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Letter to the Editor

Antioxidants cinnamaldehyde and *Galactomyces* fermentation filtrate downregulate senescence marker CDKN2A/p16INK4A via NRF2 activation in keratinocytes

Dear Editor,

Senescence is a state wherein cells are metabolically active but unable to replicate due to the increased expression of cell cycle checkpoint proteins such as cyclin-dependent kinase inhibitor 2A (CDKN2A or p16INK4A), which induce cell cycle arrest [1,2]. The accumulation of CDKN2A protein is a major and stable senescence marker in various cell types including keratinocytes and is induced in vitro and in vivo after successive replication and/or upon exposure to various stressors such as oxidative stress [1,2]. It is known that senescent keratinocytes have a 30-fold-higher intracellular concentration of reactive oxygen species (ROS) than those in the growth phase [1]. Therefore, continuous treatment with the antioxidative enzyme catalase or the general antioxidant *N*-acetyl-cysteine delays the accumulation of CDKN2A protein and subsequent senescence [1]. Senescence is generally linked to carcinogenic potential [1]. In addition, the increase in CDKN2A+ senescent keratinocytes is associated with epidermal atrophy seen in the elderly [2].

Nuclear factor-erythroid 2-related factor-2 (NRF2) is a master switch for antioxidant signaling [3] and its activation reduces intracellular ROS levels by upregulating antioxidative enzymes such as glutathione peroxidase 2 (GPX2) and NAD(P)H quinone oxidoreductase 1 (NQO1) [4,5]. Various antioxidative phytochemicals and microbial products activate the NRF2 system and exert antioxidative function [3]. Cinnamaldehyde, an active component of cinnamon, and *Galactomyces* fermentation filtrate (GFF) are such potent natural antioxidants acting via NRF2 activation [6,7]. However, whether these natural NRF2 activators downregulate CDKN2A expression remains elusive.

In this study, we stimulated normal human epidermal keratinocytes (NHEKs; Lonza, Basel, Switzerland) with cinnamaldehyde (50 μM; Sigma-Aldrich, St. Louis, MO) [6] or GFF (10%, Pitera®; P&G Innovation Godo Kaisha, Kobe, Japan) [7] for 24 h. Western blot analysis revealed that both cinnamaldehyde (Fig. 1A, D, and E) and GFF (Fig. 1B, G, and H) upregulated the protein expression of GPX2 (detected by rabbit polyclonal GPX2 antibody, ab137431; Abcam, Cambridge, UK) and NQO1 (mouse monoclonal NQO1 antibody, ab28947; Abcam), suggesting their NRF2-activating capacity. In parallel with this, both antioxidative agents appeared to inhibit the protein expression of CDKN2A (rabbit monoclonal, p16INK4A, #80772; Cell Signaling Technology, Danvers, MA, USA) (Fig. 1A–C and F). β-Actin (mouse monoclonal β-actin antibody, #3700; Cell Signaling Technology) protein level served as an internal housekeeping protein control. Output TIFF images were analyzed using ImageJ (http://imagej.nih.gov/ij) to quantify antibody expression.

To examine the dependence of these behaviors on NRF2, we performed a similar experiment using NHEKs transfected with NRF2 small interfering (si)RNA (s9492; Ambion, Austin, TX, USA) or control siRNA (Ambion). Transfection of NRF2 siRNA reduced the protein level of NRF2 (82.5 ± 4.6% reduction). The basal expression and cinnamaldehyde-induced upregulation of GPX2 and NQO1 proteins were downregulated in NRF2-silenced keratinocytes compared with the levels in those transfected with control siRNA (Fig. 2A, D, and E). The basal expression and GFF-induced upregulation of GPX2 and NQO1 proteins were also downregulated in NRF2-silenced keratinocytes compared with the levels in those transfected with control siRNA (Fig. 2B, G, H). Both cinnamaldehyde and GFF again inhibited the CDKN2A protein expression (Fig. 2A-C, F). Notably, NRF2 silencing enhanced the CDKN2A protein expression in untreated, cinnamaldehyde-treated and GFF-treated keratinocytes (Fig. 2A-C, F). These results indicate that the natural antioxidants cinnamaldehyde and GFF are capable of inhibiting protein expression of the senescence marker CDKN2A in a NRF2-dependent manner.

