**Dermal Fibroblast CCN1 Expression in Mice Recapitulates Human Skin Aging**

Taihao Quan1, Yaping Xiang, Yingchun, Liu, Zhaoping Qin, George Bou-Gharios, John J Voorhees, Andrzej A. Dlugosz, Gary J Fisher

Department of Dermatology, University of Michigan Medical School, Ann Arbor, Michigan, USA

Institute of Ageing and Chronic Diseases, University of Liverpool, Liverpool UK

1To whom correspondence should be addressed:

Taihao Quan, [thquan@med.umich.edu](mailto:thquan@med.umich.edu)

1150 W. Medical Center Drive

Medical Science I, Room 6447

Ann Arbor, Michigan 48109 USA

Telephone: (734) 615-2403

**Conflict of Interest:** The authors have declared that no conflict of interest exists.

**Abstract**

The aging process deleteriously alters the structure and function of dermal collagen fibrils that confer strength and resiliency to skin. These alterations result in thinning, fragility, wrinkles, laxity, impaired wound healing, and a microenvironment conducive to cancer. However, the key factors responsible for these changes have not been fully elucidated. CCN1, a secreted extracellular matrix (ECM) associated matricellular protein, is significantly elevated in dermal fibroblasts in aged human skin. We demonstrate that transgenic mice with expression of CCN1 selectively in dermal fibroblasts (*COL1A2-CCN1*) display accelerated skin aging. The aged phenotype in *COL1A2-CCN1* mice resembles aged human skin: the skin is wrinkled, and the dermis is thin and composed of loose, disorganized and fragmented collagen fibrils. These dermal alterations reflect reduced production of collagen due to impaired TGF-β signaling and increased expression of matrix metalloproteinases. Importantly, similar mechanisms are known to drive dermal aging in human skin. Taken together, the data demonstrate that elevated expression of CCN1 by dermal fibroblasts functions as a key mediator of skin aging. The *COL1A2-CCN1* mouse model provides a new tool for understanding mechanisms of skin aging and age-related skin disorders.

**Introduction**

As the average expected lifespan increases, the consequences of aging are becoming an increasingly prominent public health issue (1). Aging affects all individuals and is the highest risk factor for most human disease (2), including skin diseases (3). Histological and ultrastructural studies of aged skin have revealed prominent alterations in the collagen-rich dermal extracellular matrix, which comprises the bulk of skin (4, 5). These deleterious alterations include loss of dermal mass, due to reduced production (6) and increased fragmentation (7) of dermal collagen fibrils.

Collagen fibrils are responsible for the structural and mechanical support provided by the dermis. Collagen fibrils also serve as a substrate for cellular attachment, which critically regulates cell function (8, 9). In addition, the dermal extracellular matrix serves as repository for a variety of bioactive proteins. Therefore, age-related degeneration of dermal collagen fibrils broadly impacts skin function (10). Age-related alterations of collagen fibrils also create a tissue microenvironment that is directly related to age-related skin pathologies, such as increased fragility (11), impaired vasculature support (5), poor wound healing (12), and promotion of skin cancer (13).

We previously reported that the matricellular protein CCN1 is markedly elevated in aged human skin(14). CCN1 is the first member of the CCNprotein family, which comprises six members, CCN1 to CCN6 (15). Members of the CCN family exhibit diverse cellular functions, including regulation ofcell proliferation, chemotaxis, apoptosis, adhesion, motility, ion transport and ECM regulation (16). CCN1 has been reported to regulate cell adhesion, migration, chemotaxis, inflammation, cell-matrix interactions, synthesis of ECM proteins and wound healing (17). Knockout of CCN1 in mice is embryonic lethal. This lethality is primarily due to impairment of ECM homeostasis which leads to failure of vascular development (18).

CCN1 is expressed predominantly in dermal fibroblasts in human skin (19), the major cells responsible for collagen homeostasis. Elevated expression of CCN1 in cultured primary adult human dermal fibroblasts causes alterations in expression of ECM genes similar to those observed in the dermis of aged human skin (19).

