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Age-related reduction of dermal fibroblast size up-regulates multiple matrix metalloproteinases as observed in aged human skin *in vivo*

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Running Title: Fibroblast size upregulates multiple MMPs in aging skin

Key words: matrix metalloproteinases; skin aging, fibroblast; cell size; AP-1

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What is already known about this topic?

- Collagen is the major structure protein in skin, and its fragmentation is a hallmark of dermal aging.
- Matrix metalloproteinases (MMPs) are largely responsible for fragmentation of collagen fibrils.
- However, the expression of all known mammalian MMPs and the mechanism underlying altered expression of MMPs in chronologically aged human skin are less understood.

What does this study add?

- Among all 23 known mammalian MMPs, multiple MMPs are elevated in aged human skin dermis.
- As dermal fibroblasts are the major MMPs producing cells in the dermis, reduction of dermal fibroblast size, which is observed in aged human skin, contributes to elevation of age-related multiple MMPs.
- Reduction of fibroblast size up-regulates c-Jun/c-Fos and activates AP-1, the major regulator of multiple MMPs.
- These data provide a foundation for understanding the cellular and molecular basis of age-related collagen fragmentation, the characteristic feature of aged human skin.

Summary

Background: Fragmentation of collagen fibrils, the major structure protein in skin, is a hallmark of dermal aging. Matrix metalloproteinases (MMPs) are largely responsible for fragmentation of

collagen fibrils. However, the alteration of all known mammalian MMPs and the mechanism underlying altered expression of MMPs in chronologically aged human skin are less understood.

Objectives: To quantify gene expression of all 23 known mammalian MMPs in sun-protected young and aged human skin *in vivo*, and investigate the potential mechanism underlying age-related alteration of multiple MMPs.

Methods: MMPs mRNA expression levels and MMPs activity in sun-protected young and aged human skin *in vivo* were determined by real-time RT-PCR and *in situ* zymography, respectively. The relative contributions to elevated MMPs in epidermis and dermis were quantified by laser capture microdissection (LCM) coupled real-time RT-PCR. Dermal fibroblast morphology and collagen fibrils fragmentation in human skin *in vivo* were assessed by second harmonic generation microscopy and atomic force microscopy, respectively. In vitro cell morphology was assessed by CellTracker® fluorescent dye and Phalloidin staining. Protein levels were determined by ProteinSimple capillary electrophoresis immunoassay.

Results: Among all 23 known mammalian MMPs, multiple MMPs are elevated in aged human skin dermis. Consistent with this finding, increased MMPs activity and collagen fibrils fragmentation were observed in aged skin dermis. As dermal fibroblasts are the major MMPs producing cells in the dermis, reduction of dermal fibroblast size, which is observed in aged human skin, contributes to elevation of age-related multiple MMPs. Reduction of fibroblast size up-regulates c-Jun/c-Fos and activates AP-1, the major regulator of multiple MMPs.

Conclusions: Combined actions of the wide variety of MMPs that are constitutively elevated in aged dermis may be involved in progressive degradation of dermal collagen fibrils. Age-related elevations of multiple MMPs are likely resulted from the reduction of fibroblast size via activation of AP-1.

Introduction

Collagen is the most abundant protein in mammals, making up about one-third of the whole-body protein content [1]. In human skin, collagen is the main component of the dermis that provides structural and functional support of the skin [2]. During aging, dermal collagen fibrils undergo progressive alterations due to age-related fragmentation and disorganization of collagen fibrils [3,4]. Fragmentation of collagen fibrils is essentially responsible for aged-appearing skin, the most visible signs of aging such as wrinkles and fragile atrophic skin. Alterations of dermal collagen impair skin structural integrity and are associated with age-related disorders, such as increased fragility [5], impaired vasculature support [6,7], poor wound healing [8,9], and promotion of skin cancer [10,11].

In human skin, dermal fibroblasts are responsible for collagen homeostasis [12]. Dermal fibroblasts are embedded in a collagen-rich microenvironment and physically interact with collagen fibrils to maintain normal cell shape and size. Accumulating evidence demonstrates that cell shape and size regulate essential cell functions [13,14]. Cell shape and size are largely regulated by interactions of cells with surrounding extracellular matrix (ECM). The ECM provides both binding sites for cells and mechanical resistance to cellular traction forces [14,15]. In young healthy skin, dermal fibroblasts interact with intact collagen fibrils; and exert traction forces to achieve normal cell shape and size. However, in aged dermis collagen fibrils are fragmented, which impairs fibroblast-collagen interaction and results in reduced fibroblast spreading, shape and size [3,16,17]. While cell shape and size are known to regulate many cellular functions, the molecular basis of their impact on dermal fibroblast function and dermal aging are not well understood.

Matrix metalloproteinases (MMPs) are a large family of proteinases that are essentially responsible for fragmentation of collagen fibrils [18,19]. We previously reported that dermal fibroblasts in aged human skin expresses elevated MMP-1, which leads to initiation of collagen fibril fragmentation [3,20]. However, the complete profile of all 23 known mammalian MMPs in aged human skin is unknown. Here we investigated the expression of all 23 known mammalian MMPs in sun-protected young and aged human skin *in vivo*. We found that multiple MMPs are constitutively elevated in aged human skin dermis. As reduction of fibroblast size is a prominent feature of aged human skin [3,16,17], we found that reduction of fibroblast size activates transcription factor AP-1, the major regulator of multiple MMPs [21-23], leading to up-regulation of

multiple age-related MMPs observed in aged human skin. Our data suggest insights into mechanisms of collagen fibrils fragmentation, which is a defining feature of the pathophysiology of human dermal aging.

