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A 1,1′-biuracil from *Epidermidibacterium keratini* EPI-7 shows anti-aging effects on human dermal fibroblasts

Yeong-Geun Lee[1], Dong-Geol Lee[2], Jung Eun Gwag[1], Misun Kim[2], Minji Kim[2], Hyoung-Geun Kim[1], Jung-Hwan Ko[1], Hyeonju Yeo[2], Seunghyun Kang[2*] and Nam-In Baek[1*]

Abstract

Our previous study we isolated novel bacterial stain, *Epidermidibacterium keratini*, called EPI-7^T from skin samples. Repeated column separation yielded one new pyrimidine compound, 1,1′-biuracil, from EPI-7^T culture solutions grown in R2A medium. Its chemical structure was determined based on spectroscopic data, IR, FAB/MS, and NMR. And 1,1′-biuracil and EPI-7^T culture solutions showed regulating effects of anti-aging associated mRNA expressions in UV-irradiated fibroblasts without toxicity in Hs68 cells. These results demonstrates the cosmetic potential of 1,1′-biuracil and EPI-7^T as anti-aging agents.

Keywords: *Epidermidibacterium keratini* EPI-7, Keratinocyte, Skin bacteria, Human skin, 1,1′-Biuracil

Introduction

Human skin provides a habitat for various microorganisms that stably maintain communities through commensal relationships. The symbiotic relationships between the skin and the microbiome produce complex protective barriers against external environmental factors. Diverse metabolites produced by skin microbiome provide favorable efficacy to human skin [1]. In this study, we collected skin-microbiome samples from two different age groups of females. One group was in their 20 s, and the other was in their 40 s. Analysis of 16S rRNA gene sequences showed a newly found bacterium that shares 93.4% homology with the genus *Sporichthya*, indicating the discovery of a novel genus. We isolated a novel bacterial stain, *Epidermidibacterium keratini*, called EPI-7^T [2], from skin samples. Additionally, the younger skin appeared to have high proportion of EPI-7^T, while the older skin had no EPI-7^T but rather other types of bacteria. Skin probiotic strain EPI-7^T stained gram-positive, was aerobic and heterotrophic, and consisted of non-motile, non-spore-forming, rod-shaped cells (Fig. 1). Good growth was obtained on R2A agar but not on NA, ISP 2, or TSA.

Skin microorganisms produce various metabolites and influence skin cells directly or indirectly [3]. Among these microorganisms, the most common are of the genus *Staphylococcus*, which has been reported to be involved in Toll-like receptor (TLR) signaling in inflammation and wound regeneration [4, 5]. Indeed, several species in the genus *Staphylococcus* have been linked to various inflammatory diseases of the skin; however, no study has linked these organisms to aging. In addition, no report has described the relationships between aging and skin microorganisms. Therefore, in the current study, we analyzed the distribution of EPI-7^T by age and investigated mechanisms related to aging.

Experimental

Materials

Epidermidibacterium keratini EPI-7 in R2A medium was provided by COSMAX R&I Center, Seongnam, Republic of Korea, in May 2018. A voucher specimen (KHU-NPCL-201805) has been deposited at the Laboratory of Natural Products Chemistry, Kyung Hee University.

*Correspondence: shyunk@cosmax.com; nibaek@khu.ac.kr
†Yeong-Geun Lee and Dong-Geol Lee made equal contributions to this work (co-first author)
[1] Department of Oriental Medicine Biotechnology, Graduate School of Biotechnology, Kyung Hee University, Yongin 17104, Republic of Korea
[2] R&I Center, COSMAX BTI, Seongnam, Republic of Korea

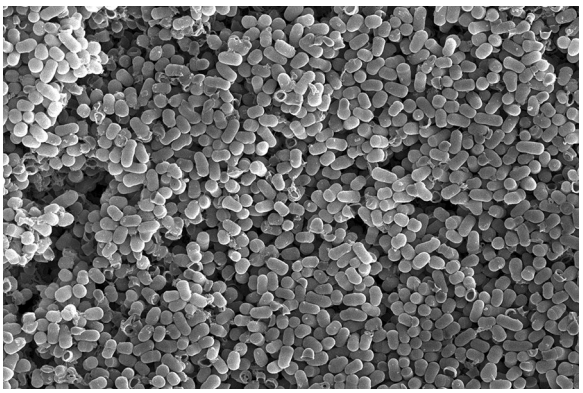

Fig. 1 Morphology of strain EPI-7^T viewed by SEM

Table 1 ^{1}H- and ^{13}C-NMR data of 1,1′-biuracil (1) and uracil (2) (600 MHz, DMSO-d_6, δ_H; 150 MHz, DMSO-d_6, δ_C)

