Aging Cell (2013) **12**, pp661–671 Doi: 10.1111/acel.12089

Expression of catalytically active matrix metalloproteinase-1 in dermal fibroblasts induces collagen fragmentation and functional alterations that resemble aged human skin

Wei Xia, Craig Hammerberg, Yong Li, Tianyuan He, Taihao Quan, John J. Voorhees and Gary J. Fisher

Department of Dermatology, University of Michigan, 1301 E Catherine, R6447 Med Sci I, Ann Arbor, MI 48109-5609, USA

Summary

Increased expression of matrix metalloproteinase-1 (MMP-1) and reduced production of type I collagen by dermal fibroblasts are prominent features of aged human skin. We have proposed that MMP-1-mediated collagen fibril fragmentation is a key driver of age-related decline of skin function. To investigate this hypothesis, we constructed, characterized, and expressed constitutively active MMP-1 mutant (MMP-1 V94G) in adult human skin in organ culture and fibroblasts in three-dimensional collagen lattice cultures. Expression of MMP-1 V94G in young skin in organ culture caused fragmentation and ultrastructural alterations of collagen fibrils similar to those observed in aged human skin *in vivo*. Expression of MMP-1 V94G in dermal fibroblasts cultured in three-dimensional collagen lattices caused substantial collagen fragmentation, which was markedly reduced by MMP-1 siRNA-mediated knockdown or MMP inhibitor MMI270. Importantly, fibroblasts cultured in MMP-1 V94G-fragmented collagen lattices displayed many alterations observed in fibroblasts in aged human skin, including reduced cytoplasmic area, disassembled actin cytoskeleton, impaired TGF-β pathway, and reduced collagen production. These results support the concept that MMP-1-mediated fragmentation of dermal collagen fibrils alters the morphology and function of dermal fibroblasts and provide a foundation for understanding specific mechanisms that link collagen fibril fragmentation to age-related decline of fibroblast function.

Key words: aging; extracellular matrix; matrix metalloproteinase; molecular biology of aging.

Introduction

Type I collagen fibrils comprise the bulk of skin connective tissue (dermis) extracellular matrix (ECM). Type I collagen and other ECM proteins are synthesized primarily by dermal fibroblasts. Binding of fibroblasts to collagen fibrils enables them to exert mechanical force and thereby influence the three-dimensional organization of the ECM. Counteracting mechanical resistance of the cross-linked collagenous ECM creates a state of dynamic force. Abundant evidence demonstrates that fibroblast morphology and function are dependent on the mechanical properties of the ECM microenvironment (Choquet *et al.*, 1997; Balaban *et al.*, 2001; Fisher *et al.*, 2009).

The dermal ECM in young human skin is composed of long, intact, tightly packed bundles of collagen fibrils. In contrast, collagen fibrils in aged/photoaged human skin are sparse, fragmented, and disorganized (Fisher *et al.*, 2009). We have proposed that collagen fibril fragmentation, with attendant alterations of mechanical properties in the dermal ECM microenvironment, disrupts fibroblast-ECM interactions, resulting in reduced spreading and shifting collagen homeostasis in favor of ECM degradation (Fisher *et al.*, 2009).

Collagen fibril fragmentation can be initiated by matrix metalloproteinase (MMP) -1, -8, -13, and -14. MMP-1 appears to be primarily responsible for initiation of collagen fragmentation in aged human skin. MMP-1 levels are very low in healthy young skin. MMP-1 mRNA, protein, and activity are constitutively increased with aging, and transiently increased with exposure to ultraviolet irradiation (Fisher *et al.*, 1996; Fisher *et al.* 1997) or wounding (Orringer *et al.*, 2004). MMP-1 is secreted as a catalytically inactive proenzyme. An N-terminal region of approximately 100 amino acids containing a cysteine switch domain interacts with the active site, thereby inhibiting enzyme activity. Proteolytic removal of the N-terminal inhibitory domain results in activation of catalytic activity. Several proteases can activate MMP-1 *in vitro*, including serine proteases (i.e., plasmin, kallikrein, trypsin, neutrophil elastase) and activated stromelysins (MMP-3 and MMP-10) (Saunders *et al.*, 2005). These proteases are also believed to activate MMP-1 *in vivo*, although definitive proof remains elusive. Activated MMP-1 cleaves type I collagen fibrils at a single site, generating characteristic one-fourth and three-fourth length fragments. Once cleaved, collagen fibrils are susceptible to further proteolysis by other MMPs such as MMP-3 and MMP-9.

Activation of full-length MMP-1 is an essential step for initiation of collagen fibril proteolysis. However, activation of endogenous MMP-1 is nearly nonexistent in human dermal fibroblast cultures, making it difficult to directly investigate mechanisms by which fragmentation of the collagenous ECM alters fibroblast function. In order to overcome this difficulty, we have generated a mutant form of MMP-1 that undergoes auto-activation, following expression and secretion by human dermal fibroblasts. We employed this active MMP-1 to investigate the impact of collagen fibril cleavage on human skin fibroblast function. MMP-1-mediated collagen fibril fragmentation resulted in disassembly of the actin cytoskeleton, reduced cell spreading, impaired expression of TGF-beta pathway,

Correspondence
Gary Fisher, Department of Dermatology, University of Michigan Medical School, Medical Science I, Room 6447, 1150 W. Medical Center Drive, Ann Arbor, MI 48109-5609, USA. Tel.: (734) 763 1469; fax: (734) 647 0076; e-mail: dianemch@umich.edu

Accepted for publication *11 April 2013*

Aging Cell

© 2013 John Wiley & Sons Ltd and the Anatomical Society

and decreased collagen production. These alterations closely resemble those observed in fibroblasts in aged human skin *in vivo* (Varani *et al.*, 2006; Yaar & Gilchrest, 2007; Fisher *et al.*, 2008, 2009).

Materials and methods

Materials

Lipofectamine 2000, pcDNA3.1 TOPO vector, nickel-charged resins, CellTracker Red CMTPX, Alexa Fluor 546 phalloidin, streptavidin-conjugated Alexa Fluor 594, Dulbecco's-modified Eagle's medium (DMEM; Invitrogen Life Technology, Carlsbad, CA, USA), fetal bovine serum, trypsin solution, penicillin, and streptomycin were purchased from Invitrogen Life Technology (Carlsbad, CA, USA). Fibroblast basal medium was purchased from Lonza (Anaheim, CA, USA). MMP-1 antibody and CTGF antibody were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Human MMP-1 was purchased from Calbiochem (La Jolla, CA, USA). Rat tail type I collagen was purchased from BD Biosciences (San Jose, CA, USA). MMP-1 Activity Assay kit, ECF Western blotting reagents, donkey antirabbit antibody were purchased from GE Health Care. PVDF membrane, AMICON filter devices with molecular weight cutoff of 10K were purchased from Millipore (Billerica, MA, USA). β-actin antibody, control nontargeting siRNA was obtained from Sigma (St. Louis, MO, USA). DsiRNA specific for human MMP-1 was purchased from Integrated DNA Technologies (San Diego, CA, USA). Adeno-X Adenoviral Expression System was purchased from Clontech (Mountain View, CA, USA). Biotinylated secondary antibodies and Vectashield mounting medium contained DAPI were purchased from Vector Laboratories, Inc. (Burlingame, CA, USA). QuikChange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA, USA). Protease Inhibitor Cocktail, pepstatin, PMSF were purchased from Roche Applied Science (Indianapolis, IN, USA). 1 percent osmium tetroxide was purchased from Electron Microscopy Sciences (Hatfield, PA, USA). Unless otherwise stated, all other reagents were purchased from the Sigma Chemical Company.

Plasmid constructs and production of recombinant adenovirus

Wild-type human MMP-1 and its deletion mutants were generated by overlap extension PCR (PCR splicing) using full-length MMP-1 as template. PCR products containing stop codon were cloned into pcDNA3.1 TOPO. MMP-1 V94G point mutant (a mutant substituting valine for glycine at position (94) was generated with the QuikChange site-directed mutagenesis kit, using pcDNA-MMP-1 wild-type as template. His- and V5-tagged MMP-1 V94G construct, used for protein expression and purification, was made by deleting the stop codon and cloned into pcDNA3.1 TOPO in-frame with His and V5-tag.