To explore the role of CCN1 in skin aging we created a transgenic mouse model that selectively expresses CCN1 in dermal fibroblasts using the collagen1A2 (*COL1A2)* enhancer and promoter sequences. *COL1A2-CCN1* mice exhibit accelerated aging of the dermis, characterized by loss of and fragmentation of collagen fibrils, which closely resembles aged human skin. Mechanistic investigations revealed that CCN1-associated impairment of TGF-β signaling, the major regulator of collagen and other ECM components, contributes to dermal thinning through reduced production of collagen. Furthermore, upregulation of the transcription factor c-Jun/AP-1 contributes to collagen fibril fragmentation, through elevated expression of multiple matrix metalloproteinases (MMPs), in *COL1A2-CCN1* mice. Thus, fibroblast-derived CCN1 expression orchestrates key age-related changes in dermal ECM by dysregulating both production and homeostasis of collagen.

**Results and Discussion**

**Fibroblast expression of CCN1 induces age-related alterations in the dermal extracellular matrix.**

*COL1A2-CCN1* mice were generated using a DNA construct containing the protein coding sequences of human CCN1 under the control of the regulatory sequences of the human COL1A2 proximal gene promoter and upstream enhancer (Figure 1A), which is selectively expressed in fibroblasts (20, 21). PCR-based genotyping identified nine *COL1A2-CCN1* transgenic founders, and extensive CCN1 transgene expression was observed throughout the dermis as shown by immunohistology (Supplemental Figure 1A), quantitative RT-PCR (Supplemental Figure 1B) and Western blot (Supplemental Figure 1C). Three lines with similar CCN1 transgene expression in skin (Supplemental Figure 1C) were used for the studies presented in this report.

Newborn *COL1A2-CCN1* mice were grossly normal in appearance, and histological evaluation revealed normal skin morphology (Supplemental Figure 1A). However, by six months of age, all three lines of *COL1A2-CCN1* mice exhibited an aged appearance, characterized by hunched back (Figure 1B), reduced weight (Figure 1C, 20% less weight compared to control littermates), and wrinkles on their dorsal skin (Supplemental Figure 2A). Development of the aged appearance began at approximately two months of age and gradually became more apparent during the following four months. The degree of the aged appearance paralleled the accumulation/increases of CCN1 protein in the skin (Figure 1D).

Histological examination revealed dermal thinning in *COL1A2-CCN1* mice by six months of age. Dermal thickness was reduced 46%, compared to non-transgenic sex and aged-matched littermates (Figure 1E). Masson’s trichrome (Figure 1F) and Sirius red (Supplemental Figure 2B) staining, which visualize collagen fibrils, revealed reduced density of collagen fibrils in *COL1A2-CCN1* mice. Nanoscale analysis by atomic force microscopy (AFM) indicated that collagen fibrils were significantly fragmented and disorganized in *COL1A2-CCN1* transgenic mice (Figure 1G). Quantitative analysis demonstrated that the average roughness of collagen fibrils, an indicator of collagen fibril organization, was 2.6-fold greater in *COL1A2-CCN1* mice than in non-transgenic littermates (25 nm vs. 66 nm, Figure 1H).

Importantly, the dermal features observed in *COL1A2-CCN1* mice closely resemble alterations that are observed in the dermis of aged human skin, such as aberrant collagen homeostasis (14, 22, 23). This finding supports the concept that elevated dermal expression of CCN1 is a critical driver of age-related dermal alterations.

**Impaired TGF-β signaling reduces type I collagen and leads to dermal thinning in *COL1A2-CCN1* mice.**

To examine possible molecular pathways that promote dermal thinning, we cultured dermal fibroblasts from *COL1A2-CCN1* mice and control littermates. We found that fibroblasts from *COL1A2-CCN1* mice express significantly less type I collagen mRNA (Figure 2A) and protein (Figure 2B). These data are consistent with reduced collagen fibril content and dermal thinning in *COL1A2-CCN1* mice.

