proliferation and secretion of type I, type II and type III collagens in KFs. The pathogenesis of keloids is not yet clear, and siRNA targeting multiple proteins may be the most effective treatment. Complications associated with the clinical application of siRNA still remains to be solved before such a treatment becomes possible, but EDIL3 is expected to become a promising target for siRNA therapy of keloids.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

Ethical statement Full ethical approval for this study was obtained from Seoul St Mary's Hospital, Republic of Korea(KCMC06BR067).

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