Adenovirus-mediated gene transfer was performed to express MMP-1 V94G in human dermal fibroblasts. Adeno-MMP-1 V94G viral DNA was constructed by excising MMP-1 V94G expression cassette from pcDNA3.1 TOPO and ligating it into Adeno-X viral DNA. Then adeno-MMP-1 V94G was packaged into infectious adenovirus by transfection into HEK-293FT cells. Recombinant adenovirus was amplified by infection of HEK-293FT cells. Adenovirus was purified by sucrose gradient centrifugation (Gerard & Meidell, 1995) and stored at −80 °C.

Cell culture and transfection

Human embryonic kidney cells (HEK-293FT, American Type Culture Collection, Manassas, VA, USA), cultured in DMEM, 10% fetal bovine serum, at 37 °C with 5% CO_2, were transiently transfected with MMP-1 expression constructs using lipofectamine 2000. Twenty-four hours after transfection, cells were washed with phosphate-buffered saline (PBS) and then cultured in serum-free DMEM. Conditioned media were collected after 48 h and concentrated with AMICON filter devices with molecular weight cutoff of 10K. Aliquots of the conditioned media were incubated with three-dimensional collagen lattices for 48 h to measure collagenase activity.

Primary adult human dermal fibroblasts were isolated from 6-mm, full-thickness punch biopsies of buttocks/hip skin of volunteers 21–30 years of age and cultured as previously described (Fisher *et al.*, 2009). Fibroblasts were used for experiments between passages 4–10. The participation of human subjects in this study was approved by the University of Michigan Institutional Review Board, and all subjects provided written-informed consent prior to their inclusion in the study.

MMP-1 activity assay

Matrix metalloproteinase-1 activity in conditioned media was measured using the Amersham MMP-1 Activity Assay kit (GE HealthCare, Piscataway, NJ, USA) according to the manufacturer's instructions. Briefly, activated MMP-1 in the conditioned media was measured by first capturing total MMP-1 by immobilized anti-MMP-1 antibody and then assaying its enzymatic activity relative to an activated MMP-1 standard curve.

Protein expression and purification

Human recombinant MMP-1 V94G-His-V5 was expressed and purified as described previously (Kamei *et al.*, 2008). Briefly, MMP-1 V94G-His-V5 was purified from conditioned media of HEK-293FT cells stably transfected with pcDNA3.1-MMP-1-V94G-His-V5 plasmid. Recombinant protein was purified using nickel-charged resins according to manufacturers' instructions and stored at −80 °C.

Three-dimensional collagen lattice fibroblast cell culture

Collagen lattices were prepared in a sterile tube by mixing an appropriate volume of rat tail type I collagen to yield a final concentration of 2 mg mL^{-1} with medium cocktail [DMEM, $NaHCO_3$ (44 mM), L-glutamine (4 mM), folic acid (9 μM), and neutralized with 1 N NaOH to pH 7.2]. Primary adult human dermal fibroblasts were resuspended with neutralized collagen solution and placed in an incubator at 37 °C for 30 min to allow gelling of the collagen. After gelling, the collagen lattices were detached from the sides and bottom of the culture dish, and 2 mL of fibroblast basal medium were added to the cultures (Fisher *et al.*, 2009).

© 2013 John Wiley & Sons Ltd and the Anatomical Society

RNA interference (siRNA)

Fibroblast cultures were transfected with 40 nM siRNA (Control nontargeting siRNA or MMP-1 DsiRNA), using Lipofectamine 2000. Fibroblasts cultures were analyzed 72 h after transfection.

Immunoblot

Western blot was performed as previously described (Bank *et al.*, 1997; Xu *et al.*, 2011). Primary antibodies were incubated with PVDF membrane for one hour at room temperature, and immunoreactive protein bands were imaged and quantified by enhanced chemifluorescence, using a STORM Molecular Imager, with ImageQuant software (GE HealthCare, Piscataway, NJ, USA). β-actin protein levels served as a loading control.

Hydroxyproline assay

Conditioned media from three-dimensional collagen lattice fibroblast cultures were subjected to acid hydrolysis (6 N HCL), derivatized with OPA/FMOC, and analyzed for hydroxyproline content by HPLC, as previously described (Bank *et al.*, 1997).

Cell Tracker staining

CellTracker™ Red CMTPX (5 μM) was added to three-dimensional collagen lattice fibroblast cultures for one hour. Dye-containing media was then replaced with fresh, prewarmed culture medium, and cultures were incubated for an additional 30 min. After extensive washes with PBS, lattices were fixed with 2% paraformaldehyde and mounted with mounting medium containing DAPI to stain nuclei. Cells were visualized by confocal fluorescence microscopy using an Olympus FluoView 500 laser-scanning confocal microscope (Tokyo, Japan) at the Morphology and Image Analysis Core of the University of Michigan Diabetes Center.

F-actin labeling and immunofluorescence staining

After 3 days of culture, collagen lattices were fixed with 2% paraformaldehyde in PBS for 30 min, followed by incubation with 1% BSA for 60 min, to block non-specific binding. Fibroblasts were labeled with Alexa Fluor 546 phalloidin for one hour and then washed with PBS (three times for 5 min each). Alternatively, samples were incubated with antibody diluent containing primary antibodies (Anti-MMP-1 or Anti-CTGF) for 1 h, then with biotinylated secondary antibodies for 30 min, followed by incubation with streptavidin conjugated to Alexa Fluor 594 for 30 min. Mounting medium containing DAPI was added to each sample to stain cell nuclei. Cells were visualized by confocal fluorescence microscopy using an Olympus FluoView 500 laser-scanning confocal microscope (Tokyo, Japan).

Electron microscopy

Full-thickness punch biopsies (2-mm diameter) of sun-protected buttocks skin were obtained from healthy, nonsmoking individuals, as previously described (Varani *et al.*, 2006). Skin samples from three young (21–30 years of age) and three aged (> 80 years of age) subjects were analyzed by electron microscopy, as previously described (Fisher *et al.*, 2008). Briefly, skin biopsies were fixed overnight in 2% glutaraldehyde in 0.1 mM cacodylate buffer at pH 7.4. Specimens were then treated with 2% osmium tetroxide buffered in 0.1-mM cacodylate buffer and dehydrated with graded ethanol and propylene oxide. The samples were embedded in pure Epon resin. Ultrathin sections were cut from areas of interest, stained with lead citrate and uranyl acetate, and observed in a Philips 400 Transmission Electron at the University of Michigan Microscopy and Image Analysis Core.

Statistical analysis

Comparisons were made with paired *t*-test or the repeated measures of analysis of variance. All stated *P*-values are two-tailed and considered significant when < 0.05.

Results

Generation and expression of MMP-1 mutants

Full-length inactive MMP-1 is highly expressed in cultured adult human dermal fibroblasts. It is not catalytically active due to lack of proteolytic processing, which cleaves the N-terminal inhibitory domain. In order to express catalytically active MMP-1, we introduced mutations within the N-terminal inhibitory region (Fig. S1) and assessed their effects on enzymatic activity. Three MMP-1 truncation mutants (Δ20-54, Δ20-83, and Δ20-86) were made based on putative cleavage sites for plasma kallikrein, plasmin, or APMA, respectively (Suzuki *et al.*, 1995). Based on homology of MMP-1 with MMP-3 and MMP-7, we also introduced a point mutation in the cysteine switch domain, changing Val^{94} to Gly^{94}. Equivalent point mutations in MMP-3 and MMP-10 have been shown to stimulate catalytic activity (Sanchez-Lopez *et al.*, 1988; Park *et al.*, 1991; Krampert *et al.*, 2004). All mutants and wild-type MMP-1 cDNAs were cloned into the pcDNA3.1 and transiently transfected into HEK-293FT cells. Media were collected 48 h after transfection, and Western blot analysis of MMP-1 was performed. As shown in Fig. 1A, wild-type MMP-1 and point mutation MMP-1 V94G were expressed at similar levels. These proteins remained largely in the full-length ~52-KDa unprocessed inactive form, although a small fraction of MMP-1 V94G was processed to smaller molecular weight forms. Wild-type and V94G MMP-1 appeared as doublets, representing glycosylated and nonglycosylated forms (Wilhelm *et al.*, 1986). The levels of all three deletion mutants in the media were very low, approximately 2–7 percent of the level of wild-type or MMP-1 V94G (Fig. 1A). These low levels of MMP-1 deletion mutant proteins detected in the media correlated with their low intracellular expression (data not shown). In contrast, transcript levels of truncation mutants were similar to those of wild-type and V94G MMP-1 (data not shown), suggesting that low expression of truncation mutants may reflect reduced intracellular stability. Due to their low expression levels, truncation mutants were not further characterized.

