Biochemical and Biophysical Research Communications 493 (2017) 1102–1108

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Exosomes derived from human umbilical cord blood mesenchymal stem cells stimulates rejuvenation of human skin

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ARTICLE INFO

Article history:
Received 5 September 2017
Accepted 12 September 2017
Available online 14 September 2017

Keywords:
Conditioned media from hUCB-MSCs
USC-CM
Exosomes
Skin permeation
Cosmetics

ABSTRACT

Human umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs) play an important role in cutaneous wound healing, and recent studies suggested that MSC-derived exosomes activate several signaling pathways, which are conducive in wound healing and cell growth. In this study, we investigated the roles of exosomes that are derived from USC-CM (USC-CM Exos) in cutaneous collagen synthesis and permeation. We found that USC-CM has various growth factors associated with skin rejuvenation. Our *in vitro* results showed that USC-CM Exos integrate in Human Dermal Fibroblasts (HDFs) and consequently promote cell migration and collagen synthesis of HDFs. Moreover, we evaluated skin permeation of USC-CM Exos by using human skin tissues. Results showed that Exo-Green labeled USC-CM Exos approached the outermost layer of the epidermis after 3 h and gradually approached the epidermis after 18 h. Moreover, increased expressions of Collagen I and Elastin were found after 3 days of treatment on human skin. The results showed that USC-CM Exos is absorbed into human skin, it promotes Collagen I and Elastin synthesis in the skin, which are essential to skin rejuvenation and shows the potential of USC-CM integration with the cosmetics or therapeutics.

1. Introduction

The skin Extracellular Matrix (ECM) is crucial for skin morphology and functions such as that of growth and elasticity [1]. Collagen is responsible for mechanical protection of the body, prevention of the skin dehydration, maintenance of elasticity, firmness of the tissues and minimization of the skin wrinkles. Elastin is a major structural protein of the body tissues and a fibrous protein that is reduced in thickness from deeper to superficial dermis layers of the skin. Elastin provides strength, natural elasticity, and plays an important role in tissue reparation [2].

Functional cosmetics, the collaboration of stem cell technology with cosmetics, are now emerging trend in the cosmetics industry. Stem cells are a population of immature tissue precursor cells capable of self-renewal and provision of multi-lineage differentiable cells for tissues. Among the stem cells, Mesenchymal Stem Cells (MSCs) are used most actively because of their feasibility and safety. MSCs are isolated from various tissues including bone marrow, adipose tissues, placenta and umbilical cord blood. MSCs secrete cytokines and various growth factor such as Epithelial Growth Factor (EGF) and Basic Fibroblast Growth Factor (bFGF), which are well known to be important for skin rejuvenation and wound healing by collagen synthesis of Human Dermal Fibroblasts (HDFs) [3–6]. Recently, paracrine effects are evaluated as one of the main mechanisms of action in MSCs [7], but their beneficial roles in growth factors for skin rejuvenation require further studies [8].

Umbilical Cord Blood Derived Mesenchymal Stem Cells (UCB-MSCs) are considered as the most primitive and youngest cells among various tissues-derived MSCs. UCB-MSCs secrete higher content of wound healing factors, compared to other MSCs [9]. UCB-MSCs promote migration, proliferation and collagen synthesis of fibroblasts [10]. In addition, UCB-MSCs conditioned media also promote wound closing and re-epithelialization via animals subcutaneous injected [11]. Due to such benefits derived from the usage of UCB-MSCs, the development of cosmetic products using stem cells are highlighted. In particular, the studies to enhance

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https://doi.org/10.1016/j.bbrc.2017.09.056
0006-291X/

penetrability of effective factors of stem cells are recently carried out.

Bilayer vesicle systems are one of the most popular strategies to increase penetrability through the skin [12]. Exosomes are small extracellular membrane vesicles with 30–100 nm in diameter with a mechanism of cell-to-cell communication. Exosomes are the most extensive class of secreted membrane vesicles that carry proteins and RNAs for intercellular communication [13–15]. A few mechanisms of exosome-mediated cell-to-cell communication have been reported: (i) interaction between exosome membrane proteins and their receptors on target cells, (ii) interaction between soluble fragments which are derived from exosome membrane proteins and cell surface receptors, (iii) internalization of contents of exosomes by target cells [16].