Methods

Procurement of human skin samples and laser capture microdissection (LCM)

All procedures involving human subjects were approved by the University of Michigan Institutional Review Board, and all subjects provided written informed consent. Sun-protected buttock human skin punch biopsies were obtained from clinically normal adult volunteers; 25-30 years for young group (N=12, mean age 26±3 years) males and 80+ years for aged group (N=12, mean age 83±4 years). Skin samples were 4mm in diameter, full thickness skin. To quantify the relative levels of epidermal and dermal MMPs, epidermis and dermis were captured by laser capture microdissection (LCM)on (15μm), and were collected in lysis buffer (RNeasy Micro kit, Qiagen), followed by RNA extraction and RT-PCR, as described below.

Cell culture and three-dimensional collagen lattice cell cultures

Young (21-30 years) and aged (>80 years) human skin primary dermal fibroblasts were isolated from punch biopsies of sun-protected buttock skin by digestion skin with bacterial collagenase (Worthington Biochemical Corporation, Lakewood, NJ). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified incubator with 5% CO2 at 37°C. Cells were utilized between passages 4 and 9, and replicate experiments used fibroblasts from different donors. In some cases, dermal fibroblasts were cultured in three-dimensional collagen lattices, based on previous publication with minor modification[17]. Briefly, neutralized collagen lattices were prepared by mixing appropriate volume of rat tail type I collagen (BD Biosciences) with medium cocktail [DMEM, NaHCO3 (44mmol/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 2mg/ml. $0.5x10^6$ cells were suspended in 2 ml collagen solution in 35mm culture dish, and placed in an incubator at 37°C for 30 minutes to allow polymerization of the

collagen. For the constrained culture system, cells were cultured in collagen gel in which a nylon mesh (0.5 mm pore size) was placed on the bottom of culture dish to provide cytoskeletal stability and tension. For the unconstrained culture system, cells were cultured in collagen lattices without nylon mesh. The collagen gels were then incubated with 2 ml media (DMEM, 10% FBS) at 37^0C, 5% CO_2. For analyses, cells were harvested by digesting collagen gel with bacterial collagenase (1 mg/ml, Sigma, St. Louis, MO, USA) for 30 minutes at 37°C. Fibroblasts were collected by centrifugation and recovery of viable fibroblasts (>80%) was confirmed by trypan blue staining. For latrunculin-A (Lat-A) treatment, cells were treated with Lat-A at a concentration of 30 nM for 24 hours or the indicated times.

RNA isolation, laser capture microdisection, quantitative real-time RT-PCR

Total RNA was extracted, using a commercial kit (RNeasy midikit, Qiagen, Chatsworth, CA), and reverse transcription was performed using Taqman Reverse Transcription kit (Applied Biosystems, Foster City, CA). Real-time RT-PCR was performed using a Taqman Universal PCR Master Mix kit (Applied Biosystems, Foster City, CA) and 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). PCR procedures were performed using a robotic workstation (Biomek 2000; Beckman Coulter, Inc., Hialeah, FL) to ensure accuracy and reproducibility. All primers and probes were purchased from Applied Biosystems (Assays-on-Demand™ Gene Expression Products). Multiplex PCR reactions contained primers and probes for the target gene and 36B4, a ribosomal protein used as an internal normalization control for quantitation.

ProteinSimple capillary electrophoresis immunoassay

Dermal fibroblasts were lysed in whole cell extraction buffer (25mM HEPES [pH 7.7]; 0.3 M NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 0.1% Triton X-100; 0.5 mM DTT' 20 mM β-glycerolphosphate; 0.1 mM Na_3VO_4; 2 µg/ml leupeptin; and 100 µg/ml PMSF). Whole cells extract was prepared by centrifugation and protein concentration was determined by the Bio-Rad protein assay (Bio-Rad laboratories, Hercules, CA, USA). ProteinSimple capillary electrophoresis immunoassay was performed according to the ProteinSimple user manual. In brief, whole cell extract samples (800 ng/lane) were mixed with a master mix (ProteinSimple) to a final concentration of 1× sample buffer, 1× fluorescent molecular weight markers, and 40 mM

dithiothreitol (DTT) and then heated at 95 °C for 5 min. The samples, blocking reagent, primary antibodies, HRP-conjugated secondary antibodies, chemiluminescent substrate, and separation and stacking matrices were also dispensed to designated wells plate. The electrophoresis and immunodetection steps took place in the capillary system (ProteinSimple Wes, ProteinSimple, Santa Clare, CA, USA) and were fully automated with instrument default settings. The digital image was analyzed and quantified with Compass software (ProteinSimple, Santa Clare, CA, USA) after normalization by and β-actin (loading control).

CellTracker, Phalloidin staining, Immunohistology, and second harmonic generation microscopy

Cell morphology in 3D collagen lattices was assessed by incubation of cultures with CellTracker® fluorescent dye (Molecular Probes, Eugene, OR, USA) for one hour. The cells were washed with PBS and were fixed in 2% paraformaldehyde for 30 minutes. Cell morphology in monolayer culture was assessed by Phalloidin staining. Cells were washed with PBS and were fixed in 2% paraformaldehyde for 30 minutes followed by Phalloidin stain (Sigma, St. Louis, MO, USA) for one hour. Images were obtained using Zeiss fluorescence microscopy. Second harmonic generation microscopy was performed using a Leica SP8 Confocal Microscope with 2-Photon, at University of Michigan Microscopy and Image Analysis Laboratory.