The TGF-β pathway is the major regulator of dermal ECM production (6). We investigated the potential involvement of TGF-β signaling in reduced expression of type I collagen in *COL1A2-CCN1* mice fibroblasts. We first examined TGF-β-dependent Smad3 phosphorylation, which plays a critical role in type I procollagen gene expression (24). Interestingly, TGF-β-dependent Smad3 phosphorylation was significantly lower in dermal fibroblasts from *COL1A2-CCN1* mice (Figure 2C). To elucidate the mechanism of impaired TGF-β signaling, we determined gene expression of the major TGF-β pathway components, including TGF-β ligands, TGF-β receptors, and Smads. We found that levels of type II TGF-β receptor (TβRII) mRNA (Figure 2D) and protein (Figure 2E) were selectively and significantly decreased by 57% and 70%, respectively, in *COL1A2-CCN1* mice fibroblasts. In contrast, type I TGF-β receptor (TβRI) expression (Figures 2D and 2E) and expression of other TGF-β pathway components remained unchanged (Supplemental Figure 3).

These data support the conclusion that specific down-regulation of TβRII impairs TGF-β/Smad signaling, which leads to reduction of type I collagen in *COL1A2-CCN1* mice fibroblasts. Consistent with this finding, we previously reported that TGF-β signaling is decreased in aged human skin, largely due to a reduction in TβRII (19, 25). Given that the TGF-β pathway is the major regulator of collagen production, it is likely that reduced collagen production and attendant dermal thinning in *COL1A2-CCN1* mice is mediated, at least in part, by impaired TGF-β signaling in dermal fibroblasts.

Impairment of TGF-β signaling, due to loss of TβRII in stromal fibroblasts, results in epithelial tumors in animal models (26). In the case of skin cancer, ultraviolet (UV) irradiation from the sun is thought to be the major risk factor. As it relates to this study, UV irradiation simultaneously induces CCN1 (~15-fold) (27) and inhibits expression of TβRII (reduced ~70%) in human skin dermal fibroblasts both *in vivo* and *in vitro* (28). These data suggest that a concomitant increase of CCN1 and reduction of TβRII in aged dermal fibroblasts could promote age-related keratinocyte skin cancer, which is the most common cancer in adult Caucasians (29).

**Elevated MMP expression contributes to dermal collagen fibril damage in *COL1A2-CCN1* mice.**

We next explored potential mechanisms leading to fragmentation of collagen fibrils in *COL1A2-CCN1* mice. We measured proteolytic activity, in conditioned media from *COL1A2-CCN1* and non-transgenic littermate fibroblasts, by collagen zymography. As shown in Figure 3A, *COL1A2-CCN1* mice fibroblasts expressed significantly greater proteolytic activity than control fibroblasts. The family of mammalian MMPs is primarily responsible for degradation of the collagen-rich ECM. Therefore, we examined the ability of the MMP inhibitor GM6001 to suppress proteolytic activity in *COL1A2-CCN1* mice fibroblast conditioned media. GM6001 substantially inhibited *COL1A2-CCN1* mice fibroblast proteolytic activity, in a dose-dependent manner (Figure 3A). This suppression suggests that *COL1A2-CCN1* mice fibroblasts express increased levels of MMPs. To ascertain which MMP family members are expressed by *COL1A2-CCN1* mice fibroblasts, we determined gene expression levels of the 24 mammalian MMPs. We found that expression of MMP-9, -10, -13, -24, and -27 were significantly elevated in fibroblasts from *COL1A2-CCN1* mice compared to control fibroblasts (Figure 3B). Importantly, these changes resemble those in aged human skin, in which elevated CCN1 upregulates expression of MMPs (22, 30).

MMP-13, or collagenase-3, can initiate type I collagen fibril degradation by cleavage at a single site within the triple helix to generate 3/4 and 1/4 length fragments (31). The fragments can be further degraded by MMP-9, also known as gelatinase B (32). MMP-10, or stromelysin-2, degrades other ECM components as well as activates proMMP-13 and proMMP-9 by limited proteolysis (33). MMP-24 and -27 are relatively less well characterized. MMP-27 contains a transmembrane domain with a unique C-terminal extension (CTE) that promotes its retention in the endoplasmic reticulum (34), while MMP-24 is a membrane-type MMP, also known as MT5-MMP, which can activate progelatinase A (proMMP-2) in tumors (35). Therefore, *COL1A2-CCN1* fibroblasts express elevated levels of MMPs that together have the capacity to initiate and further degrade type I collagen fibrils.

