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Emodin-mediated cross-linking enhancement for extracellular matrix homeostasis

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ABSTRACT

The extracellular matrix (ECM) is an essential element of mammalian organisms, and its cross-linking formation plays a vital role in ECM development and postnatal homeostasis. Defects in cross-link formation caused by aging, genetic, or environmental factors are known to cause numerous diseases in mammals. To augment the cross-linking formation of ECM, the present study established a ZsGreen reporter system controlled by the promoter of lysyl oxidase-like 1 gene (LOXL1), which serves as both a scaffold element and a cross-linking enzyme in the ECM. By using this system in a drug screen, we identified emodin as a strong enhancer of LOXL1 expression that promoted cross-linking formation of ECM in all the tested systems, including human fibroblast cells, cultured human skin tissues, and animals that received long-term emodin treatment. Collectively, the results suggest that emodin may serve as an effective drug or supplement for ECM homeostasis.

1. Introduction

Elastic fibers and collagens are two major components of the extracellular matrix (ECM). Elastic fibers confer resilience and elasticity to lung, skin, pelvic organs, and blood vessels while collagen bundles provide stiffness for tissue support [1–4]. Defects in elastogenesis are known to cause numerous diseases, such as emphysematous lung, loose skin, cardiovascular abnormalities, and age-related macular degeneration [5–7]. In addition, we have recently found that elastic fiber dyshomeostasis confers susceptibility to choroidal neovascularization (CNV), pelvic organ prolapse (POP) and POP-associated urinary disorders, including urinary incontinence and retention [8–10]. Collagen defects are associated with Ehlers–Danlos syndrome and Alport syndrome [11,12].

One major process of ECM development and homeostasis is the polymerization of elastin and collagen monomers. These cross-linking reactions are catalyzed by a series of enzymes, including lysyl oxidase (LOX) family proteins and lysyl hydroxylase. Mammalian genomes have up to five LOX members that encode prototypic LOX and LOX-like proteins 1 through 4 (LOXL1–LOXL4) [13]. While the individual roles of these LOX members remain to be fully elucidated, disruption of the prototypic LOX gene reduces cross-linking in both elastic fibers and collagen bundle [14–17]. Recently, we have demonstrated that LOXL1 deficiency leads to defective elastin deposition [8–10]. We and another research team have also found that LOXL1 down-regulation is associated with aging [9,18], suggesting that LOXL1 enhancement either by LOXL1 gene delivery or by small molecule supplementation may attenuate disease conditions. To explore drugs or supplements that would be useful for elastic fiber homeostasis, we established a LOXL1 promoter activity drug screen. Using this system, we found that emodin up-regulates LOXL1 gene expression, which enhances cross-linking formation in ECM.

2. Materials and methods

2.1. Drug library for screening

The drug library of 31 monomeric compounds and 351 traditional Chinese medicines (TCMs) was kindly provided by Professor Xuexun Fang (College of Life Science, Jilin University, Changchun, China). The details for the drug library refer to the Supplementary materials (Supplementary Tables S1 and S2).

2.2. Constructs and stable cells for drug screening

The upstream non-coding region of the human LOXL1 gene (~3 kb in length), predicted to contain the LOXL1 promoter, was cloned into the pLVX-ZsGreen vector (Biovector, Beijing, China) by

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substituting its original CMV promoter, resulting in a lentiviral vector pLenti-P_{LOXL1}-ZsGreen. The 2nd generation lentiviral packaging system was used to produce lentiviral particles. To generate stable cells containing the pLOXL1-ZsGreen component, the lentiviral particles were used to infect a human fibroblast cell line, and then flow cytometry was used to sort out the cells stably labeled with ZsGreen fluorescence. We plated 293T cells at a density of 6×10^6 cells per 100 mm dish and incubated the cell overnight before transfection with 10 μg pLenti-P_{LOXL1}-ZsGreen along with 15 μg Virapower packaging mix using a high-efficiency transfection kit (Invitrogen). Two days after transfection, the supernatant of transfected cells was collected and filtered through a 0.45 μm pore-size cellulose acetate filter (BD Falcon). Human fibroblasts were seeded at a density of 8×10^5 cells per 100-mm dish 1 day before transduction. The medium was replaced with virus-containing supernatant supplemented with 10 μg/ml polybrene (Nacalai Tesque) and incubated for 8 h. Two days after infection, approximately 80% of the cells exhibited ZsGreen fluorescence. ThehSF-pLenti-PLOXL1-ZsGreen cells stably labeled with ZsGreen were collected by flow cytometry sorting (BD FACS Aria II cell sorter) for drug screening experiments.

