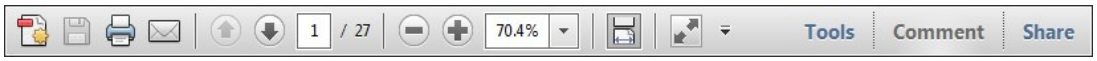
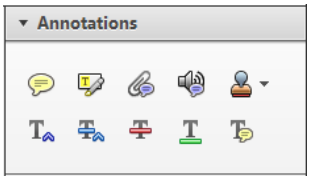
Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:



This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We've picked out some of these tools below:

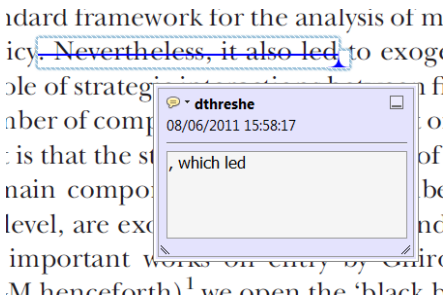


1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

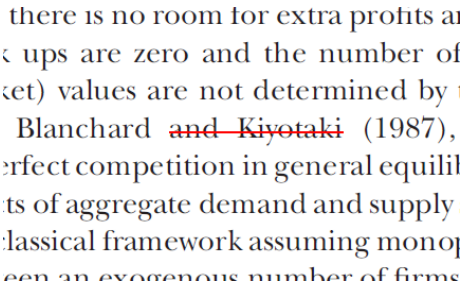


2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

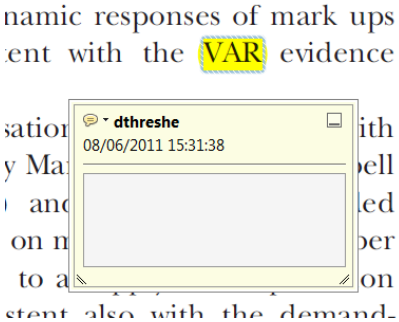


3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

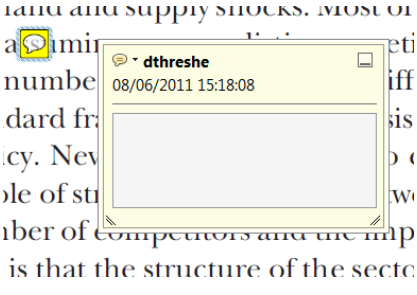


4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.

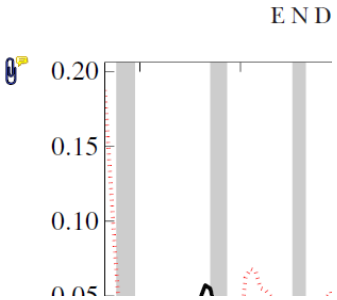


5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate place in the text.

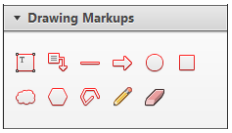
How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.



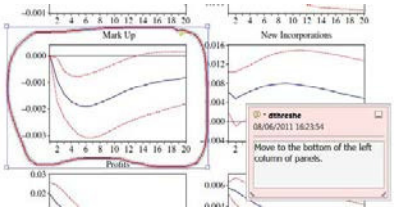
6. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks.



How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.



Photodermatology, Photoimmunology & Photomedicine

ORIGINAL ARTICLE

Conditioned medium from human bone marrow-derived mesenchymal stem cells promotes skin moisturization and effacement of wrinkles in UVB-irradiated SKH-1 hairless mice

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Key words:
antiwrinkle; MSC-CdM; photoaging; UVB radiation

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The authors declare no conflict of interest.

*These authors contributed equally to this study.

SUMMARY

Background
Mesenchymal stem cells (MSCs) are promising therapeutic agents for various diseases.

Aims
To investigate the effects of conditioned medium from human bone marrow-derived mesenchymal stem cells (MSC-CdM) on pro-collagen production and wrinkle formation, we performed *in vitro* and *in vivo* experiments.

Methods
We assessed the effects of MSC-CdM on proliferation and photo-aging in human dermal fibroblasts after UVB exposure using enzyme activity assays for collagen type I secretion and MMP-1. To determine the effect of topically applied MSC-CdM on wrinkle formation, MSC-CdM (1% and 10%) and vehicle (propylene glycol: ethanol, 7 : 3) were applied to the dorsal skin of UVB-irradiated hairless mice for 8 weeks. We examined the effects on wrinkle formation by assessing visual skin grading, replica, tape stripping, transepidermal water loss (TEWL), and skin hydration measurement. We also examined histology of the lesions using hematoxylin–eosin, Masson's trichrome, and immunohistochemical staining.

Results
MSC-CdM markedly reduced UV-induced matrix metalloproteinase-1 expression and increased pro-collagen synthesis in a dose-dependent manner. Our findings suggest that MSC-CdM induces repair of dermal damage and effacement of wrinkles on UVB-irradiated hairless mice through protective effect of hydration.