We next cultured *COL1A2-CCN1* and control littermate fibroblasts in three-dimensional collagen lattices and examined collagen fibril fragmentation by AFM. AFM images revealed that collagen fibrils in lattices containing control fibroblasts were intact and well-organized, displaying characteristic periodic D-banding (Figure 3C, left panel). In contrast, collagen fibrils in lattices containing *COL1A2-CCN1* mice fibroblasts were fragmented and disorganized (Figure 3C, middle left), similar to collagen fibrils in lattices treated with recombinant human MMP-1 (Figure 3C, right panel). *COL1A2-CCN1* mice fibroblast-mediated collagen fibril disruption was blocked by GM6001 (Figure 3C, middle right).

AFM three-dimensional surface topography mapping indicated that collagen fibrils in lattices containing *COL1A2-CCN1* mice fibroblasts were more disorganized (Figure 3D). Quantitative analysis of AFM data indicated that the average roughness (a measure of fibril organization) of collagen fibrils in lattices containing *COL1A2-CCN1* mice fibroblastswas 2-fold greater than lattices containing control fibroblasts (66 nm vs. 127 nm).

**c-Jun/AP-1 contributes to dermal collagen damage in *COL1A2-CCN1* mice through elevation of MMPs.**

Three of the five MMPs that are elevated in *COL1A2-CCN1* mice fibroblasts (MMP-9, -10 and -13) are regulated by the transcription factor AP-1 (36). We determined expression of c-Jun, a component of AP-1, and found significant increases in c-Jun mRNA (Figure 4A) and protein (Figure 4B). In addition, AP-1 transcriptional activity, measured by a luciferase reporter, was elevated four-fold in *COL1A2-CCN1* mice fibroblasts (Figure 4C). We have previously reported that multiple MMPs increase with age in human dermis (7, 37), and this increase is associated with elevated expression of c-Jun/AP-1 (38). Taken together, these data indicate that CCN1 induction of MMPs likely involves upregulation of c-Jun/AP-1.

**Summary**

We demonstrate that selective expression of CCN1 in dermal fibroblasts drives an accelerated aged phenotype in the dermis that closely resembles human aging (Figure 4D). This finding supports a causal link between CCN1 and skin aging. The *COL1A2-CCN1* mouse model provides a unique opportunity to identify and investigate initial molecular mechanisms and early markers of skin aging. Such studies could lead to development of novel preventative and ameliorative therapeutic strategies to improve skin health and reduce age-related maladies in the elderly.

**Methods**

For complete methods, see supplemental material.

**Statistical analysis.** Data are expressed as mean ± SEM. Comparisons were made with the paired t-test (two groups). All p values are two-tailed and considered significant when <0.05.

*Study approval*. Protocols for mouse experimentation were approved by the University of Michigan Institutional Animal Care and Use Committee.

**Author Contributions**

TQ, GB-G and GF designed the experiments and supervised the study. YX, YL, and ZQ performed the experiments and acquired the data. TQ and GF analyzed and interpreted the data. AD, JJV, GF and TQ discussed the data and reviewed and edited the manuscript.

**Acknowledgments**

The authors thank Diane Fiolek for administrative assistance, as well as Joel Maust, for writing/editorial support. Additional thanks to the University of Michigan Transgenic Core for production of *COL1A2-CCN1* mice, and Dr. Shonali Majumdar (University of Texas MD Anderson Cancer Center) for the pCD3 plasmid containing the 6-kb enhancer and promoter of the Col1a2 gene. This work was supported by the National Institutes of Health (AG054835 to GJF and TQ).