2.3. Cell culture

Human skin fibroblast (hSF, Shanghai Inst Biol Sci) and human dermal fibroblast (hDF, HUM-CELL-0069, PriCells) cells were maintained in Dulbecco's modified Eagle medium (Hyclone) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS, Hyclone) at 37 °C in 5% CO_2.

2.4. Measurement of ZsGreen intensity and screening procedure

Human skin fibroblast (hSF-PLenti-PLOXL1-ZsGreen) cells (4000) were seeded into wells of black, clear-bottom, tissue culture surface 96-well plate (3882, Corning) to minimize background fluorescence. The cells were treated with a compound for 24 h. The compounds were dissolved in DMSO at the concentration of 50 mM as stock solutions. Prior to use, the compounds were further diluted with cell culture medium. The final concentration of test compounds in human fibroblast cell wells was approximately 5 μM. DMSO at a concentration of 1% alone was added to control wells. One day after compound treatment, the cells were then fixed with PBS containing 4% paraformal-dehyde for 30 min at room temperature and then washed twice with 200 μl PBS for 5 min each time. For cell quantification, cells were stained with DAPI (1 μg/ml) for 5 min, and viable cells were counted. The cells were then washed with PBS, and 200 μl PBS was added to the wells prior to fluorescence measurement. Measurement of ZsGreen or DAPI fluorescence was performed using a fluorescence microplate reader (SoftMax Pro5; Molecular Devices Corp.) with excitation/emission at 485 nm/530 nm and 358 nm/461 nm, respectively [19].

2.5. RNA extraction and quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from human skin fibroblast cells after 8-h treatment with different concentrations of emodin dissolved in 1% DMSO. The cells were homogenized in 1 ml TRIzol® (Invitrogen) and then extracted according to the operating instructions. RNA purity was determined by reading the optical density of each sample at 260 and 280 nm using Nanodrop 2000 (Thermo Scientific). Prime Script RT-PCR system (DRR036A, TaKaRa) was utilized for first strand cDNA synthesis. PCR primers for the amplification of the human LOXL1 cDNA fragment were (5′-AAGGCCAGTTTCTG CCTGGA-3′) and (5′-AGGCTGCACGTCGGTTATGTC-3′). PCR primers for amplification of the mouse LOXL1 gene were (5′-ACTATGACCT CCGAGTGCTATTGC-3′) and (5′-AGGTCGTAGTGGCTGAACTCGTC-3′). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified as an internal standard for quantification. PCR primers for human GAPDH were (5′-GCACCGTCAAGGCTGAGAAC-3′) and (5′-TGGTGAAGACGCCAGTGGA-3′). PCR primers for mouse GAPDH were (5′-CTTTGGCATTGTGGAAGGGCTC-3′) and (5′-GCAGGGATG-ATGTTCTGGGCAG-3′). For quantitative PCR (qPCR), the reaction was performed with a SYBR® Premix Ex Taq (DRR820A, TaKaRa) and analyzed with the 7500 real-time PCR system (Applied Biosystems).

2.6. Protein extraction and immunoblotting analysis

After the human skin fibroblast cells treaded with different concentrations of emodin dissolved in 1% DMSO or 1% DMSO as control for 24 h, the total cellular protein was extracted from human fibroblast cells using RIPA buffer containing protease inhibitor (Thermo Scientific). The cellular proteins were separated by 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The proteins were then visualized in an imager (Luminescent image analyzer, Image Quant LAS4000 mini) using enhanced chemiluminescence (ECL) detection reagent substrate (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific). To ensure equal protein loading, the same blot was subsequently developed for actin expression. The primary antibodies used for Western blotting were anti-LOXL1 1:1000 (sc-166632, Santa Cruz), anti-actin 1:5000 (HC201, TransGen Biotech) and anti-elastin 1:500 (T3281, Epitomics). The secondary antibodies used were goat-anti-rabbit-HRP and goat-anti-mouse-HRP 1:10,000 (Jackson ImmunoResearch).

2.7. Desmosine and hydroxyproline analysis

Desmosine and hydroxyproline levels were determined with desmosine and hydroxyproline enzyme-linked immunosorbent assay (ELISA) kits (Blue Gene). Specimens were prepared according to the kit instructions. For cell samples, 24 h after emodin treatment, washed the cells twice with ice-cold PBS. 500 μl distilled water was added to each well and cells were collected using a scraper. The samples were then centrifuged for 5 min. The pellets were hydrolyzed with 6 M HCl aqueous solution at 110 °C for 24 h [20]. Hydrolyzed samples were lyophilized, and the residue was dissolved in deionized water [21]. Finally, the samples were dissolved in 0.1 mL distilled water for analysis. Desmosine and hydroxyproline levels were determined by the corresponding ELISA kits (Blue Gene) and normalized to total protein in the dishes. For tissue samples, thorough ice-cold PBS washing was performed to remove excess blood, and samples were weighed prior to hydrolyzation. For each sample 0.5 g tissue was placed into Pyrex tubes and hydrolyzed by refluxing with 6 M HCl aqueous solution at 110 °C for 24 h for desmosine determination by ELISA as described above.

