

# Cysteine-Rich Protein 61 (CCN1) Mediates Replicative Senescence-Associated Aberrant Collagen Homeostasis in Human Skin Fibroblasts

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## ABSTRACT

Dermal fibroblasts produce a collagen-rich extracellular matrix, which confers mechanical strength and resiliency to human skin. During aging, collagen production is reduced and collagen fragmentation is increased, which is initiated by matrix metalloproteinase-1 (MMP-1). This aberrant collagen homeostasis results in net collagen deficiency, which impairs the structural integrity and function of skin. Cysteine-rich protein 61 (CCN1), a member of the CCN family, negatively regulates collagen homeostasis, in primary human skin dermal fibroblasts. As replicative senescence is a form of cellular aging, we have utilized replicative senescent dermal fibroblasts to further investigate the connection between elevated CCN1 and aberrant collagen homeostasis. CCN1 mRNA and protein levels were significantly elevated in replicative senescent dermal fibroblasts. Replicative senescent dermal fibroblasts also expressed significantly reduced levels of type I procollagen and increased levels of MMP-1. Knockdown of elevated CCN1 in senescent dermal fibroblasts partially normalized both type I procollagen and MMP-1 expression. These data further support a key role of CCN1 in regulation of collagen homeostasis. Elevated expression of CCN1 substantially increased collagen lattice contraction and fragmentation caused by replicative senescent dermal fibroblasts. Atomic force microscopy (AFM) further revealed collagen fibril fragmentation and disorganization were largely prevented by knockdown of CCN1 in replicative senescent dermal fibroblasts, suggesting CCN1 mediates MMP-1-induced alterations of collagen fibrils by replicative senescent dermal fibroblasts. Given the ability of CCN1 to regulate both production and degradation of type I collagen, it is likely that elevated-CCN1 functions as an important mediator of collagen loss, which is observed in aged human skin. *J. Cell. Biochem.* 113: 3011–3018, 2012. © 2012 Wiley Periodicals, Inc.

**KEY WORDS:** CCN1; COLLAGEN; MMP-1; SENESCENT CELLS; AGED HUMAN SKIN

CCN1 belongs to a group of proteins known as CCN family, which at present consists of six distinct members, CCN1-6 [Lau and Lam, 1999; Perbal, 2004; Leask and Abraham, 2006]. Members of the CCN family exhibit diverse cellular functions such as regulation of cell proliferation, chemotaxis, apoptosis, adhesion, motility, ion transport, and extracellular matrix (ECM) production [Lau and Lam, 1999; Brigstock, 2003; Perbal, 2004; Chen and Lau, 2009]. Like other members of CCN family, CCN1 is a secreted, ECM-associated matricellular protein. CCN1 protein has been reported to

regulate cell adhesion, migration, cell-matrix interactions, and synthesis of ECM proteins in variety of cells in culture [Kireeva et al., 1996; Chen et al., 2001; Mo et al., 2002].

Type I collagen is the most abundant component of the dermal ECM in human skin. The collagenous ECM comprises the bulk of skin and is critical for structure and function. Reduced collagen production and increased fragmentation of collagen fibrils are prominent features of aged human skin. These deleterious alterations of collagen homeostasis largely account for thinning

Abbreviations: CCN1, cysteine-rich protein 61; CCN family (cysteine-rich protein 61, connective tissue growth factor, nephroblastoma overexpressed); Col-1, type I collagen; MMP-1, matrix metalloproteinase-1; ECM, extracellular matrix; PCR, polymerase chain reaction.

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and increased fragility of skin in elderly individuals [Fisher et al., 2002; Varani et al., 2006; Fisher et al., 2009]. Little is known regarding the underlying mechanisms of age-related aberrant collagen homeostasis in human skin [Fisher et al., 2008].

We have previously reported that CCN1 is elevated in chronologically aged human skin, and increased levels of CCN1 negatively regulate collagen homeostasis in human dermal fibroblasts [Quan et al., 2006]. Fibroblasts are the major collagen-producing cell type in human skin. Their response to elevated CCN1 is down-regulation of type I collagen production and up-regulation of matrix metalloproteinase-1 (MMP-1), which initiates collagen degradation [Quan et al., 2006, 2010]. These data prompted us to further explore the connection between CCN1 expression and collagen homeostasis in human skin fibroblasts. To address this question, we have employed replicative senescent dermal fibroblasts, which have been extensively studied as an *in vitro* aging model.