Exosomes derived from stem cells in conditioned media (CM) are nano-sized and may make the regeneration effect higher via penetration into the skin epidermis [17]. MSC-derived exosomes have been found to play significant roles in cutaneous wound healing via animals injected locally or intravenously [18,19]. However, it is still unclear if MSC-derived exosomes can enhance skin regeneration via human applied. In this study, we investigated if USC-CM derived exosome (USC-CM Exos) could be internalized by HDFs and promote HDFs migration and collagen synthesis, which can benefit skin regeneration. This study confirmed that USC-CM Exos can promote collagen synthesis via human skin permeation. These data present strong *in vitro* and *ex vivo* evidence that UCB-MSCs-derived exosomes such as USC-CM Exos have potentials for development and application in cosmetics.

2. Materials and methods

2.1. Preparation of USC-CM

UCB-MSCs were isolated from Human umbilical cord bloods approved by the FORMIZ WOMEN's Hospital (IRB No.219255-201305-BR-001, Seoul, Korea) with previously described method [20]. UCB-MSCs were cultured and expanded up to passage 5 at 37 °C and 5% CO_2 in KSB-3 (Irvine scientific, Santa Ana, CA) with 10% fetal bovine serum (FBS) (Gibco). UCB-MSCs (1.89×10^5 cells/Flask) were seeded in T-25 flask and cultured for 48 h in KSB-3 with 10% FBS. After PBS washing twice, the culture medium was changed to KSB-2 media; DMEM (Gibco) containing EGF (10 ng/ml) and bFGF (10 ng/ml), followed by incubation period of 96 h. Conditioned media of UCB-MSCs (USC-CM) were collected, centrifuged at 1500 rpm for 5 min, and finally filtered using a 0.22 μm syringe filter.

2.2. Isolation and identification of exosomes derived from USC-CM

Exosomes were isolated from USC-CM using exoEasy Maxi kit (QIAGEN). The method is being distributed by QIAGEN (QIAGEN GmbH, Hilden, Germany) as exoEasy Maxi Kit, and the standard protocol is described in the exoEasy Handbook. Briefly, prefiltered USC-CM was mixed 1:1 with 2x binding buffer (XBP) and added to the exoEasy membrane affinity column to bind the exosomes to the membrane. After centrifugation, the flow-through was discarded and wash buffer (XWP) was added to the column to wash off non-specifically retained material. After another centrifugation and discarding of the flow-through, the vesicles were eluted by adding elution buffer to the spin column, and the eluate was collected by centrifugation. This procedure allows concentrating the exosome from 15 ml USC-CM into a final volume of 200 μl of elution buffer. The particle number of exosomes was determined by using a CD81 ExoELISA kit (System Biosciences, Mountain View, CA) following the instructions provided. Briefly, a standard curve was prepared by serially diluting the ExoELISA protein standard with exosome binding buffer, and results were measured at 450 nm using a microplate reader (Tecan, Mannedorf, Switzerland). The collected exosomes morphologies were observed by 100 kv Transmission Electron Microscopy (TEM). The size, concentration and particle size distribution of exosomes were identified by NanoSight LM10 (Malvern, UK) and Nanoparticle Tracking Analysis software version 3.0 (NanoSight).

2.3. Human growth factor antibody array

Human Growth Factor Antibody Array was performed as described in the Supplementary Materials and Methods section.

2.4. Collagen synthesis of HDFs with exosomes stimulation

HDFs (2×10^5 cells/well) were seeded in 6-well plates and cultured for 24 h in KSB-3 medium. After washing, different concentrations of USC-CM Exos (Exos1; 1×10^8 Particles/ml, Exos2; 1×10^9 Particles/ml) with serum-free culture medium (DMEM) were changed for additional 24 h culture, and cells were collected and extracellular matrix (ECM) production expression level was examined by qRT-PCR. The primer sequences were listed in Table 1. The supernatants were collected and measured with Procollagen Type I C-peptide ELISA (Takara, Tokyo, Japan) and MMP-1 ELISA (R&D systems, Minneapolis, MN) according to the manufacturer's protocol.

2.5. Scratch assay

7×10^5 HDFs were seeded into the cell culture system by using the ibidi Culture-Insert (No. 81176, ibidi GmbH, Munich, Germany). This approach provides two cell culture reservoirs with a separation wall of 500 μm thick. For the measurement of cell migration, the silicon inserts were removed after 24 h. The gaps created were washed and each well was filled with fresh serum-free culture medium containing USC-CM Exos (1×10^9 Particles/ml). We took images of the closing area at 0 and 4 days, and measured rate of migration on different days by InCuCyte ZOOM System (Essen BioScience, Ann Arbor, MI).

2.6. Human skin permeation of USC-CM Exos

Abdominal skin samples were obtained from healthy female African donors (32 years of age) through Biopredic International (Rennes, France). The skin samples were set in a 4-well plate and dipped in Skin Culture Medium (Biopredic International) at 37 °C and 5% CO_2. USC-CM Exos were fluorescently labeled with Exo-Green (System Biosciences, Mountain View, CA) according to the manufacturer's protocol. Labeled exosomes were applied with a micropipette on the skin. The skins fixed with 4%

Table 1
Primers used in research.