2.8. Knockdown of the LOXL1 expression in human skin fibroblast cell line (hSF)

Four putative candidate shRNA sequences targeting human LOXL1 (GenBank accession no: NM_005576) and negative control vector with a scrambled non-targeting shRNA sequence were (Genechem, Shanghai, China). hFS cells (6×10^4) were seeded on 6-well dishes, and transfected with LOXL1 shRNA and negative control using Lipofectamine™ reagent (Invitrogen, Carlsbad, CA, USA) according to manufacture protocol. Cells were harvested at 48 h after transfection and the most interference efficient shRNA was identified by RT-PCR for further treatment with 4 μM emodin.

2.9. Artificial human skin tissues

Activivskin (Shaanxi Aierfu ActivTissue Engineering Co., Ltd.) is a type of artificial human skin tissue that serves as a three-dimensional model for drug tests [22,23]. The tissues were cut into the same-sized pieces, and each piece was placed in a 70-μm Nylon tamis cellulaire (BD Falcon), which was placed into a well with 4 ml culture medium. The tissue pieces were cultured at the air–liquid interface in six-well-plates in a basic culture medium contain Dulbecco's modified Eagle medium (Hyclone) supplemented with 1% penicillin/streptomycin and 10% FBS (Hyclone) [18]. We added 4 μM emodin and 1% DMSO to the experimental and control groups, respectively. After the tissues were treated for 1 or 3 days, total RNA and protein extracts were prepared for further testing. The artificial skin tissue samples were rinsed in ice-cold PBS and then minced into small pieces, and homogenized in 200 μl lysis buffer. The samples were frozen in liquid nitrogen and then thawed by gentle mixing in a 37 °C water bath. After three freeze/thaw cycles, samples were centrifuged at 10,000*g* for 10 min, and the supernatants were collected for protein assays. For mRNA determination, the tissue sample is homogenized with 1 mL TRIzol® Reagent (Invitrogen), and then extracted the tissues total RNA according to the operating instructions.

2.10. Animal and drug treatment

ICR female mice (8 weeks old) were obtained from Shanghai SLAC Laboratory Animal Co. Ltd. The mice were bred within the Animal Unit of Tongji University. All experiments involving animals were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Biological Research Ethics Committee, Chinese Academy of Sciences. The mice received drug through their drinking water, which supplemented with 1 mg/L emodin that was freshly prepared every other day. The mice were euthanized after emodin treating for 1 or 2 months, and their uterine tissues were obtained for the following experiments. For mRNA determination, the samples were prepared as described above for artificial skin tissues. Desmosine and hydroxyproline were evaluated after HCl hydrolysis with mouse desmosine and hydroxyproline ELISA kits (Blue Gene).

2.11. Statistical analysis

Results were analyzed by Student's *t*-test followed by Bonferroni test (GraphPad Prism 5.01, GraphPad Software, Inc.) when applicable to evaluate differences between groups. Each experiment was reproduced at least three times. Differences were considered significant when $P < 0.05$.