Conclusion
These results support an anti-wrinkle effect of MSC-CdM that involves increased collagen synthesis and suggest that MSC-CdM might be a potential candidate for preventing UV-induced skin damage.

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Aging is a process in which intrinsic and extrinsic factors lead to a progressive loss of physiological functions and structures (1). The symptoms of skin aging, such as wrinkles and loss of elasticity, are caused by various physiological and environmental factors, including ultraviolet (UV) radiation (2). Among the factors responsible for mediating UV-induced skin aging are matrix metalloproteinases (MMPs) (3). These factors damage the integrity of the dermis by disrupting the production of extracellular matrix proteins or by enhancing their degradation. The development of effective anti-aging agents that can reduce the risk of UV-induced skin aging is required to address pressing public cosmetic issues (4).

Ultraviolet radiation, which can be classified as electromagnetic radiation, is divided into the UVA (320–400 nm), UVB (280–320 nm), and UVC (200–280 nm) wavebands. These UV rays can penetrate and change the structure of skin cells and also penetrate deeply into the dermis, degenerating collagen and causing wrinkles (5). UV radiation injures the stratum corneum, reducing its barrier functionality and causing water loss from the skin, resulting in dryness (6). Collagen has been used as a functional skin treatment owing to its moisturizing and elasticity-enhancing efficacies. A pivotal component of the skin dermis is type 1 collagen, which performs the crucial function of maintaining the structure of the dermis (7). Reductions in the level of type 1 collagen result in degradation of the dermal structure and are a primary cause of skin aging (8).

In recent years, stem cell-derived conditioned medium has shown good prospects for being produced as a pharmaceutical for regenerative medicine (9). For example, many studies have examined the effects of conditioned medium in various kinds of diseases/conditions, including alopecia (10), acute and chronic hind limb ischemia (11, 12), acute and chronic wound healing (13), spinal cord injury (14), lung injury (15), and bone defect (16). Importantly, recent studies have shown that adipose tissue-derived mesenchymal stem cells are effective skin moisturizing and anti-wrinkle agents (17, 18). Various conditioned media contain different growth factors and cytokines that had been secreted by stem cells grown in the media. Therefore, this study aimed to investigate the UV-protective effects of a conditioned medium from human bone marrow-derived mesenchymal stem cells (MSC-CdM) on a UV-induced aging mouse model. Although several experimental studies have suggested that topical treatment with conditioned medium inhibits damage in such a model, there has been no study on the influence of MSC-CdM on the *in vivo* and *in vitro* photoaging aspects.

In this study, we investigated the effect of MSC-CdM on the expression levels of UV irradiation-induced matrix metalloproteinase-1 (MMP-1) in cultured human skin fibroblasts. Moreover, we showed an attenuation of wrinkle formation, a decrease in transepidermal water loss (TEWL), and an increase in skin hydration in a mouse model. The results of this study indicate that MSC-CdM may be useful for the control of photoaging.

MATERIALS AND METHODS

Culture of human dermal fibroblasts

Human dermal fibroblasts were isolated from foreskin. The epidermis and dermis were separated by incubation in medium containing 0.9 U/ml dispase at 4°C (19). After the epidermis and dermis were mechanically separated, the dermis was minced, plated on the surface of a tissue culture flask. The explants were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) for 1–2 weeks. Dermal fibroblasts were cultured in DMEM containing 10% FBS and 1% antibiotics at 37°C in a 5% CO_2 incubator.

CCK-8 assay

Human dermal fibroblasts were plated at a density of 5×10^3 cells/well in 96-well plates, and their proliferation was measured using a cholecystokinin (CCK)-8 assay (Dojindo, Rockville, MD, USA). Cells were treated with various concentrations of MSC-CdM (0.1%, 1%, and 10%) for 24 and 48 h. CCK-8 solution (10 μl) was added to the cells in 1 ml DMEM and incubated for 2 h at 37°C. Absorbance was measured at 450 nm using a microplate reader (SpectraMax 340; Molecular Devices, Sunnyvale, CA, USA).

Quantitative determination of collagen type I secretion

Quantitative determination of collagen type I secretion was indirectly performed using a pro-collagen type 1 C-peptide (PIP) enzyme immunoassay (Takara Bio Inc., WI, USA). In brief, human dermal fibroblasts were plated in microtiter plates (96 wells) at a density of 1×10^5 cells per well and incubated with MSC-CdM at concentrations of 0.1%, 1%, and 10% for 24 h. Transforming growth factor beta (TGF-β; 10 nM) was employed as a positive control. After the incubation period, 100 μl of antibody-peroxidase

conjugate solution was added to each well, followed by addition of 20 µl of diluents or standard solution. After incubation for 3 h, the contents were removed by aspiration, and all the wells were washed four times with 400 µl of PBS. Next, 100 µl of substrate solution was added to each well and the plates were incubated at room temperature for 15 min. The reaction was stopped by the addition of 100 µl of stop solution to each well. Absorbance was measured at 450 nm using an ELISA reader (SpectraMax 340; Molecular Devices, Sunnyvale, CA, USA).