Gene	Species	Primer
Collagen I	Human	F 5′-cacagaggtttcagtggtttgg-3′ R 5′-gcaccagtagcaccatcatttc -3′
MMP-1	Human	F 5′-ttgagaaagccttccaactctg-3′ R 5′-ccgcaacacgatgtaagttgta-3′
Fibronectin	Human	F 5′-aagattggagagaagtgggacc -3′ R 5′-gagcaaatggcaccgagata-3′
Elastin	Human	F 5′-gggttgtgtcaccagaagca-3′ R 5′-caaccccgtaagtaggaatgc3′
RPL13A	Human	F 5′-gcacgaccttgagggcagcc -3′ R 5′-catcgtggctaaacaggtactg-3′

paraformaldehyde at indicated time points 0 h, 3 h and 18 h. Skin tissues were embedded in OCT-compound (Tissue-Tek, Sakura, Japan) and were sectioned into 4-μm sections. These sections were stained with hematoxylin and eosin according to standard protocols. The nuclei were stained with Hoechst33342 (1:1000, Molecular Probes). Images were sequentially acquired with fluorescent microscope (Nikon, Tokyo, Japan).

2.7. Collagen synthesis of human skin with exosomes stimulation

As mentioned above, Human skin samples were cultured and divided into three groups: untreated group, USC-CM group, USC-CM Exos group. USC-CM and USC-CM Exos were topically applied twice a day with a micropipette on the skin for 3 days. After 3 days, skin tissues were collected for qRT-PCR analysis.

2.8. Statistical analysis

All data were shown as means ± standard deviation (SD). The statistically significant differences between groups were assessed by *t*-test using GraphPad Prism 5 software. P values < 0.05 were considered significant. *P < 0.05, **P < 0.01, ***P < 0.0001. The values are shown in the figures.

3. Results

3.1. Characterization of USC-CM Exos

We isolated exosomes from USC-CM. The round vesicles morphologies of exosomes were observed by electron microscopic analysis, and the most of these particles yielded sample sizes ranging from 50 nm to 150 nm (Fig. 1A). The ELISA confirmed that the expression of exosome marker CD81 in these particles (Fig. 1B). Moreover, the particle size distribution, concentration and dynamic tracking were measured by using NanoSight analysis (Fig. 1C). We compared USC-CM Exos with each Exosomes derived from two different conditioned media (AD-MSC-CM Exos, HDF-CM Exos). Mean particle diameters were reported as 120 nm, 230 nm and 160 nm, respectively. Also, exosome particles were measured as $1.23 \times 10^{11} \pm 2.42 \times 10^{9}$, $1.09 \times 10^{11} \pm 3.19 \times 10^{9}$ and $7.03 \times 10^{10} \pm 6.98 \times 10^{9}$ Particles/ml, respectively (Table 2). The results indicated that exosomes from USC-CM were successfully isolated and consistent with the defined exosomes. AD-MSC-CM and HDF-CM also can secrete exosomes, but the particle sizes of USC-CM Exos were smaller than those of AD-MSC-CM Exos and HDF-CM Exos.

Table 2
Exosome particles were measured by NanoSight analysis.

	Exosome particles/ml
USC-CM Exos	$1.23 \times 10^{11} \pm 2.42 \times 10^{9}$
AD-MSC-CM Exos	$1.09 \times 10^{11} \pm 3.19 \times 10^{9}$
HDF-CM Exos	$7.03 \times 10^{10} \pm 6.98 \times 10^{9}$

3.2. USC-CM and USC-CM Exos contained high amount of growth factors

In order to determine the existence of various growth factors in USC-CM as exosome forms, we analyzed 41 different cytokines in USC-CM and USC-CM Exos using the human growth factor antibody array. The membrane was printed with antibodies for 41 growth factors and receptors, with four positive and four negative controls in the upper left corner (Fig. 2A and B, left). Results from quantification of the human growth factor antibody array showed that both USC-CM and USC-CM Exos contained significantly higher concentrations of proteins associated with skin rejuvenation such as EGF and bFGF (Fig. 2A and B, right). This analysis demonstrated that USC-CM has various growth factors associated with skin rejuvenation and relatively high amount of EGF among various growth factors that exist in USC-CM as exosome forms. In addition, we compared USC-CM Exos with each exosomes derived from two

