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Ultraviolet A Regulates the Stemness of Human Adipose Tissue-Derived Mesenchymal Stem Cells Through Downregulation of the HIF-1α via Activation of PGE_2–cAMP Signaling

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ABSTRACT

Ultraviolet A (UVA) irradiation is responsible for a variety of changes in cell biology. The purpose of this study was to investigate the effects of UVA irradiation on the stemness properties of human adipose tissue-derived mesenchymal stem cells (hAMSCs). Furthermore, we examined the UVA-antagonizing effects of L-cysteine ethylester hydrochloride (ethylcysteine) and elucidated its action mechanisms. The results of this study showed that UVA reduced the proliferative potential and stemness of hAMSCs, as evidenced by reduced proliferative activity in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and downregulation of OCT4, NANOG, and SOX2, stemness-related genes. The mRNA level of hypoxia-inducible factor (HIF)-1α, but not HIF-2α was reduced by UVA. Moreover, the knockdown of HIF-1α using small interfering RNA (siRNA) for HIF-1α was found to downregulate stemness genes, suggesting that UVA reduces the stemness through downregulation of HIF-1α. In addition, we examined the mechanisms underlying the UVA-mediated effects and found that UVA induced production of prostaglandin (PG) E2 and 3′-5′-cyclic adenosine monophosphate (cAMP), and that this effect was mediated through activation of activating protein-1 (AP-1) and nuclear factor-κB (NF-κB). The UVA effects were antagonized by ethylcysteine, and the effects were found to be mediated by reduced production of PGE2 through the inhibition of JNK and p42/44 MAPK. Taken together, these findings show for the first time that UVA regulates the stemness of hAMSCs and its effects are mediated by downregulation of HIF-1α via the activation of PGE_2–cAMP signaling. In addition, ethylcysteine may be used as an antagonizing agent to mitigate the effects of UVA. J. Cell. Biochem. 113: 3681–3691, 2012.

KEY WORDS: HUMAN ADIPOSE TISSUE-DERIVED MESENCHYMAL STEM CELLS; STEMNESS GENE; HIF; PGE_2

Regulation of the cell cycle is the key process involved in determining the fate of stem cells, including renewal and differentiation. The PIT-OCT-UNC (POU) transcription factor OCT4 plays a critical role in regulating the differentiation of embryonic stem (ES) cells and maintaining the pluripotent nature of the blastocyst inner cell mass. OCT4 was also shown to function in a complex with NANOG and SOX2 to activate and repress genes controlling stem cell identity and differentiation [Boyer et al., 2005]. Hypoxia-inducible factors (HIFs) were also reported to affect the self-renewal and differentiation processes of stem cells by specific

Abbreviations used: ethylcysteine, L-cysteine ethylester hydrochloride; PG, prostaglandin; HIF, hypoxia-inducible factor; UV, Ultraviolet; hAMSCs, human adipose tissue-derived mesenchymal stem cells; AP-1, activator protein-1; JNK, Jun N-terminal kinase; cAMP, 3′-5′-cyclic adenosine monophosphate; NF-κB, nuclear factor-κB.

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regulation of relevant genes and the key transcription factors involved in these processes [Forristal et al., 2010; Moreno-Manzano et al., 2010].

Ultraviolet (UV) A (320–380 nm) is believed to be deleterious or beneficial to cells and tissues [Halliday and Rana, 2008; Lee et al., 2010; Situm et al., 2010]. In addition, several reports suggest that ultraviolet A (UVA) may affect the hypodermis where adipose tissue-derived mesenchymal stem cells, preadipocytes, and adipocytes exist [Horn et al., 1994; Mitani et al., 1999; Sunderkötter et al., 2006]. However, the direct effects of UVA irradiation on adipose tissue-derived mesenchymal stem cells (hAMSCs), especially the stemness of hAMSCs, have not yet been examined. Here, we demonstrate that UVA irradiation reduced the stemness of hAMSCs, and that this effect was mostly due to the reduced expression of OCT4, NANOG, and SOX2, which are stemness genes. This reduced expression was mediated by the activation of PGE_2–cAMP–HIF-1α signaling.

To attenuate the negative effects of UVA irradiation on stemness, a cell-based, compound library screen that was intentionally biased to select compounds with relatively low toxicity and high activity was conducted. A cell proliferation assay was used as the screening tool to evaluate the UVA-antagonizing effects of single compounds in hAMSCs. During this screening, L-cysteine ethylester hydrochloride (ethylcysteine) was selected as a candidate for use as an UVA-antagonizing agent. In this study, we found that ethylcysteine antagonized the effects of UVA on stemness by reducing the production of PGE_2 via inhibition of JNK and p42/44 MAPK.

MATERIALS AND METHODS

HUMAN ADIPOSE TISSUE-DERIVED STEM CELL CULTURE

Three kinds of hAMSCs were purchased from Invitrogen (Carlsbad, CA), ATCC (Manassas, VA), and Thermo Fisher Scientific, Inc. (Waltham, MA), respectively. The cryopreserved cells were thawed at 37°C and then immediately cultured in MesenPRO RS™ medium (Gibco, Carlsbad, CA). The cells were then expanded using MesenPRO RS™ medium to five passages. The medium was changed every 3 days until the cells were 70% confluent, at which time they were passaged.

UVA IRRADIATION

For UVA exposure, when the cells were 70% confluent, the medium was removed, and the cells were washed with PBS and gently overlaid with Dulbecco's modified Eagle's medium (DMEM) devoid of phenol red (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The cells were then irradiated for 21 s to 36 min at 0.05–5 J/cm^2, which was not cytotoxic. UVA irradiation was conducted in DMEM devoid of phenol red using a Vilber-Lourmat UVA table centered on 365 nm (TF-20L) at 25°C which was controlled during the irradiation. In addition, during irradiation, the lid of the dish was opened to minimize UVA absorption by plastic materials. A piece of glass with a thickness of 4-mm was placed above the table to absorb the residual UVB radiation.

CELL PROLIFERATION

hAMSCs were irradiated with the indicated doses of UVA and then incubated with 1–100 μM of ethylcysteine (Sigma–Aldrich, St. Louis, MO) for 3 days under serum-free conditions (in DMEM devoid of serum, at 37°C and 5% CO_2). Serum-free conditions were chosen to exclude unknown effects of exogenous serum, which can have compositions that vary based on the donor species, the age of the animal the serum was obtained, its feedstock and season. After 3 days, the cell proliferation was evaluated using the MTT assay, which is based on the conversion of a substrate containing a tetrazolium ring into blue formazan by mitochondrial dehydrogenases [Lee et al., 2007]. The level of blue formazan was then measured at a wavelength of 570 nm spectrophotometrically and used as an indirect index of cell proliferation.

ASSESSMENT OF THE PERCENTAGE OF APOPTOTIC CELLS AND NECROTIC CELLS

To detect apoptotic and necrotic cells, Apoptotic/Necrotic/Healthy Cells Detection Kit was used (PromoCell GmbH, Heidelberg, Germany). Cells were stained with Hoechst 33342, FITC-Annexin V and Ethidium Homodimer. This assay was conducted after the incubation of hAMSCs under the conditions described above. After washing with 1× binding buffer, the cells were observed under a fluorescence microscope (Zeiss Axiophoto 2, Carl Zeiss, Germany). In this assay, while apoptotic cells were stained both green and blue, necrotic cells were stained both red and blue. A minimum of 500 cells were scored from each sample.

SMALL INTERFERENCE RNA (siRNA) AND EXPRESSION PLASMID FOR HIF-1α

ON-TARGETplus SMARTpool human siRNAs against HIF-1α (L-004018-00-0020), HIF-2α (L-004814-00-0020), COX-2 (L-004557-00-0005), EP-1 (L-005711-00-0005), EP-2 (L-005712-00-0005), EP-3 (L-005713-02-0005), and EP-4 (L-005714-00-0005) and ON-TARGETplus non-targeting siRNA (D-001810-10-05) were synthesized by Thermo Fisher Scientific, Inc. Expression plasmid for HIF-1α was purchased from OriGene (Rockville, MD). Cells were then transfected with the indicated siRNAs at 50 nM or along with HIF-1α expression plasmid for 48 h using DharmaFECT transfection agent (Dharmacon Research, CO) according to the manufacturer's instructions.