Transfection AP-1 reporter activity assay

Primary adult human dermal fibroblasts were transiently transfected with AP-1 reporter construct (pAP1-TA-Luc) purchased from BD Biosciences Clontech (Palo Alto, CA). Cells were transfected by electroporation using human dermal fibroblasts nucleofector kit (Amaxa Biosystems, Gaithersburg, MD) according to the manufacturer's protocol. After 48 hours of transfection, AP-1 luciferase activity was measured by luciferase assay using an enhanced luciferase assay kit (PharMingen International, San Diego, CA) according to the manufacturer's protocol. β-galactosidase expression vector was used as an internal control for transfection efficiency and aliquots containing identical β-galactosidase activity were used for each luciferase assay.

Zymography

In situ zymography was performed as described previously [24]. Briefly, frozen skin sections were placed on glass slides coated with fluorescently-labeled collagen (Elastin products, Owensville, MO) as substrate. Skin sections were incubated for 24 hours to allow MMPs in the tissue to degrade the fluorescein-labeled collagen in the slide.

Atomic force microscopy (AFM) imaging

OCT embedded human skin samples were sectioned (50 µm) and mounted on glass coverslips (1.2 mm diameter, Fisher Scientific Co., Pittsburgh, PA). These AFM samples were allowed to air dry for at least 24 hours before AFM analysis[25]. The scan positions of the collagen lattices were determined by light optical image. Images were obtained by AFM with ScanAsyst mode (Dimension Icon, Bruker-AXS, Santa Barbara, CA) in air using a silicon etched cantilever (NSC15/AIBS, MikroMasch, San Jose, CA) with a full tip cone angle ~40º and the tip radius of curvature ~10nm. AFM images were acquired at a scan rate of 0.977 Hz, 512x512 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

Statistical significance between groups was determined with the Student's t-test. All p-values are two-tailed and considered significant when $p < 0.05$.

Results

Elevation of multiple MMPs in aged human skin dermis *in vivo*

Measurement of all 23 known mammalian MMPs indicated that eight MMPs were significantly elevated in sun-protected (buttock) aged (83±4 years) human skin, compared to sun-protected

young (26±3 years) skin (relative fold-increase from high to low: MMP-10, MMP-1, MMP-9, MMP-27, MMP-24, MMP-11, MMP-3, MMP-23) (Fig 1A). MMPs and tissue inhibitors of metalloproteinases (TIMPs) are often coordinately regulated as a means to control excess MMP activity, we also investigated whether TIMPs are elevated in aged skin. However, no differences in mRNA levels of all four known tissue inhibitors of metalloproteinases (TIMPs) were found between young and aged human skin (Fig 1B). To quantify the relative contributions to elevated MMPs, epidermis and dermis were separated by laser capture microdissection (LCM) (Fig 1C). Figure 1D shows that all MMPs that are elevated in aged skin were primarily expressed in the dermis (MMP-10, 88%; MMP-1, 78%; MMP-9, 70%; MMP-27, 91%; MMP-24, 91%; MMP-11, 92%; MMP-3, 84%; MMP-23, 81%). To access MMPs activity, we performed *in situ* zymography, in which unfixed skin sections are placed over a layer of fluorescently-labeled collagen. As shown in Fig 1E, elevated MMPs activity in aged skin resulted in breakdown of the collagen, resulting in loss of fluorescence. These data demonstrate that multiple MMPs are elevated in sun-protected aged human skin dermis, suggesting that the combined actions of the wide variety of MMPs may contribute to degradation of dermal collagen fibrils.

Age-related reduction of dermal fibroblast size up-regulates multiple MMPs observed in aged human skin

Next, we investigated the potential mechanisms of age-related elevation of multiple MMPs using primary dermal fibroblasts, since dermal fibroblasts are major MMPs producing cells in the dermis and primarily responsible for collagen turnover. To this end, we first isolated the dermal fibroblasts from sun-protected young (mean age 26±3 years) and aged (mean age 83±4 years) human skin and examined the mRNA expression of all known mammalian MMPs in standard monolayer culture. Interestingly, we found that all 23 MMPs are similarly expressed in the fibroblasts isolated from young and aged skin (Fig 2A). These data suggest that age-related elevation of multiple MMPs might be induced by epigenetics rather than intrinsic genetic alteration.

We previously reported that a prominent characteristic of aged dermal fibroblasts in human skin *in vivo* is reduced size; with decreased elongation and a more rounded, collapsed morphology due to collagen fragmentation [3,16,17]. These data prompted us to explore the connection between

reduced fibroblast size and age-related elevation of multiple MMPs. Using second harmonic generation microscopy, we confirmed that the fibroblasts in aged dermis revealed reduced size and collapsed morphology (Fig 2B, upper right panel), compared to elongated and stretched fibroblasts from young skin dermis (Fig 2B, upper left panel). Atomic force microscopy indicated that collagen fibrils in young skin dermis were intact, tightly packed and well organized (Fig 2C, lower left panel). In contrast, collagen fibrils in aged skin dermis were fragmented, sparse and disorganized (Fig 2C, lower right panel), suggesting that fragmented and disorganized collagen fibrils appear to be unable to support normal fibroblasts morphology. Consequently, we confirmed that the morphology of the isolated dermal fibroblasts from young (Fig 2D, left panel) and aged (Fig 2D, right panel) were similar in standard monolayer culture *in vitro*, suggesting that dermal ECM microenvironment is largely responsible for fibroblasts shape and size.