The skin is continuously exposed to various oxidative stressors including ultraviolet rays and environmental pollutants [1–3]. As such, the accumulation of CDKN2A protein in keratinocytes is observed in normal aged skin as well as neoplastic and inflammatory skin disorders [1,2,8]. Numerous natural antioxidative agents activate the cellular NRF2 system and protect the body from oxidative stress [3]. Very recently, it was reported that endogenous hormones such as 1,25-dihydroxyvitamin D and oxytocin alleviate cellular senescence via NRF2 activation [9,10]. The present study proved that the natural NRF2 activators cinnamaldehyde and GFF downregulate the expression of CDKN2A protein in keratinocytes. These results support the notion that the

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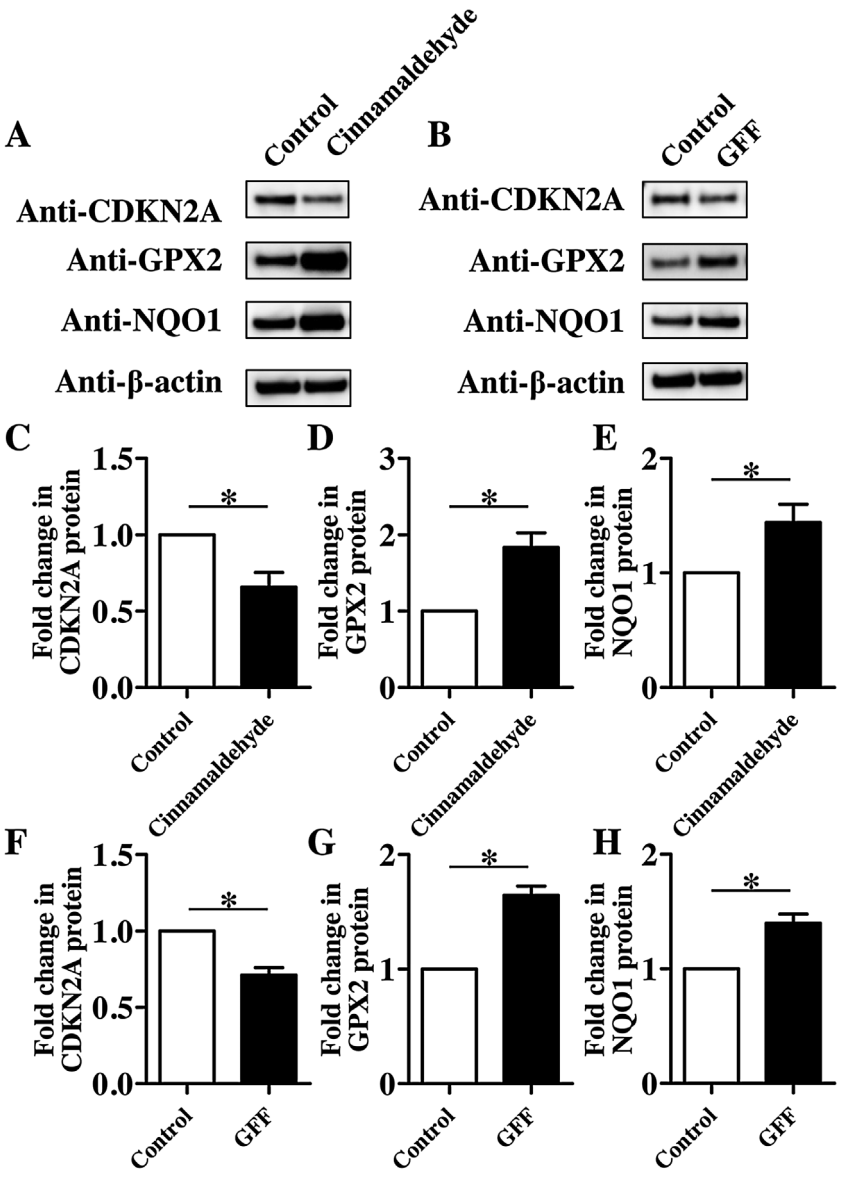

