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Anti-wrinkle effect of bone morphogenetic protein receptor 1a-extracellular domain (BMPR1a-ECD)

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Bone morphogenetic proteins (BMPs) have diverse and important roles in the proliferation and differentiation of adult stem cells in our tissues. Especially, BMPs are well known to be the main inducers of bone formation, by facilitating both proliferation and differentiation of bone stem cells. Interestingly, in skin stem cells, BMPs repress their proliferation but are indispensable for the proper differentiation into several lineages of skin cells. Here, we tested whether BMP antagonists have an effect on the prevention of wrinkle formation. For this study we used an *in vivo* wrinkle-induced mouse model. As a positive control, retinoic acid, one of the top anti-wrinkle effectors, showed a 44% improvement compared to the non-treated control. Surprisingly, bone morphogenetic protein receptor 1a extracellular domain (BMPR1a-ECD) exhibited an anti-wrinkle effect which was 6-fold greater than that of retinoic acid. Our results indicate that BMP antagonists will be good targets for skin or hair diseases. [BMB Reports 2013; 46(9): 465-470]

INTRODUCTION

In our body, when any tissue is lost or damaged, stem cells in each tissue start to replicate and then differentiate in a specific spatiotemporal manner so as to effect the repair of lost or damaged tissues (1, 2). In the skin stem cells keep dividing and differentiating in a series of steps, and undergoing apoptosis prior to shedding from the skin only to be replaced newly differentiated cells, which allows our skin to be rejuvenated (3, 4).

The occurrence of wrinkles is one of the spontaneous phenomena of the aging process (5). Many diverse factors such as the synthesis of collagen or elastin, UV exposure, and integrity of the skin epidermis layer are known to be involved in wrinkle formation. The synthesis and degradation of collagen or elastin in the skin dermis layer is one of main factors involved in wrinkle biology (6). UV irradiation exposure is also known to be an inducer of wrinkles (7). Nowadays new biological roles of known or unknown proteins are being identified by many research groups, using advanced techniques in proteomics. Diverse cell signal transduction pathways such as Wnt, FGF (fibroblast growth factor), Notch, TGF-β (transzforming growth factor beta) are involved in skin stem cell proliferation and differentiation (8, 9). Recent research articles have supported that inappropriate skin stem cell biology is one of major inducers of wrinkle formation (3, 8).

Homeostasis in the skin epidermis layer is repeated throughout life, as stem cells continue to undergo a series of replication, stepwise differentiation, apoptosis, and shedding from the skin (4). The homeostasis interval time in our skin increases with age (10). For example, the homeostasis interval time for someone in their twenties is about 20 days whereas it is increased to almost 40 days for someone in their forties, which indicates that the turnover interval time increase with age also affects wrinkle formation, hair whitening, acne, hair growth, and atopic dermatitis.

Wnt ligands and BMPs (bone morphogenetic proteins) are the two main regulators of skin stem cell proliferation and differentiation (11, 12). In a dormant state, activated BMP2 and BMP4 signals repress skin stem cell proliferation. When BMP2 and BMP4 activity is repressed by endogenous antagonists or exogenous antagonist treatment, skin stem cell proliferation signal is activated and starts to produce multiple undifferentiated skin cells, an effect which is further augmented by an activated WNT signaling pathway, mainly induced by the wnt5a ligand. Interestingly, WNT and BMP signals are absolutely required for the proper differentiation into several lineages of finally differentiated skin cells (4).

Our aim is to screen proteins which prevent wrinkle formation or reduce the effect of pre-existing wrinkles. In this experiment, we used an *in vivo* hairless mouse model to test for anti-wrinkle effects. As a positive control, retinoic acid showed a 44% anti-wrinkle improvement activity. Surprisingly bone morphogenetic protein receptor 1a extracellular domain

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(BMPR1a-ECD) showed an extremely strong anti-wrinkle effect with an approximate 300% anti-wrinkle improvement, or about 6-fold greater than that of retinoic-acid.