© 2013 John Wiley & Sons Ltd and the Anatomical Society

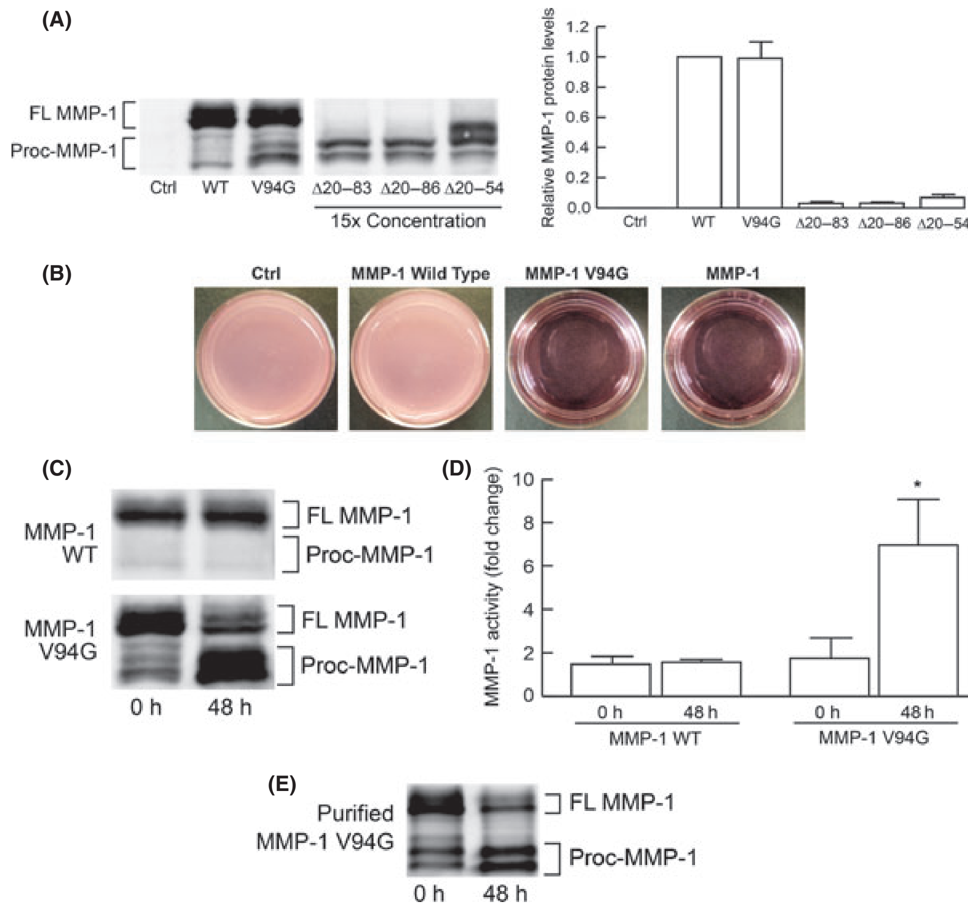

Fig. 1 Matrix metalloproteinase-1 (MMP-1) V94G displays enhanced enzymatic activity. HEK-293FT cells were transiently transfected with control vector (Ctrl) or the indicated MMP-1 expression constructs. Conditioned media were collected after 2 days, and MMP-1 enzymatic activity and expression were measured. (A) Levels of expressed MMP-1 proteins, in conditioned media, were analyzed and quantified by Western blot. Full-length (FL) and proteolytically processed (Proc) MMP-1 bands are indicated in the upper panel. Note: Conditioned media from cells transfected with deletion constructs were concentrated 15-fold prior to Western blot. (B) Three-dimensional type I collagen lattices were incubated with indicated conditioned media or media containing purified active MMP-1 for 48 h at 37 °C. Note, intact collagen lattices appear whitish (left two panels); the darker color (right two panels) is the surface under the culture dish, which is seen due to complete dissolution of collagen lattices. (C) Western blot analysis of conditioned media from HEK-293FT cells transfected with wild-type (WT) MMP-1 or MMP-1 V94G before (0 h) and after incubation with three-dimensional type I collagen lattices for 48 h at 37 °C. FL and proteolytically processed (Proc) MMP-1 bands are indicated. (D) Enzymatic activity of wild-type MMP-1 and MMP-1 V94G before (0 h) and after incubation with three-dimensional type I collagen lattices for 48 h at 37 °C. MMP-1 activity was measured with an immunocapture assay system. (E) His-tagged MMP-1 V94G was purified by nickel chelate chromatography and stored frozen (0 h) or incubated for 48 h at 37 °C. MMP-1 processing was analyzed by Western blot. Cell number and total protein were used for normalizing the total amount of conditioned medium/purified protein. All data are representative of three independent experiments, enzymatic activity and quantification results are expressed as means ± SEM, $^*P < 0.05$.

Analysis of MMP-1 V94G enzymatic activity

To determine enzymatic activity, conditioned media from cultures transfected with MMP-1 V94G or wild-type MMP-1 were added to three-dimensional type I collagen lattices. Purified, trypsin-activated MMP-1 was used as positive control. As shown in Fig. 1B, MMP-1 V94G completely dissolved the collagen lattice, similar to purified active MMP-1. In contrast, wild-type MMP-1 had no visible effect on the collagen lattice.

The high level of enzymatic activity displayed by MMP-1 V94G was surprising in view of the modest amount of proteolytic processing observed by Western blot, described above (see Fig. 1A). The results suggested that full-length MMP-1 V94G might undergo continued processing to the active form during the 48 h of incubation with the collagen lattice. To investigate this possibility, we compared the relative processing of MMP-1 V94G and wild-type MMP-1 before and after incubation with collagen lattices for 48 h at 37 °C. As expected, prior to incubation, both MMP-1 V94G and wild-type MMP-1 were primarily full-length proenzymes. However, during incubation, the majority of MMP-1 V94G underwent processing from ~52 to ~42 kDa, whereas wild-type MMP-1 remained largely unprocessed (Fig. 1C).

To confirm that processing of MMP-1 V94G leads to activation of enzymatic activity, we measured activity using an antibody capture assay. Prior to incubation, MMP-1 V94G and wild-type MMP-1 displayed similar low activity. In contrast, after 48 h at 37 °C, MMP-1 V94G showed significant increase in MMP-1 activity, while wild-type MMP-1 did not change (Fig. 1D). These data indicate that the V94G mutation promotes processing of MMP-1 to activate enzymatic activity.

© 2013 John Wiley & Sons Ltd and the Anatomical Society

MMP-1 V94G auto-activates in a time and temperature-dependent manner

To further characterize MMP-1 V94G activation, we assessed its time- and temperature dependence. MMP-1 V94G cleavage to lower molecular weight forms was nearly complete by 48 h at 37 °C. Processing was about 50% complete at 30 °C, substantially less complete at 22 °C, and very little processing occurred at 4 °C. (Fig. S2A and S2B) The kinetics of cleavage was nonlinear, with the majority of the processing occurring between 24 and 48 h.