	1,1′-Biuracil (1)			Uracil (2)	
	δ_C	δ_H*		δ_C	δ_H*
1, **1′**	–	–	**1**	–	10.82, br. s
2, **2′**	151.5	–	**2**	152.0	–
3, **3′**	–	10.98, br. s	**3**	–	11.02, br. s
4, **4′**	164.3	–	**4**	164.8	–
5, **5′**	100.2	5.44, br. d, 7.2	**5**	100.7	5.45, ddd, 6.6, 1.8, 1.2
6, **6′**	142.2	7.38, d, 7.2	**6**	142.7	7.40, dd, 6.6, 4.8

* Coupling pattern, *J* in Hz

General experimental procedures

Instruments and chemicals used in this study were prepared based on the previously described methods [6].

Extraction and isolation

EPI-7^T culture solutions grown in R2A medium (50 L) were centrifuged, filtrated, and evaporated under reduced pressure. The concentrates were extracted in 80% aqueous MeOH (500 mL × 3). The combined concentrates (144 g) were poured into H_2O (2.2 L) and successively extracted with EtOAc (2.2 L × 3) and *n*-BuOH (2.0 L × 3). Each layer was concentrated under reduced pressure to obtain EtOAc (EPE, 10.7 g), *n*-BuOH (EPB, 42.8 g), and H_2O (EPH, 90.5 g) fractions. Fraction EPE (10.7 g) was applied to silica gel (SiO_2) column chromatography (c.c.) (Φ 5.5 × 35 cm) and eluted with EtOAc-*n*-BuOH–H_2O (20:3:1 → 7:3:1, 2 L of both) with monitoring by TLC to provide 12 fractions (EPE-1 to EPE-12). Fraction EPE-3 [168.0 mg, elution volume/total volume (Ve/Vt) 0.200–0.250] was subjected to octadecyl SiO_2 (ODS) c.c. [Φ 2 × 10 cm, MeOH–H_2O (2:1), 400 mL] to yield four fractions (EPE-3-1 to EPE-3-4) and a purified compound **1** [EPE-3-1, 20.5 mg, Ve/Vt 0.000–0.225, TLC (SiO_2) R_f 0.70, EtOAc-*n*-BuOH–H_2O (15:3:1), TLC (ODS) R_f 0.88, MeOH–H_2O (2:1)].

1,1′-Biuracil (**1**) White amorphous powder; IR (KBr) ν_{max} 1715, 1674, 1418 cm^{-1}; low resolution positive ESI/MS *m/z* 113 $[M/2+1]^+$, 223 $[M+1]^+$, 267 $[M+2Na-H]^+$; High resolution positive ESI/MS *m/z* 223.0459 $[M+1]^+$ (Calcd. for $C_8H_7N_4O_4$ 223.0467); ^{1}H- and ^{13}C-NMR (600 and 150 MHz, DMSO-d_6, δ_H and δ_C), see Table 1.

Cell culture and treatment

The human fibroblast cell line (Hs68) was purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 1% Antibiotic Antimycotic Solution (DMEM; HyClone Laboratories, Inc., Logan, UT, USA) and 10% fetal bovine serum at 37 °C in an atmosphere of 5% CO_2. For UV irradiation and treatment, Hs68 cells were seeded at 80% confluence into 6-well plates and incubated in an atmosphere of 5% CO^2 at 37 °C. After 24 h, the cells were washed once with phosphate-buffered saline (PBS) and placed in fresh PBS. Next, 12 mJ/cm^2 of UVB (wavelength 290–320 nm, maximum peak 311 nm) was applied in the presence of crosslinker (UVP; Upland, CA, USA), and then EPI-7 (0.1– 1%) or 1,1′-biuracil (0.1–10 ppm) was administered into the cells through serum-free medium for 24 h.