## MATERIALS AND METHODS

### CELL CULTURE AND REPLICATIVE SENESCENT DERMAL FIBROBLASTS

Primary human skin dermal fibroblasts were prepared from healthy adult human skin biopsy and cultured as previously described [Fisher et al., 1991; Quan et al., 2005]. Briefly, human fibroblasts were isolated by digestion skin with bacterial collagenase (Worthington Biochemical Corporation, Lakewood, NJ), and cultured in Dulbecco's modified Eagle (DMEM) medium supplemented with 10% fetal bovine serum (v/v) in a humidified incubator with a 5% CO<sub>2</sub> atmosphere at 37°C. Participation of human subjects was approved by the institutional review board at the University of Michigan, and all subjects provided written informed consent before entering the study. All culture reagents; DMEM, fetal bovine serum, trypsin solution, and penicillin G (100 units/ml)/streptomycin (100 units/ml) were purchased from Invitrogen Life Technology (Carlsbad, CA). Pre-senescent control dermal fibroblasts used for this study were between passages 7 and 10. Replicative senescent dermal fibroblasts were generated by serial passage of primary cultures until the cells stopped growing and expressed the phenotype of senescent cells [Going et al., 2002; Cristofalo et al., 2004], including enlarged and flattened shape, and the positivity for senescence-associated- $\beta$ -galactosidase activity (see Results for details).

### THREE-DIMENSIONAL COLLAGEN LATTICE CELL CULTURES

Pre-senescent and senescent human skin dermal fibroblasts were cultured in three-dimensional collagen lattices, as previously described [Fisher et al., 2009]. Briefly, collagen lattices were prepared by mixing appropriate volume of rat tail type I collagen (BD Biosciences) with medium cocktail [DMEM, NaHCO<sub>3</sub> (44 mmol/L), L-glutamine (4 mmol/L), folic acid (9 mmol/L), and neutralized with 1 N NaOH to pH 7.2] to yield a final concentration of 2 mg/ml. Fibroblasts ( $2.5 \times 10^5$ ) were suspended in 2 ml collagen solution in 35 mm culture dish, and placed in an incubator at 37°C for 30 min to allow polymerization of the collagen. After polymerization, collagen lattices were detached from sides and bottom of the dish, covered with 2 ml of media (DMEM, 10% fetal bovine serum)

and incubated for 24 h at 37°C, 5% CO<sub>2</sub>. To activate secreted MMP-1, collagen lattices were washed extensively with PBS (at least three times for 30 min), and then treated with Trypsin-EDTA (100 ng/ml, Invitrogen) in serum-free media for 24 h. Conditioned media were collected, concentrated, and analyzed by 10% SDS-PAGE. Collagen bands were visualized by staining with SimplyBlue (Invitrogen).

### RNA ISOLATION AND QUANTITATIVE REAL-TIME RT-PCR

Total RNA was isolated using a commercial kit (RNeasy midikit, Qiagen, Chatsworth, CA) according to the manufacturer's protocol. Total RNA (100 ng) was reverse transcribed using Taqman Reverse Transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed on a 7300 Sequence Detector (Applied Biosystems) using Taqman Universal PCR Master Mix Reagents (Applied Biosystems). PCR primers and probes were purchased from Applied Biosystems. Type I procollagen, MMP-1, CCN1, and 36B4 primers and probes were described previously [Fisher et al., 2009; Quan et al., 2004, 2006, 2009]. Integrin primers were ordered from Realtimeprimers.com. Integrin  $\alpha$ 1 (ITGA1), forward primer, 5'-CAAAGTGCAGACCCCATATC-3', reverse primer, 5'-TGACTTG-GCTGATGTCAGAA-3'; Integrin  $\alpha$ 2 (ITGA2), forward primer, 5'-ATGCAGATGGACCACACTTT-3', reverse primer, 5'-AAGCAT-CACTGCTGAACTCC-3'; Integrin  $\alpha$ 10 (ITGA10), forward primer, 5'-TGATTTTCTGAAGCCAGGAG-3', reverse primer, 5'-AAAG-CATTTCCCATCATCAA-3'; Integrin  $\alpha$ 11 (ITGA11), forward primer, 5'-AAGTCACACCCACTCCCTTC-3', reverse primer: 5'-ATGTGTGA-GTTGCGGTCAAT-3'. Target gene levels were normalized to the housekeeping gene 36B4, as an internal control for quantification.

### WESTERN BLOT ANALYSIS

Western analysis was performed as previously described [Quan et al., 2001]. Briefly, whole cell extract was prepared from the cells using whole cell extraction buffer (25 mM HEPES, 0.3 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, 20 mM  $\beta$ -glycerolphosphate, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 2  $\mu$ g/ml leupeptin, and 100  $\mu$ g/ml PMSF). Whole cell extract was prepared by centrifugation, and concentrations of proteins were measured by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Proteins were resolved on 10% SDS-PAGE, transferred to PVDF membrane, and reacted with primary antibodies. Type I procollagen antibody was purchased from Southern Biotech (Birmingham, AL) and MMP-1 and CCN1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Protein bands were visualized and quantified with enhanced chemifluorescence (ECF) (Vistra ECF Western Blotting System, GE Healthcare, Piscataway, NJ) following the manufacturer's protocol. The intensities of each band were quantified by STORM PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and normalized using  $\beta$ -actin as loading control.

### ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) AND SECRETED CCN1 PROTEIN

Secreted type I procollagen and MMP-1 were determined from fibroblast culture fluids using enzyme-linked immunosorbent assay kits (type I procollagen, Takara Mirus Bio USA, Madison, WI; human MMP-1, GE HealthCare) according to manufacturer's instructions.