**Figure legends**

**Figure 1. Transgene expression of CCN1 in dermal fibroblasts causes age-related alterations of the dermal aged phenotype in mouse skin dermis.** (**A**) Schematic representation of the construct expressed in dermal fibroblasts. (**B**)Representative gross appearance of 6 months old control (left) and *COL1A2-CCN1* mouse (right). (**C**) Body weight of *COL1A2-CCN1* mice and their control littermates was measured at 6 months after birth (*n* = 6 mice per genotype). (**D**) Accumulation of CCN1 protein expression in *COL1A2-CCN1* mice over six months. Inset shows representative Western blots. (**E**) H&E staining in samples taken from control and *COL1A2-CCN1* mice, with representative images shown. (**F**) Masson’s trichrome staining, representative images. The dermal collagenous extracellular matrix is stained blue, cells are stained red, and the nuclei black. Subcutaneous fat appears white. The dermal thickness (**E**) and collagen fibril density (**F**) were quantified by computerized image analysis (Image-pro Plus software, version 4.1, Media Cybernetics, Rockville, MD). (**G**) Atomic force microscopy of collagen fibrils. Scale bar = 200 nm. Representative images shown. The blue arrow heads indicate intact collagen fibrils and red arrow heads indicate damaged collagen fibrils. (**H**) Dermal collagen fibril organization, measured as Collagen fibril average roughness (Ra, nm), was quantified using Nanoscope Analysis software (Nanoscope\_Analysis\_v120R1sr3, Bruker-AXS, Santa Barbara, CA). All results expressed as mean ± SEM, *n*=6, \*p < 0.05 by a two-tailed paired *t* test, scale bars = 100 µm, unless otherwise indicated.

**Figure 2. Reduced production of type I collagen and impaired TGF-β signaling in fibroblasts from *COL1A2-CCN1* mice.** Fibroblasts from six months old *COL1A2-CCN1* mice and non-transgenic littermates (CTRL) were cultured from dorsal skin. Fibroblasts from *COL1A2-CCN1*  and CTRL mice were analyzed for levels of (**A**) type I procollagen mRNA, (**B**) type I procollagen protein, (**C**) TGF-β induction of Smad3 phosphorylation, (**D**) type I (TβRI) and type II (TβRII) TGF-β receptor mRNA, and (**E**) TβRI and TβRII protein. mRNA and protein levels determined by real-time RT-PCR or Western analysis, respectively. mRNA or protein levels were normalized to 36B4 (internal housekeeping gene control) or β-actin (loading control), respectively. Inset shows representative Western blots. Mean ± SEM, *n*=6, \*p < 0.05, two-tailed paired *t* test.

**Figure 3. Fibroblasts from *COL1A2-CCN1* mice express elevated levels of collagen-degrading matrix metalloproteinases (MMPs).** (**A** and **B**) Fibroblasts from dorsal skin of six months old *COL1A2-CCN1* mice and non-transgenic littermates (CTRL) were cultured in monolayer. (**A**) Proteolytic activities in fibroblast conditioned media were examined by zymography. Areas of protease activity appear as clear bands. Inhibition of proteolytic activity by MMP inhibitor GM6001 identified the presence of MMP proteolytic activity. Recombinant human matrix metalloproteinase 1 (rh-MMP-1) was used as a positive control. (**B**) MMP mRNA levels were determined by real-time RT-PCR. Levels were normalized to 36B4 (internal housekeeping gene control). (**C** and **D**) Fibroblasts from *COL1A2-CCN1* and non-transgenic littermates (CTRL) were cultured in three-dimensional collagen lattices and the structure of the collagen fibrils was examined by atomic force microscopy (AFM) in different treatment conditions. (**C**) The red arrows indicate intact collagen fibrils and blue arrow heads indicate damaged collagen fibrils. (**D**) Collagen fibril organization, measured by average roughness (Ra), was quantified using Nanoscope Analysis software (Nanoscope\_Analysis\_v120R1sr3, Bruker-AXS, Santa Barbara, CA). All data expressed as mean ± SEM, *n*=6, \*p < 0.05, two-tailed paired *t* test.