MMP-1 inhibition assay

The MMP-1 activity was determined with an MMP-1 immunoassay kit (R&D Systems, Minneapolis, MN, USA). Human dermal fibroblasts were seeded in microtiter plates (96 wells) at a density of 1×10^5 cells per well. The cells were pretreated with MSC-CdM (0.1%, 1% or 10%) prior to UVB irradiation (50 mJ/cm^2) and harvested after 24 h. Before UVB irradiation, the cultures were rinsed in PBS and irradiated with the indicated fluencies in PBS to avoid absorption by the phenol red in the culture medium. All groups were kept in PBS at room temperature during the experimental procedure to ensure equal treatment conditions. The activity of MMP-1 in the culture solution was determined using the method described in the kit.

Culture of human bone marrow-derived mesenchymal stem cells

All the manufacturing and product testing procedures for the generation of MSC-CdM were performed under good manufacturing practice conditions (Pharmicell Co., Ltd., Sungnam, Korea). Approximately 20 ml of bone marrow (BM) was aspirated from the posterior iliac crest of healthy male donors under local anesthesia. BM mononuclear cells were isolated by density gradient centrifugation (Histopaque-1077; Sigma-Aldrich, St. Louis, MO, USA) and plated in 75-cm^2 flasks with low-glucose DMEM (Gibco, Grand Island, NY, USA) containing 10% FBS and 20 µg/ml gentamicin (Gibco) and were cultured at 37°C in a 5% CO_2 atmosphere. After 5 days, non-adherent cells were removed by replacing the medium. When the cultures approached 80% confluence, the cells were harvested by treatment with a trypsin/ethylenediaminetetraacetic acid solution (Gibco), replated at a density of $5–10 \times 10^5$ cells in 175-cm^2 flasks, and then serially subcultured for up to six passages (P6) using the same procedure. Harvested P6 cells were resuspended in DMEM without phenol red/FBS/antibiotic, plated at a density of $2–2.5 \times 10^6$ cells in 175-cm^2 flasks, and then cultured for 5 days in hypoxic conditions. The MSCs expressed CD73, CD105, and were lacking in CD14, CD34, and CD45 as confirmed by the conventional method. The conditioned medium (supernatant) was collected, filtered using a 0.2-µm membrane filter, and stored at −80°C until use.

Hairless mouse skin photoaging model

A total of 48 female SKH-1 hairless mice (6 weeks old) were purchased from Charles River Laboratories (Wilmington, MA, USA) and divided randomly into six groups (8 mice/group). The application study was performed as topical application of MSC-CdM on the dorsal skin of UVB-irradiated mice, three times a week for 8 weeks. The SKH-1 hairless mice were irradiated using a UVB-emitting system from Biospectra (Vilber Lourmat, Marne La Vallée, France), which has been designed for UV irradiation of test animals and can be programmed to deliver specific UV radiation dosages in energy. The irradiation dose was increased weekly by 1 minimal erythemal dose (1 MED = 50 mJ/cm^2) to 4 MED and then maintained at 3–4 MED until 8 weeks. Immediately after each session of UVB irradiation, a 200-µl aliquot of MSC-CdM (1% or 10%) or the vehicle solution (polyethylene glycol: ethanol, 7 : 3) was topically applied on the dorsal skin of UVB-irradiated mice three times a week. Wrinkle formation on the dorsal skin of the hairless mice after repeated (3 times/week for 8 weeks) exposure to UVB irradiation was evaluated. All procedures involving animals were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Chung-Ang University, Korea.

Visual skin grading

Wrinkling of the skin was evaluated by blinded investigators, using a grading scale based on the experimental model proposed by Bisset *et al.* (20).

Skin surface physiology

Clinical symptoms were measured using images captured daily with a digital camera (Canon 3000D; Canon Inc., Tokyo, Japan) and were analyzed using a phototrichogram system (Folliscope; Lead-M, Seoul, Korea). The transepidermal water loss (TEWL: mg/cm^2/h) was regu-

larly measured with the Tewameter TM 210 system (Courage + Khazaka Electronic GmbH, Köln, Germany). At the end of the study, prior to sacrificing the mice, replicas of the mouse dorsal skin were measured using the Replica full kit (Visioline VL650; Epigem, Seoul, Korea) and were analyzed with Visioline VL650 (Courage + Khazaka Electronics GmbH). The data analyzed were the number, depth, length, and total area of the wrinkles.