Secreted CCN1 protein was determined from fibroblasts culture fluids by western blot analysis as described above.

### CELLTRACKER AND $\beta$ -GAL STAINING

Fibroblast morphology was assessed by incubation of cultures with CellTracker fluorescent dye (Molecular Probes, Eugene, OR) for 1 h [Fisher et al., 2009]. The cells were washed with PBS and were fixed in 2% paraformaldehyde for 30 min. Fibroblasts were imaged by fluorescence microscopy. Senescence-associated- $\beta$ -galactosidase staining was performed using kit (Senescence  $\beta$ -gal Staining Kit, Cell Signaling Technology Inc, Danvers, MA) according to manufacturer's instructions.

### TRANSFECTION AND siRNA

Human skin fibroblasts were transiently transfected with control siRNA (5'-UUCUCCGAACGUGUCACGU-3') and CCN1 siRNA (5'-GCUGCGUUUCCUUCUACU-3') as described previously [Quan et al., 2010]. siRNAs were synthesized from Qiagen (Chatsworth, CA), and transfection was performed by electroporation (Amaxa Biosystems, Koeln, Germany) according to the manufacturer's protocols. After 48 h of transfection, whole cell extract was prepared and protein levels were determined by western blot analysis, as described above.

### ATOMIC FORCE MICROSCOPY (AFM) IMAGING

Three-dimensional collagen lattices were washed with PBS and mounted on microscope glass slides (25  $\times$  75  $\times$  1.0 mm, Fisher Scientific, Pittsburgh, PA), and allowed to air dry for at least 48 h before AFM image analysis. AFM images were obtained using a Dimension Icon AFM system (Bruker-AXS, Santa Barbara, CA), as previously described [Quan et al., 2011ab]. Briefly, the scan positions of the collagen lattices were determined by light optical image. AFM images were obtained with ScanAsyst mode in air using a silicon etched cantilever (NSC15/AIBS, MikroMasch, San Jose, CA) with a full tip cone angle  $\sim$ 40 $^\circ$  and the tip radius of curvature  $\sim$ 10 nm. AFM images were acquired at a scan rate of 0.977 Hz, 512  $\times$  512 pixel resolutions. AFM imaging was conducted at the Electron Microbeam Analysis Laboratory (EMAL), University of Michigan College of Engineering, and analyzed using Nanoscope Analysis software (Nanoscope Analysis v120R1sr3, Bruker-AXS, Santa Barbara, CA).

### STATISTICAL ANALYSIS

Data are expressed as mean  $\pm$  SEM. Student's *t*-test was used to evaluate the statistical differences between the groups. All *P*-values are two-tailed, and less than 0.05 was considered statistically significant.

## RESULTS

### ESTABLISHMENT OF REPLICATIVE SENESCENT DERMAL FIBROBLASTS CULTURES

After multiple serial passages, primary human skin dermal fibroblasts eventually stop growing and reach a state of replicative senescence [Going et al., 2002; Cristofalo et al., 2004]. We found that primary human dermal fibroblasts undergo replicative senescence

after 6 months of weekly passage, which is approximately 100 population doublings. Senescent cells display a well-defined morphological and molecular signature characterized by enlarged and flattened shape and expression of senescence-associated  $\beta$ -galactosidase activity, a biomarker of cellular senescence [Dimri et al., 1995]. A red fluorescent dye (CellTracker), which is taken up into the cell cytoplasm was used to assess the morphology of dermal fibroblasts [Fisher et al., 2009]. Figure 1A shows that replicative senescent dermal fibroblasts display an enlarged and flattened shape. Quantitative morphometric analysis revealed that surface area of senescent fibroblasts was increased threefold, compared to pre-senescent dermal fibroblasts. Figure 1B shows that senescent dermal fibroblasts displayed substantial positive staining of senescence-associated  $\beta$ -galactosidase, while pre-senescent dermal fibroblasts were negative.

### CCN1 IS INCREASED IN REPLICATIVE SENESCENT DERMAL FIBROBLASTS

CCN1 is elevated in aged dermal fibroblasts in vivo, and over-expression of CCN1 negatively regulates collagen homeostasis in