RNA PREPARATION

Total cellular RNA was extracted from hAMSCs grown in serum-free culture medium with or without the indicated concentrations of ethylcysteine in the presence or absence of the indicated doses of UVA irradiation for 3 days using the TRIzol reagent (Invitrogen) and purified with the RNeasy Mini Kit (Qiagen, CA) according to the manufacturer's instructions. All samples were DNase treated (Ambion, CA) and subsequently analyzed on an Agilent Bioanalyser (Agilent Technologies, Waldbronn, Germany) and a NanoDrop 8000 Spectrophotometer (Thermo Scientific, Schwerte, Germany) to determine RNA concentration, purity, and integrity. Samples with an appropriate RNA integrity number (RIN, ≥8.0) and RNA purity ($A_{260}/A_{280} = 1.8–2.0$) were used.

cDNA SYNTHESIS

Purified RNA (1 μg) was reverse-transcribed in a 20 μl reaction mixture using the RevertAid™ First Strand cDNA Synthesis Kit Oligo dT Primers (Fermentas, Canada) on a BioRad PTC-200 DNA Engine thermal cycler (BioRad, Hercules). Briefly, the RNA samples and oligo (dT) primers were mixed and denatured at 70°C for 10 min. The tubes were then immediately placed on ice for at least 1 min. The transcription mixture and RNase inhibitor were added, and the mixture was incubated at 37°C for 5 min. The first-strand cDNA synthesis was initiated after the addition of M-mulv, and the reverse transcriptase reaction was performed at 42°C for 1 h. Finally, the enzymes were inactivated at 70°C for 10 min. The reactions were performed in triplicate to reduce any differences in the efficiency of the reverse transcription reaction. The cDNA was stored at −80°C and diluted 1:5 with RNase-free water for use as the template in the real-time PCR analysis.

REAL-TIME RT-PCR (TaqMan™) ANALYSIS

Real-time RT-PCR analysis was conducted using an ABI7900HT machine (Applied Biosystems). All TaqMan RT-PCR reagents, including primers and probes, were purchased from Applied Biosystems. TaqMan analysis was conducted using predesigned and optimized Assays on Demand (Applied Biosystems). The following assays were used: HIF-1α (ID: Hs00936371_m1), HIF-2α (ID: Hs01026149_m1), OCT4 (ID: Hs03005111_g1), NANOG (ID: Hs02387400_g1), SOX2 (ID: Hs01053049_s1), REX1 (ID: Hs01938187_s1), GAPDH (ID: Hs00266705_g1). The reaction parameters were as follows: 2-min 50°C hold, 30-min 60°C hold, and 5-min 95°C hold, followed by 45 cycles of 20-s 94°C melting and 1-min 60°C annealing/extension. All measurements were performed in duplicate or triplicate and the results were analyzed using the ABI sequence detector software version 2.0 (Applied Biosystems). Relative quantitation was conducted using GAPDH as a reference gene, which was validated using the NormFinder software (Supporting Information in Fig. S1 and Table S1). Since all assays used were optimized for PCR efficiency by the manufacturer, mRNA expression levels were estimated by the delta C_t values.

LUCIFERASE REPORTER ASSAY

To assay for AP-1, NF-κB, and CRE promoter activities, hAMSCs were transfected with AP-1 (Stratagene, La Jolla, CA), NF-κB (Stratagene), CRE-Luc (Stratagene), or COX-2-Luc reporter (College of Pharmacy, Seoul National University, Republic of Korea) along with 1 μg of the Renilla luciferase expression vector, which was driven by a thymidine kinase promoter (Promega, Madison, WI) (internal standard), using the DharmFECT® Duo transfection reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Twenty-four hours later, the cells were cultured in MesenPRO RS™ medium for 24 h and then irradiated with the indicated doses of UVA. Following UVA irradiation, the cells were immediately incubated with the indicated concentrations of ethylcysteine (Sigma–Aldrich) in the presence or absence of forskolin (Tocris, Bristol, UK), Phorbol myristate acetate (PMA) (Sigma–Aldrich), tumor necrosis factor-α (TNF-α) (Sigma–Aldrich); PD98059 (Cell Signaling Technology, Beverly, MA), SB203580 (Cell Signaling Technology), SP600125 (Cell Signaling Technology), pyrrolidine dithiocarbamate (PDTC) (Sigma–Aldrich), *N*-(2-[*p*-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide (H89) (Sigma–Aldrich) for 24 h. The luciferase activities were then assayed using a Luciferase Assay System (Promega). The cells were harvested, lysed, and centrifuged. Next, the supernatants were assayed for luciferase activity using a Dual Luciferase Assay system (Promega) and a LB953 luminometer (Berthold, Germany). The activities were expressed as a ratio of the AP-1, NF-κB, or CRE-dependent firefly luciferase activity to the control thymidine kinase Renilla luciferase activity (% control). Results were confirmed by eight independent transfections.

IMMUNOBLOTTING

hAMSCs were irradiated with the indicated doses of UVA and then incubated with the indicated concentrations of ethylcysteine for 1 h or 3 days under serum-free conditions. The cells were washed twice with cold PBS (Sigma–Aldrich) and then lysed in 150 μl of sample buffer (100 mM Tris–HCl (Sigma–Aldrich), pH 6.8, 10% glycerol (Sigma–Aldrich), 4% sodium dodecyl sulfate (SDS) (Sigma–Aldrich), 1% bromophenol blue (Sigma–Aldrich), 10% β-mercaptoethanol (Sigma–Aldrich)). Next, the samples were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to Immobilon-P PVDF membranes (Millipore Corporation, Bedford, MA). The membranes were incubated overnight at 4°C with anti-HIF-1α antibody (Novus Biologicals, Beverly, MA), anti-HIF-2α antibody (Novus Biologicals), and anti-β-actin antibody (Sigma–Aldrich). The membranes were subsequently washed three times with Tris-buffered saline containing Tween-20 (Sigma–Aldrich) (TBST), probed with horseradish peroxidase-conjugated secondary antibody (Sigma–Aldrich), and developed using an ECL (enhanced chemiluminescence) Western blotting detection system (Amersham Biosciences).

MAPK PHOSPHORYLATION ANALYSIS

The levels of phospho-SAPK/JNK (Thr183/Tyr185), phospho-p38 MAPK (Thr180/Tyr182), JNK, and p38 MAPK were measured using a PathScan Inflammation Multi-Target Sandwich enzyme-linked immunosorbent assay (ELISA) Kit (Cell Signaling Technology) according to the manufacturer's instructions. The levels of phospho-p42/44 MAPK (Thr202/Tyr204) and p42/44 MAPK expression were also determined using a PathScan Cell Growth Multi-Target Sandwich ELISA Kit (Cell Signaling Technology) according to the manufacturer's instructions.

ELISA

hAMSCs were irradiated with the indicated doses of UVA and then incubated with the indicated concentrations of ethylcysteine for 3 days under serum-free conditions. After 3 days, the concentrations of PGE_2 or cAMP in the culture supernatant were measured using ELISA kits (ENZO Life Sciences International, Inc., PA). Culture supernatants were added to 96-well plates. Alkaline phosphatase-conjugated PGE_2 or cAMP and antibodies to PGE_2 or cAMP were added to the sample wells. The samples were incubated at room temperature for 2 h. The sample wells were then washed and the *p*-nitrophenyl phosphate (pNpp) substrate solution was added. Finally, the samples were incubated at room temperature for 1 h and

the absorbance was read according to the manufacturer's instructions.

