Cysteine-rich Protein 61 (CCN1) Domain-specific Stimulation of Matrix Metalloproteinase-1 Expression through αVβ3 Integrin in Human Skin Fibroblasts*

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Human skin largely comprises collagenous extracellular matrix. The hallmark of skin aging is fragmentation of collagen fibrils. Matrix metalloproteinases (MMPs) are largely responsible for collagen degradation. MMP-1, principally derived from dermal fibroblasts, is the major protease capable of initiating degradation of native fibrillar collagens. Presently, we report that CCN1, a secreted and extracellular matrix-associated protein, is elevated in aged human skin dermal fibroblasts in vivo and stimulates MMP-1 expression through functional interaction with αVβ3 integrin in human dermal fibroblasts. CCN1 contains four conserved structural domains. Our results indicate that the three N-terminal domains (IGFBP, VWC, and TSP1), but not the C-terminal CT domain, are required for CCN1 to stimulate MMP-1 expression. This stimulation is dependent on interaction between the active structural domains and αVβ3 integrin. The interaction of VWC domain with integrin αVβ3 is necessary and requires functional cooperation with adjacent IGFBP and TSP1 domains to stimulate MMP-1 expression. Finally, induction of MMP-1 expression in dermal fibroblasts by CCN1 N-terminal domains resulted in fragmentation of type I collagen fibrils in a three-dimensional collagen lattice model. These data suggest that domain-specific interactions of CCN1 with αVβ3 integrin contribute to human skin aging by stimulating MMP-1-mediated collagen fibril fragmentation.

Human skin, like all human organs, undergoes progressive alterations as a consequence of natural aging. Additional damage is superimposed on natural aging as a result of chronic exposure to UV irradiation from sunlight. The bulk of skin comprises collagenous extracellular matrix, and the molecular hallmark of aged human skin is altered collagen fibril in dermal connective tissue (1, 2). Age-related, progressive alterations of dermal connective tissue collagen correlate with a decline of skin functions including increased fragility and reduced wound healing and with increased cancer incidence (2–6).

In human skin, collagen turnover is carried out by matrix metalloproteinases (MMPs), a family of zinc-containing proteases that specifically degrade extracellular matrix including type I collagen fibrils. To date, 23 members of the MMP family are known in humans. 18 members are expressed in human skin. Of these, MMP-14 is most highly expressed (7). Although MMP-14 has been shown to degrade type I collagen (8–10), its expression is not altered by stimuli, such as UV irradiation and reactive oxygen species, or wounding, which greatly increase collagen fibril fragmentation (7, 11). Also, MMP-14 is not increased in aged or photoaged human skin, which have elevated levels of collagen defragmentation (12). In contrast, MMP-1 (also known as collagenase 1) is produced principally in fibroblasts and appears to be the major protease capable of initiating degradation of native fibrillar collagens (13–18). MMP-1 cleaves native type I collagen fibrils at a single site, resulting in fragments that are one fourth and three fourths of the total length of the native molecule. MMP-1 expression is very low in normal human skin but is elevated in aged human skin and is associated with fragmented and disorganized collagen fibrils in the dermis (2). However, the precise molecular mechanisms underlying elevated MMP-1 expression in aged human skin are not well understood.

CCN1 is a secreted, extracellular matrix-associated protein that belongs to the CCN (cysteine-rich protein 61, connective tissue growth factor, nephroblastoma overexpressed) family (19, 20). CCN proteins play fundamental roles in growth, differentiation, angiogenesis, migration, and extracellular matrix

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2 The abbreviations used are: MMP, matrix metalloproteinase; AFM, atomic force microscopy; AP-1, activator protein 1; CCN1, cysteine-rich protein 61, connective tissue growth factor, nephroblastoma overexpressed; CCN1, cysteine-rich protein 61; CT, C-terminal cysteine knots; DEL, deletion; ECM, extracellular matrix; FAK, focal adhesion kinase; FL, full-length; IGFBP, insulin-like growth factor-binding proteins; LCM, laser capture microdissection; NS, nonsecreted; TSP1, thrombospondin type I repeat; VWC, von Willebrand factor type C repeat.

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regulation (19, 21). We previously reported that CCN1 is expressed in human skin dermis and is substantially elevated in the dermis of naturally aged, photoaged, and acutely UV-irradiated human skin (22, 23). In cell culture models, CCN1 recombinant protein regulates cell adhesion, cell migration, cell-matrix interactions, and the synthesis of extracellular matrix (24–26). Furthermore, elevated CCN1 up-regulates MMP-1 protein expression in cultured primary human skin dermal fibroblasts (22, 23).