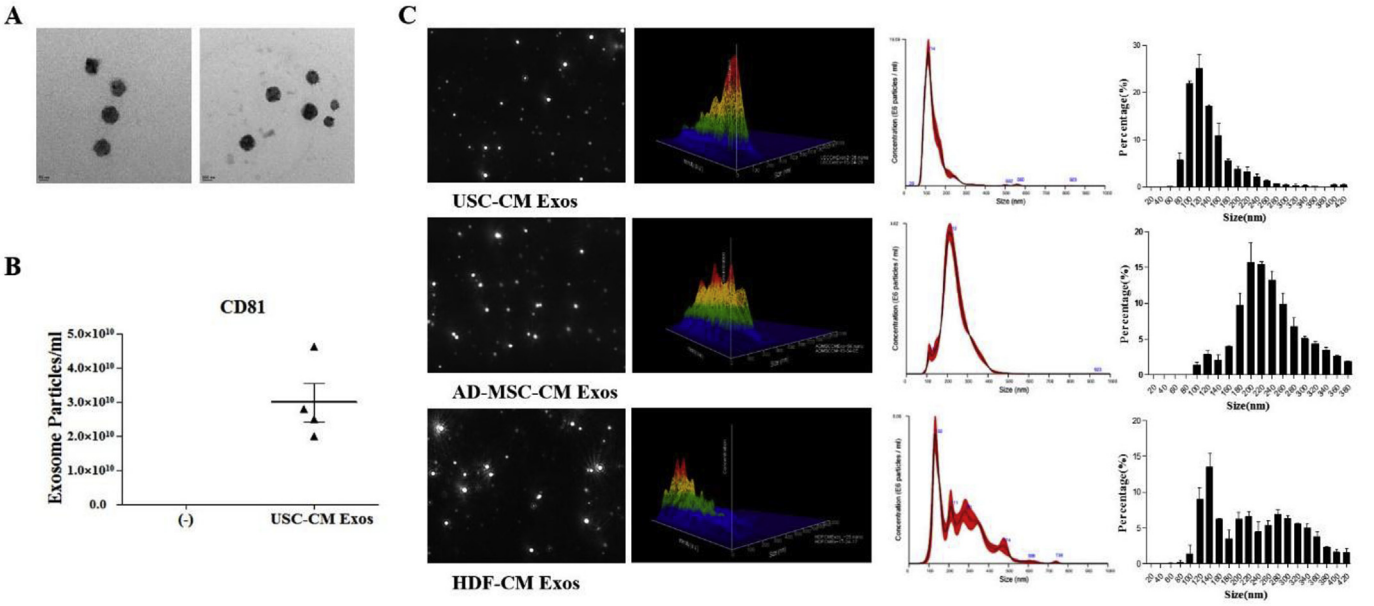

Fig. 1. Characterization of USC-CM Exos.
(A) Morphologic analysis of USC-CM Exos by transmission electron microscopy (scale bar = 50 nm, 100 nm). (B) Detection of CD81 expression in exosomes by ExoELISA. (C) Characterizations of exosomes were measured by NanoSight analysis. Dynamic tracking video capture (C-i), Concentration analysis (C-ii), Size distribution (C-iii), Particles size distribution of USC-CM Exos, AD-MSC-CM Exos and HDF-CM Exos displayed about 60% range from 40 to 120 nm, 60% range from 80 to 230 nm and 30% range from 40 to 160 nm, respectively (C-iv).