Tape stripping

For each of the group sampling sites, standard adhesive disks (D-Squame; CuDerm, Dallas, TX, USA) with a 2.2 cm diameter were placed on the mouse dorsal skin under a standard weight of 500 g for 3 s and were then removed from the skin using forceps. Tape strips were collected every week, and image analysis was used to quantify the total tissue area within this region, followed by determination of the area with remaining stratum corneum using Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD, USA).

Biopsy specimens and histological measurements

The biopsies were fixed with 4% paraformaldehyde and embedded in paraffin. Sections were then transferred to Probe-On-Plus slides (Fisher Scientific, Pittsburgh, PA, USA), and deparaffinized skin sections were stained with Masson's trichrome and hematoxylin–eosin stains. For immunohistochemical analysis, sections were stained for immunohistochemical markers using monoclonal antibodies against type I collagen (1:200, ab292; Abcam, Cambridge, MA, USA), as described previously (21).

Two independent, blinded pathologists evaluated each serial section. Each pathologist assigned each section a score according to the following scale: 0 = negative control, + = moderately increased staining, ++ = considerably increased staining), based on percentages of stained cells in each category.

Statistical analyses

The data are expressed as the mean ± standard deviation (SD). The groups treated with MSC-CdM were compared with the control group (untreated) and UVB-irradiated group. All experiments were performed at least three times and were scored by two investigators. Statistical analysis was performed with one-way analysis of variance, followed by the Tukey multiple comparisons post hoc test, using SPSS software. The statistical level of significance was $P < 0.05$.

RESULTS

Effect of MSC-CdM on procollagen production in cultured human dermal fibroblasts

To evaluate the effects of MSC-CdM on human dermal fibroblasts, we performed a cell proliferation assay. Cells were treated with various concentrations of MSC-CdM (0.1%, 1%, and 10%) and FBS (10%) for 24 and 48 h. The Cell Counting Kit-8 (CCK-8) assay confirmed an increase in human dermal fibroblast proliferation by MSC-CdM, with a significant increase observed at 10% MSC-CdM (Fig. 1a). Next, we studied the effects of MSC-CdM on the expression of MMP-1 and type 1 procollagen in UV-irradiated cultured human skin fibroblasts. Human skin fibroblasts were treated with 0.1%, 1%, or 10% MSC-CdM for 72 h, after which the expression levels of MMP-1 and type I procollagen were determined in the culture media using ELISA. We demonstrated that MSC-CdM decreased the expression of MMP-1 and increased the expression of type I procollagen in a dose-dependent manner in UVB-irradiated cultured human skin fibroblasts. That is, the type 1 procollagen expression level increased by 176%, 198%, and 215% in the presence of 0.1%, 1%, and 10% MSC-CdM, respectively (Fig. 1b), whereas the UV-induced MMP-1 expression was inhibited by 4.7% and 16.2% at 1% and 10% MSC-CdM, respectively (Fig. 1c). These results indicate that MSC-CdM inhibits UV-induced MMP-1 expression and increases procollagen synthesis in human skin fibroblasts, thus suggesting the possibility of using MSC-CdM as a therapeutic agent for UV-induced skin aging.

Effect of MSC-CdM on UVB-induced wrinkle formation on the dorsal skin of hairless mice

The goal of our study was to determine whether topical treatment with MSC-CdM prevents wrinkle formation in UVB-irradiated hairless mice. Therefore, the dorsal skin of UVB-irradiated hairless mice was treated three times a week for 8 weeks with MSC-CdM (1% or 10%) or vehicle solution (polyethylene glycol:ethanol, 7 : 3). Adenosine (0.04%) was used as a positive control. After 4 to 8 weeks, visual assessment and