STATISTICAL ANALYSIS

All data are expressed as the means ± SD. Comparison between the control and the treated group was evaluated by one-way analysis of variance followed with the Tukey's multiple comparison test using GraphPad Prism (5.0) (GraphPad, La Jolla, CA). Significance was considered at P values less than 0.05.

RESULTS

UVA IRRADIATION INHIBITS PROLIFERATIVE POTENTIAL AND SELF-RENEWAL OF hAMSCs

In this study, we first examined the effect of UVA irradiation on the proliferation of hAMSCs using a MTT assay under serum-free conditions. In these experiments, UVA was shown to reduce the proliferation of hAMSCs under serum-free conditions in a dose-dependent manner. Specifically, UVA at a dose of 5 J/cm^2 induced a 30% decrease in the cell growth rate of hAMSCs when compared with the serum-free group (Fig. 1A). In addition, Figure 1B,C demonstrated that this effect was not attributed to the apoptotic and necrotic effects of UVA irradiation (Fig. 1B,C). We also examined the expression of stemness genes in hAMSCs using a real-time PCR assay. As shown in Figure 1D, expression of OCT4, NANOG, and SOX2, but not REX1, was reduced by UVA irradiation. In addition, to investigate whether the UVA effects are attributed to serum-free condition which is stressful for cells, the same experiments were performed. In these experiments, we found that UVA induced the same effects in the presence of serum as under serum-free condition (data not shown). These results suggest that UVA irradiation may be involved in the attenuation of self-renewal via a decrease in the proliferative capacity and stemness signals of hAMSCs.

EFFECTS OF UVA IRRADIATION ON THE EXPRESSION OF HYPOXIA-INDUCIBLE FACTORS (HIFS)

As previously mentioned, we found the possibility that UVA irradiation may reduce the stemness of hAMSCs. It has also been reported that HIFs are involved in the self-renewal and differentiation of stem cells [Forristal et al., 2010; Moreno-Manzano et al., 2010]. Specifically, HIFs have been reported to regulate the

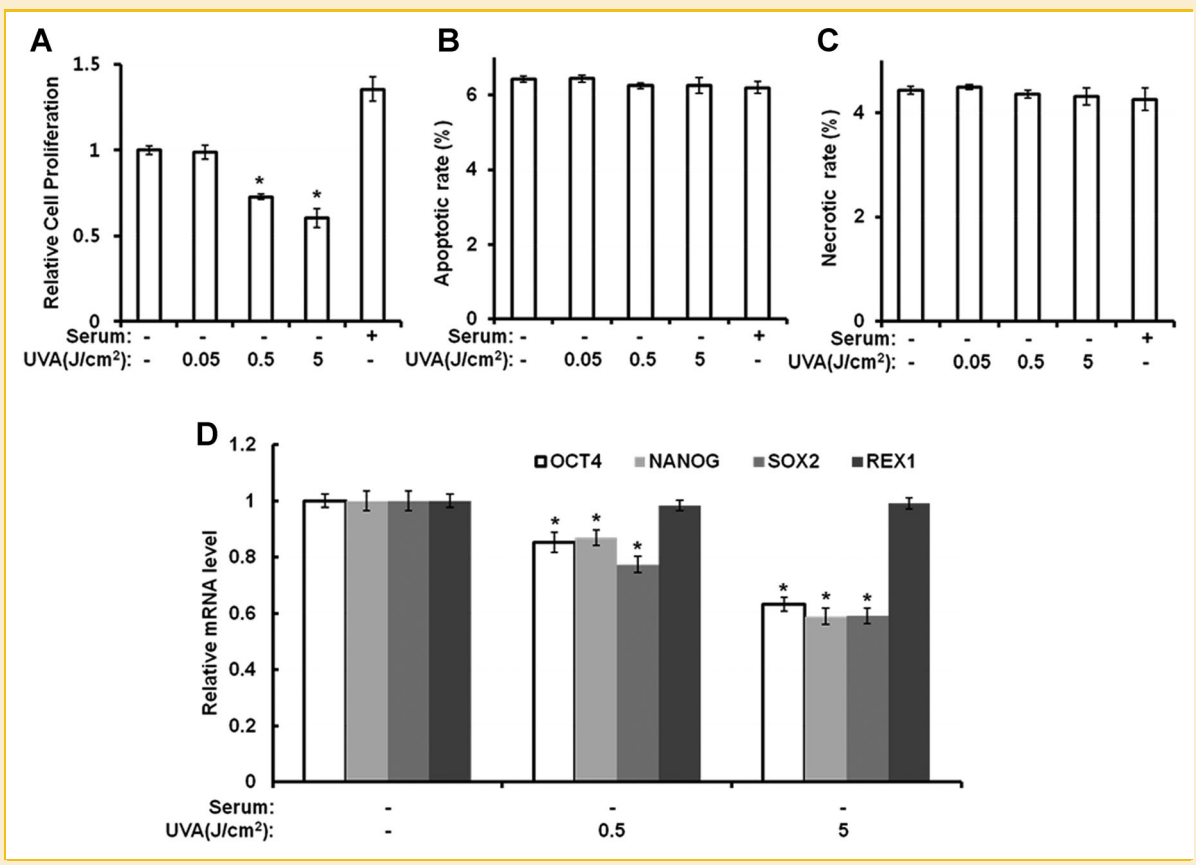

Fig. 1. UVA irradiation inhibits proliferative potential and self-renewal of hAMSCs. hAMSCs were irradiated with the indicated doses of UVA and then incubated for 3 days under serum-free conditions. After 3 days, the cell proliferation and apoptotic effects of UVA irradiation were evaluated using the MTT assay (A), apoptotic assay (B), and necrotic assay (C), respectively. The results were confirmed by five independent experiments, which were each conducted in duplicate. Data are expressed as the means ± SD. $^*P < 0.05$ versus controls. D: After 3 days, total RNA was isolated and the mRNA levels of the OCT4, NANOG, SOX2, and REX1 genes were measured by real-time quantitative RT-PCR. The results are expressed relative to untreated cells after normalization against the GAPDH. Data are expressed as the means ± SD. $^*P < 0.05$ versus controls. The results were verified by repeating the experiments four times, each of which was conducted in duplicate.

expression of stemness-related genes. Therefore, we examined the effects of UVA irradiation on the expression of HIFs [Chen et al., 2011]. As shown in Figure 2A, the mRNA level of HIF-1α but not HIF-2α was significantly lower in cells that were treated with UVA irradiation relative to the control cells. These results were confirmed by Western blot analysis for HIF-1α and HIF-2α, which revealed that UVA irradiation inhibited HIF-1α protein expression, but not HIF-2α expression in a dose-dependent manner (Fig. 2B). These findings suggest that the effect of UVA irradiation on stemness may be HIF-1α-dependent. To test this hypothesis, we used siRNA for HIFs to knock down the expression of HIFs in hAMSCs. As shown in Figure 2C,D, similar to the UVA irradiation, siRNA for HIF-1α reduced the expression levels of OCT4, NANOG, and SOX2, but not REX1. The inhibitory effects of siRNA for HIF-1α were also more enhanced by UVA irradiation (Fig. 2D). In addition, overexpression of HIF-1α attenuated the inhibitory effects of UVA irradiation on stemness genes (Fig. 2D). These results suggest that UVA irradiation may inhibit stemness through the downregulation of HIF-1α.