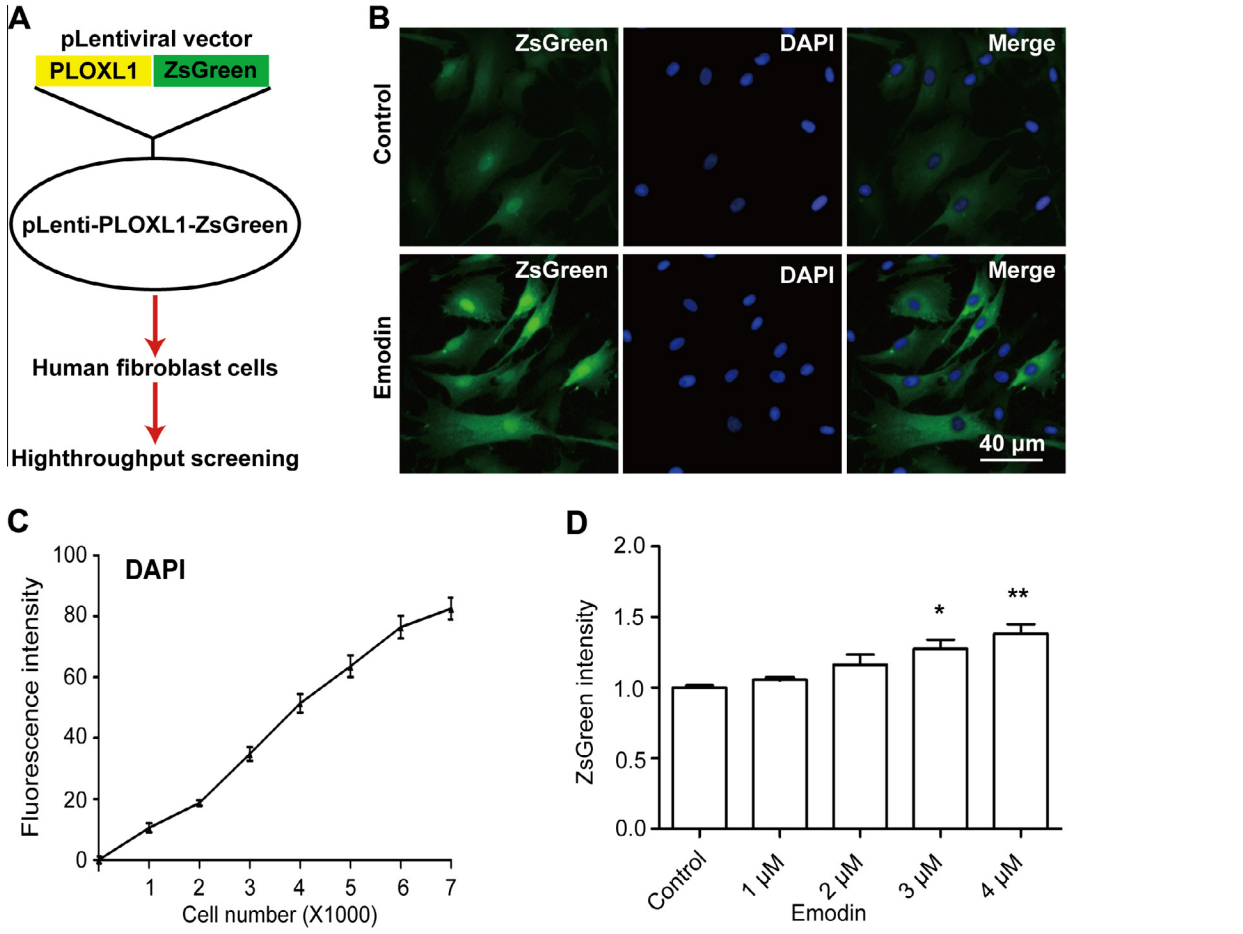

Fig. 1. Drug screening strategy for LOXL1 promoter activation and the effects of emodin inhuman skin fibroblast cells. (A) Drug screening: a lentiviral vector containing the pLenti-P_{LOXL1}-ZsGreen component was packaged into viral particles. Human skin fibroblast cell line (hSF) was infected, and cells with ZsGreen fluorescence were sorted out for high-throughput drug screening. (B) 4 μM emodin exerted an obvious activation effect for the LOXL1 promoter as indicated by increased ZsGreen fluorescence. DMSO served as a control. (C) ZsGreen fluorescence intensity was correlated with the number of cells plated, and DNA well content was based on DAPI fluorescence. Values shown are means ± SEM for five determinations. (D) ZsGreen fluorescence intensity linearly increased as the emodin concentration increased. ZsGreen fluorescence intensity was normalized to the level of DAPI fluorescence and then expressed as the fold increase of the control. Results were analyzed statistically by *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$ when compared to the control group. Error bars represent SEM of the mean from three independent experiments.