To further understand mechanisms of MMP-1 V94G processing, we determined the effects of inhibitors that target serine, cysteine, aspartate, or cation-dependent proteases. Neither PMSF (inhibitor of serine proteases and cysteine proteases), nor pepstatin (inhibitor of aspartate proteases) had any effect (Fig. S2C). The catalytic activity of MMPs is dependent on a Zn2 + ion, which is bound by three histidine residues, found in the conserved sequence HExxHxxGxxH, within the catalytic domain (Kontogiorgis *et al.*, 2005). EDTA, which chelates divalent cations including Zn2 +, inhibited MMP-1 V94G activation, suggesting that it may undergo self-processing.

To examine this possibility, we expressed and purified His-tagged MMP-1 V94G from transfected HEK-293FT cells. As shown in Fig. 1E, purified His-tagged MMP-1 V94G underwent self-cleavage to ~42-kDa forms during incubation for 48 h at 37 °C.

MMP-1 V94G generates collagen fragmentation that resembles aged human skin

Having characterized self-activation of MMP-1 V94G, we next determined its impact on collagen fibril fragmentation in human skin. Sun-protected buttocks skin samples were obtained from healthy nonsmoker young (21–30 years old) and aged (over 80 years old) individuals. Skin collagen fibril ultrastructure was assessed by transmission electron microscopy. Collagen fibrils in young skin dermis are intact and tightly packed (Fig. 2A), while collagen fibrils in aged skin dermis are fragmented, sparse, and disorganized (Fig. 2B). Infection of young skin to MMP-1 V94G adenovirus caused fragmentation and alterations in the structure and organization of collagen fibrils (Fig. 2C) that were qualitatively similar to those observed in aged skin (Fig. 2B). Structure and organization of collagen fibrils in young skin treated with empty virus similar to that seen in the nontreated young skin (Fig. 2D).

Dermal fibroblast expression of MMP-1 V94G in collagen lattice cell culture model

We next employed a three-dimensional collagen lattice model to investigate the impact of collagen fibril fragmentation of fibroblast morphology and function. Human dermal fibroblasts were infected with adenovirus encoding human MMP-1 V94G or with empty control adenovirus and subsequently cultured in type I collagen lattices.

Western blot analysis of the conditioned media showed time-dependent accumulation of endogenous MMP-1 in cultures infected with control empty virus. As expected, endogenous MMP-1 in these cultures was predominately full-length enzyme (Fig. 3A). In contrast, conditioned media from MMP-1 V94G adenovirus-infected fibroblasts showed substantial accumulation of ~42-kDa active form of MMP-1 (Fig. 3A). During the 3-day time course, MMP-1 V94G expression resulted in marked changes in the collagen lattices (Fig. 3B). Fibroblast expression of MMP-1 V94G caused lattices to become thin, transparent, and soft compared to collagen lattices containing fibroblasts infected with control adenovirus.

We then measured the ability of fibroblasts embedded in collagen lattices to degrade surrounding collagen. Western blots of conditioned media revealed accumulation of collagen fragments in cultures containing MMP-1 V94G-expressing fibroblasts (Fig. S3). No collagen fragments were detected in control fibroblast cultures. Furthermore, we quantified collagen released from three-dimensional lattices into the media by measurement of hydroxyproline. Hydroxylation of proline residues in procollagen occurs during biosynthesis and is a validated measure of collagen content (Bank *et al.*, 1997). As shown in Fig. 3C, fibroblasts expressing MMP-1 V94G released significantly more hydroxyproline into the media than control fibroblasts.

We next used siRNA-mediated knockdown to further investigate the dependence of collagen lattice fragmentation on MMP-1 V94G expression. MMP-1 siRNA markedly reduced MMP-1 mRNA (Fig. S4A) and protein (Fig. S4B) levels in dermal fibroblasts by 80% and 70%, respectively, compared to control siRNA-transfected fibroblasts. Introduction of MMP-1 siRNA into fibroblasts that expressed MMP-1 V94G in three-dimensional collagen lattices substantially prevented thinning/softening of the lattices and release of hydroxyproline (Fig. 3D). Similar results were obtained by inhibition of MMP-1 V94G activity with the MMP inhibitor (MMI270, 33 nM) (Fig. 3E).

Taken together, these results demonstrate that time-dependent expression of active MMP-1 V94G by fibroblasts in three-dimensional collagen lattices leads to collagen fragmentation.

Collagen lattice fragmentation by expression of V94G MMP-1 alters fibroblast morphology and promotes disassembly of F-actin fibers

Having established a model in which human dermal fibroblasts express active MMP-1 that cleaves surrounding collagen fibrils, we next investigated the impact of collagen fragmentation on fibroblast morphology and function. Control and MMP-1 V94G adenovirus-infected fibroblasts were cultured for 3 days in three-dimensional collagen lattices and then stained with MMP-1 antibody to confirm expression. As shown in Fig. 4A, MMP-1 protein levels, detected by immunofluorescence, were significantly elevated in MMP-1 V94G adenovirus-infected cells (lower panel), compared with empty virus-infected cells (upper panel). In order to investigate the effects of MMP-1 V94G-catalyzed collagen fragmentation on cell morphology, fibroblasts were stained with CellTracker™ Red CMTPX. Control fibroblasts, residing in intact collagen lattices, displayed long; thin; and stretched morphology with abundant cytoplasm area (Fig. 4B, top panel). In contrast, MMP-1 V94G-expressing fibroblasts

© 2013 John Wiley & Sons Ltd and the Anatomical Society

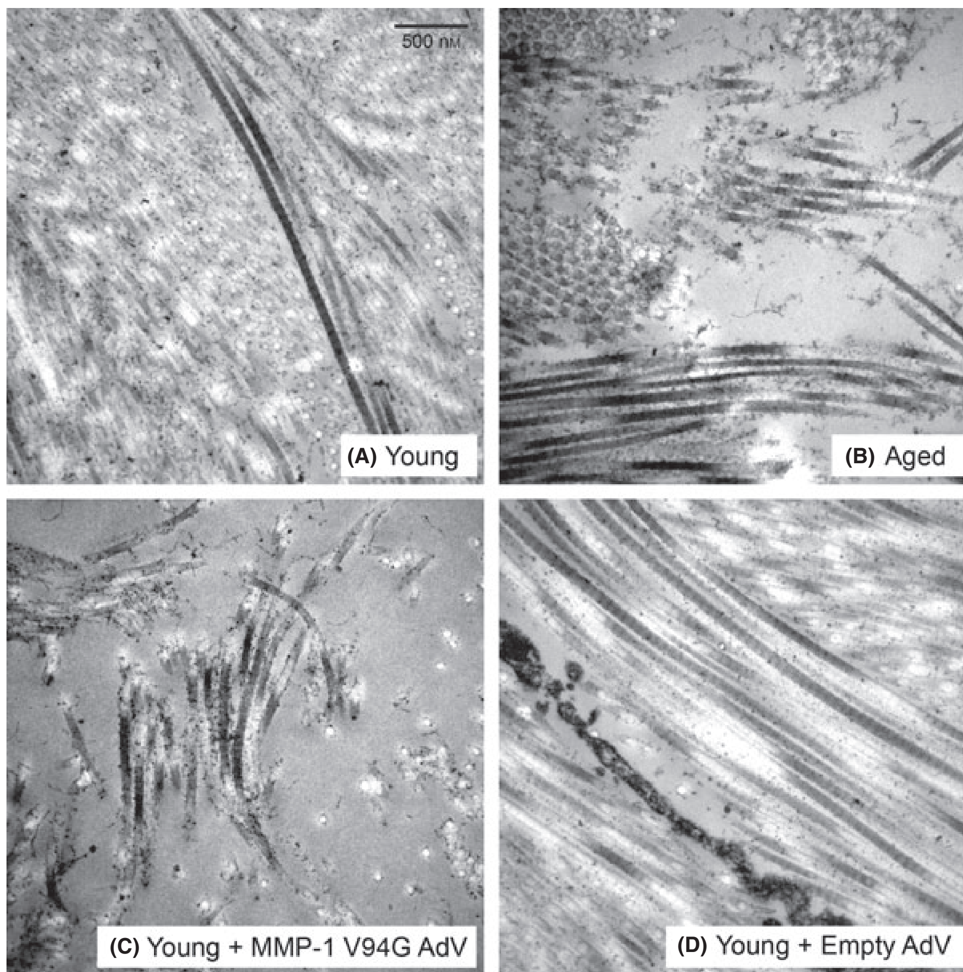

Fig. 2 Expression of matrix metalloproteinase-1 (MMP-1) V94G in young human skin generates collagen fragmentation that resembles aged human skin. (A) Representative electron micrography showing uniform distribution of intact collagen fibrils in young human skin (21–30 years old). (B) Representative electron micrography showing fragmented, sparse, and disorganized collagen fibrils in aged human skin (over 80 years old). Young human skin samples were cultured with (C) MMP-1 V94G adenovirus or (D) empty adenovirus for 4 days. MMP-1 V94G causes alterations in the structure and organization of collagen fibrils in young skin, similar to those seen in aged human skin. Images are representative of three young and three aged individuals.