Based on above observations, we investigated the relationship between reduced fibroblast size and age-related elevation of multiple MMPs using young dermal fibroblasts (mean age 26±3 years). First, we modulated dermal fibroblast size by culturing young dermal fibroblasts in three dimensional (3D) collagen lattices, under constrained and unconstrained 3D collagen lattices to model young and aged dermal fibroblasts, respectively. A red fluorescent dye (Cell Tracker), which is taken up into the cell cytoplasm was used to assess the morphology of fibroblasts in 3D collagen lattices. Dermal fibroblasts cultured in constrained collagen lattices displayed spread flattened appearance (Fig. 3A, left). In contrast, fibroblasts in unconstrained collagen gels, had reduced cytoplasmic area, and a contracted appearance (Fig 3B, right), similar to fibroblasts in aged human skin *in vivo* (Fig 2B, upper right panel). Quantification indicated that fibroblast size was reduced by approximately 50% in unconstrained collagen gel (Fig 3A, right panel). Interestingly, measurement of all 23 mammalian MMPs indicated that the majority MMPs that were found to be elevated in aged dermis *in vivo*, except MMP-23 and MMP-24, were elevated by reduction of fibroblasts size (Fig 3B). To further confirm this fibroblast-size-dependent elevation of MMPs, we modulated young dermal fibroblasts size by disrupting the actin cytoskeleton with latrunculin-A (Lat-A), which rapidly blocks actin polymerization [26]. As expected, disruption of the actin cytoskeleton resulted in loss of actin cytoskeletal fibers and reduced fibroblasts size approximately 70% (Fig 3C, right panel). Importantly, reduction of

fibroblast size resulted in elevation of multiple MMPs that were found to be elevated in aged dermis *in vivo* (Fig. 2E). As shown in Table 1, reduction of fibroblasts size either in 3D collagen lattices or in monolayer culture resulted in elevation of the majority MMPs that were elevated in aged dermis *in vivo*, except MMP-23 and MMP-24.

Next, we assessed whether elevated MMPs are reversible by restoration of fibroblast size. For these studies, Lat-A-containing media was withdrawn 24 hours after its addition, and the cells were extensively washed, and fresh culture media was added. With removal of Lat-A, fibroblasts converted from a small, rounded morphology (Fig. 3E middle panel) to typical elongated morphology (Fig. 3E, right panel). Consistent with recovery of cellular size, elevated MMPs were normalized to basal level (Fig. 3F). These data suggest that reduced fibroblast size as observed in aged human skin likely contributes to age-related elevation of multiple MMPs.

Reduced fibroblast size up-regulates c-Jun/c-Fos and activates AP-1, the major regulator of multiple MMPs

We next investigated the mechanism by which reduced fibroblast size up-regulates multiple MMPs expression. We focused on transcription factors c-Jun and c-Fos, which typically comprise the AP-1 transcription factor complex. AP-1 transcription factor has been shown to principal regulator of multiple MMPs transcription, under a variety of conditions[21,22,27]. As shown in Figure 4, reduction of fibroblast size in either 3D collagen lattices (non-contraction vs contraction) or monolayer culture (DMSO vs latrunculin-A) resulted in significant increase of c-Jun mRNA (Fig 4A) and protein (Fig 4B), and c-Fos mRNA (Fig 4C) and protein (Fig 4D). Furthermore, reduction of fibroblast size markedly increased AP-1 reporter activities (Fig 4E). Finally, we asked that whether c-Jun and c-Fos expression are elevated in aged human skin dermis *in vivo*, the major source of elevated multiple MMPs. To address this question, the dermis was prepared by cutting off epidermis at a depth of 1 mm by cryostat. Indeed, both c-Jun and c-Fos mRNA (Fig 5A) and protein (Fig 5B) levels were elevated in aged dermis, compared to young dermis. These data suggest that reduction of fibroblast size may elevate multiple MMPs via up-regulation of c-Jun/c-Fos and activation of AP-1, the major regulator of multiple MMPs.

Discussion

MMPs are primarily responsible for fragmentation of dermal collagen fibrils, the hallmark of dermal aging [3,4,12]. MMPs are a large family of proteinases that are capable of degrading every type of ECM protein [18]. We demonstrate here that multiple MMPs become elevated during the aging process in human skin dermis. Elevated MMPs in aged dermis can be divided into following groups: collagenase (MMP-1), gelatinase-B (MMP-9), stromelysins (MMP-3), MMP-10, MMP-11, membrane-associated: MMP-23, MMP-24, and recently identified MMP-27. It is well-known that ultraviolet (UV) from the sun causes premature skin aging (photoaging) by transiently inducing only three MMPs in human skin *in vivo* (MMP-1, MMP-3, and MMP-9) [28-30]. We demonstrate here that compared to acute UV irradiation, a larger variety of MMPs, including UV-inducible MMPs, are constitutively elevated in aged skin. Interestingly, compared to acute UV irradiation, in which the epidermis is the primary source of transiently induced MMPs [30], the dermis is the major source of elevated MMPs in chronologically aged sun-protected skin. These observations suggest that although chronologically aged and photoaged skin share many common molecular features, such as MMPs-mediated collagen fragmentation, the primary sources (epidermis vs dermis) and cell types (keratinocytes vs fibroblasts) of elevated MMPs are different. Also, our investigations appear to reveal that collagen fragmentation in aged skin dermis results from constitutive elevation of a wide variety of dermal MMPs, rather than a transient elevation of limited epidermal MMPs as observed in acute UV irradiation in human skin.