UVA IRRADIATION UPREGULATES PRODUCTION OF PGE_2 AND cAMP

Several groups have reported that the PGE_2–cAMP signaling pathway downregulates the expression of HIFs [Torii et al., 2009; Sakata et al., 2010]. Therefore, we evaluated UVA irradiation to determine if it exerted a stimulatory effect on the production of the aforementioned proteins. As shown in Figure 3A,B, the production of PGE_2 and cAMP was induced by UVA irradiation in a dose-dependent manner. In addition, UVA-induced production of PGE_2 and cAMP was significantly reduced by siRNA for COX-2 (Fig. 3A,B). To determine if the stimulatory effect of UVA irradiation on the production of PGE_2 and cAMP was mediated by the activation of AP-1, NF-κB, or CRE, luciferase reporter assays were conducted. As shown in Figure 3C–E, UVA irradiation increased the reporter activities of AP-1, NF-κB, and CRE promoters in hAMSCs, suggesting that these promoters may be involved in the UVA-induced production of PGE_2 and cAMP. To further confirm these results, a COX-2 promoter luciferase assay was performed. UVA-induced expression of COX-2 was reduced by PD98059, a p42/44

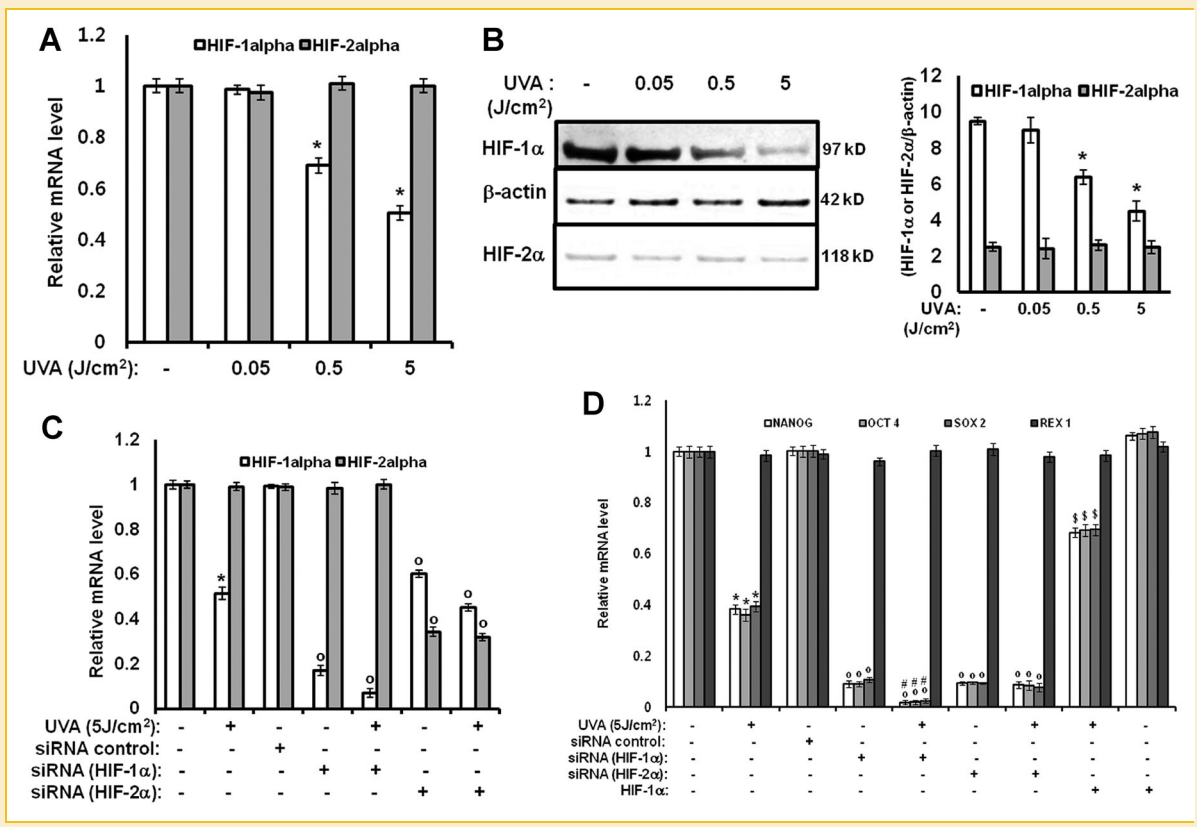

Fig. 2. Effects of UVA irradiation on the expression of the hypoxia-inducible factors (HIFs). hAMSCs were irradiated with the indicated doses of UVA and then incubated for 3 days under serum-free conditions. A,B: After 3 days of incubation, the mRNA and protein levels of the HIF-1α and HIF-2α genes were measured by real-time quantitative RT-PCR (A) and Western blot analysis (B), respectively. Data are expressed as the means ± SD. $^{*}P < 0.05$ versus controls. The results were verified by repeating the experiments four times, each of which was conducted in duplicate. C,D: The cells were transfected with siRNA for HIF-1α and HIF-2α or siRNA control. Following transfection, the cells were irradiated with 5 J/cm^2 UVA and subsequently incubated for 3 days. Total RNA was then isolated and the mRNA levels of the HIF-1α and HIF-2α genes (C) and the OCT4, NANOG, SOX2, and REX1 genes (D) were measured by real-time quantitative RT-PCR. Data are expressed as the means ± SD. $^{*}P < 0.05$ versus untreated controls; $^{\circ}P < 0.05$ versus siRNA control-transfected controls; $^{\$}P < 0.05$ versus siRNA HIF-1α-transfected controls. The results were verified by repeating the experiments four times, each of which was conducted in duplicate.