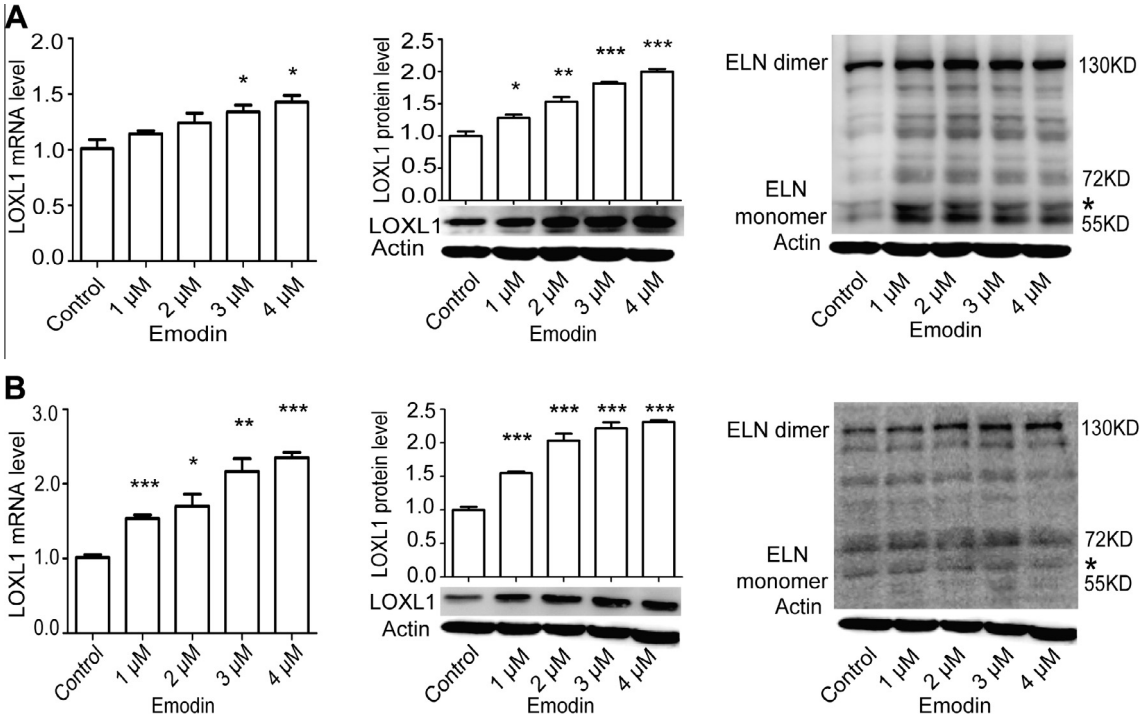

Fig. 2. Loxl1 expression and elastin dimerization in human fibroblast cells after emodin treatment. (A and B) Emodin enhanced LOXL1 levels as determined by RT-PCR and immunoblotting in both human skin cells hSF (A) and hDF (B). The level of LOXL1 mRNA linearly increased as the concentration of emodin increased compared to the untreated group (left). Up-regulation of LOXL gene activity after emodin treatment was further confirmed by immunoblotting (middle). Elastin dimerization was also enhanced after emodin treatment (right). The mRNA and protein levels were normalized according to those of GAPDH and actin control, respectively. Results were analyzed statistically by *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ when compared to the control group. Error bars represent SEM of the mean from 3 to 6 independent experiments.

3. Results

3.1. Emodin-mediated activation of the human LOXL1 gene promoter

Lentivirus containing the P_{LOXL1}-ZsGreen component was used to infect a human fibroblast cell line (hSF), and flow cytometers were used to sort out cells stably labeled with ZsGreen fluorescence (Fig. 1A). Drugs that enhanced ZsGreen fluorescence intensity was predicted to up-regulate LOXL1 gene expression. By preliminary drug screen, emodin was found to activate the LOXL1 promoter at a low concentration. As shown in Fig. 1B, ZsGreen intensity was significantly enhanced after treated with 4 μM emodin, indicating that emodin activated the human LOXL1 gene. Given that drugs could become toxic at certain concentrations and thus suppress cell growth, we standardized the observed ZsGreen fluorescence level to some indicator of cell number by measuring the DNA content of each well as an internal standard. For screening simplicity, we used the DNA staining ability of the intercalating molecule DAPI for the rapid quantitative measurement of DNA content. Thus, ZsGreen expression levels for each drug were normalized to the level of DAPI fluorescence found in each well to determine a final ZsGreen value per cell. We observed an excellent correlation between DAPI intensity and cell number (Fig. 1C). After this correction, ZsGreen intensity was linearly correlated with the concentration of emodin, and we determined that 4 μM emodin was sufficient to significantly activate human LOXL1 gene promoter activity (Fig. 1D).