Cell viability test

Hs68 cells were seeded in 48-well plates, incubated for 24 h, and then treated with the indicated concentrations of EPI-7 or 1,1′-biuracil for another 24 h. After removing the culture medium, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solution was added to each well and incubated for 4 h. After termination of the reaction, the medium was discarded, and dimethylsulfoxide (DMSO) was added to dissolve the formazan crystals. Optical density (OD) values were measured at 570 nm using a microplate reader and normalized to that of the control.

RNA isolation and real-time PCR

Total RNA was isolated from cells using TRIzol reagent according to the manufacturer's instruction (TaKaRa, Shiga, Japan). cDNA was synthesized from 1 μg of total RNA using Reverse Transcription Premix (Elpis-biotech, Daejeon, Korea) under the following reaction conditions: 45 °C for 45 min and 95 °C for 5 min. Gene expression signals were quantified with real-time PCR, and the data were analyzed using StepOne PlusTM system software (Applied Biosystems, Foster City, CA, USA). Real-time PCR amplification reactions were performed using SYBR

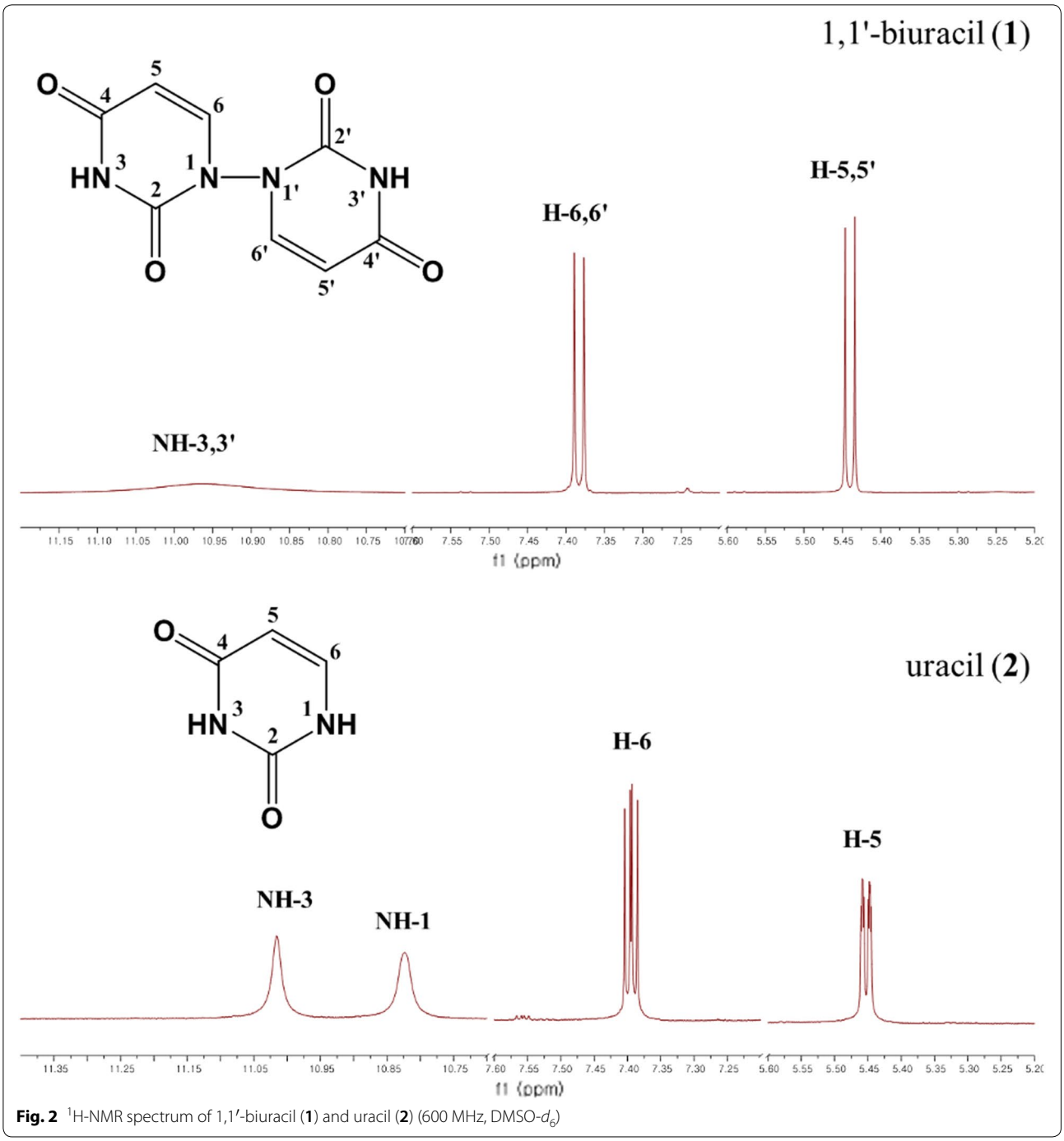

Fig. 2 ^{1}H-NMR spectrum of 1,1′-biuracil (**1**) and uracil (**2**) (600 MHz, DMSO-d_6)

Green PCR Master Mix with premixed ROX (Applied Biosystems, Foster City, CA, USA). The following primer pairs (Bioneer, Daejeon, Korea) were used in the reactions performed in an ABI 7300 following the manufacturer's protocol: β-actin (F: 5′-GGCCATCTCTTGCTCGAAGT-3′ and R: 5′-GACACCTTCAACACCCCAGC-3′), type I procollagen (F: 5′-CTCGAGGTGGACACCACCCT-3′ and R: 5′-CAGCTGGATGGCCACATCGG-3′), fibrillin (F: 5′-AATGTCAGACGAAGCCAGGG-3′ and R: 5′-GATTTGGTGACGGGGTTCCT-3′), MMP-1 (F: 5′-CGAATTTGCCGACAGAGATGA-3′ and R: 5′-GTCCCTGAACAGCCCAGTACTT-3′), MMP-3 (R: 5′-ATTCCATGGAGCCAGGCTTTC-3′ and R: 5′-CATTTGGGTCAAACTCCAACTGTG-3′). The

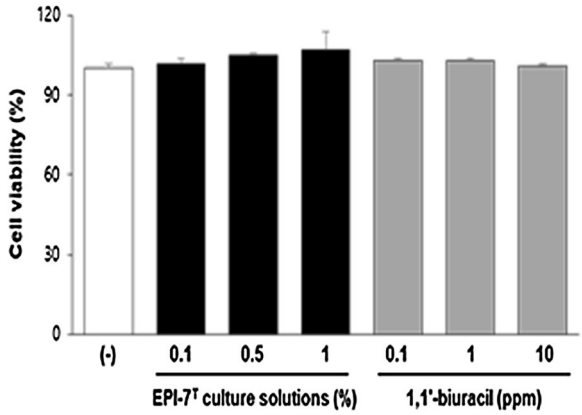