RESULTS

Recombinant BMP2, noggin, and BMPR1a-ECD protein production

Recombinant BMP2 and noggin were purified by the published protocols (13, 14) (Fig. 1A, B). Recombinant BMPR1a-ECD is itself not well folded and is unstable when expressed in *E. coli*. To solve this problem, the fusion protein TRX-BMPR1a-ECD has been constructed by attaching the thioredoxin (TRX) gene as a fusion partner. TRX is commonly used as a fusion domain of an interest target protein to improve several shortcommmings such as poor solubility, incorrect folding and a low expression level (15). BMPR1a-ECD was purified to a high degree through the established protocol (Fig. 1C, lane 2), where TRX is clearly removed.

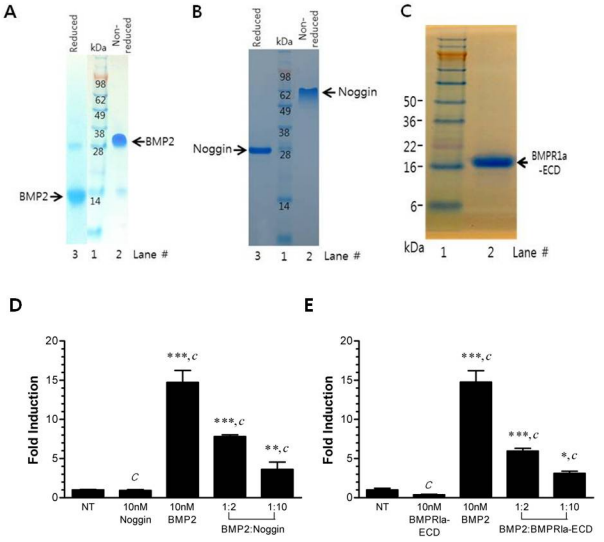

Fig. 1. Production and biological activity of BMP2, Noggin or BMPR1a-ECD protein. (A-C) 12% SDS-PAGE images. Lane 1 contains the protein ladder, lanes 2 and 3 indicate purified BMP2, Noggin or BMPR1a-ECD solution in the presence or absence of reducing agent, DTT. The active form of BMP2 or Noggin is a dimer. The migration of BMPR1a-ECD is not affected by the addition of DTT because it is a monomeric form. (D, E) Smad-1 luciferase reporter assay. C2C12 cells were transfected with Id1-Luc, Smad1 and beta-galactosidase plasmids using Fugene6. Luciferase activity was measured and normalized by beta-Gal value. Fold induction values are mean ± standard error from triplicated experiments, the χ axis shows the Noggin and BMP2 molar ratio in nM (D) or the BMPR1a-ECD and BMP2 molar ratio in nM (E), while the y axis shows the relative luciferase fold induction activity. *P < 0.05, **P < 0.01 and ***P < 0.001 indicate significant differences as compared to the NT group. ^{a}P < 0.05, ^{b}P < 0.01 and ^{c}P < 0.001 means significant different as compared to the 10 nM BMP2 (only) treated group.

Noggin or BMPR1a-ECD protein efficiently inhibits the SMAD1 signal triggered by BMP2 ligand in a concentration dependent manner

BMP ligands send distinct signals through sequential bindings of type II and type I receptors (16). Several kinds of type II and type I receptors have been identified, and distinct combinations of type II and type I receptors for each BMP ligand are necessary for the full signal transduction (17). BMP2 or BMP4, which are the main regulators of skin stem cell proliferation and differentiation, bind to bone morphogenetic protein receptor type 1a (BMPR1a) to send appropriate biological signals (18). Many endogenous BMP antagonists including Noggin, Chordin, Cerberus, glypican-3 and Follistatin have been identified, among which noggin is known to have a strong inhibition activity for the BMP signal pathways (19).