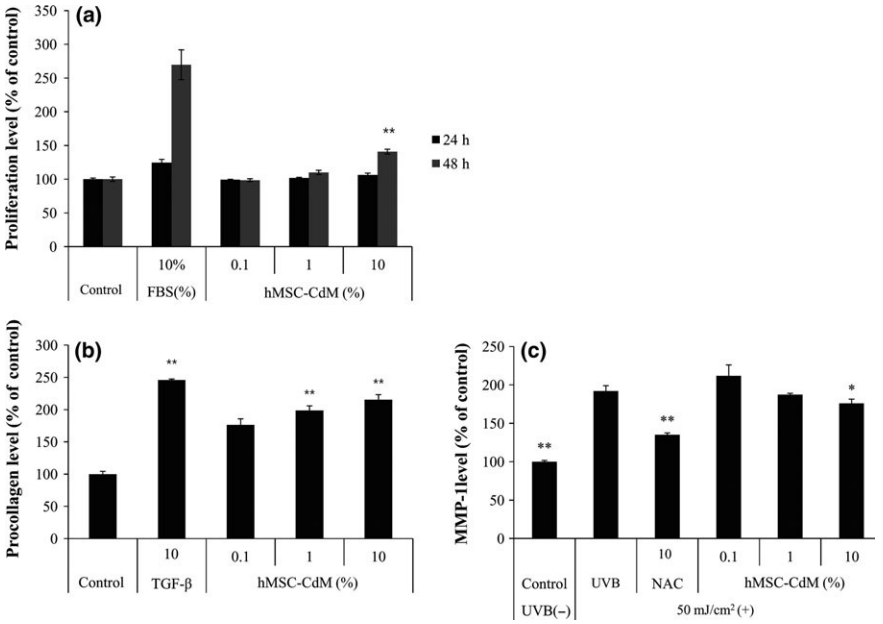

Fig. 1. Effect of conditioned medium from human bone marrow-derived mesenchymal stem cells (MSC-CdM) on human dermal fibroblast proliferation, pro-collagen secretion, and matrix metalloproteinase 1 (MMP-1) activity. (a) The effect of MSC-CdM on human dermal fibroblast proliferation. Human fibroblasts were treated with various concentrations of MSC-CdM (0.1%, 1%, or 10%). Proliferation of the cells was measured using a CCK-8 assay. (b) Pro-collagen biosynthesis was determined by ELISA using a type I C-peptide enzyme immunoassay (EIA) kit. The cells were treated with transforming growth factor beta (TGF-β; 10 ng/ml), or various concentrations of MSC-CdM (0.1%, 1%, or 10%). (c) MMP-1 expression was evaluated by ELISA using an MMP-1 EIA kit after UVB irradiation. The cells were treated with N-acetyl-L-cysteine (NAC; 10 μM), in various concentrations of MSC-CdM (0.1%, 1%, or 10%). Each bar represents the mean ± SD of three experiments. $*P < 0.05$ vs. control.

evaluation of the wrinkle score were performed on each mouse at every 2 weeks. According to the visual assessment, the UVB-induced wrinkle formation on the skin of MSC-CdM-treated or adenosine-treated mice was reduced in comparison with that on the skin of vehicle-treated UVB-irradiated mice (Fig. 2). These results indicate that UVB irradiation induces more wrinkles on the hairless mouse skin than the unirradiated mouse skin. To quantitatively assess the skin roughness, a skin visiometer was applied through the replicas. The results of the quantitative wrinkle scores, which represent the number, depth, length, and total area of the wrinkles, were consistent with the photographs. Figure 3 represents the mean values of the total wrinkle area. All the roughness values in the UVB group were significantly higher than those in the unirradiated group. The MSC-CdM group exhibited significantly reduced levels of total wrinkle area compared with the vehicle-treated group. These data demonstrate that topical treatment with MSC-CdM diminished rough and thick wrinkles caused by skin exposure to UVB irradiation.

Changes in skin barrier function in UVB-irradiated mice after topical application of MSC-CdM

The stratum corneum forms the outer layer of the skin and plays an important role in maintaining the barrier function and water-holding capacity of the skin (22). UVB irradiation causes skin damage, leading to skin dehydration and an increase in TEWL. First, we performed *in vivo* studies of stratum corneum intercellular lipids, by conducting microscopy analysis of progressively tape-stripped mouse dorsal skin. Quantitative analysis was performed to evaluate the relative percentage of the remaining stratum corneum (Fig. 4a). The results showed that the MSC-CdM- and adenosine-treated groups exhibited significantly reduced contents of keratin compared with the UVB group. Furthermore, the TEWL level of the UVB group was increased by 1.2-fold compared with the non-irradiated group, but the TEWL levels of the 1% and 10% MSC-CdM-treated groups were reduced by as much as 1.19- and 1.32-fold, respectively, compared with the vehicle group (Fig. 4b).

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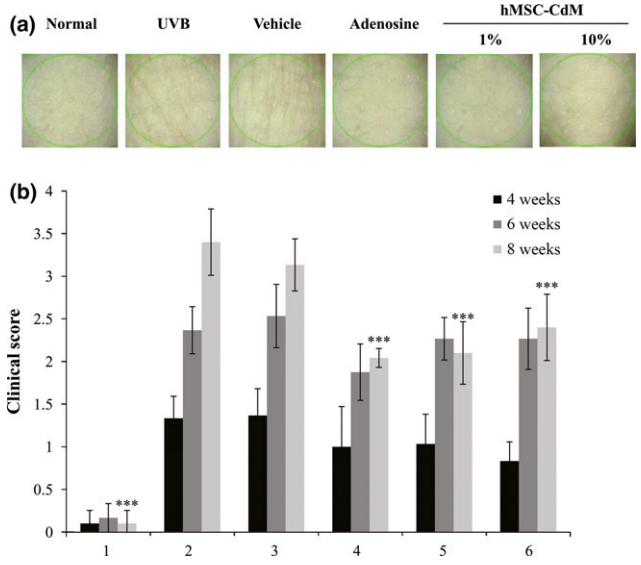

4 Fig. 2. Effect of topically applied conditioned medium for human bone marrow-derived mesenchymal stem cells (MSC-CdM) on wrinkle formation in UVB-irradiated mice. To evaluate changes in dorsal skin wrinkle formation, images were taken using a folliscope (a), and the wrinkle score (b) was analyzed (n = 8). MSC-CdM inhibited wrinkle formation. Clinical results for the following groups: 1, Normal (non-irradiated); 2, UVB; 3, Vehicle; 4, Adenosine; 5, 1% MSC-CdM; 6, 10% MSC-CdM. *** $P < 0.001$.