Fig. 3 Cell viability of EPI-7^{T} culture solutions and 1,1′-biuracil in Hs68 human fibroblasts

reaction conditions were as follows: initiation at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 1 min. The expression of β-actin was used as an internal control.

Results and discussion

Concentrated R2A medium including *E. keratini* EPI-7^{T} were successively partitioned into EtOAc, *n*-BuOH, and aqueous fractions. Repeated SiO_2 and ODS c.c. of the *n*-BuOH fraction yielded one new uracil derivative.

Compound **1**, white amorphous powder, showed an IR absorbance band of carboxyl (1715, 1674 cm^{-1}), aromatic (1674 cm^{-1}), and amide group (1418 cm^{-1}). IR spectrum of it was very similar to uracil with the exception for the absence of an amide moiety [7]. Its molecular weight and molecular formula were respectively 222 Da and $C_8H_6N_4O_4$ from the molecular ion peak *m/z* 223.0459 $[M+1]^+$ (Calcd. for $C_8H_7N_4O_4$ 223.0467) in positive HR ESI/MS. The four carbon signals at δ_C 164.3 (s, C-4,4′), 151.5 (s, C-2,2′), 142.2 (d, C-6,6′), and 100.2 (d, C-5,5′) in the ^{13}C-NMR spectrum suggested that compound **1** was very similar to uracil (**2**), one of the pyrimidine bases of nucleic acids [8, 9]. The carbon signals of two amide carbonyls [δ_C 164.3 (C-4,4′) and δ_C 151.5 (C-2,2′)], a nitrogenated olefine methine [δ_C 142.2], and an olefine methine [δ_C 100.2] were observed. The above mentioned ^{13}C-NMR and ESI/MS data suggested compound **1** to be a dimer of uracil (**2**, molecular weight 112 Da) with a symmetrical structure. The ^{1}H-NMR spectrum (600 MHz, DMSO-d_6, δ_H) showed amine (δ_H 10.96, 2H, br. s, H-NH-3,3′) and aromatic (δ_H 7.38, 2H, d, $J=7.2$ Hz, H-6,6′; δ_H 5.40, 2H, br. d, $J=7.2$ Hz, H-5,5′) proton signals. As shown in Fig. 2, the uracil (**2**) showed amine proton signals at two different chemical shifts in the ^{1}H-NMR spectrum, while