CCN1 (and other CCN family proteins) is composed of an N-terminal signal peptide followed by four conserved structural/functional domains (19, 21). These domains share a high degree of sequence homology with (i) insulin-like growth factor-binding proteins (IGFBP), (ii) Von Willebrand factor type C repeat (VWC), (iii) thrombospondin type I repeat (TSP1), and (iv) C-terminal cysteine knots (CT). Interestingly, these four distinct structural domains are separated by protease-sensitive residues, and each of these domains is encoded by a separate exon. The multimodular structural organization of CCN1 protein suggests that its diverse biological functions are programmed by the combinatorial actions of individual domains, either acting independently or interdependently (27–29). CCN1 exerts a range of functions by interacting with numerous integrins, in a cell type- and function-specific manner (27). In this study, we determined the role of CCN1 structural domains and their binding to specific integrins in the regulation of MMP-1 expression, in human skin fibroblasts.

EXPERIMENTAL PROCEDURES

Procurement of Human Skin Samples and Laser Capture Microdissection (LCM)—Punch biopsies (4 mm) of sun-protected buttock human skin were obtained from young (25–30 years) and aged (≥80 years) healthy volunteers as described previously (30, 31). Human skin dermal fibroblasts were subsequently captured by LCM as described previously (7, 30). Briefly, OCT-embedded human skin cryosections (15 μm) were stained with hematoxylin and eosin, and ~200 dermal fibroblasts were captured by LCM (Leica ASLMD system; Leica Microsystems, Wetzlar, Germany). Total RNA was prepared from LCM-captured dermal fibroblasts using a commercial kit (RNasy Micro kit, Qiagen). A TaqMan PreAmp Master Mix kit (Applied Biosystems) was used to preamplify cDNA for quantitative real-time PCRs. The quality and quantity of amplified cDNA were determined by Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA). Quantitation of transcript levels of several different genes in samples of total RNA and amplified cDNA yielded essentially identical results (data not shown).

All procedures involving human subjects were approved by the University of Michigan Institutional Review Board, and all participating subjects provided written informed consent prior entering the study.

Immunohistology—Immunohistology was performed as described previously (18). Briefly, slides were fixed in 2% paraformaldehyde and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline (PBS), blocked with rabbit serum (5% in PBS), and incubated for 1 h at room temperature with CCN1 primary antibody (Santa Cruz Biotechnology) followed by incubation of secondary antibody for 1 h at room temperature. After staining, the slides were examined using a digital imaging microscope (Zeiss). Isotype-control immunoglobulin substituted for the CCN1 primary antibody yielded no detectable staining (data not shown).

RNA Isolation and Quantitative Real-time RT-PCR—Total RNA was extracted using TRizol reagent (Invitrogen). cDNA for PCR templates was prepared by reverse transcription of total RNA (100 ng) using a Taqman Reverse Transcription kit (Applied Biosystems). Real-time PCR was performed on a 7300 Sequence Detector (Applied Biosystems) using Taqman Universal PCR Master Mix Reagents (Applied Biosystems). MMP-1, CCN1, and 36B4 primers and probes were purchased from Applied Biosystems. Target gene mRNA expression levels were normalized to the housekeeping gene 36B4 as an internal control for quantification.

Cell Culture—Primary human skin dermal fibroblasts were established from healthy adult human skin biopsy and cultured as described previously (32).

CCN1 Constructs and Transfection—CCN1 domain-specific or domain-specific deletion expression vectors were generated by PCR using domain-specific primers. PCR products were inserted into a pCDNA3.1 expression vector, and constructs (2 μg) were transiently transfected into human skin dermal fibroblasts by electroporation (Amaxa Nucleofector, Koeln, Germany).

Western Blot Analysis, Immunoprecipitation, and Integrin Neutralization—Forty-eight h after transfection with CCN1 expression constructs, conditioned medium, cellular protein, and ECM were extracted for analysis. To prepare ECM, cells were removed by 5 mm EDTA for 1 h at 37°C. ECM was collected by scraping the tissue culture plates with a Cell Lifter Polyethylene (Costar®; Fisher Scientific) in whole cell extraction buffer (25 mm HEPES (pH 7.7), 0.3 mm NaCl, 1.5 mm MgCl₂, 0.2 mm EDTA, 0.1% Triton X-100, 0.5 mm DTT, 20 mm β-glycerophosphate, 0.1 mm Na₂VO₄, 2 μg/ml leupeptin, and 100 μg/ml PMSF). Protein concentrations of whole cell extract, ECM proteins, and conditioned medium were determined by Bradford assay (Bio-Rad Laboratories). Western blotting was performed as described previously (33). From each sample, total cellular protein (~50 μg/lane), ECM protein (~50 μg/lane), and 50 μl of conditioned medium were subjected to electrophoresis in a 4–20% gradient SDS-polyacrylamide gel and transferred to a PVDF membrane (Millipore). MMP-1 and CCN1 antibodies were purchased from Santa Cruz Biotechnology. Protein bands were visualized and quantified with enhanced chemifluorescence (Vistra ECF Western blotting System; GE Healthcare) following the manufacturer’s protocol. The intensities of each band were quantified by a STORM Molecular Imager (Molecular Dynamics) and normalized using β-actin as a loading control. The intensity of protein bands was quantified by ImageQuant software (an integral application of the STORM Molecular Imager system). For immunoprecipitation experiments, cellular and ECM extracts were incubated overnight at 4°C with HA antibody (Pierce) to capture HA-tagged αVβ3 integrin. After immunoprecipitation, proteins were separated in a 4–20% gradient SDS-polyacrylamide gel followed by Western blotting with V5 tag antibody (Invitrogen).
to detect tagged CCN1 proteins. Integrin neutralization assays were performed using Integrin Antibody Investigation Kits (α Integrin Blocking Kit (ECM430) and β Integrin Blocking Kit (ECM440); Chemicon) or αVβ3 integrin neutralization antibody (MAB1976B). Briefly, cells were transfected with control vector (pCDNA3.1) or relevant CCN1 expression vectors. After 24 h, cells were harvested and then incubated with neutralization antibodies (10 μg/1 × 10^6 cells) for 1 h at 37 °C. The cells were then cultured for 2 days prior to analysis.