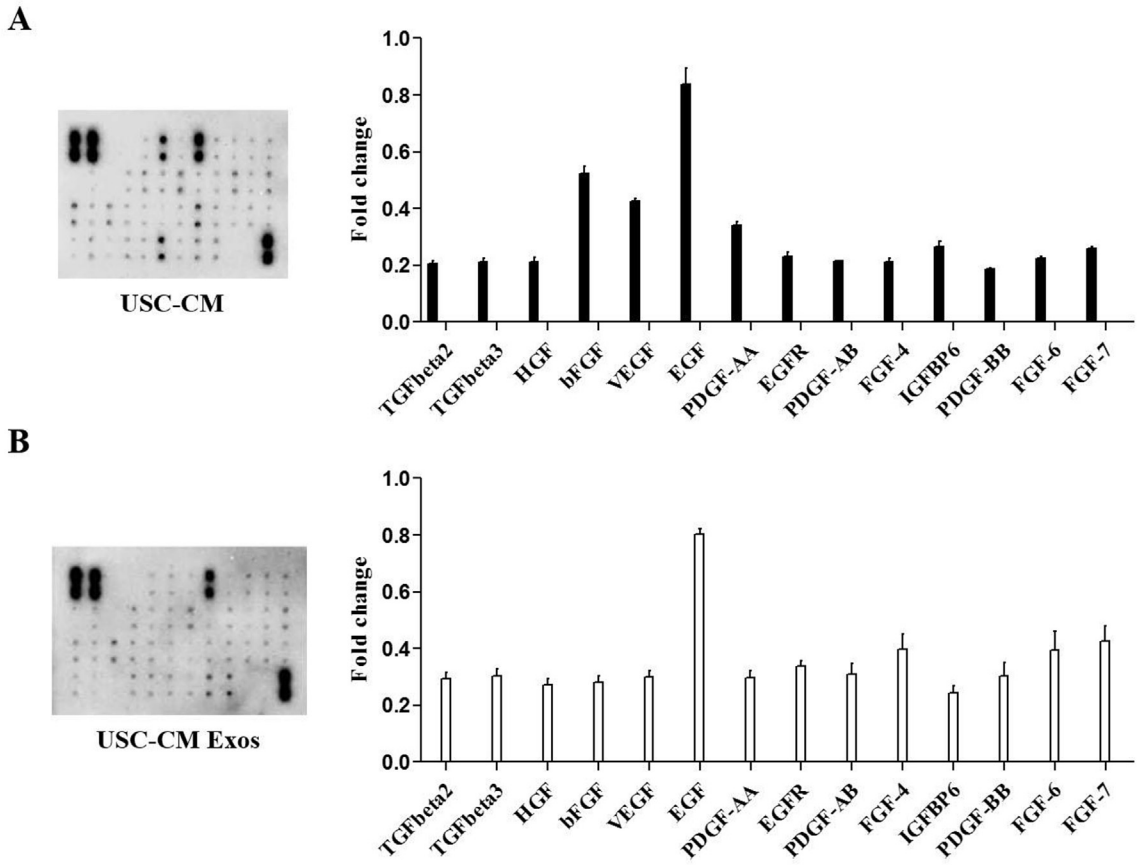

Fig. 2. Quantitative Analysis of USC-CM Exos.
Human growth factor antibody array analysis of USC-CM (A) and USC-CM Exos (B). Densitometric analysis of proteins associated with skin rejuvenation.

different MSC-CM (AD-MSC-CM Exos, BM-MSC-CM Exos) (Fig. S1, Supplementary material). USC-CM Exos strongly contained skin-related proteins compared with AD- and BM-MSC-CM Exos. Quantification of the human growth factor antibody array results showed that USC-CM Exos strongly contained skin-related proteins compared with AD- and BM-MSC-CM Exos.

3.3. Integration of USC-CM Exos by HDFs

To examine the exosome intracellular trafficking, we used the PKH26 Fluorescent Cell Linker Kits to label the exosomes and the Exo-Glow kits for internal exosome proteins with green. After incubating HDFs with USC-CM Exos for 24 h, the cellular localization of USC-CM Exos were analyzed using fluorescence microscopy and observed in the perinuclear region of HDFs (Fig. 3A). This analysis demonstrated that internal exosome proteins as well as USC-CM Exos can be integrated with HDFs.

3.4. USC-CM Exos promoted HDFs migration and collagen synthesis in vitro

In order to determine the effects of USC-CM Exos on growth and collagen synthesis, we evaluated the ECM gene expression of HDFs treated with various concentrations of exosomes (Exos1; 1×10^8 Particles/ml, Exos2; 1×10^9 Particles/ml) for 24 h. The results of Collagen I, Fibronectin and Elastin gene expression were significantly increased. Therefore, the optimal concentration of exosomes was decided as Exos2 (Fig. 3B). The protein levels of Procollagen Type 1 C-peptide were significantly increased in Exos2 group compared to control group. MMP-1 protein expression decreased in Exos1 and 2 group compared to control group (Fig. 3C). We further investigated HDFs growth ability of USC-CM Exos. To determine the effect of USC-CM Exos (Exos2) on migration of HDFs, scratch assay was performed. Images of the closing area at 0 and 4 days is shown in Fig. 3D. In addition, automated live cell imaging with InCuCyte ZOOM is shown in Fig. S2. The migration rate was measured on different times by InCuCyte ZOOM System (Fig. 3E). Consecutively, migration rate consistently increased in USC-CM Exos compared to control (Fig. 3D and E). All data demonstrate that USC-CM Exos promoted HDFs migration, collagen synthesis *in vitro*.