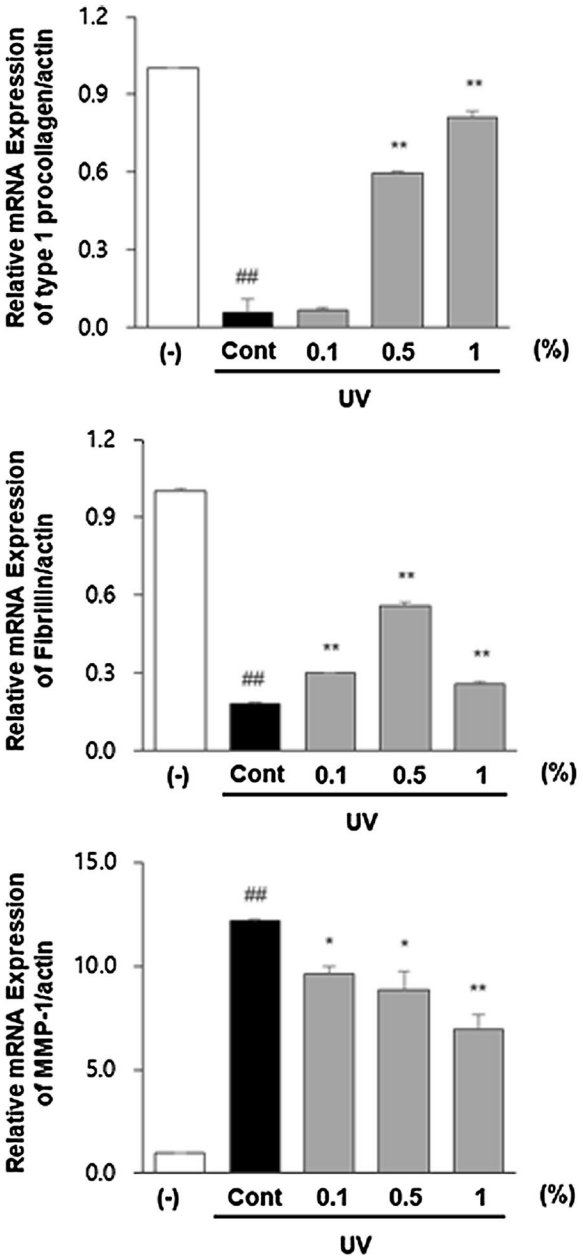

Fig. 4 The effect of EPI-7^{T} culture solutions on the regulation of anti-aging associated mRNA expressions in UV-irradiated fibroblasts

compound **1** had only one chemical shift, indicating the uracil dimer was linked through an N–N linkage. *J* values (7.2 Hz) of the nitrogenated aromatic proton signals (H-6,6′) confirmed the proton signals to show only one ^{3}J coupling in the heterocyclic structure, which also indicated an N–N linkage between NH-1 and NH-1′. As shown in Fig. 2, the nitrogenated aromatic proton signals (H-6,6′) in uracil (**2**) were split as dd through two ^{3}J couplings with H-5 and NH-1. Also, the gHMBC