3.2. LOXL1 expression and ECM deposition enhancement in human fibroblast cells after emodin treatment

To investigate the effects of emodin on LOXL1 expression and ECM cross-linking, both human skin fibroblast (hSF) and human primary dermal fibroblast (hDF) cells were treated with emodin. It was clear that 4 μM emodin promoted LOXL1 expression, and that 3–4 μM emodin maximally augmented LOXL1 mRNA levels in both hSF and hDF cells (Left panels, Fig. 2A and B). Immunoblotting confirmed that LOXL1 expression was doubled both in hSF and hDF cells treaded by 3–4 μM emodin (Middle panels, Fig. 2A and B). Our previous study has revealed that LOXL1 deficiency results in an accumulation of elastin monomer or/and an decrease of elastin dimer (~130 kDa) in tissues and that the molecular weight of elastin monomer may range from 55 kDa to 72 kDa in different tissues. Mature elastic fibers are spatially defined polymers of elastin molecules. Thus, elastin dimer, as an intermediate product for elastin deposition, could serve as a parameter to evaluate the elastin deposition [8]. Here we found that the levels of elastin dimer (~130 kDa) were increased following emodin treatment, indicating emodin promote the crosslink formation of elastic fiber (Right panels, Fig. 2A and B). To further evaluate the cross-linkage of elastic fiber after emodin treatment, the levels of desmosine were measured after HCl hydrolysis. Consistent with the increase in LOXL1 protein levels in these cells, desmosine levels were significantly increased after emodin treatment, and 3–4 μM emodin was sufficient to enhance elastin cross-linking (Left panels, Fig. 3A and B). We also evaluated the stability of collagens, the other major components of ECM, by detecting the levels of hydroxylproline. The increment of hydroxylproline indicated that emodin treatment may also promote the collagen deposition in the ECM (Right panels, Fig. 3A and B). To determine whether emodin treatment enhanced the ECM cross-linkage by improving the expression level of LOXL1, we utilized four shRNA plasmids targeting the LOXL1 gene to knockdown the LOXL1 expression in human fibroblast cells. By RT-PCR, we found that S1 shRNA plasmid worked best and reduced the LOXL1 transcript level by approximate 60% (Left panel, Fig. 3C). We further determined the desmosine levels of S1 treated cells and found that the cross-linking formation of elastic

