Supplementary Materials and Methods

Human skin biopsies

Skin tissue from healthy subjects (8 males and 7 females) was collected at Fujita Health University School of Medicine. This study was approved by the ethics committee of Fujita Health University and Nippon Menard Cosmetic Research Laboratories, and was conducted after informed consent had been obtained from each subject.

Laser microdissection and microarray analysis

A normal skin biopsy sample obtained from a person was embedded in OCT compound, frozen, and sectioned on a cryostat (Carl Zeiss, Thornwood, NY, USA), and samples were attached to slide glasses (Leica Microsystems, Wetzler, Germany). Frozen skin sections were fixed with 75% ethanol and stained with 0.05% toluidine blue. After the sections were dried, the papillary and reticular dermis were collected separately using a laser microdissection system (Leica LMD6000, Leica Microsystems), and total RNA was extracted with the RNeasy micro kit (Qiagen, Hilden, Germany). The Ovation PicoSL WTA system V2 (NuGEN Technologies, San Carlos, CA) was used to amplify

cDNA. After the cDNA had been labeled with a SureTag DNA labeling kit (Agilent Technologies, Santa Clara, CA), the expression level of each gene was measured using the SurePrint G3 human GE microarray 8x60K (Agilent Technologies). The Subio Platform 1.18 (Subio, Kagoshima, Japan) was used for the analysis. The genes whose papillary dermis:reticular dermis expression level ratios were >2 were selected for further analysis. Undetectable genes were eliminated before this selection process. Microarray data are deposited in the NIH GEO repository (GEO ID: GSE87551).

Immunostaining

Human skin tissue was fixed with paraffin and sectioned. After subjecting the sections to deparaffinization, the antigens within them were activated with proteinase K (Dako, Glostrup, Denmark). The samples were then incubated overnight at 4°C with primary antibodies against CD271 (Acris Antibodies, Herford, Germany), collagen type 1 (LSL, Tokyo, Japan), collagen type 3 (LSL), collagen type 5 (NOVOTEC, Bron, France) or beta III tubulin (Abcam, Bristol, UK) in phosphate-buffered saline (PBS) solution containing 0.1% bovine serum albumin (BSA). For isotype control antibodies, rabbit IgG

isotype control (R&D Systems, MN, USA) or mouse IgG isotype control (Cell Signaling Technology, MA, USA) were used. After being rinsed with PBS, the samples were incubated with Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies (Life Technologies, Carlsbad, CA, USA) for 60 minutes at 37°C. Nuclear staining was performed with 4’ 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA).

The resultant fluorescent images were analyzed using the image analysis software Metamorph® (Molecular Devices, CA, USA). To compare the expression levels of collagen types 1, 3, and 5 between the papillary and reticular dermis, the fluorescence intensity per unit area of each tissue sample was measured. To assess the distribution of CD271+ cells, the number of stained cells was counted in the same areas in which the expression level of collagen was examined. To visualize the distributions of collagen types 1, 3, and 5 and CD271+ cells relative to the distance from the epidermal basal layer (down to 500 μm), the fluorescence intensity produced by each molecule was plotted on a histogram, and the threshold at which the fluorescence intensity level differed significantly was determined using statistical analysis.

Cell culture

Human fibroblasts were obtained from the normal tissue of 10 patients at Fujita Health University. This study was approved by the ethics committee of Fujita Health University and Nippon Menard Cosmetics Research Laboratories. Skin tissues were reacted overnight in 200 U/ml of Dispase II (Godo Shusei, Tokyo, Japan) at 4 °C. On the following day, epithelial and adipose tissues were peeled off from the skin tissues and shredded. The shredded dermal tissues were reacted in 0.2% collagenase (Sigma-Aldrich, MO, USA) at 37 °C for one hour, before being filtered through a 100 μm mesh. Then, the collagenase was removed by diluting the mixture with PBS (-) and centrifuging for five minutes at 1500 rpm, which was done twice. The resultant pellet was suspended in High-Yield Lyse (Invitrogen, NY, USA) and incubated to remove any contaminating red blood cells. Normal human fibroblasts were cultured in DMEM (Nacalai Tesque, Kyoto, Japan) containing 10% FBS (fetal bovine serum, Sigma-Aldrich). Twelve-well plates were incubated with Cellmatrix type I-A (Nitta Gelatin, Osaka, Japan), Cellmatrix type III (Nitta Gelatin, Osaka, Japan), and collagen 5 (CORNING, NY, USA) at 1 μg/cm^2 for one hour at 37°C. In the dishes coated with each