residing in fragmented collagen lattices were rounded and small with reduced cytoplasm area (Fig. 4B, middle panel). This collapsed morphology is similar to that of fibroblasts seen in the aged human skin *in vivo* (Varani *et al.*, 2006). To confirm the observed changes of cell morphology that were due to collagen fragmentation, MMI-270 was added to inhibit MMP-1 catalytic activity. As shown in Fig. 4B bottom panel, inhibition of collagen fragmentation yielded normal fibroblasts morphology, similar to that of control fibroblasts.

Fibroblast elongation and spreading depends on attachment to the surrounding collagen ECM and assembly of the cellular cytoskeleton (Yeung *et al.*, 2005). Mechanical force resulting from cell-ECM attachment is transmitted throughout the cell by an intact cytoskeleton (Quan *et al.*, 2010a; Provenzano & Keely, 2011). To assess cytoskeleton organization, dermal fibroblasts in intact (empty virus-infected) and fragmented (MMP-1 V94G virus-infected) three-dimensional collagen lattices were stained with phalloidin to visualize F-actin. Control fibroblasts, in intact collagen lattices, displayed stretched morphology with fine, long F-actin filaments (Fig 4C, upper panel). In contrast, MMP-1 V94G-expressing fibroblasts in fragmented collagen lattices displayed shortened, disassembled actin filaments. (Fig. 4C, lower panel).

Collagen lattice fragmentation by MMP-1 V94G impairs TGF-β signaling and reduces procollagen production

TGF-β pathway is the major regulator of collagen production in human dermal fibroblasts. In aged human skin, TGF-β pathway is impaired due to specific down-regulation of TGF-β type II receptor (TβRII) (Quan *et al.*, 2004). This impairment represses TGF-β target genes including CCN2 and type I collagen (Quan *et al.*, 2004, 2010a,b). Therefore, we determined the impact of MMP-1 V94G expression on TGF-β-signaling pathway, and target genes in fibroblasts cultured in three-dimensional collagen lattices. As shown in Fig. 5A, MMP-1 V94G-mediated collagen fibril fragmentation resulted in specific reduction of TβRII. Gene expressions of other components of the TGF-β pathway, including TβRI and Smad2/3/4,

© 2013 John Wiley & Sons Ltd and the Anatomical Society

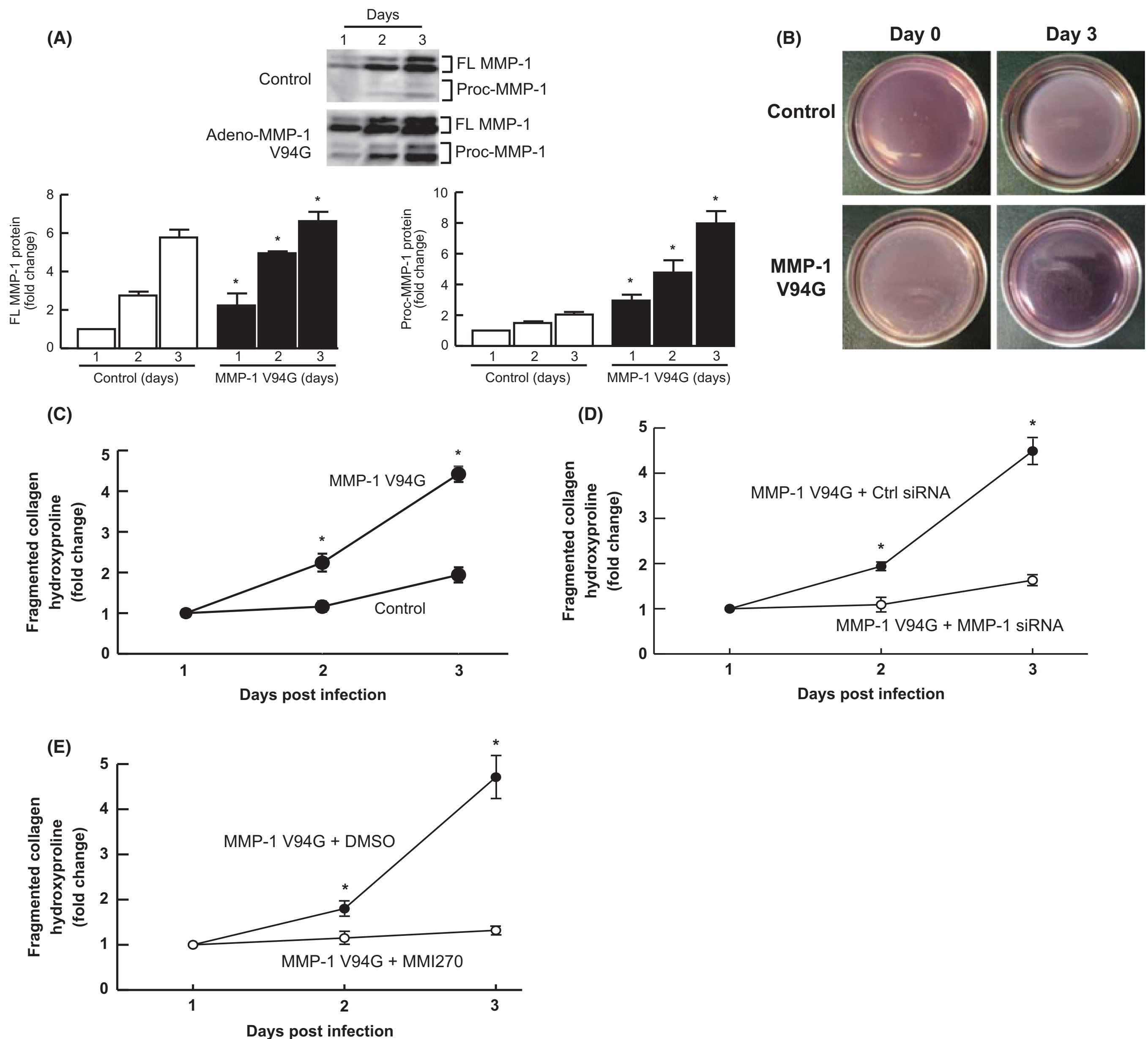

Fig. 3 Expression of matrix metalloproteinase-1 (MMP-1) V94G in dermal fibroblasts degrades three-dimensional type I collagen lattices creating a fragmented extracellular matrix. Human skin fibroblasts infected with control or MMP-1 V94G adenovirus were cultured in three-dimensional collagen lattices. Media were collected and three-dimensional collagen lattices were photographed at the indicated times. (A) Full-length (FL) and proteolytically processed (Proc) MMP-1 protein in media were quantified by Western blot. Inset shows a representative blot. (B) Photographs of three-dimensional collagen lattices were taken at day 0 and day 3 postinfection. Note, thinning, transparent appearance of collagen lattices containing fibroblasts expressing MMP-1 V94G at 3 days postinfection. (C) Hydroxyproline in media was determined by HPLC analysis. (D) Human dermal fibroblasts were infected with MMP-1 V94G adenovirus and transfected with control or MMP-1 siRNA. Hydroxyproline in media was determined by HPLC analysis. (E) Human dermal fibroblasts were infected with MMP-1 V94G adenovirus and cultured the presence of DMSO (control) or MMP inhibitor (MMI270, 33 nM). Hydroxyproline in media was determined by HPLC analysis. All results are expressed as means ± SEM, $n = 3$, $*P < 0.05$.

were not altered. Consistent with the observed impairment of the TGF-β pathway, expression of target genes CCN2 and type I procollagen was significantly reduced (Fig. 5B).