### RESULTS

**CCN1, a Positive Regulator of MMP-1 Expression, Is Elevated in Human Skin Dermal Fibroblasts in Vivo**—We previously reported that CCN1 is elevated in full thickness aged human skin which includes epidermis and dermis (22). However, expression levels of CCN1 in dermal fibroblasts, the major cells responsible for collagen homeostasis, in young and aged human skin are unknown. To address this question, we isolated dermal fibroblasts in young and aged human skin by LCM (Fig. 1A). Total RNA was prepared from LCM-captured dermal fibroblasts, and CCN1 mRNA levels were determined by quantitative real-time PCR. As shown in Fig. 1B, CCN1 mRNA in dermal fibroblasts in aged skin was elevated 3-fold compared with dermal fibroblasts in young skin. Consistent with this observation, CCN1 protein was markedly elevated in aged human skin dermis compared with young skin dermis (Fig. 1C). Importantly, MMP-1, which is induced by CCN1 in human dermal fibroblasts (22), was significantly increased in LCM-captured aged dermal fibroblasts, compared with dermal fibroblasts young skin (Fig. 1D). These data indicate that elevated CCN1 correlates with increased expression of MMP-1 in dermal fibroblasts in aged human skin.

**Characterization of CCN1 Structural Domain(s) Involved in Stimulation of MMP-1 Expression in Human Dermal Fibroblasts**—Although CCN1 is elevated in aged human skin and CCN1 can up-regulate MMP-1 in human dermal fibroblasts (22), it is unknown which CCN1 domain(s) regulate MMP-1 expression. To dissect domain-specific function, we created expression vectors for individual CCN1 domains and domain-specific deletions, as shown in Fig. 2A. These constructs were transiently transfected into human dermal fibroblasts, and Western blotting was performed to determine expression levels in fibroblast cell extracts, culture media, and ECM extracts (Fig. 2B). CCN1 lacking signal peptide (NS-CCN1) was detected only in the cell extract. All other constructs were detected in both cell extract and culture medium, indicating that CCN1 domain-specific constructs were expressed and secreted into the media. Single domain constructs were undetectable in the ECM fraction, whereas all other constructs were detectable in the ECM extracts.

Importantly, only full-length CCN1 (FL-CCN1) and CCN1 in which the CT domain was deleted (CT-DEL) were able to up-regulate MMP-1 mRNA and protein expression (Fig. 2, C and D). Deletion of the IGFBP, VWC, or TSP1 domain, either alone or in combination, abrogated the ability of CCN1 to induce MMP-1 expression. Interestingly, whereas the IGFBP, VWC, or TSP1 domain alone was unable to regulate MMP-1 expression (Fig. 2, C and D), combined expression of these three individual domains, which mimics expression of the CT domain deletion protein, resulted in stimulation of MMP-1 expression (Fig. 2E). This effect required expression of all three structural domains. Combined expression of any two domains had no effect on MMP-1 expression (Fig. 2E). Collectively, these data demonstrate that stimulation of MMP-1 expression by CCN1 requires IGFBP, VWC, and TSP1 structural domains, but not the CT domain.

**CCN1 Domains Up-regulate MMP-1 through Interaction with αVβ3 Integrin in Human Dermal Fibroblasts**—To identify integrins that mediate CCN1 stimulation of MMP-1 expression, we performed integrin antibody neutralization assays. The use of function-blocking, anti-integrin antibodies revealed that αV and β3 integrins are critical for CCN1 stimulation of MMP-1 (Fig. 3, A and B). Antibodies that neutralized α1, α2, α3, α5, α6, β1, β2, β4, or β4 integrins had no effect on CCN1-induced MMP-1 mRNA expression (Fig. 3A).
We next performed immunoprecipitation assays to explore the mechanism by which αVβ3 integrin mediates CCN1 regulation of MMP-1 expression, HA-tagged αV and β3 integrin proteins were co-expressed with V5-tagged full-length, CT domain deletion or single domain proteins, in human dermal fibroblasts. As shown in Fig. 3C, both full-length (FL-CCN1) and the CT domain deletion proteins (CT-DEL) co-immunoprecipitated with αVβ3 integrin. The VWC, but not other single domain proteins, also interacted with αVβ3 (Fig. 3D). Interestingly, whereas the VWC single domain is able to interact with αVβ3 integrin (Fig. 3D), the VWC domain alone was unable to stimulate MMP-1 expression (Fig. 2, C–E). CCN1 lacking secretion signal peptide (NS-CCN1) did not interact with αVβ3 integrin (Fig. 3C).