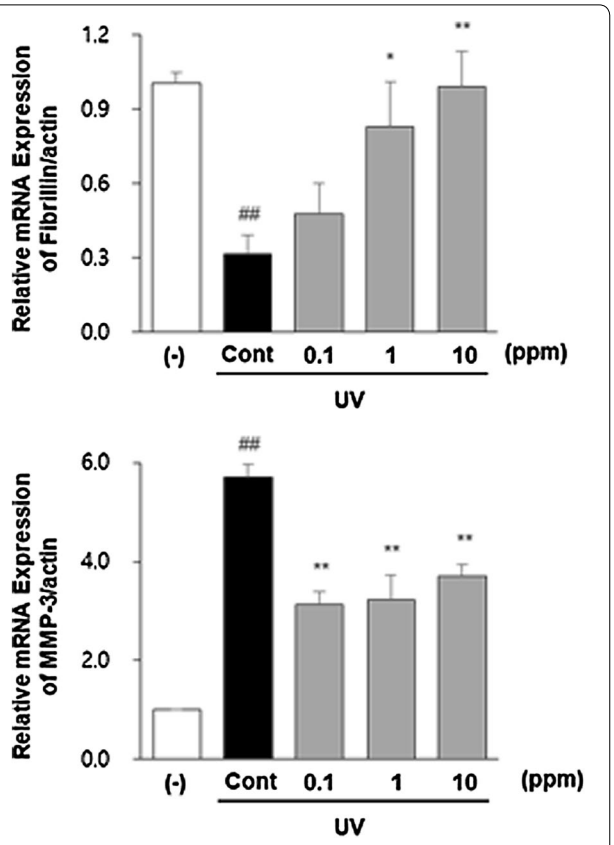

Fig. 5 Anti-aging functions of 1,1′-biuracil in UV-irradiated fibroblasts

spectrum (Additional file 1: Fig. S5) showed correlations between the nitrogenated aromatic proton signal (H-6,6′) and two amide carbonyl carbons [(C-4,4′) and (C-2,2′)] as well the olefine methine carbon (C-5,5′) and between the aromatic proton (H-5,5′) and the amide carbonyl carbon (C-4,4′) as well the nitrogenated olefine methine carbon (C-6,6′). Based on these findings, compound **1** was identified as 1,1′-biuracil, which was revealed to be a new compound. A similar pyrimidine compound, 5,5′-biuracil, has been reported as a synthetic compound [10].

To determine the effects of EPI-7^T culture solutions and 1,1′-biuracil, we first examined the effects of EPI-7^T culture solutions and 1,1′-biuracil on cell viability of Hs68 human fibroblasts using MTT assay (Fig. 3), which showed no cytotoxicity at concentrations less than 1% and 10 ppm, respectively.

Next, we evaluated the expression levels of MMP-1 and the skin aging-associated factors type I procollagen, fibrillin, in UV-irradiated fibroblasts [11, 12]. Hs68 human fibroblasts were irradiated with UVB (12 mJ/cm^2) and then treated with EPI-7^T culture solutions ranging from 0.1 to 1% or 1,1′-biuracil ranging from 0.1 to 10 ppm for 24 h. The mRNA expression levels were measured using RT-qPCR. As shown in Fig. 4, EPI-7^T culture solutions increased type I procollagen and fibrillin mRNA expression, which were suppressed by UV irradiation. In addition, the mRNA expression level of MMP-1, the major collagen-degrading proteinase, was significantly reduced by EPI-7^T culture solutions. These results indicated that EPI-7^T culture solutions exerted skin anti-aging effects.

Furthermore, we measured the mRNA expression levels of type I procollagen, fibrillin, and MMP-1 after treatment with 1,1′-biuracil in UV-irradiated Hs68 fibroblasts to clarify the anti-aging effects of 1,1′-biuracil derived from EPI-7^T culture solutions. Application of 1,1′-biuracil did not regulate type I procollagen or MMP-1 mRNA expression (data not shown), whereas it significantly increased fibrillin mRNA expression and reduced that of MMP-3, the fibrillin-degrading proteinase [13], in the UV-irradiation condition (Fig. 5). Taken together, these results suggest that 1,1′-biuracil is a key molecule in EPI-7^T culture solutions, exerting protective effects against UV-irradiated skin aging.

Additional file

Additional file 1. Spectroscopic data of 1,1′-biuracil are available as supplementary material.

Authors' contributions

Y-GL, D-GL, SHK, and N-IB planned study and made paper. Y-GL, JEG, H-GK, J-HK, and N-IB isolated biuracil. Y-GL and N-IB identified compound. D-GL, MSK, MJK, HJY, and SHK performed anti-aging experiments. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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