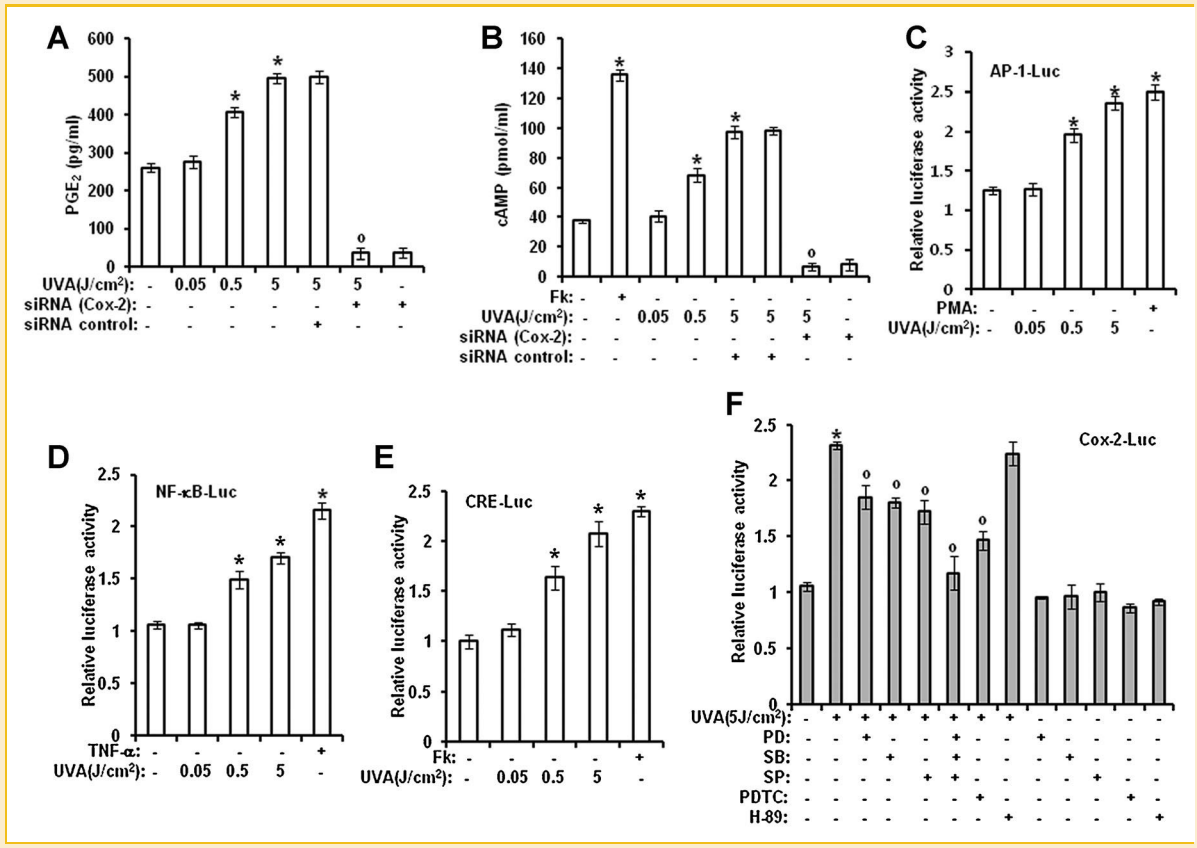

Fig. 3. UVA irradiation upregulates production of PGE_2 and cAMP. hAMSCs were irradiated with the indicated doses of UVA and then incubated for 3 days under serum-free conditions. A,B: After 3 days of incubation, the supernatants were harvested for PGE_2 (A), and cAMP (B) measurement. Data are expressed as the means ± SD. $^*P < 0.05$ versus controls; $^\circ P < 0.05$ versus siRNA control-transfected controls. The results were verified by repeating the experiments three times, each of which was conducted in duplicate. Fk, forskolin (5 μM). C–F: hAMSCs were transfected with AP-Luc, NF-κB-Luc, or CRE-Luc reporters along with a Renilla luciferase expression vector driven by a thymidine kinase promoter using DharmFECT® Duo transfection reagent according to the manufacturer's instructions. After incubation for 24 h, the cells were irradiated with the indicated doses of UVA and then treated with the indicated drugs. The cells were further incubated under serum-free conditions for 14 h. The cells were then harvested, lysed, and assayed. The results were confirmed by three independent transfections. Data are expressed as the means ± SD. $^*P < 0.05$ compared to the untreated control; $^\circ P < 0.05$ versus UVA-irradiated controls. Fk, forskolin (5 μM); PMA, Phorbol myristate acetate (100 nM); TNF-α, tumor necrosis factor-α (20 ng/ml); PD, PD98059 (20 μM); SB, SB203580 (1 μM); SP, SP600125 (20 μM); PDTC, pyrrolidine dithiocarbamate (100 μM); H-89, H-89 (10 μM).

MAPK inhibitor, SB203580, a p38 MAPK inhibitor, SP600125, a JNK inhibitor, and PDTC, a NF-κB inhibitor, but not H-89, a PKA inhibitor (Fig. 3F). In addition, co-treatment of PD98059, SB203580, and SP600125 was found to synergistically reduce expression of COX-2 induced by UVA (Fig. 3F). These findings indicate that, while AP-1 and NF-κB were involved in the expression of the COX-2 gene, CRE was only involved in the downstream signaling of PGE_2 and did not have a direct effect on COX-2.

UVA-INDUCED PGE_2 SIGNALING IS MEDIATED BY EP-2 AND EP-4

Our previous results suggested that UVA enhances the PGE_2 signaling pathway by upregulating the expression of COX-2 through activation of AP-1 and NF-κB. These results were further confirmed by experiments using siRNA for COX-2. As shown in Figure 4A,B, knock-down of the COX-2 gene attenuated the effects of UVA on the expression of stemness-related genes. Specifically, the reduced expression of HIF-1α, OCT4, NANOG, and SOX2 genes by UVA irradiation was increased by knock-down of the COX-2 gene.

Four subtypes of the PGE_2 receptor (EP-1, EP-2, EP-3, and EP-4) have been defined pharmaceutically. To determine the involvement of these receptors in UVA-induced PGE_2 signaling, a cAMP production assay and real-time PCR assay for HIF-1α were conducted using siRNAs for EP-1, EP-2, EP-3, and EP-4. As shown in Figure 4C,D, the increase of cAMP induced by UVA irradiation was reduced by knock-down of EP-2 and EP-4, but not by EP-1 and EP-3. Similarly, while reduced expression of HIF-1α by UVA irradiation was attenuated by knock-down of EP-2 and EP-4, knock-down of EP-1 and EP-3 had no impact on the UVA effects. In addition, knock-down of EP-2 and EP-4 at the same time synergistically attenuated the effects of UVA irradiation on cAMP production and HIF-1α expression (Fig. 4C,D). These results indicate that UVA-induced PGE_2 signaling is dependent on EP-2 and EP-4.

ETHYLCYSTEINE ATTENUATES THE EFFECTS OF UVA IRRADIATION ON PROLIFERATIVE POTENTIAL AND SELF-RENEWAL OF hAMSCs

To attenuate the negative effects of UVA on stemness, a cell-based, compound library screen that was intentionally biased to select

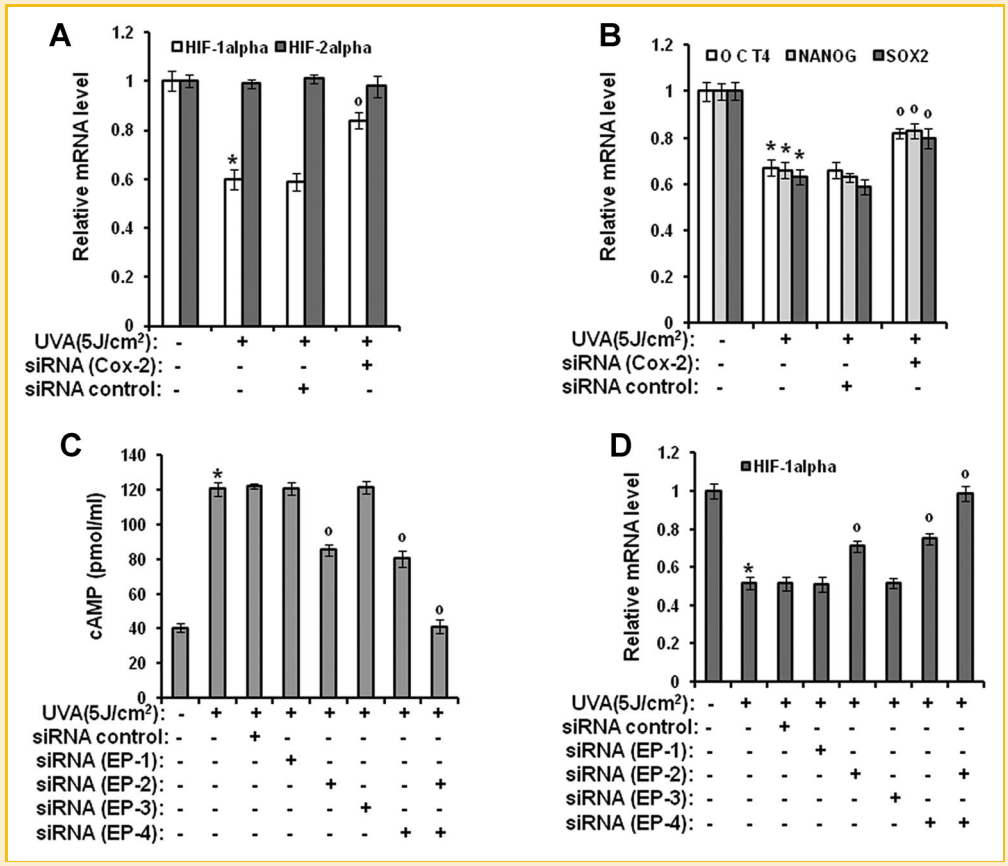

Fig. 4. UVA-induced PGE_2 signaling is mediated by EP-2 and EP-4. A,B: hAMSCs were transfected with siRNA for COX-2 or siRNA control. Following transfection, the cells were irradiated with 5 J UVA and subsequently incubated for 3 days. Total RNA was then isolated and the mRNA levels of the HIF-1α and HIF-2α genes (A) and the OCT4, NANOG, and SOX2 genes (B) were measured by real-time quantitative RT-PCR. The results are expressed relative to untreated cells after normalization against GAPDH. Data are expressed as the means ± SD. $^{*}P < 0.05$ versus untreated controls; $^{\circ}P < 0.05$ versus UVA (5 J/cm^2)-irradiated and siRNA control-transfected controls. The results were verified by repeating the experiments four times, each of which was conducted in duplicate. C,D: hAMSCs were transfected with siRNA for EP-1, EP-2, EP-3, and EP-4 or siRNA control. Following transfection, the cells were irradiated with 5 J/cm^2 UVA and subsequently incubated for 3 days. After 3 days of incubation, the supernatants were harvested for cAMP (C) measurement. Data are expressed as the means ± SD. The results were verified by repeating the experiments three times, each of which was conducted in duplicate. D: After 3 days of incubation, total RNA was isolated and the mRNA level of the HIF-1α gene was measured by real-time quantitative RT-PCR. The results are expressed relative to untreated cells after normalization against GAPDH. Data are expressed as the means ± SD. $^{*}P < 0.05$ versus untreated controls; $^{\circ}P < 0.05$ versus siRNA for EP-1, EP-2, EP-3, and EP-4-transfected controls. The results were verified by repeating the experiments four times, each of which was conducted in duplicate.