The production of these MMPs is the responsibility of dermal fibroblasts, which are the major MMP-producing cells and control collagen homeostasis in the dermis. We demonstrate here that reduced fibroblast size up-regulates multiple MMPs as observed in aged human skin dermis *in vivo*. Reduced fibroblast size is a prominent feature of dermal fibroblasts in aged human skin [3,16,17]. We found that isolated primary dermal fibroblasts from aged (>80 years) or young (25-30 years) individuals are indistinguishable from each other with respect to morphology (Fig 2D) and expression of all known mammalian MMPs (Fig 2A). In contrast, human dermal fibroblasts, obtained from individuals of any age (21-86 years of age), cultured in the conditions of reduced cell size, result in elevation of multiple MMPs (Table 1). These data suggest that the elevation of

multiple MMPs in aged human skin arises, at least in part, from reduced size of dermal fibroblasts. We are unable to confirm fibroblast-size-dependent increase of MMP-23 and MMP-24, which are elevated in aged human skin dermis. As both MMP-23 and MMP-24 are cell membrane-associated MMPs (type II transmembrane MMPs), we found that the basal mRNA expression levels of these two MMPs are extremely low in dermal fibroblasts, suggesting that dermal fibroblasts are not major source of elevated MMP-23 and MMP-24 in aged human skin dermis.

Further investigation indicates that reduced fibroblast size is closely associated with elevated c-Jun/c-fos and increased transcription factor AP-1 activity, the major driving for multiple MMPs [21,22,27]. Transcription factor AP-1, typically composed of c-Jun and c-Fos is one of the first mammalian transcription factors to be identified [31,32]. We and others, previously reported that stress-activated MAP Kinase pathways and c-Jun mRNA and protein are increased in aged, compared to young human skin *in vivo* [17,33,34]. In this study, we confirmed that c-Jun and c-Fos expression are significantly elevated in aged human skin dermis *in vivo*, the major source of elevated multiple MMPs. These data suggest that reduced fibroblast size induces c-Jun and c-Fos, which in turn elevates multiple MMPs in aged human skin dermis.

In skin dermis, fibroblast spreading and size, which are mediated by cytoskeletal and intracellular structural machinery, largely depends on cellular interactions with surrounding ECM microenvironment. In young human skin dermis, binding of fibroblasts to intact collagen fibrils allows generation of traction forces that are necessary for spreading, mechanical stability, normal function. However, in aged human skin dermis, collagen fibril binding sites are lost and mechanical resistance to traction forces is reduced due to fragmentation. In this state, the ECM microenvironment is unable to provide sufficient mechanical stability to maintain normal cell spreading/mechanical force [3,16,17]. Therefore, age-related fragmentation of the collagen fibril microenvironment deleteriously alters fibroblast size and function.

Also, cell shape and size impacts multiple cellular processes including signal transduction, gene expression, and metabolism [14,35-37]. Currently, mechanisms by which reduced cells size induces c-Jun/AP-1 are not well-understood. AP-1 activity is regulated by a wide range of stimuli

including reactive oxygen species (ROS) [32]. We previously reported that fibroblasts that have reduced spreading/size due to fragmentation of surrounding collagen fibrils display increase levels of ROS [17]. ROS is considered to be a major driving force for the aging process [38,39]. Indeed, we observed that protein oxidation, a marker of oxidative stress, is increased in aged human dermis *in vivo* [17]. These data suggest that elevated ROS may be mediate induction of c-Jun/AP-1 activity in response to reduced fibroblast spreading and size. Clearly, further studies are needed to understand the connections among fibroblast size, oxidative stress, AP-1 activity, and MMPs.

We have previously reported that reduction of fibroblast size by disruption of cytoskeleton reduced cellular mechanical force, which substantially induced MMP-1 expression[40] and consequent collagen fibril fragmentation[17]. Reduced mechanical force induced transcription factor c-Jun to bind to a canonical AP-1 binding site in the MMP-1 proximal promoter. Blocking c-Jun function with dominant negative mutant c-Jun significantly reduced induction of MMP-1 expression in response to reduced cell size and mechanical force. Current study added new information that reduced fibroblast size not only induces MMP-1, but also induces multiple MMPs (Fig.1A). Furthermore, we demonstrated that restoration of fibroblast size led to reverse elevated multiple MMPs to a basal level (Fig. 3F). We also previously reported that reduced fibroblast size inhibits type I procollagen synthesis, the major structural protein in skin, via impairment of TGF-β/Smad signaling[41]. Further investigation reveals that reduced fibroblast size specifically down-regulates TGF-β type II receptor, and this down-regulation largely mediates the reduction of type I procollagen and other ECM production. Taken together, these data suggest that reduced fibroblast size could be a marker of cellular aging and of aging human skin dermis.

Impaired wound healing is common in the elderly and presents a significant clinical and economic problem. It has been reported that fibroblast dysfunction is a key factor in the non-healing of chronic wounds in the elderly[42,43]. Dermal fibroblasts are largely responsible for collagen turnover and remodeling in healing of wounds. Although MMPs play essential and beneficial roles in normal wound healing, MMPs must be present at the right amount and in the right time frame (duration) during the wound healing process. Substantial evidence has amassed

that MMPs in general are highly elevated in the delayed wound healing[44-47], and that treatments that lower MMP activities accelerate healing of wounds[48-50]. It is conceivable that constitutive elevations of multiple MMPs, as identified in current paper, result in a hostile tissue microenvironment that promotes age-related skin diseases, such as delayed wound healing in elderly. Clearly, additional studies are warranted to uncover the precise molecular mechanism(s) by which constitutive elevations of multiple MMPs may contribute to impaired wound healing in elderly.