Consistent with these mRNA data, immunofluorescence demonstrated reduced levels of CTGF (Fig. 5C) and type I procollagen (Fig. 5D) proteins by MMP-1 V94G expressing in fragmented collagen lattice cultures (Fig. 5C,D, lower panel), compared to fibroblasts in intact collagen lattices (Fig. 5C,D, upper panel).

Discussion

Elevated expression and activity of MMP-1 are associated with fragmentation of the collagenous ECM in aged human skin (Fisher *et al.*, 1997, 2009; Varani *et al.*, 2006; Yaar & Gilchrest, 2007). This fragmentation impairs the mechanical properties and function of the collagen-rich dermis. Fibroblasts in aged skin display impairment of the TGF-β pathway, due to down-regulation of TβRII, decreased expression of CCN2, and repression of type I collagen expression.

© 2013 John Wiley & Sons Ltd and the Anatomical Society

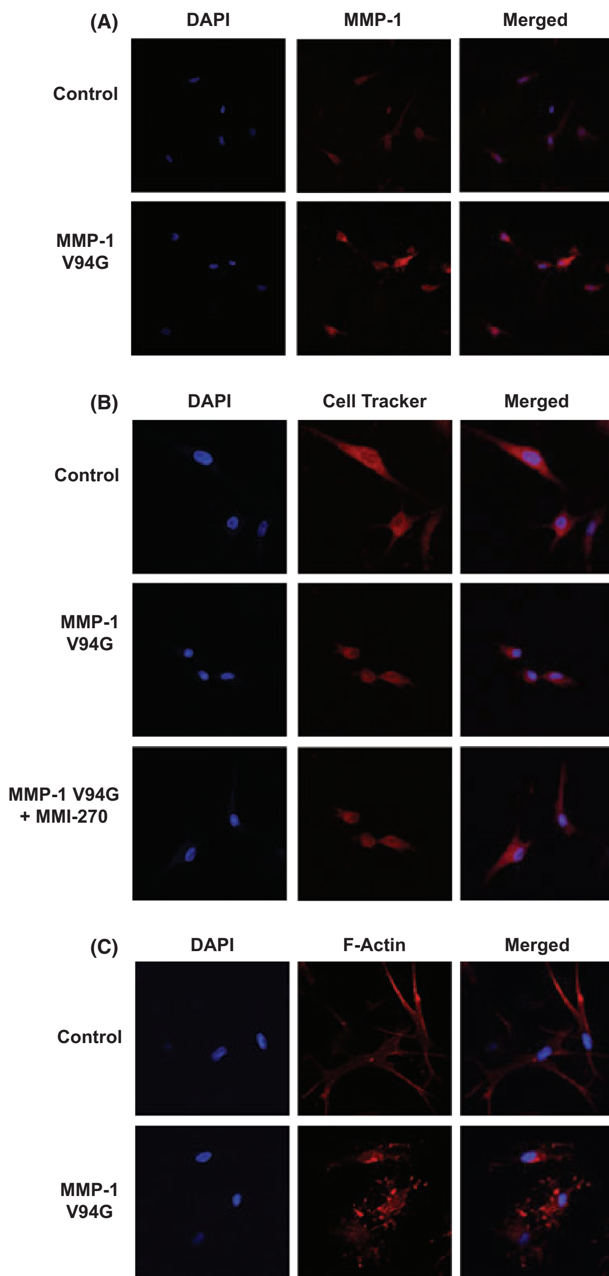

Fig. 4 Expression of matrix metalloproteinase-1 (MMP-1) V94G reduces fibroblast spreading and promotes disassembly of F-actin filaments. Human dermal fibroblasts were infected with control or MMP-1 V94G adenovirus with or without MMI-270 and cultured in three-dimensional collagen lattices for 3 days. (A) MMP-1 expression level was determined by immunofluorescence. Reddish fluorescence is MMP-1 protein; blue fluorescence is nuclei. (B) Fibroblast morphology was assessed by incubation of cultures with CellTracker™ Red CMTPX fluorescent dye. Reddish fluorescence delineates cell cytoplasm; blue fluorescence delineates nuclei. (C) F-actin was visualized by Alexa Fluor 546 phalloidin. Reddish fluorescence delineates F-actin; blue fluorescence delineates nuclei. Fibroblasts were imaged by confocal microscopy. Images are representative of three independent experiments.

However, the mechanisms by which fibroblast function is deleteriously affected in the context of fragmentation of the collagenous ECM in aged human skin are largely not known. To help address this lack of knowledge, we sought to generate a model system in which fibroblasts were cultured in three-dimensional collagen lattices, composed of collagen fibrils, and expressed active MMP-1, which caused collagen fibril fragmentation. To implement this model, we needed to overcome lack of conversion of inactive proMMP-1 to active MMP-1, which requires proteolytic cleavage of the inhibitory N-terminal domain, in culture. Therefore, we investigated modifications of MMP-1 that could enhance the activation process. We found that introduction of a point mutation within the N-terminal cysteine switch region of proMMP-1 most efficiently resulted in auto-proteolytic activation and demonstrated that expression of this active MMP-1 in young human skin generated collagen fragmentation similar to that observed in aged skin.

In aged human skin, it is likely that MMP-1 is activated by endogenous proteases, rather than age-related somatic mutations, for which there is no evidence. Irrespective of the mechanism of activation, our data support the hypothesis that MMP-1-mediated collagen fibril fragmentation is a major contributor to the physiology of skin aging.

Accumulating evidence demonstrates that association of cells with ECM provides important instructions for regulation of cell function (Varani *et al.*, 2002). Microenvironmental cues from the ECM affect intracellular signaling networks and control cellular activities including proliferation, survival, and expression of tissue-specific genes (Ingber, 1997). In aged human skin, fibroblasts within fragmented collagenous ECM have decreased attachment to ECM, which results in a collapsed morphology, and by inference, experience reduced mechanical force (Varani *et al.*, 2006; Fisher *et al.*, 2009; Streuli, 2009). In our three-dimensional collagen lattice culture model, fibroblast expression of MMP-1 V94G caused fragmentation of surrounding collagen fibrils. This collagen fragmentation was associated with decreased cell-collagen fibril interactions, with concomitant reduction of spreading, and disassembly of the actin cytoskeleton. These morphological alterations are similar to those observed in fibroblasts within the fragmented ECM in aged human skin (Varani *et al.*, 2001; Dupont *et al.*, 2011). Mine *et al.* have reported that fibroblasts in the upper (papillary) dermis have a higher proportion of small cells with low granularity, compared to fibroblasts in the lower (reticular) dermis. However, these differences became less marked with increasing age (Mine *et al.*, 2008). Our primary fibroblast cultures were generated from whole dermis of young individuals and may have contained fibroblasts of both papillary and reticular origin.

Mechanical force is normally generated within cells through interactions between the ECM and cell surface adhesion receptors, primarily integrins (Streuli, 2009; DuFort *et al.*, 2011). These transmembrane receptors are linked intracellularly to the actin cytoskeleton within specialized focal adhesion structures. In the present study, staining of F-actin revealed substantial disassembly of actin fibers in fibroblasts within fragmented collagen lattices, which were created by expression of MMP-1 V94G. These results indicate that reduced interaction between fibroblasts and the fragmented collagen ECM resulted in disassembly of F-actin, which would be expected to alter signals encoded by mechanical tension.