3.5. Human skin permeation of USC-CM Exos

To study the USC-CM Exos permeation through human abdominal skin, representative permeation imaging of human abdomen skin treated with exosomes were collected at indicated time points 0 h, 3 h and 18 h. Since obvious fluorescent images could not lasted long times, observation was limited on 18 h. Human abdominal skin penetration assays were evaluated by histology. In H&E staining, stratum corneum, epidermis and dermis in skin layers were well observed. In fluorescent microscope, Exo-Green reached the outermost layer of the epidermis after 3 h of exposure (Fig. 4A) and that started reaching epidermis progressively at 18 h. This analysis suggested that USC-CM Exos could be reached epidermis and the absorption of USC-CM Exos progressed with the course of time on human skin.

3.6. USC-CM Exos promoted collagen synthesis in human skin

qRT-PCR analysis was performed to assess Collagen I (Fig. 4B),

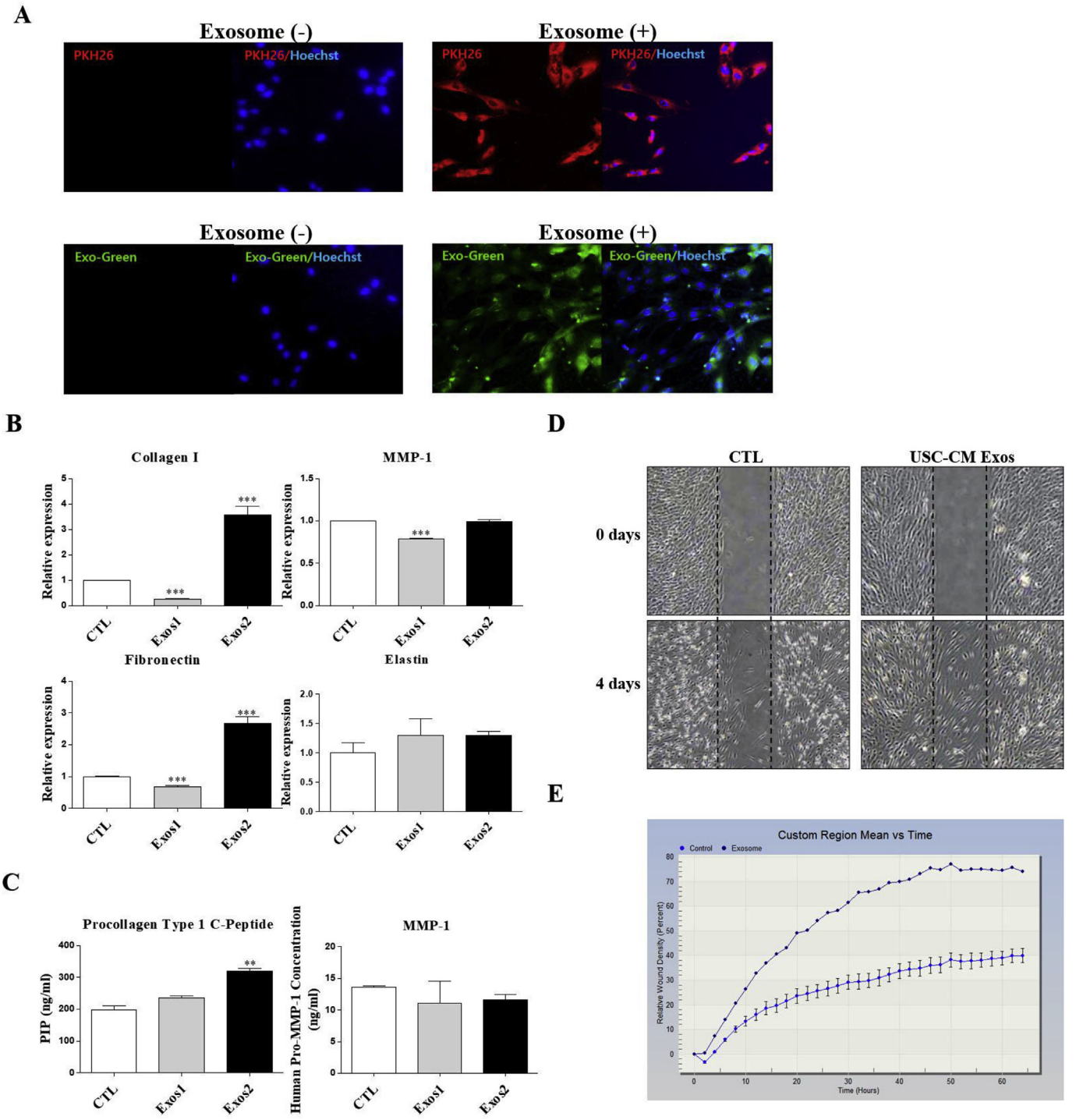

Fig. 3. Cellular internalization of USC-CM Exos by HDFs, and USC-CM Exos promoted HDFs migration, ECM gene expression *in vitro*.
(A) Exosome membrane-labeled PKH 26 was integrated in HDFs (x400) (upper). Exosome protein-labeled EXO-green was perinuclear region of HDFs (x400) (lower). (B) ECM gene expression of HDFs treated with different concentrations of exosomes (CTL; DMEM only, Exos1; 1×10^8 Particles/ml, Exos2; 1×10^9 Particles/ml) for 24 h*P < 0.05, **P < 0.01, ***P < 0.001. (C) Extracellular (supernatant) collagen content was determined by means of Procollagen Type I C-peptide (left) and MMP-1 (right) ELISA. (D) Scratch assay of HDFs following treated with 1×10^9 Particles/ml USC-CM Exos for 4 days. (E) The migration rate on different times by InCuCyte ZOOM System. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MMP-1 (Fig. 4C), Fibronectin (Fig. 4D) and Elastin (Fig. 4E) expressions in human skin after 3 days treatment of USC-CM and USC-CM Exos. The expression of Collagen I and Elastin were significantly increased in both of USC-CM and USC-CM Exos treatment groups. Particularly, the expression of these genes were higher in USC-CM Exos treatment groups comparing to USC-CM. Furthermore, MMP-1 was expressed lower in USC-CM Exos treatment group compared to that of control group. Therefore, it is suggested that USC-CM Exos can penetrate human skin and can promote the expression of Collagen I and Elastin after 3 days treatment.