**Figure 4. c-Jun/AP-1 contributes to damaged dermis in *COL1A2-CCN1* mice through elevation of MMPs.** Fibroblasts from dorsal skin of six months old control and *COL1A2-CCN1* mice were cultured in monolayer. (**A**) c-Jun mRNA and (**B**) c-Jun protein levels were determined by real-time RT-PCR and Western analysis. Levels were normalized to 36B4 (internal housekeeping gene control) and β-actin (loading control). Inset shows representative Western blots. (**C**) An AP-1 reporter construct (pAP1-TA-Luc) was transiently transfected by electroporation into control and *COL1A2-CCN1* fibroblasts. After 48 hours, luciferase activity was measured to determine AP-1 activity. (**D**) Illustrated diagram showing mechanisms by which elevated CCN1 leads to dermal ECM aging. All data expressed as mean ± SEM, *n*=6, \*p < 0.05 by two-tailed paired *t* test.

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**Methods (SupplementaL information)**

**Generation of *COL1A2-CCN1* transgenic mice**. The *COL1A2-CCN1* transgenic mice used in this study were produced by standard pronuclear injection of linear DNA into a fertilized mouse egg (C57BL/6, Jackson Labs) at University of Michigan Transgenic Core. Briefly, a Sal1 fragment containing the human CCN1 was PCR amplified and cloned in the plasmid pCD3 containing the 6-kb enhancer and promoter of the Col1A2 gene, an IRES-lacZ reporter, and the murine protamine polyA signal (20, 21). All cloning was verified by sequencing. Integration of the CCN1 transgene was assessed by genotyping of mouse tail DNA with specific primers for CCN1 transgene (5’-AAC-CCT-GAG-TGC-CGC-CTT-GTG-AAA-3’ and 5’-ATT-GGC-ATG-CGG-GCA-GTT-GTA-GTT-3’) and mouse β-globin (5'-CCA-ATC-TGC-TCA-CAC-AGG-ATA-GAG-AGG-GCA-GG-3' and 5’-CCT-TGA-GGC-TGT-CCA-AGT-GAT-TCA-GGC-CAT-CG 3') as a positive control. The *COL1A2-CCN1* founders were crossed with C57BL/6 background breeders (Jackson Labs) for at least 6 generations. Three lines with similar expression of CCN1 transgene (Supplemental Figure 1C) were used for the studies presented in this report. All protocols for mouse experimentation were approved by the University of Michigan Institutional Animal Care and Use Committee.

**Histology and Immunohistology.** Mouse skin was embedded in OCT and cryo-sections (7µm) were stained with hematoxylin and eosin (H&E), Masson’s trichrome and Sirius red by the standard procedures. The dermal thickness and collagen density (blue in Masson’s trichrome and red in Sirius red) were quantified by computerized image analysis (Image-pro Plus software, version 4.1, Media Cybernetics). Immunohistology was performed as described previously using antibodies against CCN1 (Santa Cruz Biotechnology, Santa Cruz, CA) (19), type I procollagen (EMD Millipore, Temecula, CA). Briefly, skin samples embedded in OCT were sectioned (7 µm), fixed in 2% paraformaldehyde, permeabilized with 0.5% Triton X-100 in phosphate-buffered saline (PBS), blocked with rabbit serum (5% in PBS) and incubated for one hour at room temperature with primary antibodies, followed by incubation with secondary antibody for one hour at room temperature. After staining, the slides were examined using a digital imaging microscope (Zeiss, Germany). Specificity of staining was determined by substituting isotype-control immunoglobulin (mouse IgG2a) for the primary antibodies. No detectable staining was observed with isotype-controls.

**Laser capture microdissection (LCM) coupled quantitative real-time RT-PCR.** LCM was performed as previously described (7). Briefly, human skin punch biopsies were embedded in OCT, sectioned and stained with hematoxylin. Epidermis and dermis were captured using LCM (Leica ASLMD System, Leica Microsystems, Germany). Total RNA was extracted and CCN1 mRNA levels were determined by quantitative real-time RT-PCR.