To ensure the biological activity of Noggin or BMPR1a-ECD, and to test the repression of this signal pathway by its treatment, we used the SMAD-1, 5, 8 luciferase assay, in which SMAD 1/5/8 signal is converged by BMP ligands (20). As expected, the SMAD 1/5/8 signal induced by BMP2 treatment was repressed by the addition of serially increased Noggin or BMPR1a-ECD, confirming their good BMP signal inhibition effects (Fig. 1D, E). When 20 nM Noggin was added to a 10 nM BMP2 treated C2C12 cell culture, the SMAD-1 signal index induced by BMP2 was about 50% decreased and similarly, was approximately 59% decreased by treatment of 20 nM BMPR1a-ECD.

Preparation of liposome-encapsulated protein samples

The skin follicle is a reservoir of stem cells and a location in which stem cell proliferation and differentiation actively occur (11). The bulge stem cells, located at the upper right side of skin follicles, are multi-potent progenitor cells and all the skin cells are derived from these bulge stem cells when aged through repeated turnover or when damaged. Also epidermis stem cells are continuously connected to the follicle stem cells to relay and share signals triggered by protein ligands in skin follicles. The orifice of a skin follicle is wide enough for protein delivery but is filled with fatty acid mixtures secreted by sebaceous gland, located in the upper side of the skin follicle (21). To allow for the successful movement of target proteins into skin follicles, hydrophilic proteins may be delivered by hydrophobic delivery vehicles. In this study, we used liposomes as a delivery carrier. Hydrogenated lecithin, one of several kinds of liposomes, was mixed with BMP2, Noggin or BMPR1a-ECD and was homogenized several times at 800bar to encapsulate each target protein into liposomes until liposome sizes ranged 100-200 nm (Fig. 2). It has been reported that 100-200 nm liposomes suitable for efficient delivery into skin follicles, based on accumulated data revealed by many groups, including our own (22). In Lipo/BMP2, Lipo/Noggin and Lipo/BMPR1a-ECD samples, the liposome sizes ranged 100-200 nm (Fig. 2).

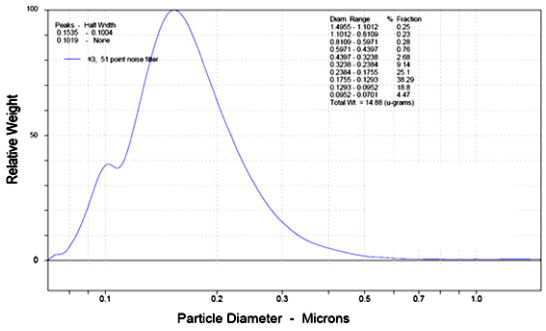

Fig. 2. Liposome size measurement in Lipo/BMP2, Lipo/Noggin or Lipo/BMPR1a-ECD sample. Hydrogenated lecithin comprised of more than 75% phosphocholine was mixed with BMP2, Noggin or BMPR1a-ECD protein solution to a final concentration of 1% lecithin and 0.001% of each test protein, in a total volume of 1 liter. These mixtures were homogenized at 800bar until the liposome size ranged from 100 to 200nm. In this figure, used here as an example, the liposome size in Lipo/BMPR1a-ECD samples was determined.