relative compounds with low toxicity and high activity was conducted. From this screen, L-cysteine ethylester hydrochloride (ethylcysteine) was selected as a candidate for use as an UVA-antagonizing agent. As shown in Figure 5A, ethylcysteine recovered UVA-induced reduction of proliferation in a dose dependent manner. Ethylcysteine also did not show any apoptotic effects at the treated concentration (Fig. 5B). In addition, as shown in Figure 5C, reduced expression levels of OCT4, NANOG, and SOX2 by UVA irradiation were all increased by ethylcysteine, suggesting that ethylcysteine attenuates the effects of UVA irradiation on hAMSCs.

UVA IRRADIATION-INDUCED DOWNREGULATION OF HIF-1α IS RESTORED BY ETHYLCYSTEINE

The results described above demonstrated that expression of the HIF-1α gene plays an important role in the UVA-induced reduction of stemness. To characterize the effects of ethylcysteine on the reduction in expression of HIF-1α by UVA irradiation, real-time PCR and Western analyses of HIF-1α and HIF-2α were conducted. Treatment with ethylcysteine attenuated the reduction in the expression of HIF-1α that occurred in response to treatment with UVA irradiation (Fig. 6A). However, ethylcysteine did not effect the expression of HIF-2α (Fig. 6A). Consistent with these findings, the reduction in the protein level of HIF-1α that was induced by UVA irradiation was also significantly recovered by ethylcysteine (Fig. 6B). Taken together, these findings suggest that ethylcysteine recovered the reduced stemness of hAMSCs due to UVA irradiation through the upregulation of HIF-1α.

ETHYLCYSTEINE REDUCES UVA-INDUCED PRODUCTION OF PGE_2 AND cAMP THROUGH INHIBITION OF JNK AND P42/44 MAPK

As previously described, several articles have reported that the PGE_2–cAMP signaling pathway downregulates the expression of

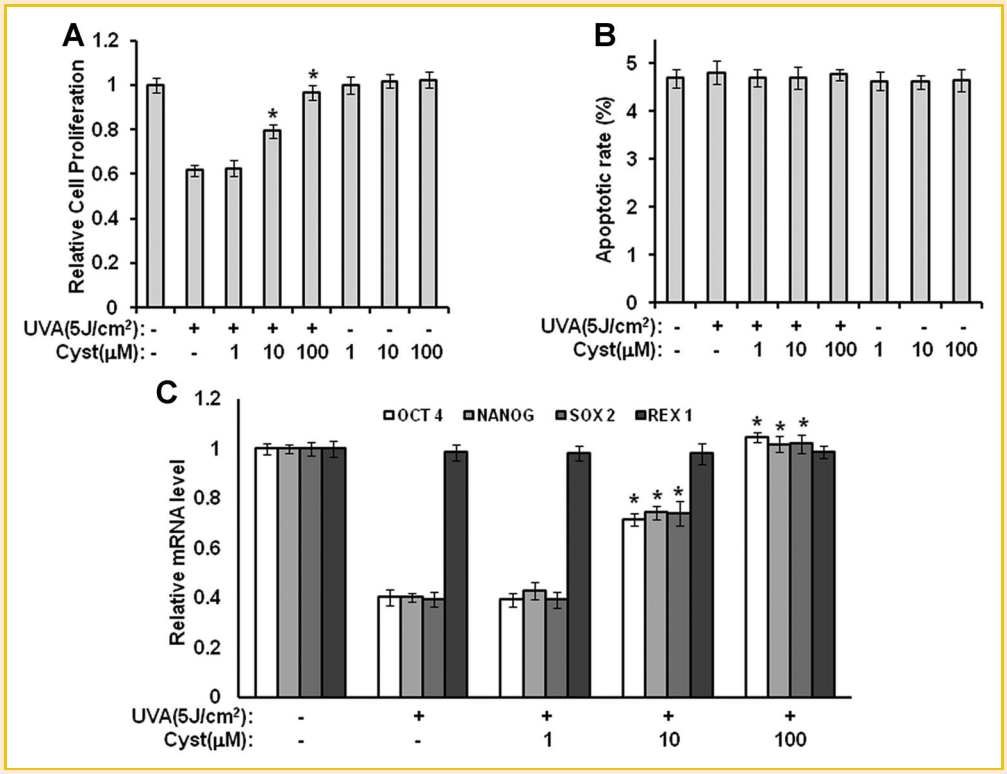

Fig. 5. Ethylcysteine attenuates the effects of UVA irradiation on proliferative potential and self-renewal of hAMSCs. hAMSCs were irradiated with 5 J/cm^2 UVA and then incubated for 3 days in the presence of the indicated concentrations of ethylcysteine under serum-free conditions. A: After 3 days, cell proliferation was evaluated using the MTT assay. The results were confirmed by five independent experiments, which were each conducted in duplicate. Data are expressed as the means ± SD. $^*P < 0.05$ versus controls. B: The apoptotic effects of UVA irradiation were determined by Hoechst 33332 staining. The results were verified by repeating the experiments four times, each of which was conducted in duplicate. Data are expressed as the means ± SD. $^*P < 0.05$ versus controls. C: After 3 days of incubation under serum-free conditions, total RNA was isolated and the mRNA levels of the OCT4, NANOG, SOX2, and REX1 genes were measured by real-time quantitative RT-PCR. The results are expressed relative to untreated cells after normalization against the GAPDH. Data are expressed as the means ± SD. $^*P < 0.05$ versus UVA (5 J)-treated controls. The results were verified by repeating the experiments four times, each of which was conducted in duplicate. Cyst: ethylcysteine.

HIFs [Torii et al., 2009; Sakata et al., 2010]. The results of the present indicate that UVA irradiation-induced reduction of HIF-1α was restored by ethylcysteine. Therefore, we investigated the effects of ethylcysteine on the increased production of PGE_2 and cAMP by UVA irradiation. In this study, treatment of hAMSCs with ethylcysteine was found to lead to a significant decrease in the production of PGE_2 and cAMP when compared to the UVA-irradiated controls (Fig. 7A,B). To investigate the action mechanisms of ethylcysteine, luciferase reporter assays for AP-1, NF-κB, or CRE were conducted. As shown in Figure 7C–E, while ethylcysteine had no effect on the UVA-induced activation of the NF-κB promoter, activation of AP-1 and CRE by UVA irradiation was reduced. These results suggest that ethylcysteine attenuated UVA-induced production of PGE_2 through inhibition of AP-1 activity. In addition, among the three types of MAPKs evaluated, the activities of JNK and p42/44 MAPK, but not p38 MAPK, were found to be reduced by ethylcysteine when compared to the UVA-irradiated controls (Fig. 7F). Collectively, these results suggest that ethylcysteine attenuates the UVA-induced reduction of stemness by reducing the production of PGE_2 and cAMP via the inhibition of JNK and p42/44 MAPK.