Functional analysis indicated that stimulation of MMP-1 expression by full-length CCN1 (FL-CCN1) and CT domain deletion proteins (CT-DEL) was significantly reduced by αVβ3 integrin blocking antibody (Fig. 3E). These data indicate CCN1 proteins containing IGFBP, VWC, and TSP1 domains interact with αVβ3 integrin and that this interaction mediates stimulation of MMP-1 expression in human dermal fibroblasts.

CCN1 Domain-specific Fragmentation and Lattice Contraction of Three-dimensional Collagen Lattices—Finally, we examined the functional consequences of expression of full-length CCN1 (FL-CCN1, CT domain deletion CCN1 (CT-DEL), positive control) and nonsecreted CCN1 lacking secretion signal peptide (NS-CCN1, negative control) by human dermal fibroblasts in three-dimensional collagen lattices. As shown in Fig. 4, expression of FL-CCN1 and CT-DEL but not NS-CCN1 or control vector, resulted in collagen gel contraction (Fig. 4A). This contraction was blocked by αVβ3 integrin neutralizing antibody (Fig. 4B). Collagen gel contraction in response to expression of FL-CCN1 or CT-DEL, was accompanied by increased levels of MMP-1-generated collagen fragments (Fig. 4C). This collagen fragmentation was blocked by neutralizing αVβ3 integrin antibody (Fig. 4D). AFM images show that collagen fibrils in lattices with control vector (Fig. 4E) or expression of NS-CCN1 (Fig. 4F) were intact and well organized. In contrast, collagen fibrils were fragmented and disorganized in lattices with expression of FL-CCN1 (Fig. 4G) and CT-DEL (Fig. 4H). This collagen fibril disruption was blocked by αVβ3 neutralizing antibody (Fig. 4, I and J). These data suggest that the CT domain deletion CCN1 is functionally equivalent to full-length CCN1 and that collagen lattice contraction, and collagen fibril fragmentation, and disorganization are associated with CCN1 stimulation of MMP-1. Finally, we show that CCN1 activates focal adhesion kinase (FAK) and paxillin, two key effectors of integrin signaling, and the downstream target MAPK/ERK (Fig. 4K), which is a major driving force for stimulation of MMP-1 expression (35).

FIGURE 1. Elevated CCN1 and MMP-1 in aged human skin dermal fibroblasts in vivo. A, illustration of LCM of dermal fibroblasts from human skin section coupled with real-time RT-PCR. Human skin dermal fibroblasts were captured from young and aged skin dermis by LCM. Total RNA was isolated from LCM-captured dermal fibroblasts, and quantitative real-time PCR was performed as described under “Experimental Procedures.” White arrows indicate dermal fibroblasts before LCM. Black arrows indicate removal of fibroblasts by LCM. Scale bars, 100 μm. B, dermal fibroblasts from young and aged human skin captured by LCM. CCN1 and 36B4 (internal control housekeeping gene) mRNA levels were quantified by real-time RT-PCR. Data are mean ± S.E., n = 10; *, p < 0.05. C, immunohistology of CCN1 protein in young and aged human skin. Data are representative of five subjects. Arrows indicate CCN1 positive staining cells in dermis of aged human skin. Scale bars, 100 μm. D, dermal fibroblasts from young and aged human skin captured by LCM. MMP-1 and 36B4 (internal control housekeeping gene) mRNA levels were quantified by real-time RT-PCR. Data are mean ± S.E., n = 10; *, p < 0.05.
**DISCUSSION**