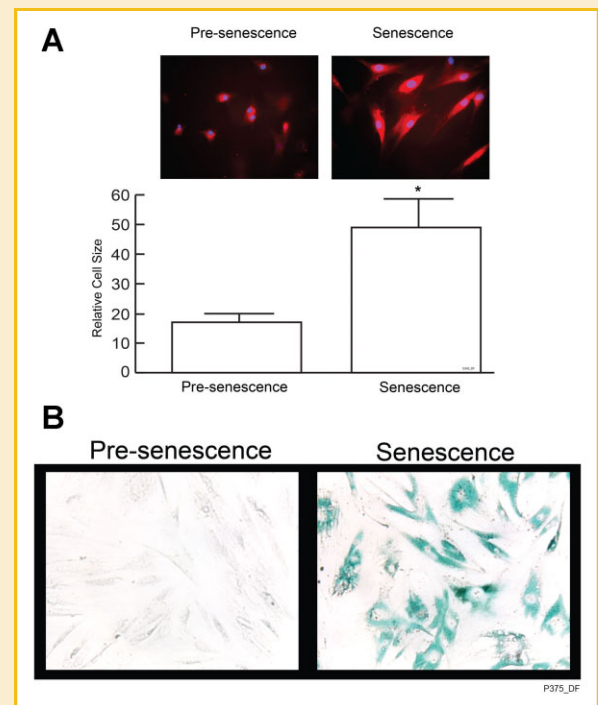


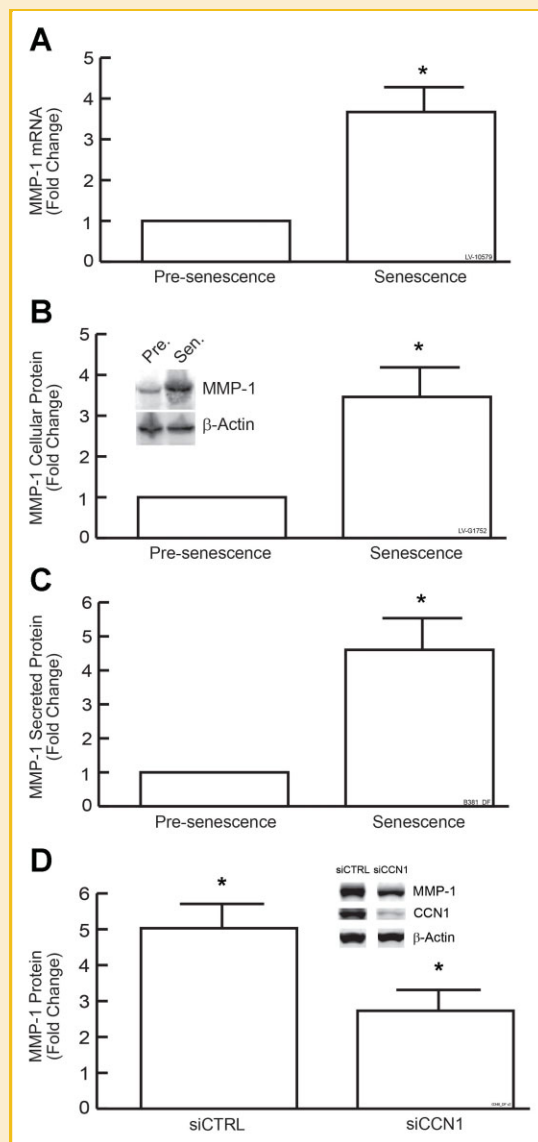
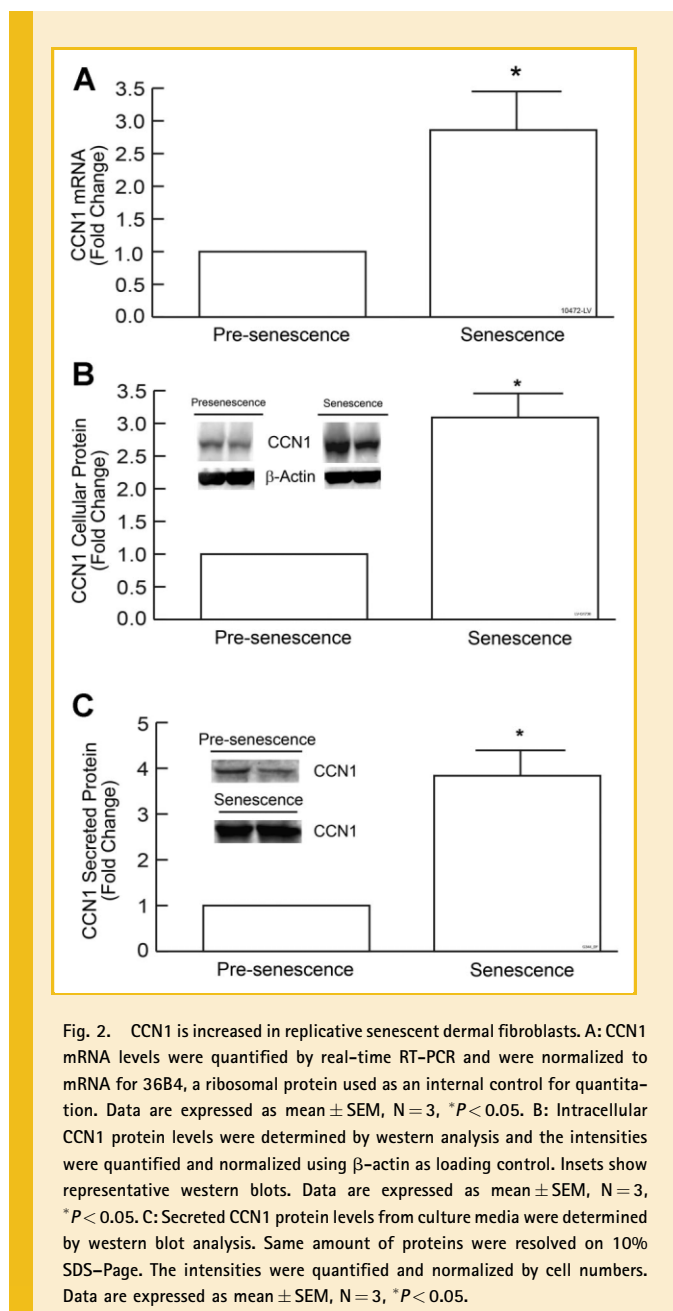
Fig. 1. Establishment of replicative senescent dermal fibroblasts cultures. Replicative senescent cells were prepared from primary normal human skin dermal fibroblasts. The cells were cultured by multiple serial passages until they stopped growing and reached senescence. A: Cell morphology was assessed by incubation of cells with CellTracker fluorescent dye (Molecular Probes, Eugene, OR) for 1 h. Cells were imaged by fluorescence microscopy. Reddish fluorescence delineates cell cytoplasm; blue fluorescence delineates nuclei. Relative red fluorescence levels were quantified by imageJ (NIH). Results are means  $\pm$  SEM. N = 3, \**P* < 0.05. B: Senescence-associated- $\beta$ -galactosidase staining was performed using kit (Cell Signaling Technology Inc, Danvers, MA) according to manufacturer's instructions. Images are representative of three experiments.