type of collagen, normal human fibroblasts were seeded at a concentration of 1.0×10^4 cells/cm^2 and cultured for three days. The cells were collected, and total RNA was extracted with TRI reagent (MRC, OH, USA).

qPCR analysis

cDNA was labeled with SYBR select master mix (Thermo Fisher Scientific, MA, USA), and gene expression was analyzed with the 7300 real-time PCR system (Thermo Fisher Scientific). The primers used are described below. The expression level of each molecule was normalized to that of GAPDH.

GAPDH:	F	CCGTGTTCCTACCCCCAAT
	R	TGCCTGCTTCACCACCTTCT
NGFR:	F	CATCCTGGCTGCTGTGGTT
	R	TGCAGCTGTTCCACCTCTTG
COL1A1:	F	GGAAACCCGAGGTATGCTTGA
	R	CACTCGCCCTCCCGTCTT
COL3A1:	F	TTCCTGAAGATGTCGTTGATGTG
	R	TGTTTTTGCAGTGGTATGTAATGTTC

COL5A1:	F	GCCCATCGTGGACATCAT
	R	GCCCCACTTCAAATCCAA
NANOG:	F	CCTTCCTCCATGGATCTGCTT
	R	AAGTGGGTTGTTTGCCTTTGG
SOX2:	F	GAGAACCCCAAGATGCACAACT
	R	CGCTTAGCCTCGTCGATGA
POU5F1:	F	AGTTTGTGCCAGGGTTTTTG
	R	ACTTCACCTTCCCTCCAACC
MKI67:	F	AGACGCCTGGTTACTATCAAAAG
	R	GGAAGCTGGATACGGATGTCA

Cell sorting by FACS

Normal human fibroblasts were cultured in DMEM containing 15% FBS (Sigma-Aldrich), rinsed in PBS, and collected with a cell scraper (Sumitomo Bakelite, Tokyo, Japan). The cell suspension was incubated with primary antibodies against CD271 (Acris Antibodies) for 30 minutes on ice, rinsed in PBS, and then incubated with Alexa Fluor 488-conjugated secondary antibodies (Life Technologies). After being rinsed in PBS, the cells were sorted

using a FACS ARIA™ (BD, NJ, USA). The sorted CD271+ and CD271- cells were rinsed in PBS, and total RNA was extracted using TRI reagent (MRC).

In vitro differentiation

Differentiation inductions to adipocytes, osteoblasts, and chondrocytes were performed by modifying the methods which has been reported (Stem Cells. 25 (2007) 1610-7). For induction to adipocytes, CD271+ cells were cultured for six days in DMEM (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich), 33 µM Biotin (Sigma-Aldrich), 10 µg/mL Insulin (Sigma-Aldrich), 1 µM dexamethasone (Sigma-Aldrich), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), 0.2 mM indomethacin (Sigma-Aldrich), and 1% Antibiotic-Antimycotic (Gibco, MA, USA). The cells were cultured further for adipogenic differentiation in the medium consisting of DMEM (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich), 3 µM Biotin (Sigma-Aldrich), 10 µg/mL Insulin (Sigma-Aldrich) and 1% Antibiotic-Antimycotic (Gibco) for four days. The medium was replaced every 2–3 days. Adipogenesis was assayed by lipid accumulation in differentiated cells with Oil Red O staining. To induce osteoblast differentiation, CD271+ cells were cultured for 21 days.