Although fragmentation of existing collagen fibrils is central to the deleterious changes observed in aged human skin, failure to replace damaged collagen also contributes to the decline of function (Varani

© 2013 John Wiley & Sons Ltd and the Anatomical Society

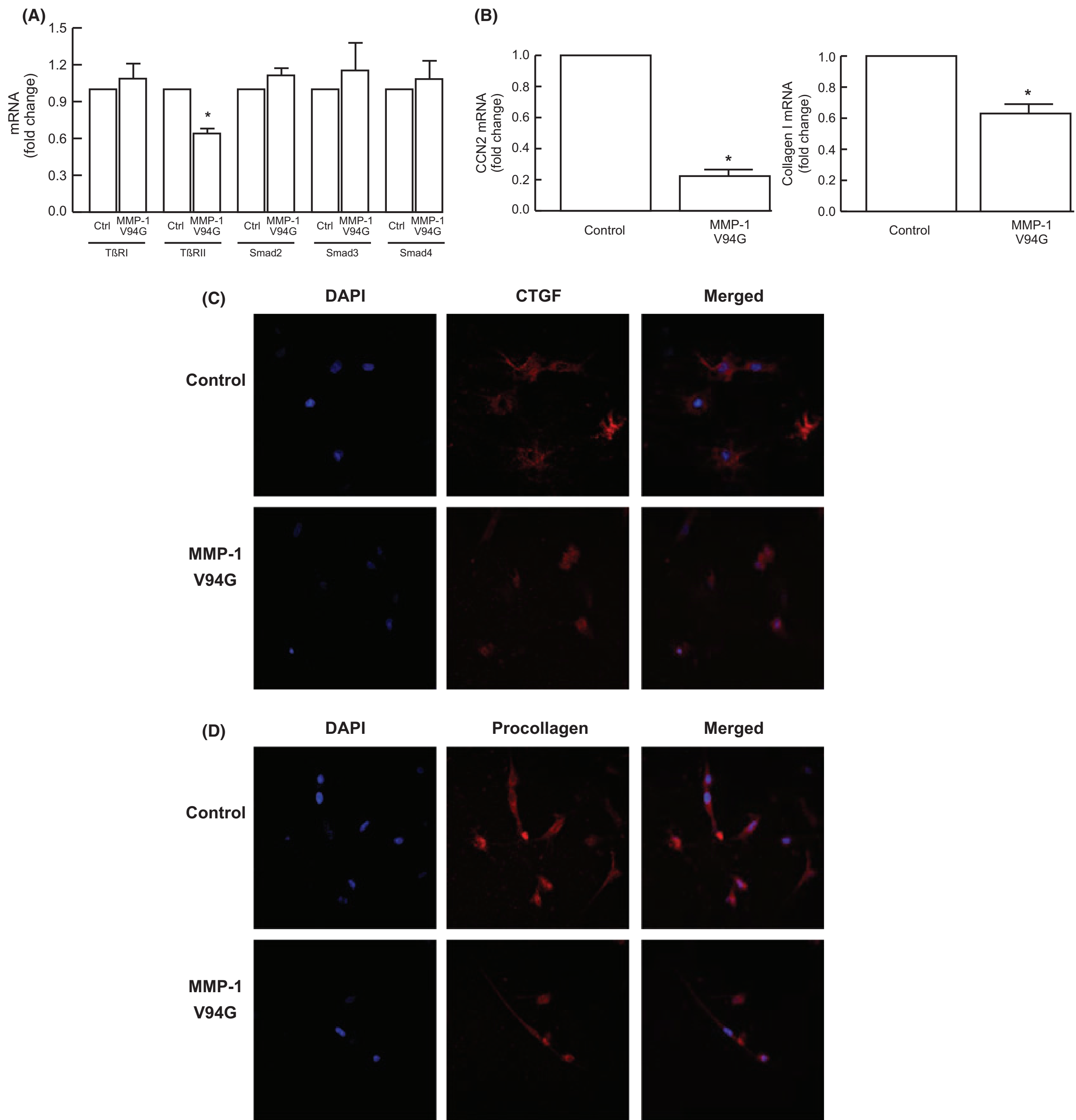

Fig. 5 Collagen lattice fragmentation by matrix metalloproteinase-1 (MMP-1) V94G reduces expression of type II TGF-β receptor, CCN2, and type I collagen. Human dermal fibroblasts infected with control (Ctrl) or MMP-1 V94G adenovirus were cultured in three-dimensional collagen lattices for 3 days. (A) Type I TGF-β receptor (TβRI), type II TGF-β receptor (TβRII), and Smad 2, 3, and 4 mRNA levels. (B) CCN2 and collagen Iα1 mRNA levels. Gene expression was quantified by real-time PCR and normalized to internal control housekeeping gene 36B4. All results are expressed as means ± SEM. $n = 3$, *$P < 0.05$. (C) CTGF and (D) type I procollagen protein expressions were determined by Immunofluorescence. Reddish fluorescence is CTGF/type I Procollagen protein; blue fluorescence is nuclei. Images are representative of three independent experiments.

et al., 2001, 2004; Quan *et al.*, 2004). Evidence suggests that this decreased collagen production is linked to collagen fragmentation, which reduces fibroblast–ECM interactions, and creates a micro-environment in which fibroblasts experience altered mechanical force (Varani *et al.*, 2004; Fisher *et al.*, 2008, 2009). Our studies revealed collagen fibril fragmentation by expression of MMP-1 V94G reduced type I collagen expression. This reduction was associated with reduced expression of key regulators of collagen production, TβRII

© 2013 John Wiley & Sons Ltd and the Anatomical Society

and CCN2. This down-regulation of TβRII, CCN2, and type I collagen resembles alterations that are observed in aged human skin (Quan *et al.*, 2004, 2010b; Yaar & Gilchrest, 2007).

Signaling pathways by which mechanical forces regulate fibroblast function remain to be determined. Interestingly, nuclear localization of transcriptional co-activators YAP/TAZ has recently been shown to be regulated by mechanical force and directly regulates CCN2 (Dupont *et al.*, 2011). In addition, YAP/TAZ has been shown to participate in TGF-β signaling through interaction with Smad proteins (Varelas *et al.*, 2010; Dupont *et al.*, 2011; Zhang *et al.*, 2011). These data raise the possibility that YAP/TAZ may be involved in mechano-signaling pathways that are coupled to collagen fragmentation in skin fibroblasts.

Collectively, our results support the concept that increased expression of enzymatically active MMP-1 causes fragmentation of dermal collagen and alters morphology and function of dermal fibroblasts. Our data provide a foundation for understanding molecular mechanisms that link collagen fragmentation to the decline of fibroblast function in aged human skin.

Acknowledgments

We thank Diane Fiolek for graphic support. This research was partially funded by grants from National Institutes of Health RO1-AG025186-S1, RO1-AG025186 (GF), RO1-AG031452 (GF), and RO1-AG019364 (GF).

References

Balaban NQ, Schwarz US, Riveline D, Goichberg P, Tzur G, Sabanay I, Mahalu D, Safran S, Bershadsky A, Addadi L, Geiger B (2001) Force and focal adhesion assembly: a close relationship studied using elastic micropatterned substrates. *Nat. Cell Biol.* **3**, 466–472.

Bank RA, Krikken M, Beekman B, Stoop R, Maroudas A, Lafeber FP, te Koppele JM (1997) A simplified measurement of degraded collagen in tissues: application in healthy, fibrillated and osteoarthritic cartilage. *Matrix Biol.* **16**, 233–243.

Choquet D, Felsenfeld DP, Sheetz MP (1997) Extracellular matrix rigidity causes strengthening of integrin-cytoskeleton linkages. *Cell* **88**, 39–48.

DuFort CC, Paszek MJ, Weaver VM (2011) Balancing forces: architectural control of mechanotransduction. *Nat. Rev. Mol. Cell Biol.* **12**, 308–319.

Dupont S, Morsut L, Aragona M, Enzo E, Giulitti S, Cordenonsi M, Zanconato F, Le Digabel J, Forcato M, Bicciato S, Elvassore N, Piccolo S (2011) Role of YAP/TAZ in mechanotransduction. *Nature* **474**, 179–183.