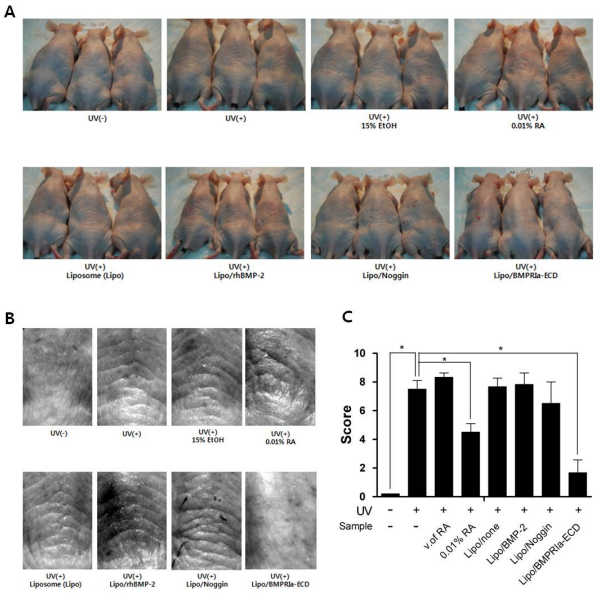

Fig. 3. Dorsal skin photographs and wrinkle scores. Each 8 group is as follows. (A, B) Each sample was topically treated on the back of mice of each group as described in Materials and Methods. Group 1; UVB(-), group 2; UVB(+), group 3-8; UVB(+) with treatment of 15% ethanol (group 3), 0.01% retinoic acid (group 4) or 1% liposome (group 5), 1% liposome-encapsulated 0.001% BMP2 (group 6), 0.001% Noggin (group 7) or 0.001% BMPR1a-ECD (Group 8). ×2 magnified representative images are displayed in (B), and wrinkle scores (skin wrinkling grade: 0-10) in (C). *P < 0.05, **P < 0.01, ***P < 0.001 vs. column. ANOVA.

Effects of BMP antagonists on prevention of wrinkle formation

11 weeks after UVB irradiation, photographs of non-treated mice showed clear and thick wrinkles transversely on the back (Fig. 3). We evaluated an anti-wrinkle effect of each test sample based on the wrinkle score given by generally established protocols. As the vehicle of retinoic acid, 15% ethanol did not make any prominent anti-wrinkle improvement (score 8.0-8.5), compared to just UVB-exposed control (score 7.8-8.4). As expected, 0.01% retinoic acid displayed a sound anti-wrinkle effect (score 3.8-4.5), about a 44% improvement compared to the control group (Fig. 3). As the other vehicle control group for liposome-encapsulated protein samples, 1% liposome treatment did not make any noticeable anti-wrinkle effect (score 8.3). As we expected, the Lipo/BMP2 treated group didn't show any anti-wrinkle effect (score 8.0), because exogenous BMP2 treatment may strengthen the SMAD 1/5/8 signal to further suppress skin stem cell proliferation. Diverse *in vitro* and *in vivo* data have supported that endogenous BMP2 is sufficient to repress the proliferation of skin stem cells in hair follicles. Thus the treatment of exogenous BMP2 does not further block the replication of skin stem cells.

Next, the Lipo/Noggin treated group exhibited only a slight anti-wrinkle activity (score 7-8), unexpectedly. We expected that Noggin would have a good anti-wrinkle effect, because Noggin is known to be one of the most powerful endogenous BMP antagonists. Surprisingly, the Lipo/BMPR1a-ECD treated group showed a wonderful anti-wrinkle effect (score 1.8-2.2) with an approximate 300% anti-wrinkle improvement, much better than that of the retinoic acid-treated group (score 3.8-4.5) (Fig. 3).

Each mouse of the six experimental groups was histologically analyzed after 11 week UVB irradiation and sample application. According to the results of H&E staining, UVB irradiated SKH-1 hairless mice showed great changes in epidermal thickness. The UVB-exposed group with no sample application showed abnormal thickening of the epidermal layer, noticeable hyperplasia and irregular basement membrane disruptions (Fig. 4). In each vehicle group, 15% ethanol or 1% liposome treatment did not prevent epidermal thickening from UVB irradiation. However, abnormal epidermal thickening and hyperplasia in dorsal skin was clearly and similarly repressed in both the retinoic acid and Lipo-BMPR1a-ECD treated groups (Fig. 4).