In summary, aged dermis constitutively expresses elevated levels of several MMPs, which likely lead to chronic, progressive degradation of the dermal collagen in aged human skin. We propose a novel mechanism by which age-related reduction of fibroblast size activates AP-1, which in turn elevates multiple MMPs as observed in aged human skin (Fig 5C). This mechanism provides a foundation for understanding the cellular and molecular basis of age-related collagen fragmentation, the characteristic feature of aged human skin.

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Author contribution

ZQ and RMB performed the experiments. TQ designed, analyzed the data, and wrote the manuscript.

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Figure Legends

Figure 1 Elevated expression of multiple MMPs in aged human dermis. Adult human skin punch biopsies were obtained from sun-protected buttock skin. (**A**) Elevated multiple MMPs in aged (83±4 years) compared to young (26±3 years) human skin. N=12 each group. Mean ± SEM. *p<0.05. (**B**) No change in TIMP mRNA expression in young and aged human skin. N=12 each group. Mean ± SEM. (**C**) Schematic representation of the dissection of human skin epidermis and dermis by Laser Capture Microdissection (LCM, see *Methods* for details). (**D**) Elevated MMPs in the dermis of aged human skin. Epidermis and dermis were captured by LCM and total RNA was prepared form epidermis and dermis. N=8, Mean ± SEM. All MMPs mRNA levels were quantified by real-time RT-PCR and were normalized by the housekeeping gene (36B4, internal control). (**E**) Elevated collagenase activity in the dermis of aged human skin determined by *in situ* zymography (see *Methods* for details). Loss of green fluorescence in aged dermis indicates degradation of fluorescein-collagen substrate. White lines indicate boundary between the epidermis (top) and dermis (bottom). N=6.

Figure 2 (**A**) MMPs are similarly expressed in the fibroblasts isolated from aged (83±4 years) and young (26±3 years) skin. Dermal fibroblasts were isolated from sun-protected young and aged buttock skin. MMPs mRNA expression levels were quantified by real-time RT-PCR and were normalized by the housekeeping gene (36B4, internal control). N=6. Mean ± SEM. (**B**) Reduced dermal fibroblast size is a prominent feature of aged dermal fibroblasts in human skin

in vivo. Representative images of dermal fibroblasts in aged (78 years, right panel) and young (26 years, left panel) human skin. Skin was sectioned, and dermal fibroblasts were identified by immunostaining with collagen chaperone heat shock protein 47 (blue). Gray/white color is collagen fibrils and green color is elastin. Note spread fibroblasts in young skin versus contracted fibroblasts in aged human skin. Images were obtained by multiphoton laser scanning fluorescence microscopy. N=6 for each group. The black and white arrow heads indicate dermal fibroblasts in young and aged human skin, respectively Bars=25µm. (**C**) Collagen fibrils in aged skin dermis were fragmented and disorganized. Nanoscale collagen fibrils were imaged by atomic force microscopy. The black and white arrow heads indicate intact and fragmented/disorganized collagen fibrils, respectively. Images are representative of six independent experiments. Bars=100 nm (**D**) The morphology of the dermal fibroblasts from young (left panel) and aged (right panel) were similar in standard monolayer culture *in vitro*. Cells were stained with CellTracker® fluorescent dye and were imaged by fluorescence microscopy. Red fluorescence delineates cell cytoplasm; blue fluorescence delineates nuclei. Bars=100 µm. Images are representative of the dermal fibroblasts from five young and aged individuals.

Figure 3 Age-related reduction of dermal fibroblast size up-regulates multiple MMPs observed in aged human skin. (**A**) To model young and aged dermal fibroblasts, dermal fibroblasts from young skin (26±3 years) were cultured under conditions of constrained (left panel) and unconstrained (right panel) 3D collagen lattices (see *Methods* for details). Cells were stained with CellTracker® fluorescent dye and were imaged by fluorescence microscopy. Red fluorescence delineates cell cytoplasm; blue fluorescence delineates nuclei. Relative cell surface areas were quantified using ImageJ software and data were expressed as % of control (non-contracted gel). Mean ± SEM, N =3, *p<0.05. Bars = 100 µm (**B**) The majority age-related MMPs were elevated by reduction of fibroblasts size. Total RNA was extracted from dermal fibroblasts cultured from 3D collagen lattices. MMPs mRNA expression levels were quantified by real-time RT-PCR and were normalized by the housekeeping gene (36B4, internal control). N=4. Mean ± SEM. *p<0.05. (**C**) Dermal fibroblasts from young skin (26±3 years) were treated with Lat-A (30 nM) for 24 hours. Dermal fibroblasts were stained with phalloidin and were imaged by fluorescence microscopy. Red fluorescence delineates cell cytoplasm; blue