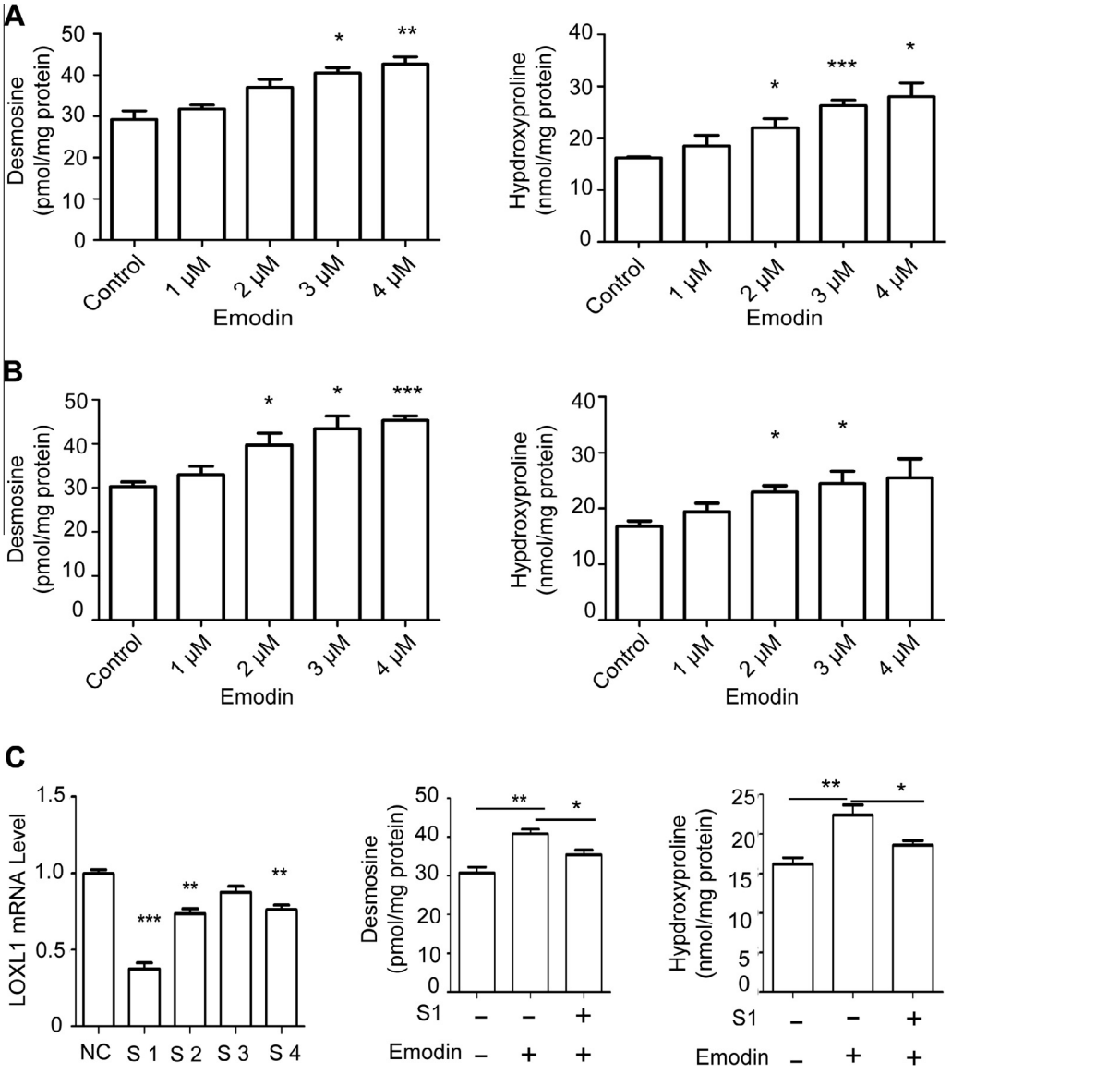

Fig. 3. Emodin promoted desmosine and hydroxyproline formation through LOXL1. (A and B) Desmosine and hypdroxyproline were increased after emodin treatment in hSF (A) and hDF (B). (Left panel of C) Four plasmids ShRNA plasmids (S1, S2, S3 and S4) against the LOXL1 gene were utilized to silence the expression of the LOXL1 gene. By RT-PCR, S1 worked more efficiently than the other three shRNA plasmids with approximate 60% silencing efficiency. (Middle panel of C) In cells with reduced LOXL1 levels by S1, desmosine was reduced by 10% when compared to cells treated only by emodin. Results were analyzed statistically by *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Error bars represent SEM of the mean from three independent experiments.

fiber was compromised in cells treated with both emodin and S1 compared to the cells incubated only by emodin, suggesting that emodin enhanced the cross-linking formation of elastic fiber by activating the expression of the LOXL1 gene.

3.3. ECM homeostasis enhancement in artificial human skin tissue and animal tissues after emodin treatment

To investigate the effects of emodin on LOXL1 expression and ECM deposition in human tissues, artificial human skin tissue was treated with emodin. Consistent with our observations in human fibroblast cells, LOXL1 mRNA levels approximately tripled after 1 μM emodin treatment. Higher emodin concentrations further increased LOXL1 levels (Fig. 4A). After 3 days of emodin treatment, we found that the LOXL1 protein level was also doubled (Fig. 4B) and was associated with an increase in elastin dimer (~130 kDa) and a decrease of elastin monomer (the star position, Fig. 4C), suggesting that emodin enhanced elastin deposition. To further evaluate cross-linkage after emodin treatment, artificial human skin tissues were hydrolyzed by HCl overnight, and levels of desmosine and hydroxyproline were determined. While neither desmosine nor hydroxyproline was noticeably increased, this may be because maturation of elastic fiber or collagen bundles may require a longer period in tissues compared to cultured cells (data not shown).

Analysis indicated that the murine and human LOXL1 genes are approximately 90% homologous. To determine whether emodin was also capable of promoting murine LOXL1 gene expression, we fed a cohort of mice emodin in their drinking water. After 1 month, LOXL1 mRNA levels were significantly increased in uterine tissues by 20% (Fig. 4D). In accordance with the increment of LOXL1 levels, desmosine was increased by approximately 20%, but hydroxyproline levels did not change compared to the untreated group (Fig. 4E and F). After 2 months, LOXL1 mRNA level was further increased by 0.9-fold, with an 87% increment in desmosine level, whereas hydroxyproline appeared unchanged