human skin dermal fibroblasts [Quan et al., 2006]. We found that CCN1 expression was significantly elevated in senescent dermal fibroblasts. Both CCN1 mRNA (Fig. 2A), and intracellular (Fig. 2B) protein were increased threefold, and extracellular secreted CCN1 protein was elevated fourfold (Fig. 2C) in senescent dermal fibroblasts, compared to pre-senescent dermal fibroblasts.

### CCN1 REGULATES INCREASED MMP-1 EXPRESSION IN REPLICATIVE SENESCENT DERMAL FIBROBLASTS

MMP-1 is the major enzyme that initiates degradation of collagen fibrils, in human skin [Fisher et al., 1997; Brennan et al., 2003; Fligel et al., 2003]. We found that MMP-1 expression was significantly elevated in senescent dermal fibroblasts. Both MMP-

1 mRNA (Fig. 3A), and intracellular (Fig. 3B) protein were increased greater than threefold, and extracellular secreted MMP-1 protein was elevated greater than fourfold (Fig. 3C) in senescent dermal fibroblasts, compared to pre-senescent dermal fibroblasts. We next investigated the connection between elevated expression of CCN1



and MMP-1 in senescent dermal fibroblasts, using siRNA-mediated knockdown. Treatment of senescence dermal fibroblasts with CCN1 siRNA reduced CCN1 protein levels greater than 90%. Knockdown of CCN1 in senescent dermal fibroblasts reduced elevated MMP-1 protein levels 50% (Fig. 3D). These data support the concept that elevated CCN1 is an important mediator of elevated MMP-1 in replicative senescent dermal fibroblasts.

### CCN1 MODULATES COLLAGEN GEL, CONTRACTION AND ALTERATIONS INDUCED BY RELICATIVE SENCENCE DERMAL FIBROBLASTS

Cleavage of collagen fibrils by MMP-1 promotes contraction of dermal equivalent cultures, which are composed of dermal fibroblasts embedded in three dimensional collagen lattices. Figure 4A demonstrates that replicative senescent fibroblasts contracted three-dimensional collagen lattices significantly greater than pre-senescent fibroblasts. This increased lattice contraction was inhibited 72% by knockdown of CCN1 (Fig. 4A) and 78% by MMP inhibitor MMI270 (33 nM), suggesting that CCN1-induced MMP-1 plays a major role in senescence-mediated collagen gel contraction. MMP-1 cleaves collagen fibrils at a single site, resulting in one-quarter and three-quarter length fragments [Fligel et al., 2003; Varani et al., 2008]. We therefore examined the pattern of collagen fragmentation in dermal equivalent cultures containing pre-senescent or replicative senescent fibroblasts. Cultures containing pre-senescent fibroblasts displayed nearly undetectable collagen fragments (Fig. 4B), consistent with lack of contraction of these cultures. Cultures containing senescent fibroblasts, however, displayed readily detectable collagen fragments, consistent with substantial contraction of these cultures. This accumulation of MMP-1-generated collagen fragments was substantially reduced by knockdown of CCN1 in senescent dermal fibroblasts (Fig. 4B), further supporting the role of CCN1 in upregulation of MMP-1 expression. Finally, we used atomic force microscopy (AFM) to assess the structure of collagen fibrils in dermal equivalent cultures containing pre-senescent or replicative senescent dermal fibroblasts. AFM revealed that collagen lattices containing pre-senescent fibroblasts were composed of long, intertwining, intact collagen fibrils (Fig. 4C). In contrast, collagen fibrils in lattices containing senescent fibroblasts were largely fragmented and disorganized, consistent with biochemical measurement of increased collagen fragmentation. This collagen fibril fragmentation was largely prevented by knockdown of CCN1 in replicative senescent dermal fibroblasts (Fig. 4C). Taken together, these data indicate that upregulation of CCN1 induces collagen fragmentation by MMP-1, which mediates collagen lattice disorganization and contraction by replicative senescent dermal fibroblasts.