The culture medium was DMEM (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich), 50 μM ascorbic acid 2-phosphate (Sigma-Aldrich), 0.01 μM dexamethasone (Sigma-Aldrich) and 1% Antibiotic-Antimycotic (Gibco), and the medium was replaced every 2–3 days. Osteogenesis was assayed by calcium content in differentiated cells and matrix mineralization with Alizarin Red S staining.

Differentiation medium into chondrocytes was DMEM (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich), 50 μM ascorbic acid 2-phosphate (Sigma-Aldrich), 0.01 μM dexamethasone (Sigma-Aldrich), 0.5 × ITS-X (Invitrogen), 10 ng/mL BMP-2 (PeproTech, NJ, USA), 10 ng/mL TGF-μ1 (PeproTech) and 1% Antibiotic-Antimycotic (Gibco). Fibroblasts were seeded in triplicate in a 15 mL polypropylene conical tube (Corning) at a density of 1×10^5. After two weeks of induction to chondrocytes, 1×10^5 cells were seeded in a 15 mL polypropylene conical tube (Corning) in triplicate to allow formation of cell aggregates. Differentiated cells formed cell masses with smooth surface characteristic of chondrocytes.

Analysis of cell growth potentials

Human fibroblasts were seeded in a 96-well plate at a concentration of 3.0×10^3, and after incubation at 37°C with 5% CO_2 overnight, images of cells were captured by IncuCyte ZOOM (Essen BioScience, MI, USA) every 24 hours for three days (PMID: 26384394). The cell growth potentials were examined by quantifying the ratio of areas covered by cells on the day 3 using IncuCyte Confluence version 2016B software. All experiments were done three times.

Statistical analysis

The Student's t-test and ANOVA were used to evaluate statistical significance.

Table S1

A list of the genes that are highly expressed in the papillary dermis.

Of the genes whose papillary dermis: reticular dermis expression level ratios were greater than 2, those encoding matrix components, papillary dermis markers, or markers of an undifferentiated state are shown.

Figure S1

Images of human skin tissue double-stained with CD271 and collagen type 5. CD271, green; collagen type 5, red; nuclei stained with DAPI, blue. When the isotype control antibodies were used, green or red signals were not seen, indicating that CD271 and collagen type 5 were stained specifically. Arrows indicate CD271+ cells (scale bar: 50 μm).

Figure S2

The expression of CD271 antigen on the surfaces of normal human fibroblasts was analyzed using a FACS Aria™. In the left graph, the expression of IgG isotype (as a control) is shown. In the right graph, the expression of CD271 is shown by the histograms, and the percentage of cells expressing CD271 is indicated.

Figure S3

Normal human fibroblasts were cultured in a 96-well plate which had been coated with collagen type 1, 3, or 5, and images of cells were taken by IncuCyte ZOOM. (A) Images of cells cultured for three days with either type of collagen. (B) The potentials of cell growth were determined by quantifying the ratio of areas covered by cells

using IncuCyte Confluence version 2016B software (mean ± SD, n=3, *P<0.05 compared with the control). (C) Normal human fibroblasts were cultured in a dish coated with either type of collagen for three days, and the gene expression of MKI67 was examined by qPCR (mean ± SD, n=5, *P<0.05 compared with the control).

Figure S4

Images of human skin tissue double-stained with antibodies against beta III tubulin and CD271. CD271, green; collagen type 5, red. Arrows and arrowheads indicate CD271+ cells and beta III tubulin+ cells, respectively (Scale bar: 50 μm).

Figure S5

CD271+ cells were sorted from normal human fibroblasts, and induced to differentiate into adipocytes, osteocytes, and chondrocytes. Adipogenesis was assayed by Oil Red O staining. Differentiation induction into osteoblasts was examined by alizarin red staining. A characteristic aggregate formed by cells induced to differentiate into chondrocytes is shown in the right panel.