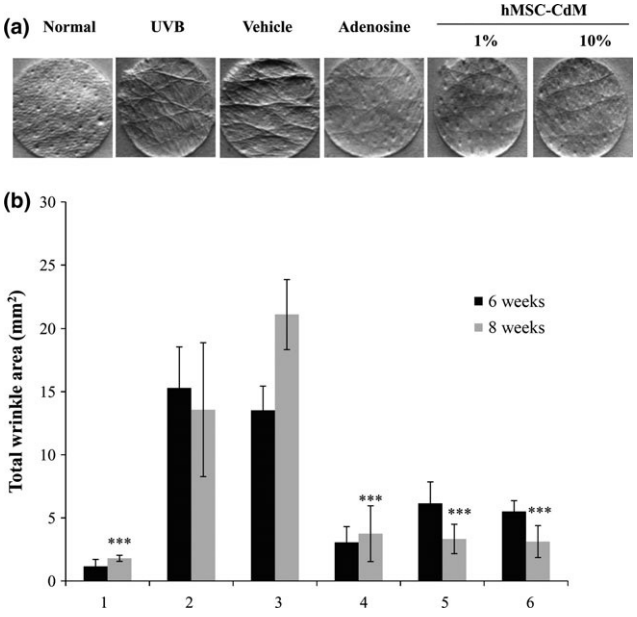

Fig. 3. Reduction in UVB-induced wrinkle formation after repeated topical treatment with conditioned medium for human bone marrow-derived mesenchymal stem cells (MSC-CdM). The dorsal skin of SKH-1 hairless mice was treated topically with 1% or 10% MSC-CdM or vehicle solution (propylene glycol:ethanol, 7 : 3). Replica (a) and image analyses (b) were performed after 6 and 8 weeks. A 0.025% adenosine solution was used as a positive control. Graphical representation of the replica image analysis (n = 8). Clinical results for the following groups: 1, Normal (non-irradiated); 2, UVB; 3, Vehicle; 4, Adenosine; 5, 1% MSC-CdM; 6, 10% MSC-CdM. ***$P < 0.001$ vs. the vehicle-treated group.

As shown in Fig. 4c, skin hydration of the UVB group was decreased by 1.23-fold compared with the unirradiated group. The 1% MSC-CdM group exhibited significantly recovered levels of hydration compared with the vehicle group. Overall, MSC-CdM had a protective effect on skin barrier function by decreasing the TEWL and increasing skin hydration, thereby reducing skin wrinkling.

Effects of topical application of MSC-CdM on elastic fibers and collagen synthesis

Histological sections of the dorsal skin were subjected to hematoxylin–eosin and Masson's trichrome staining to visualize the extracellular matrix components and morphological changes. Increased wrinkle areas in the epidermis and marked epidermal thickening were observed in the UVB group after exposure to UVB for 8 weeks (Fig. 5a,b). Interestingly, decreased epidermal thickening and increased bundles of collagen fibers were observed in the MSC-CdM-treated group. Moreover, immunohistochemical data revealed that UVB exposure enhanced the formation of bundles of collagen fibers (as revealed by the staining patterns of type 1 collagen) compared with those in the UVB exposure group and the MSC-CdM group. We found that topical application of MSC-CdM markedly enhanced the staining pattern of type 1 collagen in the mouse epidermis (Fig. 5c). Thus, our results indicate that topical application of MSC-CdM helps to attenuate wrinkle formation by increasing the collagen in the epidermis/dermis junction and the dermis.

DISCUSSION

UV irradiation contributes to wrinkle formation by enhancing collagenase activity leading to degradation of collagen in the dermal extracellular matrix (23). In recent studies, the development of novel agents with anti-photo-aging activities from various compounds such as natural extracts (24) and chemicals (25) has received a great deal of attention. As the inhibition of the production of MMPs appears to be a useful intervention for preventing collagen damage, we investigated the effects of MSC-CdM on pro-collagen production and wrinkle formation by performing experiments in *in vivo* and *in vitro* systems.