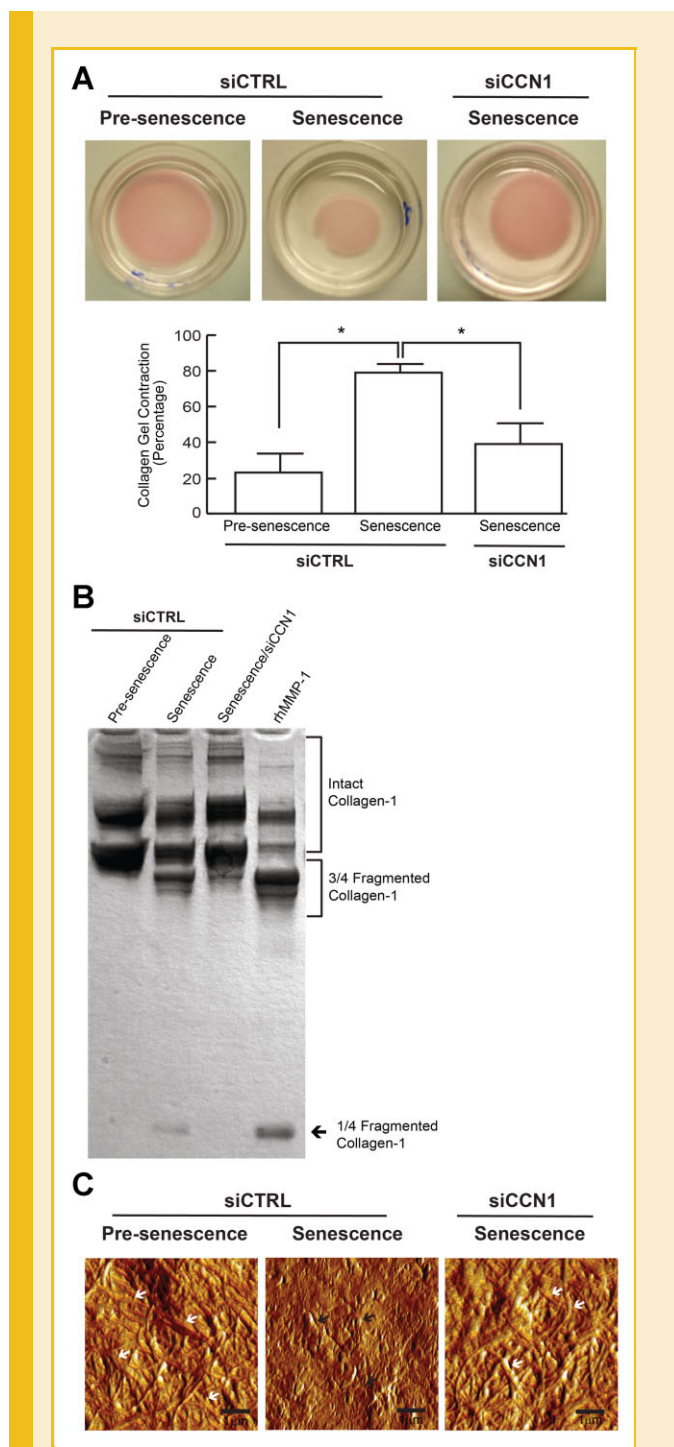


Fig. 4. CCN1 modulates collagen gel contraction and alterations induced by replicative senescent dermal fibroblasts. Pre-senescent and replicative senescent dermal fibroblasts were transfected with control siRNA or CCN1 siRNA. One day after transfection, the cells were cultured in three-dimensional collagen lattices for 24 h. Secreted MMP-1 was activated by Trypsin-EDTA for 24 h as described in Materials and Methods section. A: CCN1 modulates collagen gel contraction. Collagen gel contraction was imaged by digital camera and quantified. Data expressed as mean  $\pm$  SEM, N = 3, \* $P$  < 0.05. B: CCN1 modulates collagen fragmentations. Conditioned media were collected, concentrated, and resolved in a 10% SDS-PAGE. Intact and fragmented collagens were visualized by staining with SimplyBlue. Activated recombinant human MMP-1 (rhMMP-1) used as a positive control. C: CCN1 modulates collagen disorganization. Nanoscale three-dimensional collagen lattices were visualized by atomic force microscopy (AFM) as described in Materials and Methods section. The white arrows indicate intact and well-organized collagen fibrils and black arrows indicate damaged and disorganized collagen fibrils. AFM images are representative of three independent experiments.