CCN1 proteins are composed of an N-terminal secretion signal peptide followed by four conserved structural domains (19, 21, 27). Our data indicate that the secretion signal and the first three domains (IGFBP, VWC, and TSP1) are required for CCN1 stimulation of MMP-1 expression, in human dermal fibroblasts. The C-terminal CT domain is dispensable for this function. Interestingly, CCN family member CCN5 contains IGFBP, VWC, and TSP1 domains, but naturally lacks the CT domain (36, 37). Absence of the CCN1 CT domain may have distinct consequences for different functional roles. It has been reported that the CT domain is required for cell adhesion, but its deletion has no effect on stimulation of growth factor-induced proliferation (28). Presently, we find that CCN1 lacking the CT domain is equivalent to full-length wild type CCN1 in its ability to bind to...
αVβ3 integrin and stimulate MMP-1 expression in human dermal fibroblasts; indicating that the CT domain is nonessential for these activities. Our data demonstrate that secretion is vital for CCN1 function because signal peptide-deleted, nonsecreted CCN1 had no effect on MMP-1 expression. Our data further suggest that upon secretion, CCN1 interacts with ECM and this ECM-CCN1 interaction may be important in CCN1 stimulation of MMP-1. The modular structural organization of CCN1 protein suggests that its diverse biological functions may result from the combined actions of individual domains, either acting independently or interdependently (38). A remarkable finding of our study is that each CCN1 domain is independently stable; however, the individual domains are unable to regulate MMP-1 expression. We found that co-expression of IGFBP, VWC, and TSP1 domains, but not any combination of two individual domains, resulted in stimulation of MMP-1 expression. These data indicate that these three domains cooperate with each other to stimulate MMP-1 in human dermal fibroblasts.

CCN1 protein exerts a range of diverse functions by interacting with multiple integrins (27, 39). CCN1 interacts with distinct integrins in a cell type- and function-specific manner to mediate disparate biologic activities. Because CCN1 can stimulate MMP-1 in human dermal fibroblasts, an important question is the identity of a cell surface CCN1-binding integrin that mediates CCN1 stimulation of MMP-1.

Screening of integrins by antibody neutralization revealed that αVβ3 integrin acts as a cell surface receptor for CCN1; CCN1 physically interacts with αVβ3 integrin to mediate MMP-1 induction. αVβ3 integrin is implicated in the patho-

**FIGURE 3.** Full-length CCN1 and CCN1 lacking the CT domain up-regulate MMP-1 through interaction with αVβ3 integrin in human dermal fibroblasts. A, induction of MMP-1 expression by full-length CCN1 (FL-CCN1) is inhibited by function-blocking antibody to either αV integrin or β3 integrin in human dermal fibroblasts. mRNA (A) and protein (B) levels were quantified by real-time PCR and Western blotting, respectively. A, MMP-1 mRNA levels were normalized to mRNA levels of the housekeeping gene, 36B4 (internal control), n = 4; *, p < 0.05 versus Ctrl; **, p < 0.05 versus FL-CCN1. β-Actin was used as Western blot loading control. B, MMP-1 expression was determined by Western blotting and quantified by STORM Molecular Imager. Inset shows representative Western blot. n = 3; *, p < 0.05 versus Ctrl; **, p < 0.05 versus FL-CCN1. C, full-length CCN1 (FL-CCN1) and CCN1 lacking the CT domain (CT-DEL) interact with αVβ3 integrin. V5-tagged FL-CCN1, CT-DEL CCN1, or CCN1 lacking secretion signal peptide (NS-CCN1) was co-expressed with HA-tagged αVβ3 integrin in human dermal fibroblasts. Cell extracts were prepared, and αVβ3 integrin chains were immunoprecipitated (IP) with HA antibody, and the immunoprecipitated proteins were analyzed by Western blotting with V5 antibody. Data are representative of three independent experiments. D, VWC domain, but not other individual domains, interacts with αVβ3 integrin. V5-tagged single domains were co-expressed with HA-tagged αVβ3 integrin in human dermal fibroblasts. Cell extracts were prepared, and αVβ3 integrin chains were immunoprecipitated with HA antibody, and the immunoprecipitated proteins were analyzed by Western blotting with V5 antibody. Data are representative of three independent experiments. E, CCN1 stimulation of MMP-1 expression is reduced by function blocking antibody to αVβ3 integrin. β-Actin was used as loading control. MMP-1 expression was determined by Western blotting and quantified by STORM Molecular Imager. Inset shows representative Western blot. n = 3; *, p < 0.05.
physiology of wound healing, angiogenesis, and tumor metastasis (40, 41). Our data provide evidence that αVβ3 integrin contributes to human skin connective tissue aging by stimulating MMP-1 expression through functional interaction with CCN1. It has been reported that CCN1 interacts with αVβ3 integrin through VWC domain (27, 28). We also observed that the VWC domain is able to interact with αVβ3 integrin (Fig. 3D). However, we found that the VWC domain alone is unable to stimulate MMP-1 expression (Fig. 2, C and D). Interestingly, combined expression of the VWC domain with domains adjacent to VWC can stimulate MMP-1 expression (Fig. 2E). These data indicate that the interaction of the VWC domain with αVβ3 integrin requires functional cooperation with adjacent IGFBP and TSP1 domains. It appears that although the interaction of VWC domain with αVβ3 integrin is necessary, this interaction alone is not sufficient to stimulate MMP-1 expression. It is possible that the adjacent IGFBP and TSP1 domains may interact with other integrins, which may act together with αVβ3 integrin to mediate CCN1 stimulation of MMP-1. The nature of the possible complex interactions between CCN1 domains and integrins remains to be determined.