**Atomic force microscopy (AFM) imaging.** Mouse skin biopsies and three-dimensional collagen lattices were embedded in OCT and cryo-sections (15µm) were mounted on microscope cover glass (1.2 mm diameter, Fisher Scientific Co., Pittsburgh, PA) for AFM image analysis. AFM images were acquired using a Dimension Icon AFM system (Bruker-AXS, Madison, WI) in air using a silicon etched cantilever (NSC15/AIBS, MikroMasch, San Jose, CA) with a full tip cone angle ~40º and the tip radius of curvature ~10 nm. AFM images were acquired at a scan rate of 0.977 Hz, 512x512 pixel resolutions, as previously described (38). Collagen fibril’s average roughness (Ra) was analyzed using Nanoscope Analysis software (Nanoscope Analysis v120R1sr3, Bruker-AXS). AFM images were obtained from Electron Microbeam Analysis Laboratory (EMAL), University of Michigan College of Engineering, and analyzed using Nanoscope Analysis software (Bruker-AXS).

**Cell culture, RNA isolation and quantitative real-time PCR.** Mouse dorsal skin was washed with cold phosphate buffered saline (PBS) and sterilized with 70% ethanol twice. The skin tissue was placed in an empty 100 mm petri, then dissected and minced with surgical scissors using sterile techniques under the hood. The tissue was placed in a flask and was incubated with 10ml of 0.25% trypsinizing solution (Sigma Chemical Co., St. Louis, MO) and 0.02% ethylene diamine tetraacetate (EDTA, Sigma Chemical Co.) at room temperature for 15 minutes. Trypsinizing solution was replaced with fresh solution (10ml) and incubated for one hour. Isolated cells were collected following brief centrifugation and resuspended in Dulbecco's modified Eagle's minimal essential medium (DMEM; Life Technology Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Sigma Chemical Co.), penicillin (100 U/ml), streptomycin (100 μg/ml) in a humidified incubator with 5% CO2 at 37°C. Cells were cultured at sub-confluence and utilized between passages 2 and 4. Total RNA was isolated from mouse skin punch biopsy and dermal fibroblasts using a commercial kit (RNeasy mini kit, Qiagen, Chatsworth, CA) according to the manufacturer’s protocol. PCR template was prepared by reverse transcription using Taqman Reverse Transcription kit (Applied Biosystems, Foster City, CA). PCR was performed in duplicate with 2 μl of cDNA for the genes of interest using TaqMan Universal PCR Master Mix kit (Applied Biosystems) and a 7700 sequence detector system (Applied Biosystems). PCR procedures were performed with a robotic workstation (Biomek 2000; Beckman Coulter, Inc., Hialeah, FL) to ensure accuracy and reproducibility. Type I procollagen and MMPs mRNA levels were normalized to the mRNA levels of the housekeeping gene, 36B4 (internal control). Type I procollagen and MMPs primers were purchased from Real Time Primers (Elkins Park, PA).

**Western blot analysis.** Whole cell proteins were extracted, and equal amounts of protein (~40 μg/lane) were resolved by 6-12% gradient sodium dodecyl sulfate-polyacrylamide (SDS) gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were then blocked with PBST (0.1% Tween 20 in PBS) containing 5% nonfat milk for one hour at room temperature. Primary antibodies were incubated for one hour at room temperature. The following antibodies were used for Western blotting: type I procollagen (EMD Millipore), phospho-Smad3 (Cell Signaling Technology, Danvers, MA), total Smad3, CCN1, TβRI, TβRII, c-Jun (Santa Cruz Biotechnology) and β-actin (Sigma). The membranes were washed three times with PBST solution and incubated with appropriate secondary antibodies for one hour at room temperature. After washing three times with PBST, the membranes were developed with ECF (Vistra ECF Western blotting system, GE Health Care, Piscataway, NJ) following the manufacturer’s protocol. The membranes were scanned with a STORM PhosphorImager (Molecular Dynamics, Sunnyvale, CA), the intensities of each band were quantified using ImageQuant software (GE Health Care, Piscataway, NJ) and normalized to β-actin (control).