Effect of BMPR1a-ECD on expression of procollagen Iα and MMP-1

Collagen and elastin expressed in fibroblast cells of skin dermis layer are known to be one of main factors of wrinkle biology. It is generally supported that proteins cannot enter the dermis layer due to their size and hydrophilic property. Thus we postulated that BMPR1a-ECD maybe not be an effective treatment for collagen or elastin. However, unexpectedly BMPR1a-ECD did prevent the loss of procollagen expression triggered by UVB exposure (Fig. 4C), in which the collagen level was even further increased compared to the non-UVB irradiated group. The decreased procollagen level through UVB exposure was recovered by treatment of 0.01% retinoic acid

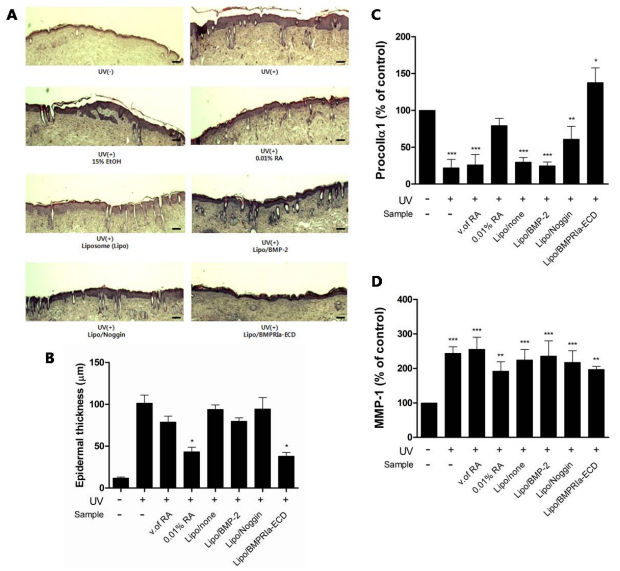

Fig. 4. BMPRIa-ECD prevents both abnormal epidermal thickness and loss of collagen triggered by UVB exposure in SKH-1 mice. SKH-1 hairless mice were irradiated with a total dose of 1.86 J/Cm^2 over the 11 week treatment period. Application of each sample is illustrated in the Materials and Methods section. Dorsal skin samples were obtained 24 h after the last sample application. Epidermal thickness was determined by H&E stained skin sections (A). The results are expressed as a mean ± SD of the thickness in μm (B). Bar size, 100 μm. *P < 0.05. The same amount of total RNA was reverse-transcribed to generate its cDNA, and these cDNAs were used for the RT-PCR analysis of procollagen Iα (C) and MMP-1 (D) expression. The results are representative of 3 independent experiments and have been normalized based on GAPDH level. *P < 0.05, **P < 0.01, ***P < 0.001 vs. controls. *ANOVA*.

up to 75 percent. Interestingly, the procollagen level was further increased by treatment of BMPR1a-ECD, approximately 30% more compared to mice not exposed to UVB in addition to recovery of its expression level. Many reports have shown that the matrix metalloproteinase-1 (MMP-1) expression is increased by UVB irradiation (23). We tested whether BMPR1a-ECD influences the MMP-1 level activated by UVB exposure, in which both BMPR1a-ECD and retinoic acid did not affect MMP-1 expression (Fig. 4D).

DISCUSSION

Investigations of the mechanism of the anti-wrinkle effect of BMPR1a-ECD protein are now underway. Based on our experimental logic and proven data, we hypothesize that BMPR1a-ECD protein competitively binds to BMP ligands and inhibits BMP signals, which allows active skin stem cells to initiate the process of proliferation and shedding from the skin. This active skin cell turnover may immediately replace damaged cells induced by UVB irradiation and maintain skin tissue replenishment and repair. BMPR1a-ECD might have additional anti-wrinkle effect by strengthening collagen expression. It is known that CD34 positive stem cells in skin follicles are increased when BMP signals are inhibited by endogenous antagonists such as noggin (11, 12). Thus we thought that a population of CD34 positive bulge stem cells might also be increased by BMPR1a-ECD treatment and we aim to test this in the future.