COLOR

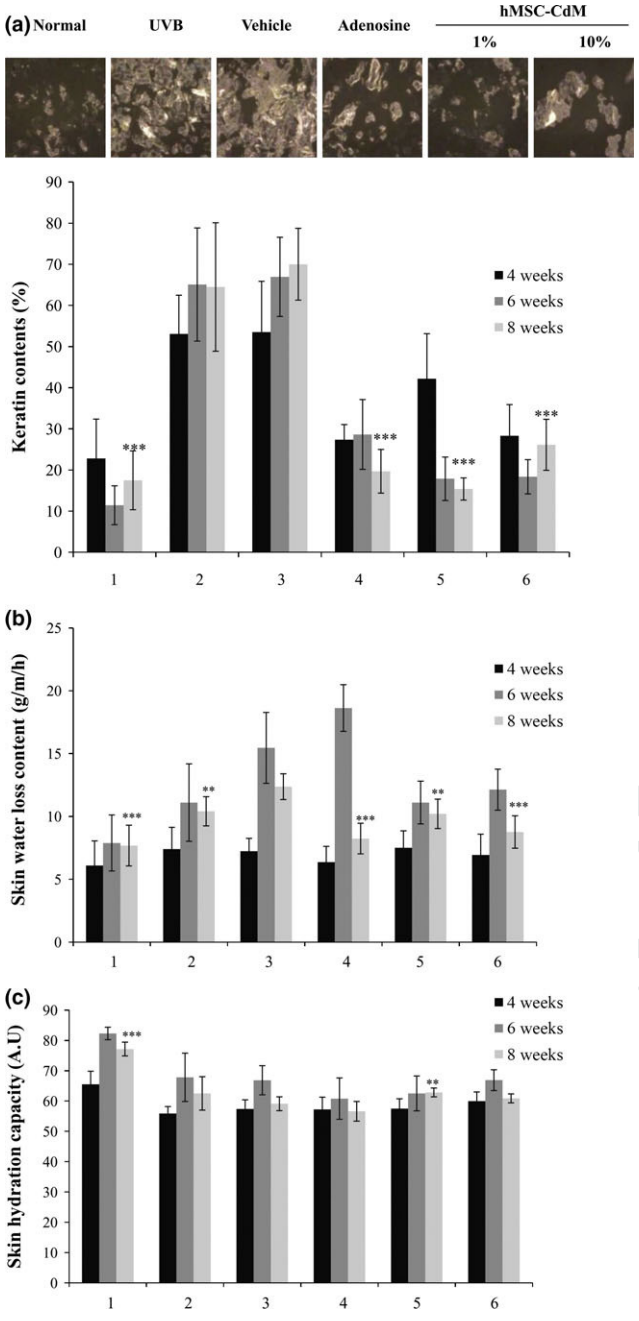

Fig. 4. Effect of conditioned medium for human bone marrow-derived mesenchymal stem cells (MSC-CdM) on transepidermal water loss (TEWL) and skin hydration in UVB-irradiated mice. (a) The effects of tape stripping on the back skin of UVB-irradiated mice. Photographs show the relative percentage of stratum corneum remaining. (b) and (c) Measurements of TEWL were taken at 4, 6, and 8 weeks. Measurements of TEWL and with the corneometer were taken under similar conditions. Results are representative of three readings taken from different regions of the back from three sets of animals. Clinical results for the following groups: 1, Normal (non-irradiated); 2, UVB; 3, Vehicle; 4, Adenosine; 5, 1% MSC-CdM; 6, 10% MSC-CdM. Values represent the mean ± SEM (n = 8). *P < 0.05; **P < 0.01; ***P < 0.001 vs. the vehicle-treated group.

To define the pathways through which topical application of MSC-CdM inhibits UV-induced skin aging, we examined the effects of the conditioned medium on skin fibroblast proliferation and the expression levels of MMP-1 and pro-collagen. Our data indicated that MSC-CdM may prevent UVB-induced MMP-1 expression and upregulate type I pro-collagen synthesis (Fig. 1). This finding suggests a mechanism whereby MSC-CdM inhibits UV-induced wrinkle formation. However, additional experiments are required to validate the mechanisms presented here.

Skin hydration, maintained by the prevention of TEWL, affects skin barrier function, and enhanced skin barrier function can improve skin wrinkling. In our efforts to develop effective anti-aging cosmetic supplements to promote skin moisturization and the effacement of wrinkles, we assessed the efficacy of topical application of MSC-CdM in an animal model. Our *in vivo* model revealed that topical application of MSC-CdM inhibited wrinkle formation (Figs 2 and 3).

Moisturizers not only increase the skin's water content, but also protect the skin. Moisturizers are available in lotion, cream, serum, and even liquid form, and they supply the skin with ingredients that maintain its water content and barrier integrity, and help cells function normally (26). Importantly, lipids are a crucial component for both water retention function and permeability barrier function in the stratum corneum. The stratum corneum contains a dense network of keratin, a protein that helps keep the skin hydrated by preventing water evaporation (27). Interestingly, we found that the MSC-CdM-treated group exhibited significantly reduced contents of keratin compared with the UVB group (Fig. 4a). Thus, we confirmed that topical application of MSC-CdM resulted in significant protection of skin barrier function by decreasing the TEWL and increasing skin hydration, thereby reducing skin wrinkling (Fig. 4b,c). Thus, topical application of MSC-CdM may serve as an effective skin-protecting agent for the prevention of wrinkle formation.