fluorescence delineates nuclei. Relative cell surface areas were quantified using ImageJ software and data were expressed as % of control (DMSO). Mean ± SEM, N=6, *p<0.05. Bars = 100 µm. (**D**) The majority age-related MMPs were elevated by reduction of fibroblast size via disruption of the actin cytoskeleton. Total RNA was extracted from dermal fibroblasts. MMPs mRNA expression levels were quantified by real-time RT-PCR and were normalized by the housekeeping gene (36B4, internal control). N=5. Mean ± SEM. *p<0.05. (**E**) Restoration of dermal fibroblast size after withdrawn Lat-A. Lat-A was withdrawn (right panel) 24 hours after Lat-A (30 nM) treatment (middle panel) by replacing with fresh culture medium, and the cells were further incubated for 48 hours. Dermal fibroblasts were stained with phalloidin and relative cell surface areas were quantified using ImageJ software. Data were expressed as % of control (DMSO, left panel). Mean ± SEM, N=6, *p<0.05. Bars = 100 µm. (**F**) Restoration of dermal fibroblast size reverses elevated MMPs. MMPs mRNA expression levels were quantified by real-time RT-PCR and were normalized by the housekeeping gene (36B4, internal control). MMPs mRNA expression levels after Lat-A withdrawn were expressed as % of control cells (DMSO). N=5. Mean ± SEM.

Figure 4 Reduced fibroblast size up-regulates c-Jun/c-Fos and activates AP-1, the major regulator of multiple MMPs. Dermal fibroblasts from young skin (26±3 years) were cultured in 3D collagen lattices or monolayer under conditions of reduced cell size, as described in *Method*. (**A**) Reduction of fibroblast size upregulates c-Jun mRNA expression. (B) Reduction of fibroblast size upregulates c-Jun protein expression. (C) Reduction of fibroblast size upregulates c-Fos mRNA expression. (D) Reduction of fibroblast size upregulates c-Fos protein expression. mRNA levels were quantified by real-time RT-PCR and were normalized by the housekeeping gene (36B4, internal control). Mean ± SEM, N=3, *p<0.05. Protein levels were determined by ProteinSimple capillary electrophoresis immunoassay (see Materials and methods for details) and normalized by β-actin (loading control). Insert shows representative digital images. Mean ± SEM, N=3, *p<0.05. (E) Dermal fibroblasts were transfected with AP-1 reporter construct (pAP-1-TA-Luc) or empty vector (CTRL). Cell lysates were prepared 48 hours after transfection. Reporter activity was determined by luciferase assay. Data are Mean ± SEM, N=3, *p<0.05.

Figure 5 Elevated expression of c-Jun and c-Fos expression in aged human skin dermis. Adult human skin punch biopsies were obtained from young (26±3 years) and aged (83±4 years) sun-protected buttock skin. Dermis was prepared by cutting off epidermis at a depth of 1 mm by cryostat. (**A**) Total RNA was prepared from dermis and mRNA levels were quantified by real-time RT-PCR and were normalized by the housekeeping gene (36B4, internal control). Mean ± SEM, N=6, *$p<0.05$. (**B**) Dermal protein levels were determined by ProteinSimple capillary electrophoresis immunoassay (see *Methods* for details) and normalized by β-actin (loading control). Insert shows representative digital images. Mean ± SEM, N=6, *$p<0.05$. (C) Reduced fibroblast size elevates age-related MMPs (see details in Discussion).