CCN1 binding to the ECM may alter fibroblasts interactions with the ECM and with associated integrins. ECM-bound CCN1 may function as a “docking” protein that coordinates interactions between ECM proteins and cell surface integrins, ultimately orchestrating the cell response to the ECM microenvironment. Consistent with this notion, we found that CCN1 activates integrin downstream targets FAK, paxillin, and MAPK/ERK. Human skin dermal fibroblasts were transfected with CCN1 expression vector. Cell extracts were prepared, and activation of FAK, paxillin, and MAPK/ERK, and β-actin protein levels were determined 2 days after transfection by Western blotting. Numbers indicate fold change compared with control (CTRL). n = 3.

**FIGURE 4.** Expression of full-length CCN1 or CCN1 lacking the CT domain induces contraction, fragmentation, and disorganization of three-dimensional collagen lattices. Human dermal fibroblasts were transfected with the indicated constructs and cultured in three-dimensional collagen lattices. For the neutralization assays, CCN1-transfected cells were treated with αVβ3 integrin antibody and cultured in three-dimensional collagen lattices, as indicated under “Experimental Procedures.” A, full-length CCN1 (FL-CCN1) or CCN1 lacking the CT domain (CT-DEL) induces collagen gel contraction, compared with control vector (Ctrl) or CCN1 lacking the secretion signal peptide (NS-CCN1). B, CCN1-induced collagen gel contraction is inhibited by function blocking antibody to αVβ3 integrin. C, FL-CCN1 or CT-DEL induces collagen fragmentation, compared with control vector (Ctrl) and NS-CCN1. D, CCN1-induced collagen fragmentation is inhibited by function-blocking antibody to αVβ3 integrin. Conditioned media were collected, concentrated, and resolved in a 10% SDS-polyacrylamide gel. Collagen bands were visualized by staining with SimplyBlue. E–J, representative images from AFM of three-dimensional collagen lattices are shown. E, control; F, NS-CCN1; G, FL-CCN1; H, CT-DEL; I, FL-CCN1 with function-blocking antibody to αVβ3 integrin; J, CT-DEL with function-blocking antibody to αVβ3 integrin. The white and red arrows indicate intact and fragmented collagen fibrils, respectively. All data (A–J) are representative of at least three independent experiments. Scale bars, 200 μm. K, CCN1 activates integrin downstream targets FAK, paxillin, and MAPK/ERK. Human skin dermal fibroblasts were transfected with CCN1 expression vector. Cell extracts were prepared, and activation of FAK, paxillin, and MAPK/ERK, and β-actin protein levels were determined 2 days after transfection by Western blotting. Numbers indicate fold change compared with control (CTRL). n = 3.
**alpha V beta 3 Integrin in CCN1 Domain-specific Stimulation of MMP-1**

Aged Dermal Fibroblasts

![Diagram of aging and integrin signaling](Image)

CCN1 can alter collagen gel contraction which is a visible readout of changes in ECM integrity and cell-matrix interactions. These data further suggest that elevated CCN1 in aged dermal fibroblasts promotes type I collagen fragmentation and thus contributes to skin aging. Our data suggest that targeting the CCN1/alpha V beta 3 integrin pathway may be an effective therapeutic strategy to reduce collagen loss and thereby lessen the deleterious impact of aging on the health of human skin.