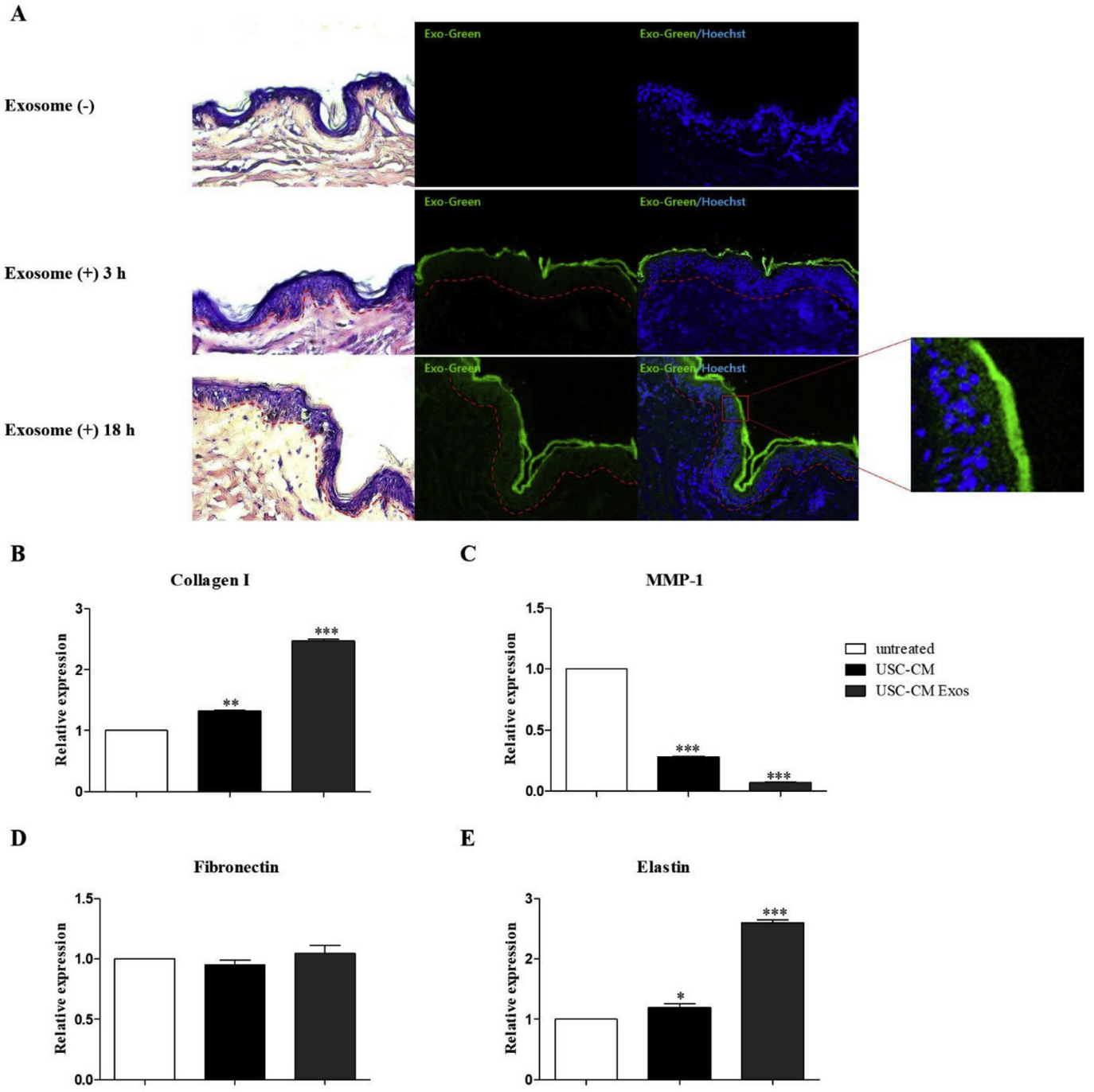

Fig. 4. Human skin permeation of USC-CM Exos, and USC-CM Exos promoted collagen expression in human skin.
(A) Tissue samples treated with USC-CM Exo were fixed and stained with H&E and fluorescent dye. Human skin tissues were observed with fluorescence microscopy on 0 h, 3 h and 18 h (x200). Tissue samples were collected at 3 days after USC-CM and USC-CM Exos treatment. The Collagen I (B), MMP-1 (C), Fibronectin (D) and Elastin (E) mRNA expression were observed. The expressions of Collagen I and Elastin were increased but MMP-1 expression was decreased in USC-CM and USC-CM Exos treated samples compared those of untreated samples. *P < 0.05, **P < 0.01, ***P < 0.001.

4. Discussion

In this paper, we tested functional roles of USC-CM and USC-CM derived exosomes in human skin and human fibroblast in respect to the skin rejuvenation effect via HDFs migration, ECM production and exosome penetration to the epidermis. Among popularly cited human MSCs, USC-CM contained the highest amount of growth factors and the penetration of USC-CM Exos was proposed when ectopically treated to the human skin.

Stem cell therapy is a safe, practical, and effective source for repairing the damaged tissue and rejuvenating the skin conditions. Much of the functional improvement and attenuation of the injury afforded by stem cells can be repeated by treatment with cell-free conditioned media derived from MSCs, which contain the useful growth factors.

Extracellular vesicles (EVs) can be isolated from cultured supernatants of many cell types including MSCs [21,22]. In the present study, we successfully isolated exosomes from USC-CM, AD-MSC-

CM and HDF-CM. Moreover, we observed that the particle size of USC-CM Exos was the smallest compared with each exosomes derived from two different cells (AD-MSC and HDFs). Several studies about permeation of nanoparticles through human skin have been reported [23,24]. This research suggests that USC-CM Exos could enhance skin permeation by reason of the smallest nano-sized. Moreover, size-distribution of USC-CM Exos showed narrower ranges compared to the exosomes derived from AD-MSC and HDFs, which means USC-CM Exos is unique and easy to standardization that is critical to commercialization.