Many different factors induce wrinkle formation in a combinational and sophisticated way (3, 5). The main targets in the treatment and prevention of wrinkles are collagen and elastin proteins, which are extracellular cross-linkers released from fibroblast cells in the skin dermis layer. In our study, BMPR1a-ECD is too big to pass through both the skin epidermis and the basement membrane. Thus we thought that BMPR1a-ECD may not have direct contact with collagen or elastin in the skin dermis layer. Unexpectedly, in this study we showed that BMPR1a-ECD did prevent the loss of collagen from UVB irradiation and further induced its expression even compared to that of non-irradiated mice. Further studies for collagen expression increased by treatment of BMPR1a-ECD will be performed in the future.

In the *in vitro* BMP2 inhibition assay, BMPR1a-ECD and Noggin showed a similar activity. Thus we expected Noggin to have a similar or better anti-wrinkle activity than BMPR1a-ECD. However, Noggin did not affect any noticeable change in wrinkle formation (Fig. 3). We postulate that BMPR1a-ECD is stably transferred to target receptors in mouse skin follicles to result in good anti-wrinkle activity. On the other hand, Noggin is thought to have limited delivery to skin follicle stem cells due to several probable restrictions, such as a short half-life, aggregation, and nonspecific bindings to endogenous proteins. Further studies are needed to determine the specific differences in the anti-wrinkle effects of Noggin and BMPR1a-ECD.

Here, we showed that protein regulators for skin stem cell proliferation and differentiation have a good anti-wrinkle effect. Many recent data have indicated other skin and hair effects such as whitening, atopic dermatitis, acne, and hair growth might be affected by skin stem cell effectors (24, 25). Thus the use of skin stem cell effectors as active ingredients for skin or hair care as well as for skin or hair disease is expected to becoming one of main branches of both functional cosmetics and skin disease therapy.

MATERIALS AND METHODS

BMP2, noggin and BMPR1a-ECD production

Human BMP2 or noggin gene was cloned into a pET21a vector and each protein was purified by published methods (13, 14). BMPR1a-ECD gene encoding 129 amino acids was synthesized and linked to a bacterial thioredoxin gene as a fusion partner which was then cloned into pET-32(a) plasmid and expressed in Origami B (DE3). It was then purified through the established protocol, where BMPR1a-ECD-thioredoxin fusion

protein was purified through a Ni-affinity column and thioredoxin was then cleaved by thrombin treatment and BMPR1a-ECD was purified via gel filtration chromatography.

Biological activity assay of BMP2, noggin and BMPR1a-ECD
The Smad1-dependent luciferase assay was performed as previously described (20). In brief, for luciferase reporter assays, C2C12 myoblast cells were cultured in Dulbecco's minimum essential medium (Hyclone) supplemented with 10% FBS, trypsinized, washed with PBS, and resuspended in Opti-minimum essential medium (Invitrogen) containing 0.1% FBS prior to plating into 96-well plates. 24 hlater, cells were transfected with three kinds of plasmids, which are 1147Id1-luciferase construct containing the Smad binding site (Id1-Luc), a Smad-1 expression construct and a CAGGS-LacZ plasmid, using Fugene 6 (Promega) as a transfection carrier according to the manufacturer's instruction. The luciferase activity was measured 24 h after treatment of test samples and different transfection rates in each well were normalized based on their β-galactosidase measures.

Liposome-encapsulated protein sample preparation and treatment
Hydrogenated lecithin was bought from Neuropid (Soya-SPL75H, Anyang, South Korea) and was used to encapsulate proteins using a homogenizer (HA-1004, Hwasung F&B, South Korea). Hydrogenated lecithin was mixed with BMP2, Noggin or BMPR1a-ECD solution in final concentrations of 1% hydrogenated lecithin and 10 μg/ml of each protein. Sample were homogenized three times at 800bar until the liposome size ranged 100-200 nm, as determined by a Particle Size Analyzer (Songwon Systems, Sungnam, South Korea).