Fisher GJ, Datta SC, Talwar HS, Wang ZQ, Varani J, Kang S, Voorhees JJ (1996) Molecular basis of sun-induced premature skin ageing and retinoid antagonism. *Nature* **379**, 335–339.

Fisher G, Wang ZQ, Datta SC, Varani J, Kang S, Voorhees JJ (1997) Pathophysiology of premature skin aging induced by ultraviolet light. *New Eng. J. Med.* **337**, 1419–1428.

Fisher GJ, Varani J, Voorhees JJ (2008) Looking older: fibroblast collapse and therapeutic implications. *Arch. Dermatol.* **144**, 666–672.

Fisher GJ, Quan T, Purohit T, Shao Y, Cho MK, He T, Varani J, Kang S, Voorhees JJ (2009) Collagen fragmentation promotes oxidative stress and elevates matrix metalloproteinase-1 in fibroblasts in aged human skin. *Am. J. Pathol.* **174**, 101–114.

Gerard R, Meidell R (1995) Adenovirus vectors. In *DNACloning- A Practical Approach: Mammalian Systems*. (Hames BD, Glover D, eds) Oxford, UK: Oxford University Press, pp. 285–307.

Ingber DE (1997) Tensegrity: the architectural basis of cellular mechanotransduction. *Annu. Rev. Physiol.* **59**, 575–599.

Kamei H, Lu L, Jiao S, Li Y, Gyrup C, Laursen LS, Oxvig C, Zhou J, Duan C (2008) Duplication and diversification of the hypoxia-inducible IGFBP-1 gene in zebrafish. *PLoS One* **3**, e3091.

Kontogiorgis CA, Papaioannou P, Hadjipavlou-Litina DJ (2005) Matrix metalloproteinase inhibitors: a review on pharmacophore mapping and (Q)SARs results. *Curr. Med. Chem.* **12**, 339–355.

Krampert M, Bloch W, Sasaki T, Bugnon P, Rulicke T, Wolf E, Aumailley M, Parks WC, Werner S (2004) Activities of the matrix metalloproteinase stromelysin-2 (MMP-10) in matrix degradation and keratinocyte organization in wounded skin. *Mol. Biol. Cell* **15**, 5242–5254.

Mine S, Fortunel NO, Pageon H, Asselineau D (2008) Aging alters functionally human dermal papillary fibroblasts but not reticular fibroblasts: a new view of skin morphogenesis and aging. *PLoS One* **3**, e4066.

Orringer JS, Kang S, Johnson TM, Karimipour DJ, Hamilton T, Hammerberg C, Voorhees JJ, Fisher GJ (2004) Connective tissue remodeling induced by carbon dioxide laser resurfacing of photodamaged human skin. *Arch. Dermatol.* **140**, 1326–1332.

Park AJ, Matrisian LM, Kells AF, Pearson R, Yuan ZY, Navre M (1991) Mutational analysis of the transin (rat stromelysin) autoinhibitor region demonstrates a role for residues surrounding the "cysteine switch". *J. Biol. Chem.* **266**, 1584–1590.

Provenzano PP, Keely PJ (2011) Mechanical signaling through the cytoskeleton regulates cell proliferation by coordinated focal adhesion and Rho GTPase signaling. *J. Cell Sci.* **124**, 1195–1205.

Quan T, He T, Kang S, Voorhees JJ, Fisher GJ (2004) Solar ultraviolet irradiation reduces collagen in photoaged human skin by blocking transforming growth factor-beta type II receptor/Smad signaling. *Am. J. Pathol.* **165**, 741–751.

Quan T, Qin Z, Xu Y, He T, Kang S, Voorhees JJ, Fisher GJ (2010a) 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.

Quan T, Shao Y, He T, Voorhees JJ, Fisher GJ (2010b) Reduced expression of connective tissue growth factor (CTGF/CCN2) mediates collagen loss in chronologically aged human skin. *J. Invest. Dermatol.* **130**, 415–424.

Sanchez-Lopez R, Nicholson R, Gesnel MC, Matrisian LM, Breathnach R (1988) Structure-function relationships in the collagenase family member transin. *J. Biol. Chem.* **263**, 11892–11899.

Saunders WB, Bayless KJ, Davis GE (2005) MMP-1 activation by serine proteases and MMP-10 induces human capillary tubular network collapse and regression in 3D collagen matrices. *J. Cell Sci.* **118**, 2325–2340.

Streuli CH (2009) Integrins and cell-fate determination. *J. Cell Sci.* **122**, 171–177.

Suzuki K, Lees M, Newlands GF, Nagase H, Woolley DE (1995) Activation of precursors for matrix metalloproteinases 1 (interstitial collagenase) and 3 (stromelysin) by rat mast-cell proteinases I and II. *Biochem J.* **305**(Pt 1), 301–306.

Varani J, Spearman D, Perone P, Fligiel SE, Datta SC, Wang ZQ, Shao Y, Kang S, Fisher GJ, Voorhees JJ (2001) Inhibition of type I procollagen synthesis by damaged collagen in photoaged skin and by collagenase-degraded collagen *in vitro*. *Am. J. Pathol.* **158**, 931–942.

Varani J, Perone P, Fligiel SE, Fisher GJ, Voorhees JJ (2002) Inhibition of type I procollagen production in photodamage: correlation between presence of high molecular weight collagen fragments and reduced procollagen synthesis. *J. Invest. Dermatol.* **119**, 122–129.

Varani J, Schuger L, Dame MK, Leonard C, Fligiel SE, Kang S, Fisher GJ, Voorhees JJ (2004) Reduced fibroblast interaction with intact collagen as a mechanism for depressed collagen synthesis in photodamaged skin. *J. Invest. Dermatol.* **122**, 1471–1479.

Varani J, Dame MK, Rittie L, Fligiel SE, Kang S, Fisher GJ, Voorhees JJ (2006) Decreased collagen production in chronologically aged skin: roles of age-dependent alteration in fibroblast function and defective mechanical stimulation. *Am. J. Pathol.* **168**, 1861–1868.

Varelas X, Samavarchi-Tehrani P, Narimatsu M, Weiss A, Cockburn K, Larsen BG, Rossant J, Wrana JL (2010) The Crumbs complex couples cell density sensing to Hippo-dependent control of the TGF-beta-SMAD pathway. *Dev. Cell* **19**, 831–844.

Wilhelm SM, Eisen AZ, Teter M, Clark SD, Kronberger A, Goldberg G (1986) Human fibroblast collagenase: glycosylation and tissue-specific levels of enzyme synthesis. *Proc. Natl Acad. Sci. USA* **83**, 3756–3760.

Xu Y, Xia W, Baker D, Zhou J, Cha HC, Voorhees JJ, Fisher GJ (2011) Receptor-type protein tyrosine phosphatase beta (RPTP-beta) directly dephosphorylates and regulates hepatocyte growth factor receptor (HGFR/Met) function. *J. Biol. Chem.* **286**, 15980–15988.

Yaar M, Gilchrest BA (2007) Photoageing: mechanism, prevention and therapy. *Br. J. Dermatol.* **157**, 874–887.

Yeung T, Georges PC, Flanagan LA, Marg B, Ortiz M, Funaki M, Zahir N, Ming W, Weaver V, Janmey PA (2005) Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. *Cell Motil. Cytoskeleton* **60**, 24–34.

© 2013 John Wiley & Sons Ltd and the Anatomical Society

Zhang H, Pasolli HA, Fuchs E (2011) Yes-associated protein (YAP) transcriptional coactivator functions in balancing growth and differentiation in skin. *Proc. Natl Acad. Sci. USA* **108**, 2270–2275.

Supporting Information

Additional Supporting Information may be found in the online version of this article at this publisher's web-site.

Fig. S1 Diagram of human MMP-1 expression constructs.

Fig. S2 Time- and temperature dependence of MMP-1 V94G auto-activation.

Fig. S3 Expression of MMP-1 V94G in dermal fibroblasts degrades three-dimensional type I collagen lattices creating a fragmented extracellular matrix.

Fig. S4 siRNA-mediated MMP-1 knockdown.

© 2013 John Wiley & Sons Ltd and the Anatomical Society