USC-CM Exos treatment increased the production of Collagens, Fibronectin and Elastin in HDFs, which are the key cell responsible for ECM structure. Collagen I is one of main proteins of ECM and Elastin is pivotal to skin elasticity [25]. Their migration, proliferation and collagen synthesis are important for the quality of skin rejuvenation. MMP-1 works as a collagenase and encodes a secreted enzyme that breaks down the I, II, and III interstitial Collagens [26]. The production of MMP-1 was decreased in USC-CM and USC-CM Exo treated HDFs significantly, which supported that USC-CM had skin rejuvenation function more strongly.

Exosomes may subsequently be internalized by other cells via direct membrane fusion, endocytosis or cell-type specific phagocytosis [17,27,28]. We found that USC-CM Exos were uptaken by HDFs and enhanced the growth and migration *in vitro*. This latter finding is in line with several reports demonstrating HDFs migration after MSC exosomes treatment. Furthermore, we observed a significant increase of permeation with USC-CM Exos in whole skin as well as stratum corneum and epidermis. Taken together, USC-CM Exos and USC-CM infiltrated to human skin in topical treatment were proved to enhance the production of Collagen I and Elastin, but decreased the MMP-1 production in *ex vivo* human skin. These are one of the demonstration why cosmetics that contain exosomes derived from USC-CM are effective to human skin.

In summary, the effective factors of USC-CM can be encapsulated with exosomes and penetrate the human skin. These findings suggest that USC-CM as exosome forms can be used for skin-regeneration materials and a key factor that stimulates growth and secretion of ECM proteins in HDFs, which are important to human skin rejuvenation.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2017.09.056.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2017.09.056.

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