## CCN1 REGULATES REDUCED TYPE I PROCOLLAGEN EXPRESSION IN REPLICATIVE SENESCENT DERMAL FIBROBLASTS

Type I collagen fibrils are the major structural component of the dermal ECM. We found that type I procollagen expression was significantly reduced in senescent dermal fibroblasts. Both type I procollagen mRNA (Fig. 5A), and intracellular (Fig. 5B) protein were reduced greater than 80%, and extracellular secreted type I procollagen protein was reduced nearly 70% (Fig. 5C) in senescent dermal fibroblasts, compared to pre-senescent dermal fibroblasts. Importantly, knockdown of CCN1 significantly increased type I procollagen protein levels in senescent dermal fibroblasts (Fig. 5D). These data support the concept that elevated CCN1 is an important mediator of type I collagen homeostasis in replicative senescent human dermal fibroblasts.

## DISCUSSION

Type I collagen fibrils form the framework of the ECM in the dermal compartment of skin. The collagenous ECM is critical for normal skin structure and function. Fibroblasts within the dermis are primarily responsible for maintaining ECM homeostasis by controlling the production and turnover of type I collagen fibrils. During the passage of time, collagen fibrils undergo progressive alterations characterized by reduced organization, thinning, and increased fragmentation [Quan et al., 2006; Varani et al., 2006; Fisher et al., 2009; Quan et al., 2010]. These alterations are associated with decreased collagen synthesis and increased expression of collagen-degrading MMPs, by dermal fibroblasts. Deleterious alterations of skin connective tissue collagen impair skin structural integrity, resulting in increased skin fragility, which impairs wound healing [Eaglstain, 1986; Holt et al., 1992; Raine-Fenning et al., 2003]. Age-dependent alterations in the dermal ECM also create a microenvironment that facilitates formation and growth of skin cancer [Weinstock, 1994; Khorramizadeh et al., 1999; Campisi, 2003].

While impaired dermal fibroblast function is largely responsible for aberrant collagen homeostasis in aged skin, the underlying mechanisms are not well understood. We find that CCN1 is a negative regulator of collagen homeostasis in dermal fibroblasts, and that CCN1 is substantially elevated in aged human skin [Quan et al., 2006, 2011a]. This observation led us to explore the relationship between age-related elevation of CCN1 and aberrant collagen homeostasis in human skin fibroblasts. To address this question, we employed a replicative senescent dermal fibroblast model. Aging of cultured cells *in vitro* is often used as a model of cellular aging [Dimri et al., 1995; Going et al., 2002; Cristofalo et al., 2004]. We find that replicative senescent dermal fibroblasts resemble fibroblasts in aged human skin *in vivo*, with respect to reduced expression of type I procollagen and elevated MMP-1, as well as elevated CCN1. Importantly, we find that aberrant collagen homeostasis in replicative senescent dermal fibroblasts is partially normalized by knockdown of elevated CCN1, indicating elevated CCN1 functions as a mediator of cellular senescence-associated alterations in collagen homeostasis.