Several cell types, including embryonic stem cells, mesenchymal stem cells, endothelial progenitor cells, and induced pluripotent stem cells, have been proposed for use in potential stem cell therapeutic strategies. In particular, human MSC-CdM represents a promising cell-free therapeutic strategy for promoting neovascularization in ischemic diseases (28). Recently, human MSC-CdM was shown to contain many paracrine angiogenesis-enhancing factors, such as vascular endothelial growth factor, stromal cell-derived factor 1, granulocyte

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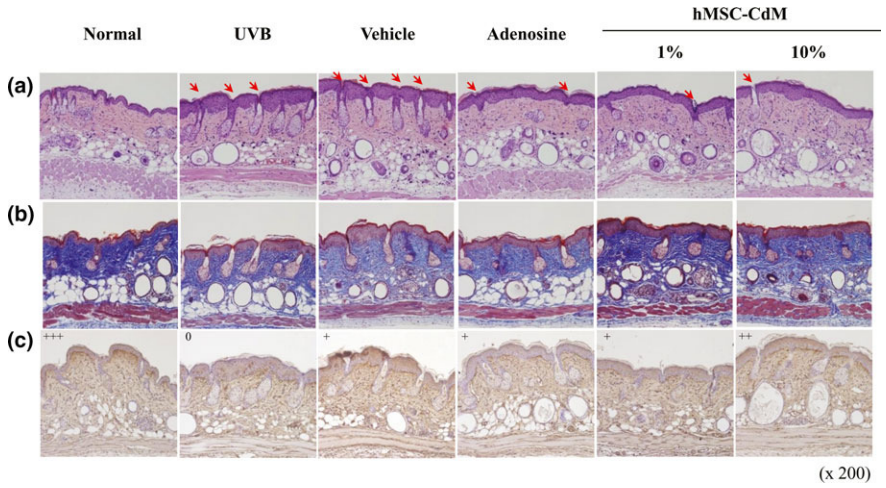

Fig. 5. Effects of topical application of conditioned medium for human bone marrow-derived mesenchymal stem cells (MSC-CdM) on UVB-induced wrinkle formation on the backs of SKH-1 hairless mice skin at the end of week 8. Histological sections of mice dorsal skins were stained with (a) hematoxylin and eosin staining and (b) Masson's trichrome stain. Wrinkle formation can be clearly seen on the epidermis (as marked by red arrows). (c) Paraffin sections of skin biopsies from each of the groups were immunostained with anti-type I collagen antibodies. Sections were counterstained with hematoxylin for visualization of nuclei. Brown dots signify positively stained cells. Each photograph is representative of at least eight animals. The IHC intensity score represents the average of the scores in each IHC staining intensity category (++ indicates pronounced findings, + moderate findings, and 0 no/scant findings) identified in each sample. Original magnification: ×200.

colony-stimulating factor, TGF-β1, and hepatocyte growth factor.

In addition, direct DNA damage in skin can occur when DNA directly absorbs a UVB photon. UVB light causes consecutive thymine base pairs in genetic sequences to bond together into pyrimidine dimers, a disruption in the strand (29). It results in sunburn and triggers the production of melanin. However, direct DNA damage such as sunburn is reduced by sunscreens. There have been a number of recent studies indicating that topical application of growth factors and cytokines is beneficial in reducing signs of facial skin aging in clinical studies (30, 31). Moreover, Jung *et al.* (32) showed that the conditioned medium from adipose-derived stem cells stimulates both collagen synthesis and the migration of fibroblasts and accelerates wound healing *in vivo*. However, the inhibitory actions of hMSC-CdM in photo-aging are not well defined and additional experiments are required.

Interestingly, hMSC are not immunologically reactive and can trespass species defense barriers in mice (33). Here, we confirm that hMSC can be used in mouse skin regeneration. Thus, use of MSC-CdM in a clinically relevant mouse model with assessment of clinically relevant end points therefore engenders direct and immediate clinical implications.

In the present study, histological data confirmed that UVB exposure resulted in enhanced formation of collagen fiber bundles, as revealed by the staining pattern of collagen I, compared with those of the UVB exposure group and the MSC-CdM group. This result may reflect the action of MSC-CdM on other mechanisms, such as skin regeneration and wound healing.

In conclusion, the present results demonstrate that MSC-CdM has effective anti-wrinkle properties, as shown by its ability to maintain the skin's moisture content. In addition, MSC-CdM alleviated the UVB-induced dermal roughening that leads to skin wrinkling. Therefore, topically applied MSC-CdM is a promising agent for use in curtailing skin wrinkling and protecting against the moisture loss associated with photoaging from UV exposure.

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