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**REFERENCES**

1. Uitto, J., and Bernstein, E. F. (1998) Molecular mechanisms of cutaneous aging: connective tissue alterations in the dermis. *J. Investig. Dermatol. Symp. Proc.* 3, 41–44
2. Fisher, G. J., Varani, J., and Voorhees, J. J. (2008) Looking older: fibroblast collapse and therapeutic implications. *Arch. Dermatol.* 144, 666–672
3. Varani, J., Warner, R. L., Gharaei-Kerami, M., Phan, S. H., Kang, S., Chung, J. H., Wang, Z. Q., Datta, S. C., Fisher, G. J., and Voorhees, J. J. (2000) Vitamin A antagonizes decreased cell growth and elevated collagen-degrading matrix metalloproteinases and stimulates collagen accumulation in naturally aged human skin. *J. Invest. Dermatol.* 114, 480–486
4. Ashcroft, G. S., Horan, M. A., and Ferguson, M. W. (1995) The effects of aging on cutaneous wound healing in mammals. *J. Anat.* 187, 1–26
5. Kudravi, S. A., and Reed, M. J. (2000) Aging, cancer, and wound healing. *In Vivo* 14, 83–92
6. Campisi, J. (2008) Aging and cancer cell biology. *Aging Cell* 7, 281–284
7. Quan, T., Qin, Z., Xia, W., Shao, Y., Voorhees, J. J., and Fisher, G. J. (2009) Matrix-degrading metalloproteinases in photoaging. *J. Investig. Dermatol. Symp. Proc.* 14, 20–24
8. Hotary, K. B., Allen, E. D., Brooks, P. C., Datta, N. S., Long, M. W., and Weiss, S. J. (2003) Membrane type I matrix metalloproteinase usurps tumor growth control imposed by the three-dimensional extracellular matrix. *Cell* 114, 33–45
9. Itoh, Y., and Seiki, M. (2006) MT1-MMP: a potent modifier of pericellular microenvironment. *J. Cell. Physiol.* 206, 1–8
10. Poincloux, R., Lázár-Farkas, E., and Chavrier, P. (2009) Matrix invasion by tumour cells: a focus on MT1-MMP trafficking to invadopodia. *J. Cell Sci.* 122, 3015–3024
11. Orringer, J. S., Sachs, D. L., Shao, Y., Hammerberg, C., Cui, Y., Voorhees, J. J., and Fisher, G. J. (2012) Direct quantitative comparison of molecular responses in photodamaged human skin to fractionated and fully ablative carbon dioxide laser resurfacing. *Dermatol. Surg.* 38, 1668–1677
12. Quan, T., Little, E., Quan, H., Qin, Z., Voorhees, J. J., and Fisher, G. J. (2013) Elevated matrix metalloproteinases and collagen fragmentation in photodamaged human skin: Impact of altered extracellular matrix microenvironment on dermal fibroblast function. *J. Invest. Dermatol.* 10.1038/jid.2012.509
13. Brauchle, M., Glück, D., Di Padova, F., Han, J., and Gram, H. (2000) Independent role of p38 and ERK1/2 mitogen-activated kinases in the up-regulation of matrix metalloproteinase-1. *Exp. Cell Res.* 258, 135–144
14. Liu, X., Wu, H., Byrne, M., Jeffrey, J., Krane, S., and Jaenisch, R. (1995) A targeted mutation at the known collagenase cleavage site in mouse type I collagen impairs tissue remodeling. *J. Cell Biol.* 130, 227–237
15. Brennan, M., Bhatti, H., Nerusu, K. C., Bhagavathula, N., Kang, S., Fisher, G. J., Varani, J., and Voorhees, J. J. (2003) Matrix metalloproteinase-1 is the major collagenolytic enzyme responsible for collagen damage in UV-irradiated human skin. *Photochem. Photobiol.* 78, 43–48
16. Fisher, G. J., Datta, S. C., Talwar, H. S., Wang, Z. Q., Varani, J., Kang, S., and Voorhees, J. J. (1996) Molecular basis of sun-induced premature skin aging and retinoid antagonism. *Nature* 379, 335–339
17. Fisher, G. J., Quan, T., Purohit, T., Shao, Y., Cho, M. K., He, T., Varani, J., Kang, S., and Voorhees, J. J. (2009) Collagen fragmentation promotes oxidative stress and elevates matrix metalloproteinase-1 in fibroblasts in aged human skin. *Am. J. Pathol.* 174, 101–114
18. Fisher, G. J., Wang, Z. Q., Datta, S. C., Varani, J., Kang, S., and Voorhees,
αvβ3 integrin in CCN1 Domain-specific Stimulation of MMP-1