Fig. 1. Cinnamaldehyde (A, C—E) and GFF (B, F—H) reduce the CDKN2A protein expression and upregulate the protein levels of antioxidative GPX2 and NQO1 enzymes. Protein expression levels were quantified using ImageJ (C–H). Data are expressed as mean ± S.E.M.; n = 3 for each group. $^{*}P < 0.05$. The data are representative of experiments repeated three times with similar results.

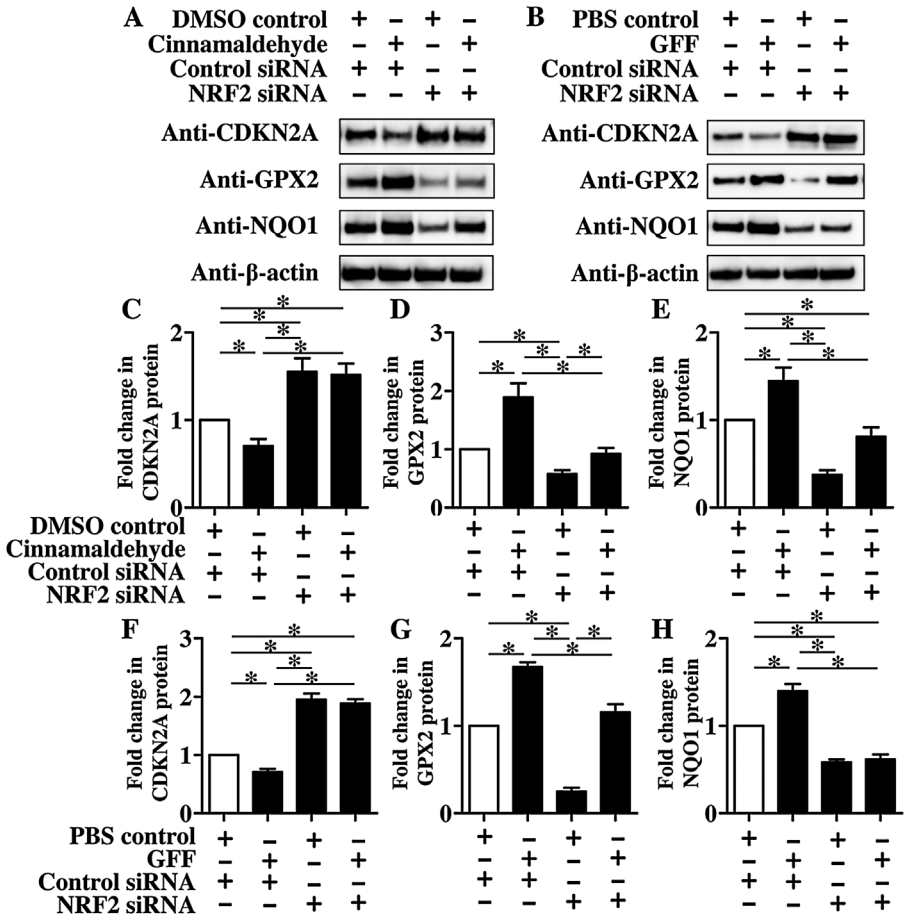

Fig. 2. Cinnamaldehyde-induced downregulation of CDKN2A protein and upregulation of GPX2 and NQO1 are canceled in the keratinocytes transfected with NRF2 siRNA (A, C—E). Moreover, GFF-induced downregulation of CDKN2A protein and upregulation of GPX2 and NQO1 are canceled in the NRF2-silenced keratinocytes (B, F—H). Protein expression levels were quantified using ImageJ (C–H). Data are expressed as mean ± S.E.M.; n = 3 for each group. $^{*}P < 0.05$. The data are representative of experiments repeated three times with similar results.

topical or systemic administration of these natural NRF2 activators may reduce the process of senescence of human epidermis.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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