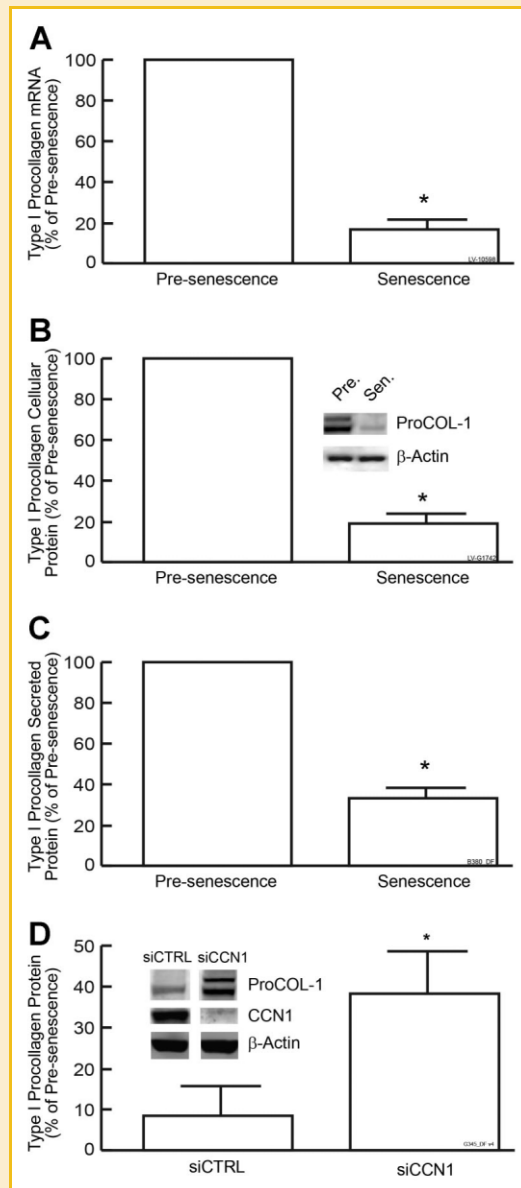


Fig. 5. Type I procollagen is reduced in replicative senescent dermal fibroblasts. A: Type I procollagen mRNA levels were quantified by real-time RT-PCR and were normalized to mRNA for 36B4, a ribosomal protein used as an internal control for quantitation. Data are expressed as mean  $\pm$  SEM,  $N = 3$ ,  $*P < 0.05$ . B: Intracellular type I procollagen (COL-1) protein levels were determined by western blot analysis and the intensities were quantified and normalized using  $\beta$ -actin as loading control. Insets show representative western blots. Data are expressed as mean  $\pm$  SEM,  $N = 3$ ,  $*P < 0.05$ . C: Secreted type I procollagen protein levels from culture media were determined by ELISA. Data are expressed as mean  $\pm$  SEM,  $N = 3$ ,  $*P < 0.05$ . D: Senescent dermal fibroblasts were transfected with control siRNA or CCN1 siRNA. Type I procollagen and CCN1 protein levels were determined 2 days after transfection by western blot analysis. Insets show representative western blots. Data are means  $\pm$  SEM,  $N = 3$ ,  $*P < 0.05$ .

We have previously reported that CCN1 down regulates TGF- $\beta$  type II receptor, thereby impairing TGF- $\beta$ 1 responsiveness in human dermal fibroblasts [Quan et al., 2006]. It is well established that TGF- $\beta$ 1 controls both collagen production and collagen degradation

[Varga et al., 1987; Chen et al., 2000; White et al., 2000; Hall et al., 2003]. TGF- $\beta$ 1 stimulates collagen synthesis and down-regulates MMP-1 expression. TGF- $\beta$ 1 also up-regulates plasminogen activator inhibitor-1 and tissue inhibitor of metalloproteinase, two major inhibitors of ECM-degrading enzymes. In addition we reported that elevated CCN1 induces AP-1 transcription factor [Quan et al., 2006], which directly drives MMP-1 transcription, and negatively regulates type I procollagen expression [Matrisian, 1994; Fisher and Voorhees, 1998; Fisher et al., 2000]. Therefore, it is likely that elevated CCN1 alters collagen homeostasis by impairment of TGF- $\beta$  signaling and induction of transcription factor AP-1, in replicative senescent dermal fibroblasts.

Molecular mechanisms responsible for the elevated expression of CCN1 in replicative senescent dermal fibroblasts remain to be determined. Interestingly, replicative senescence is associated with increased oxidative stress [Campisi, 2003; Brandl et al., 2011], and we have observed that CCN1 is markedly induced by oxidative stress (unpublished data). These data raise the possibility that CCN1 is induced by oxidative stress in replicative senescent dermal fibroblasts.

CCN1 belongs to a family of secreted proteins termed matricellular proteins [Lau and Lam, 1999; Perbal, 2004; Leask and Abraham, 2006]. Fibroblasts secrete CCN1 where it interacts with the extracellular milieu, including ECM, growth factors, proteases, and cytokines. Analysis of mechanisms by which CCN1 functions indicates that it exerts cellular actions primarily through interactions with cell surface adhesion receptors, integrins [Lau and Lam, 1999; Chen and Lau, 2009]. It appears that CCN1 interacts with distinct integrins depending on the cell type and that these interactions provide functional specificity. At least seven integrins have been shown to mediate CCN1 functions in cell type specific manner [Chen and Lau, 2009]. In fibroblasts, CCN1 mediates cell adhesion and migration through  $\alpha$ v $\beta$ 5 and  $\alpha$ v $\beta$ 3 integrins, respectively. The exact complement of integrins that mediate CCN1 regulation of collagen homeostasis in human dermal fibroblasts remains to be determined.

Binding of fibroblasts to collagen is required for lattice contraction. This binding is mediated by specific collagen receptors, integrins  $\alpha$ 1 $\beta$ 1,  $\alpha$ 2 $\beta$ 1, and  $\alpha$ 11 $\beta$ 1 [Klein et al., 1991; Tiger et al., 2001; Jokinen et al., 2004]. We find that knockdown of CCN1 does not alter expression of collagen-binding integrins, in senescent dermal fibroblasts (see supplemental figure). Collagen gel contraction operates against the mechanical properties of the collagen fibrils. Intact collagen fibrils provide greater resistance than fragmented collagen fibrils to the contractive mechanical forces exerted on them by dermal fibroblasts. MMP-1-mediated collagen fragmentation promotes collagen gel contraction [Fisher et al., 2009]. Therefore, we conclude that MMP-1-catalyzed collagen fragmentation plays a major role in CCN1-mediated collagen gel contraction.

Dermal fibroblasts in aged human skin express elevated levels of CCN1 [Quan et al., 2006], and aberrant collagen homeostasis characterized by reduced type I procollagen [Quan et al., 2010] and increased MMP-1 expression [Fisher et al., 2009]. The data presented above support the concept that aberrant collagen homeostasis in aged human skin is mediated, at least in part, by elevated CCN1. Suppression of CCN1 expression may provide a novel therapeutic

mechanism to improve the health of chronologically aged human skin and age-related skin diseases.

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