J. J. (1997) Pathophysiology of premature skin aging induced by ultraviolet light. *N. Engl. J. Med.* **337**, 1419–1428
19. Lau, L. F., and Lam, S. C. (1999) The CCN family of angiogenic regulators: the integrin connection. *Exp. Cell Res.* **248**, 44–57
20. Planque, N., and Perbal, B. (2003) A structural approach to the role of CCN (CYR61/CTGF/NOV) proteins in tumourigenesis. *Cancer Cell Int.* **3**, 15
21. Perbal, B. (2004) CCN proteins: multifunctional signalling regulators. *Lancet* **363**, 62–64
22. Quan, T., He, T., Shao, Y., Lin, L., Kang, S., Voorhees, J. J., and Fisher, G. J. (2006) Elevated cysteine-rich 61 mediates aberrant collagen homeostasis in chronologically aged and photoaged human skin. *Am. J. Pathol.* **169**, 482–490
23. Quan, T., Qin, Z., Xu, Y., He, T., Kang, S., Voorhees, J. J., and Fisher, G. J. (2010) Ultraviolet irradiation induces CYR61/CCN1, a mediator of collagen homeostasis, through activation of transcription factor AP-1 in human skin fibroblasts. *J. Invest. Dermatol.* **130**, 1697–1706
24. Kireeva, M. L., Mo, F. E., Yang, G. P., and Lau, L. F. (1996) Cyr61, a product of a growth factor-inducible immediate-early gene, promotes cell proliferation, migration, and adhesion. *Mol. Cell. Biol.* **16**, 1326–1334
25. Chen, C. C., Chen, N., and Lau, L. F. (2001) The angiogenic factors Cyr61 and connective tissue growth factor induce adhesive signaling in primary human skin fibroblasts. *J. Biol. Chem.* **276**, 10443–10452
26. Quan, T., Qin, Z., Shao, Y., Xu, Y., Voorhees, J. J., and Fisher, G. J. (2011) Retinoids suppress cysteine-rich protein 61 (CCN1), a negative regulator of collagen homeostasis, in skin equivalent cultures and aged human skin *in vivo*. *Exp. Dermatol.* **20**, 572–576
27. Chen, C. C., and Lau, L. F. (2009) Functions and mechanisms of action of CCN matricellular proteins. *Int. J. Biochem. Cell Biol.* **41**, 771–783
28. Grzeszkiewicz, T. M., Kirschling, D. J., Chen, N., and Lau, L. F. (2001) CYR61 stimulates human skin fibroblast migration through integrin αvβ3 and enhances mitogenesis through integrin αvβ3, independent of its carboxyl-terminal domain. *J. Biol. Chem.* **276**, 21943–21950
29. Kireeva, M. L., Lam, S. C., and Lau, L. F. (1998) Adhesion of human umbilical vein endothelial cells to the immediate-early gene product Cyr61 is mediated through integrin αvβ3. *J. Biol. Chem.* **273**, 3090–3096
30. Quan, T., He, T., Kang, S., Voorhees, J. J., and Fisher, G. J. (2002) Connective tissue growth factor: expression in human skin *in vivo* and inhibition by ultraviolet irradiation. *J. Invest. Dermatol.* **118**, 402–408
31. Quan, T., He, T., Kang, S., Voorhees, J. J., and Fisher, G. J. (2002) Ultraviolet irradiation alters transforming growth factor β/SMAD pathway in human skin *in vivo*. *J. Invest. Dermatol.* **119**, 499–506
32. Fisher, G. J., Henderson, P. A., Voorhees, J. J., and Baldassare, J. J. (1991) Epidermal growth factor-induced hydrolysis of phosphatidylincholine by phospholipase D and phospholipase C in human dermal fibroblasts. *J. Cell. Physiol.* **146**, 309–317
33. Quan, T., He, T., Voorhees, J. J., and Fisher, G. J. (2001) Ultraviolet irradiation blocks cellular responses to transforming growth factor-β by down-regulating its type-II receptor and inducing Smad7. *J. Biol. Chem.* **276**, 26349–26356
34. Quan, T., Qin, Z., Voorhees, J. J., and Fisher, G. J. (2012) Cysteine-rich protein 61 (CCN1) mediates replicative senescence-associated aberrant collagen homeostasis in human skin fibroblasts. *J. Cell Biochem.* **113**, 3011–3018
35. Gutman, A., and Wasylyk, B. (1990) The collagenase gene promoter contains a TPA and oncogene-responsive unit encompassing the PEA3 and AP-1 binding sites. *EMBO J.* **9**, 2241–2246
36. Zhang, R., Averboukh, L., Zhu, W., Zhang, H., Jo, H., Dempsey, P. J., Colley, R. J., Pardee, A. B., and Liang, P. (1998) Identification of rCop-1, a new member of the CCN protein family, as a negative regulator for cell transformation. *Mol. Cell. Biol.* **18**, 6131–6141
37. Pennica, D., Swanson, T. A., Welsh, J. W., Roy, M. A., Lawrence, D. A., Lee, J., Brush, J., Taneyhill, L. A., Deuel, B., Lew, M., Watanabe, C., Cohen, R. L., Melhem, M. F., Finley, G. G., Quirke, P., Goddard, A. D., Hillan, K. L., Gurney, A. L., Botstein, D., and Levine, A. J. (1998) WISP genes are members of the connective tissue growth factor family that are up-regulated in wnt-1-transformed cells and aberrantly expressed in human colon tumors. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 14717–14722
38. Holbourn, K. P., Acharya, K. R., and Perbal, B. (2008) The CCN family of proteins: structure-function relationships. *Trends Biochem. Sci.* **33**, 461–473
39. Leu, S. J., Lam, S. C., and Lau, L. F. (2002) Pro-angiogenic activities of CYR61 (CCN1) mediated through integrins αvβ3 and αvβ1 in human umbilical vein endothelial cells. *J. Biol. Chem.* **277**, 46248–46255
40. Menéndez, J. A., Mehms, I., Griggs, D. W., and Lupu, R. (2003) The angiogenic factor CYR61 in breast cancer: molecular pathology and therapeutic perspectives. *Endocr. Relat. Cancer* **10**, 141–152
41. Jin, H., and Varner, J. (2004) Integrins: roles in cancer development and as treatment targets. *Br. J. Cancer* **90**, 561–565
42. Cabodi, S., del Pilar Camacho-Leal, M., Di Stefano, P., and Delfilippi, P. (2010) Integrin signalling adaptors: not only figurants in the cancer story. *Nat. Rev. Cancer* **10**, 858–870
43. Giancotti, F. G., and Ruoslahti, E. (1999) Integrin signaling. *Science* **285**, 1028–1032