DISCUSSION

Our studies suggest that UVA irradiation suppresses the stemness properties of hAMSCs and its inhibitory mechanisms involve upregulation of PGE_2 production through activation of AP-1 and NF-κB. This is consistent with the activation of cAMP–HIF-1α signaling in response to UVA irradiation, which reduces expression of stemness genes and consequently attenuates the stemness properties of hAMSCs. These effects of UVA irradiation were found not to simply be a consequence of apoptotic and necrotic effects of UVA. These effects of UVA irradiation were also found to be attenuated by ethylcysteine, which was mediated by reducing the production of PGE_2 through the inhibition of JNK and p42/44 MAPK, which consequently inhibited PGE_2–cAMP–HIF-1α signaling and increased the stemness of hAMSCs.

Our study was designed based on the hypothesis that UVA effects may be mediated by PGE_2–cAMP–HIF-1α signaling (Fig. 8). PGE_2, one of prostanoids, was shown to induce activation of cAMP metabolism via EP-2 and EP-4 [Sakata et al., 2010]. It was also recently reported that cAMP, a downstream molecule of PGE_2, represses the expression of HIFs [Torii et al., 2009]. Consistent with

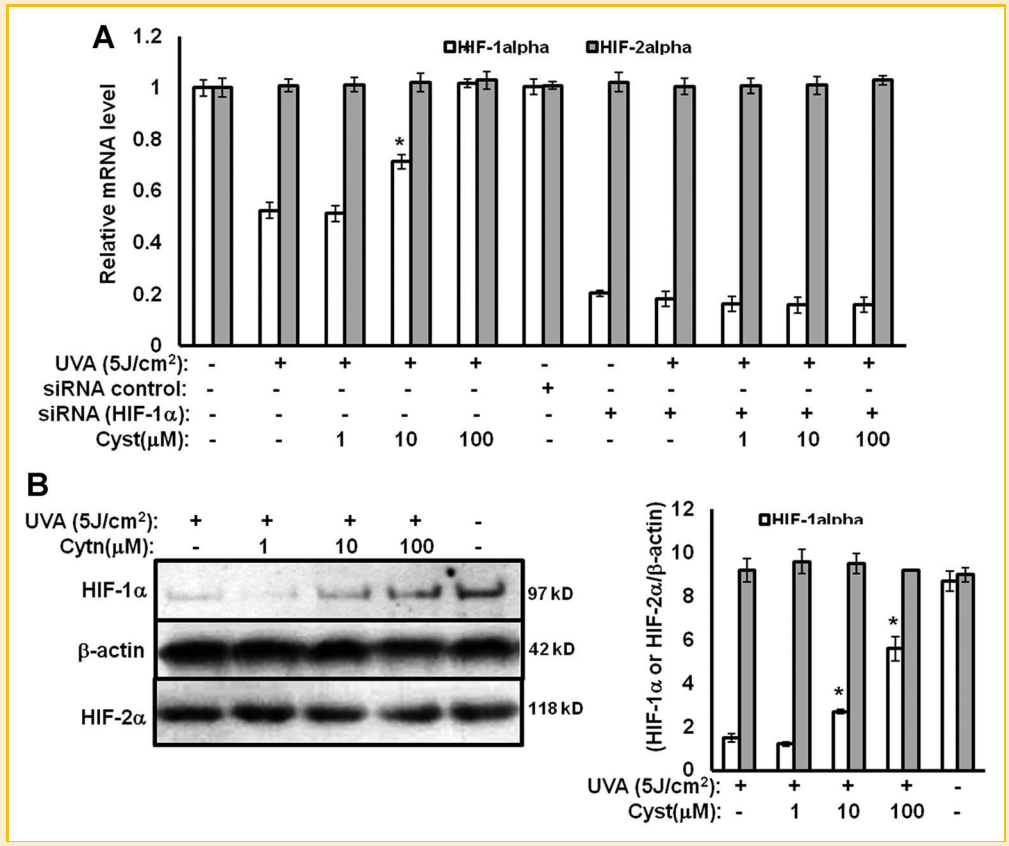

Fig. 6. Ethylcysteine increase downregulated expression of HIF-1α by UVA irradiation. hAMSCs were irradiated with 5 J/cm^2 UVA or transfected with the siRNA for HIF-1α or 2α and then incubated for 3 days in the presence of the indicated concentrations of ethylcysteine under serum-free conditions. A: After 3 days of incubation, total RNA was isolated and the mRNA levels of the HIF-1α and HIF-2α gene were measured by real-time quantitative RT-PCR. The results are expressed relative to untreated cells after normalization against GAPDH. Data are expressed as the means ± SD. $^*P < 0.05$ versus UVA (5 J/cm^2)-treated controls. The results were verified by repeating the experiments four times, each of which was conducted in duplicate. B: Total lysates were analyzed by Western blot using the HIF-1α and HIF-2α antibodies. The results were verified by repeating the experiments three times. Cyst: ethylcysteine.

these findings, the results of the present study showed that while the production of PGE_2 and cAMP was induced upon UVA irradiation, the expression of HIF-1α was reduced. The results of this study may have important implications for stem cell biology, as well as for understanding the role of PGE_2–cAMP signaling in the UVA-induced reduction of stemness.

In this study, to investigate the mechanisms of UVA-induced PGE_2 production, we found that UVA induced expression of the COX-2 gene through the activation of AP-1 and NF-κB. Specifically, three MAPKs, JNK, P38 MAPK, and p42/44 MAPK were all found to be involved in the UVA-induced expression of the COX-2 gene. In addition, NF-κB was shown to contribute to the UVA-induced expression of COX-2 gene. Furthermore, ethylcysteine suppressed UVA-induced production of PGE_2 as well as cAMP, a downstream molecule of PGE_2, through the inhibition of JNK and p42/44 MAPK. Collectively, these results indicate that UVA irradiation exerts its inhibitory effects on the stemness of hAMSCs by upregulating expression of COX-2 through the activation of AP-1 and NF-κB and that ethylcysteine attenuates the effects of UVA by selectively inhibiting activation of JNK and P42/44 MAPK. This finding suggests that ethylcysteine may be used as an UVA-antagonizing agent to recover the stemness of hAMSCs.

OCT4, a crucial transcription factor regulating stem cell self-renewal, was reported to be a specific direct target of HIF-2α, but not of HIF-1α [Covello et al., 2006]. However, in the present study, we found that expression of stemness genes such as OCT4, NANOG, and SOX2 was reduced by UVA irradiation through the downregulation of HIF-1α, but not of HIF-2α. In addition, we found that similar to HIF-2α, HIF-1α was involved in the regulation of the stemness of hAMSCs and its expression was partially dependent on the expression of HIF-2α. These results suggest that HIF-2α operates upstream of HIF-1α and affects HIF-1α-dependent functions by regulating expression of HF-1α.

UVA irradiation is an environmental factor which exerts its several effects on cells and tissues. In addition, a large area of research has recently focused on adipose tissue due to the increase in the rates of obesity. However, the effects of UVA irradiation on adipose tissue, adipocytes and adipose tissue-derived mesenchymal stem cells have not been reported, except our previous study,