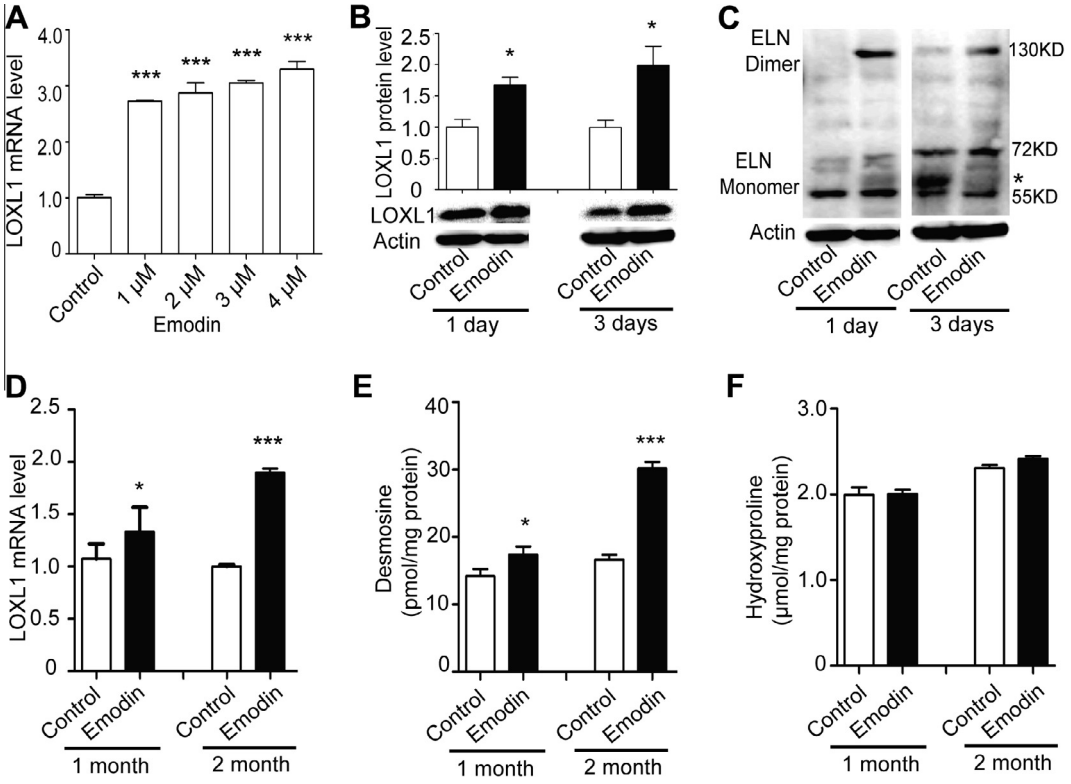

Fig. 4. LOXL1 expression and ECM homeostasis were enhanced in artificial human skin tissues and mouse uterine tissues after emodin treatment. (A) We found that 1 μM emodin increased LOXL1 mRNA by approximately twofold. (B) LOXL1 protein level was doubled after 4 μM emodin treated for 3 days. (C) There was more elastin dimer (130 kDa) and less elastin monomer (the star position) after emodin treatment, suggesting that emodin promoted elastin polymerization at both days 1 and 3. (D and E) LOXL1 mRNA and desmosine levels of mouse uterine tissues were significantly increased after emodin feeding for 1 and 2 months. (F) Hydroxyproline levels of mouse uterine tissues remained unchanged after emodin feeding. Results were analyzed statistically by *t*-test. $^{*}P < 0.05$, and $^{***}P < 0.001$ when compared to the control group. Error bars represent SEM of the mean from three independent experiments (A and B) and ten different samples (D–F).

(Fig. 4D–F). These findings support our hypothesis that supplementation of LOXL1 either by increasing its expression levels or biological activity would specifically promote the cross-linking formation of elastic fibers but not collagen deposition in vivo [8]. After emodin treatment, it should be noted that hydroxyproline level was also significantly increased in cultured cells but was unchanged in cultured human tissues and animals. These differences could be explained by the notion that cultured cells utilized emodin more effectively, resulting in higher LOXL1 levels that would likely trigger non-specific collagen deposition. Although we used lungs to do the same assays, we did not find significant changes in LOXL1 levels after 2-month emodin treatment. Probably, it could be explained by the view that the elastic fiber deposition in uterine tissues is more active than other tissues. It appears that the data obtained from uterine tissues were more consistent than other tissues.

4. Discussion

Elastic fiber is a major ECM component. It is widely known that failure in elastic fiber homeostasis results in a series of age-related disorders, including loose skin, organ prolapse, emphysematous lung, cardiovascular abnormalities, and increased susceptibility to macular degeneration [5–10]. It should be noted that the aging process is usually accompanied by the down-regulation of LOXL1 expression in organs [9,18]. Thus, it is hypothesized that LOXL1 enhancement either by LOXL1 gene delivery or by small molecule supplementation may attenuate disease conditions. Given that fibroblast is a major cell type that contributes to the deposition of both elastic fiber and collagen bundles in vivo, both fibroblast cell line and primary fibroblast cells were employed in the present drug screen to identify LOXL1 enhancers. To confirm our findings, the drugs were further evaluated in artificial skin tissues and animals. Our systemic analysis revealed that emodin was an effective small molecule for boosting LOXL1 level mRNA and protein levels and specifically promoted cross-linking of elastic fibers, suggesting that emodin may be an effective drug or supplementation for treating aging conditions associated with elastic fiber homeostasis failure.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.052.

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