**Transfection and AP-1 reporter assay.** AP-1 reporter construct (pAP1-TA-Luc) was purchased from BD Biosciences Clontech (Palo Alto, CA). Mouse skin fibroblasts were transiently transfected by electroporation using dermal fibroblasts nucleofector kit (Amaxa Biosystems, Gaithersburg, MD) according to the manufacturer’s protocol. After 48 hours of transfection, luciferase activity was measured by luciferase assay using an enhanced luciferase assay kit (PharMingen International, San Diego, CA) according to the manufacturer’s protocol. Aliquots containing identical β-galactosidase activity were used for each luciferase assay.

**Three-dimensional (3D) collagen cultures and zymography assay.** 3D collagen gels were prepared based on a previous publication with minor modification (39). Briefly, rat tail type-I collagen (2 mg/ml, BD Biosciences) was suspended in medium cocktail (DMEM, NaHCO3 [44 mM], L-glutamine [4 mM], Folic Acid [9 mM]), and neutralized with 1N NaOH to pH 7.2. Cells (0.5x106) were suspended in 2 ml collagen and medium cocktail solution and plated in a 35 mm bacterial culture dish. The collagen gels were placed in incubator at 37°C for 30 minutes to allow collagen polymerization. The collagen gels were then incubated with 2 ml media (DMEM, 10% FBS) at 370C, 5% CO2. To activate secreted MMP-1, collagen lattices were washed extensively with PBS (at least three times for 30 minutes) and then treated with Trypsin-EDTA (100 ng/ml, Invitrogen Life Technology, Carlsbad, CA) in serum-free media for 24-36 hours. 3D collagen gels were embedded in OCT and cryo-sections (15 µm) were analyzed for collagen fragmentation by AFM image analysis. For zymography assays, conditioned media from the cultured cells were concentrated and then analyzed by electrophoresis in the presence of 12% Zymogram (collagen) protein gel (Thermal Fisher Scientific, Waltham, MA). After electrophoresis, the gel was incubated in Zymogram Renaturing buffer (Thermal Fisher Scientific) for 30 minutes at room temperature with gentle agitation. After renaturing, the gel was incubated in developing buffer (Thermal Fisher Scientific) at 37°C overnight. The MMPs activities were visualized by staining Coommasie Blue R-250 (Thermal Fisher Scientific) solution. MMP inhibitor (GM6001, Santa Cruz Biotechnology) was used to test MMP-mediated proteolytic activity.

**Supplemental Figures Legend**

**Supplemental Figure 1. CCN1 expression in *COL1A2-CCN1* transgenic mice.** (**A**) Representative immunohistology of CCN1 (red) and DAPI (blue) in control and *COL1A2-CCN1* mouse skin. (**B**) CCN1 mRNA and (**C**)CCN1 protein expression isolated from dorsal skin were determined by real-time RT-PCR and Western analysis, respectively. mRNA and protein levels were normalized to 36B4 (internal housekeeping gene control) and β-actin (loading control), respectively. Panel shows representative Western blots. Mean ± SEM, *n*=6, \*p < 0.05, two-tailed paired *t* test.

**Supplemental Figure 2. Transgene expression of CCN1 in dermal fibroblasts exhibits aged phenotype in mouse skin dermis.** (**A**) Representative skin wrinkles of a 6-month old control (left) and *COL1A2-CCN1* transgenic mouse (right). (**B**) Sirius red staining, representative images shown. Collagen fibers are stained red, and white lines indicate epidermal and dermal junctions. Collagen fibril density (B) was quantified by computerized image analysis (Image-pro Plus software, version 4.1, Media Cybernetics). All results expressed as mean ± SEM, *n*=6, \*p < 0.05 by two-tailed paired *t* test, scale bars = 100 µm.

**Supplemental Figure 3. mRNA expression of TGF-β ligands and Smads in *COL1A2-CCN1* mice.** mRNA levels in *COL1A2-CCN1* mouse skin were determined by real-time RT-PCR and normalized to 36B4 (internal housekeeping gene control). Results are expressed as mean ± SEM, *n*=6, \*p < 0.05 by two-tailed paired *t* test.