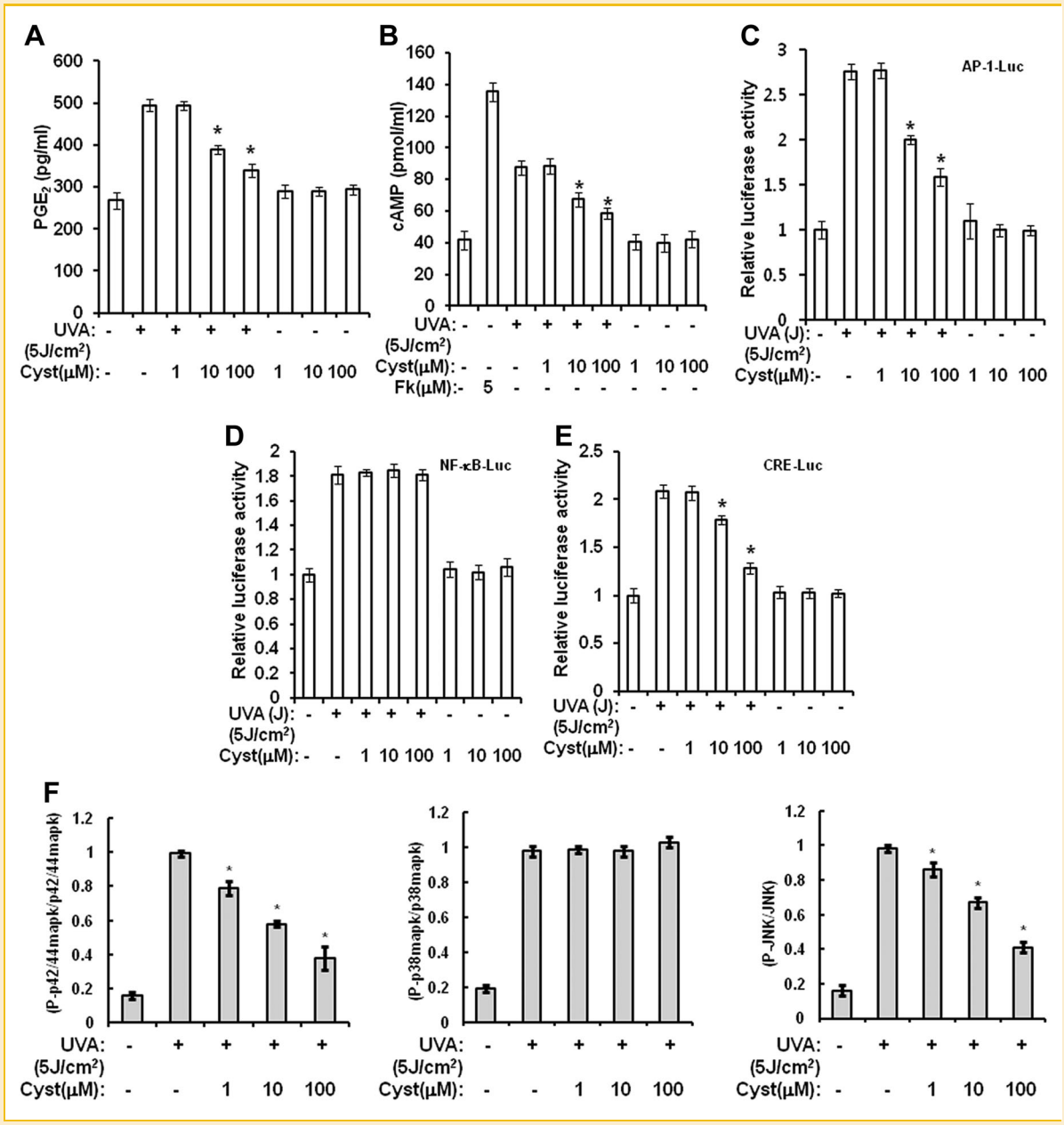

Fig. 7. Ethylcysteine reduces UVA-induced production of PGE_2 and cAMP through the inhibition of JNK and p42/44 MAPK. hAMSCs were irradiated with 5 J/cm^2 UVA and then incubated for 3 days in the presence of the indicated concentrations of ethylcysteine under serum-free conditions. A,B: After 3 days of incubation, the supernatants were harvested for PGE_2 (A) and cAMP (B) measurement. Data are expressed as the means ± SD. $^*P < 0.05$ versus UVA (5 J)-treated controls. The results were verified by repeating the experiments three times, each of which was conducted in duplicate. C–E: hAMSCs were transfected with AP-Luc, NF-κB-Luc, or CRE-Luc reporters along with a Renilla luciferase expression vector driven by a thymidine kinase promoter using DharmFECT® Duo transfection reagent according to the manufacturer's protocols. After incubation for 24 h, the cells were irradiated with 5 J UVA and then further incubated in the presence of the indicated concentrations of ethylcysteine under serum-free conditions for 14 h. These cells were then harvested, lysed, and assayed. The results were confirmed by three independent transfections. Data are expressed as the means ± SD. $^*P < 0.05$ compared to the UVA (5 J)-treated control. F: hAMSCs were irradiated with 5 J/cm^2 UVA and then treated with the indicated concentrations of ethylcysteine for 1 h under serum-free conditions. After 1 h of incubation, the cell lysates were analyzed using a Multi-Target Sandwich ELISA Kit. The results were verified by repeating the experiments three times, each of which was conducted in duplicate. Cyst: ethylcysteine.

which demonstrated that UVA irradiation inhibits adipogenic differentiation of hAMSCs [Lee et al., 2010]. In this study, we found that UVA irradiation negatively impacted the stemness of hAMSCs, suggesting UVA irradiation is deleterious to the normal stemness biology of hAMSCs, despite its anti-adipogenic effect, which may be beneficial for reducing diet-induced obesity. In addition, hAMSCs have received a lot of attention as a therapeutic candidate for cell therapy because, compared to other mesenchymal stem cells, hAMSCs are relatively easy to obtain in large quantities. Therefore, ethylcysteine, which was shown to attenuate the effects

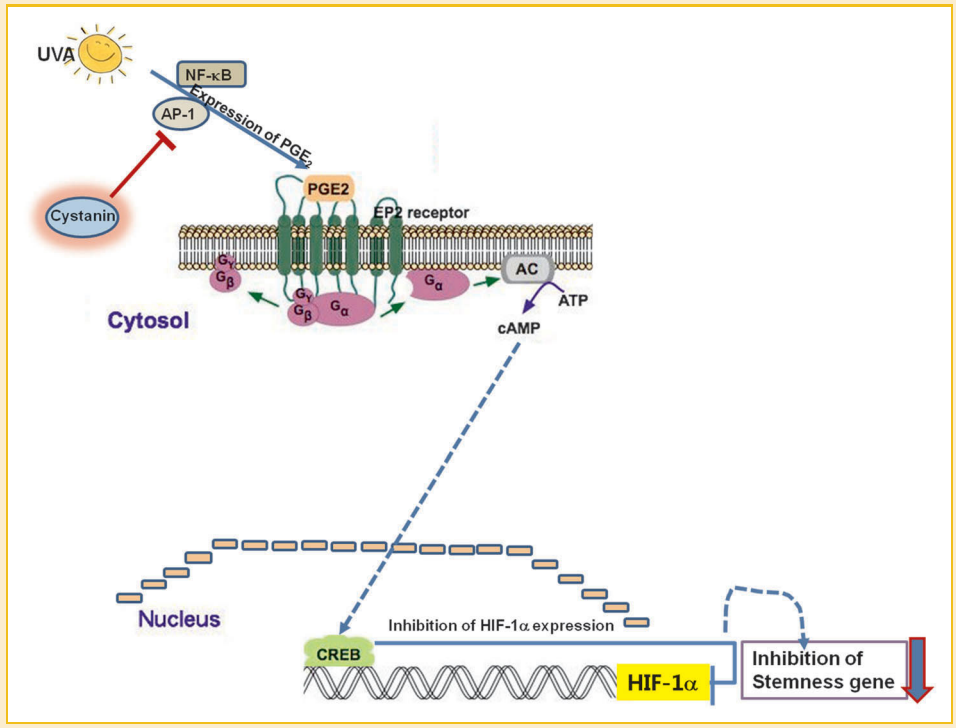

Fig. 8. Mechanisms of UVA-induced attenuation of stemness of stem cells. Cyst: ethylcysteine.

of UVA irradiation, may be used to restore the UVA-damaged stemness properties of hAMSCs.

Taken together, the results of this study demonstrate that UVA irradiation reduces the stemness of hAMSCs by downregulating the expression of HIF-1α through the activation of PGE_2–cAMP signaling. In addition, these results show that ethylcysteine attenuates the effects of UVA irradiation by reducing the production of PGE_2 through the inhibition of JNK and p42/44 MAPK, consequently recovering the stemness of hAMSCs.

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