Evaluation of wrinkle formation
Four-week old female SKH-1 hairless mice were purchased from OrientBio (Sungnam, South Korea) and were raised in a condition of 24 ± 2°C temperature, 50 ± 10% relative humidity, 12 h light: 12 h dark cycle. This animal study was approved by Institutional Animal Care and Use Committee in Lee Gil Ya Cancer and Diabetes Institute (approval number: LCDI-2010- 0081). After two weeks of domestication and acclimatization, mice were grouped into six cages, containing three mice each: Group 1; UVB(-), group 2; UVB(+), group 3-5; UVB(+) with treatment of 15% ethanol (group 3), 0.01% retinoic acid (group 4) or 1% liposome (group 5), group 6-8; UVB(+) with treatment of 1% liposome-encapsulated 0.001% BMP2 (group 6), 0.001% Noggin (group 7) or 0.001% BMPR1a-ECD (Group 8). Schematic UV irradiation was applied 3 times per week over an 11 week period, using a UVB irradiator and detector (VilberLourmat, Germany). The initial UV intensity was 40 mJ/Cm^2 (1-2 week) which was sequentially increased to 50 mJ/Cm^2 (3-4 week), 60 mJ/Cm^2 (5-9 week), finally to 70 mJ/Cm^2 (10-11 week) in a total 1.86 J/Cm^2. 300 μl of each sample was brushed on each mouse right after UVB exposure 5 times weekly during the whole experimental period. Finally, the UVB exposed dorsal area of mice of each group was photographed every week, and 3 investigators gave wrinkle scores from 0.0 (no wrinkle) to 10.0 (deep wrinkle) based on dorsal skin photographs.

H&E staining for the histological analysis
Skin specimens were obtained from the central dorsum of the mice and were fixed in 10% buffered formalin, and processed for hematoxylin and eosin (H&E) staining for microscopic evaluation. The thickness of the epidermis was measured by using an Infinity image analyzer (Lumenera, Ottawa, Canada) based on microscopic images. Statistical analysis was conducted by using 1 way AVOVA and Dunnett's multiple comparison test (*$P < 0.05$).

Expression analysis of procollagen Iα and MMP-1
Total RNA from the paraffin embedded dorsal skin of each mouse was extracted using an RNeasy FFPE kit (Qiagen, CA, USA) and each cDNA was prepared via reverse transcription of one microgram of RNA using AccuPower RT premix (Bioneer, Korea). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control gene. Each PCR reaction was performed in a total volume of 20 μl, containing 1 μl cDNA, 1pmol each primer and 17 μl RNA-free water using an MyCycler (Bio-Rad, USA) under the following steps: 5 min at 95°C followed by 35 cycles at 95°C for 20 sec, 54°C for 30 sec and 72°C for 30 sec. The reactions were performed in triplicate. The PCR products were analyzed by agarose gel electrophoresis in which the intensity of each band was normalized based on the GAPDH expression level. The primers for RT-PCR are as follows. For procollagen 1α, sense: 5′-TCTCCACTCTTCTAGTTCCT-3′, antisense: 5′-TTGGGTCAT TTCCACATGC-3′; for MMP-1, sense: 5′-GCCTTTAGAGTCT GGAGTGATG-3′, antisense: 5′-TGTGGAAGGAGAGCACAAT ATC-3′; and for GAPDH, sense: 5′-GGTCGGTGTGAACGG ATTTG-3′, antisense: 5′-ATGAGCCCTTCCACAATG-3′.

Statistical analysis
All mouse experiments were performed in triplicate. Results are expressed as mean ± SD. Differences between groups were evaluated with one way *ANOVA* and post test was done Dunnett's multiple comparison (*$P < 0.05$).

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