Figure 1

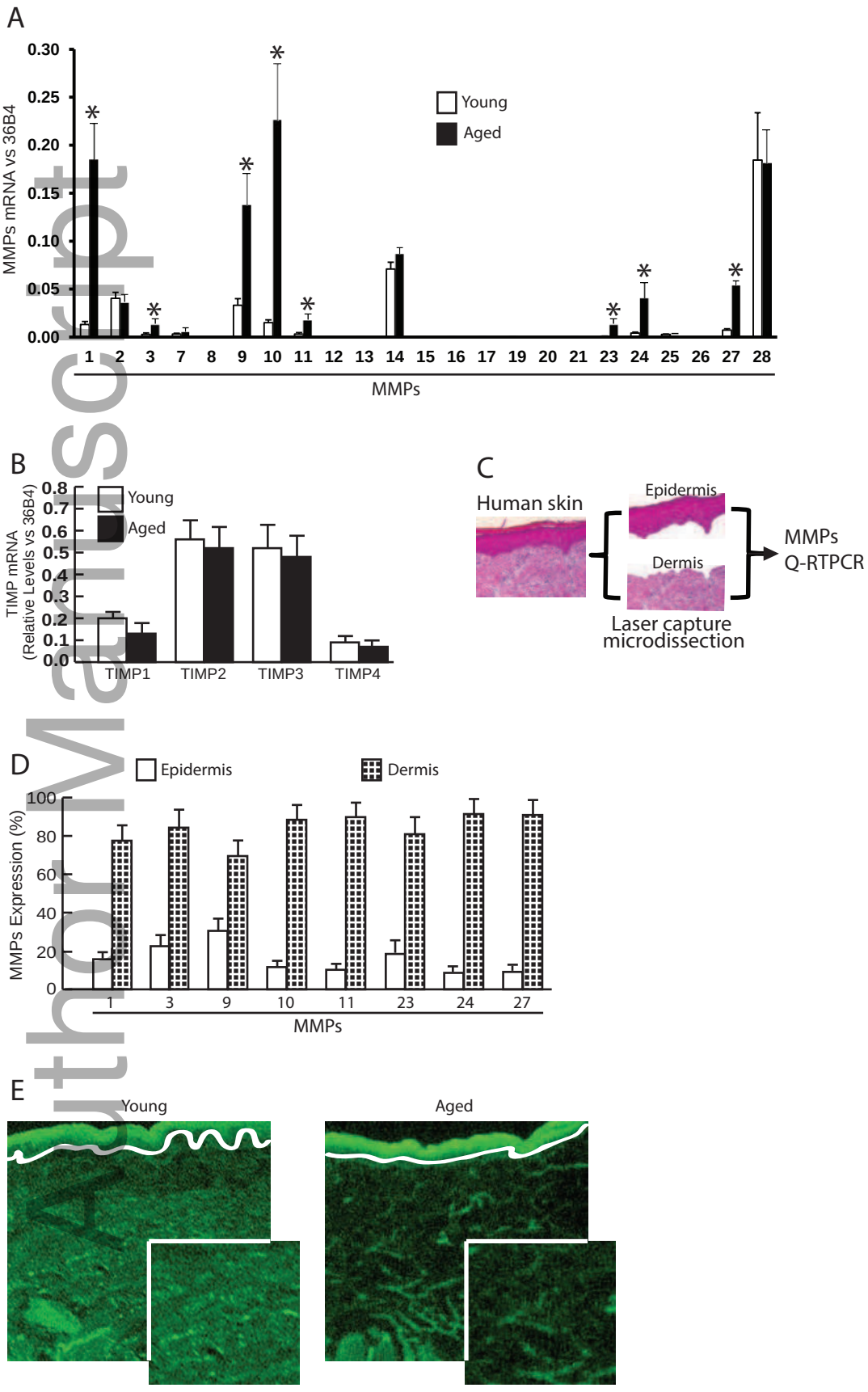


Figure 2

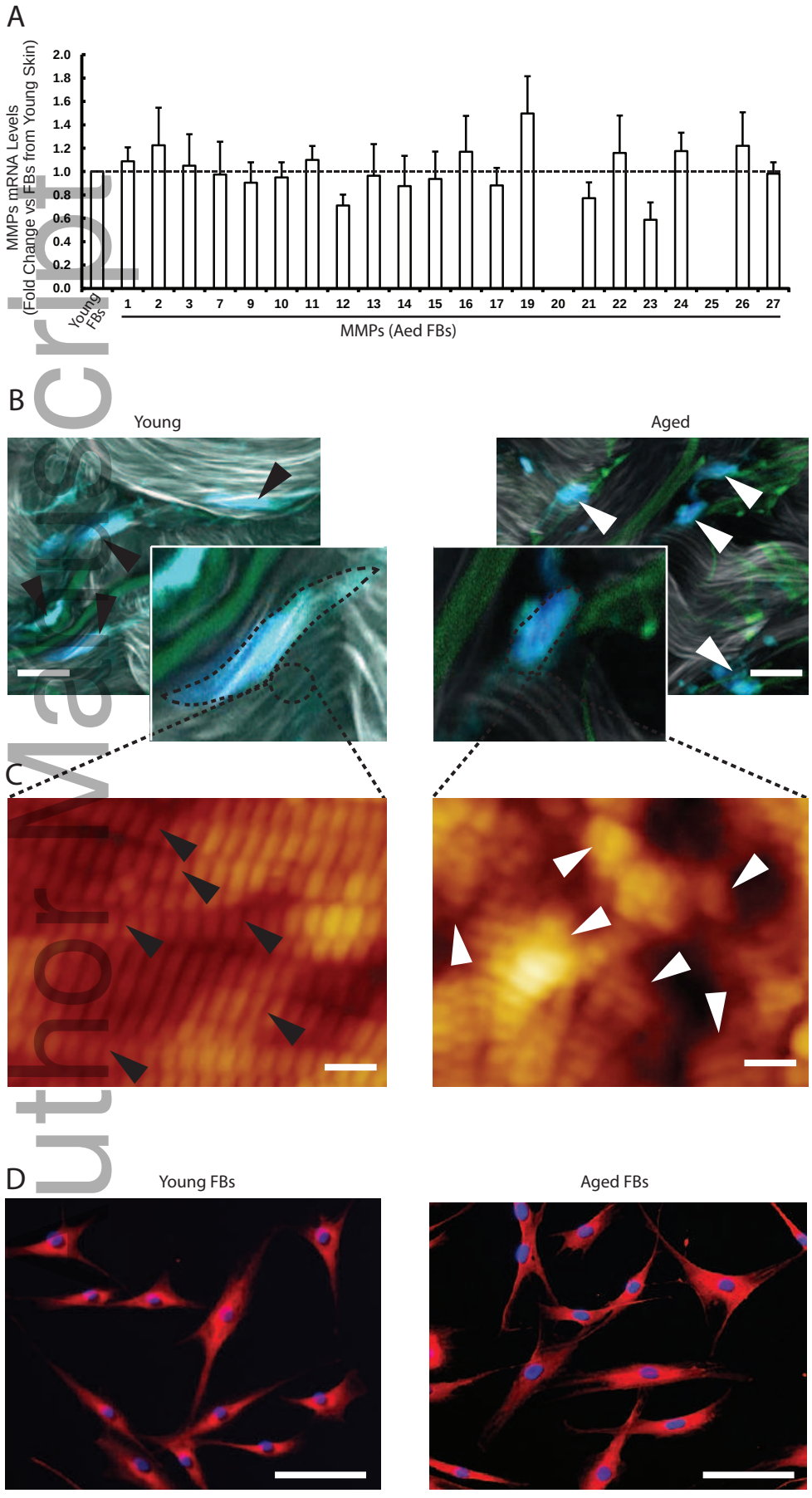


Figure 3

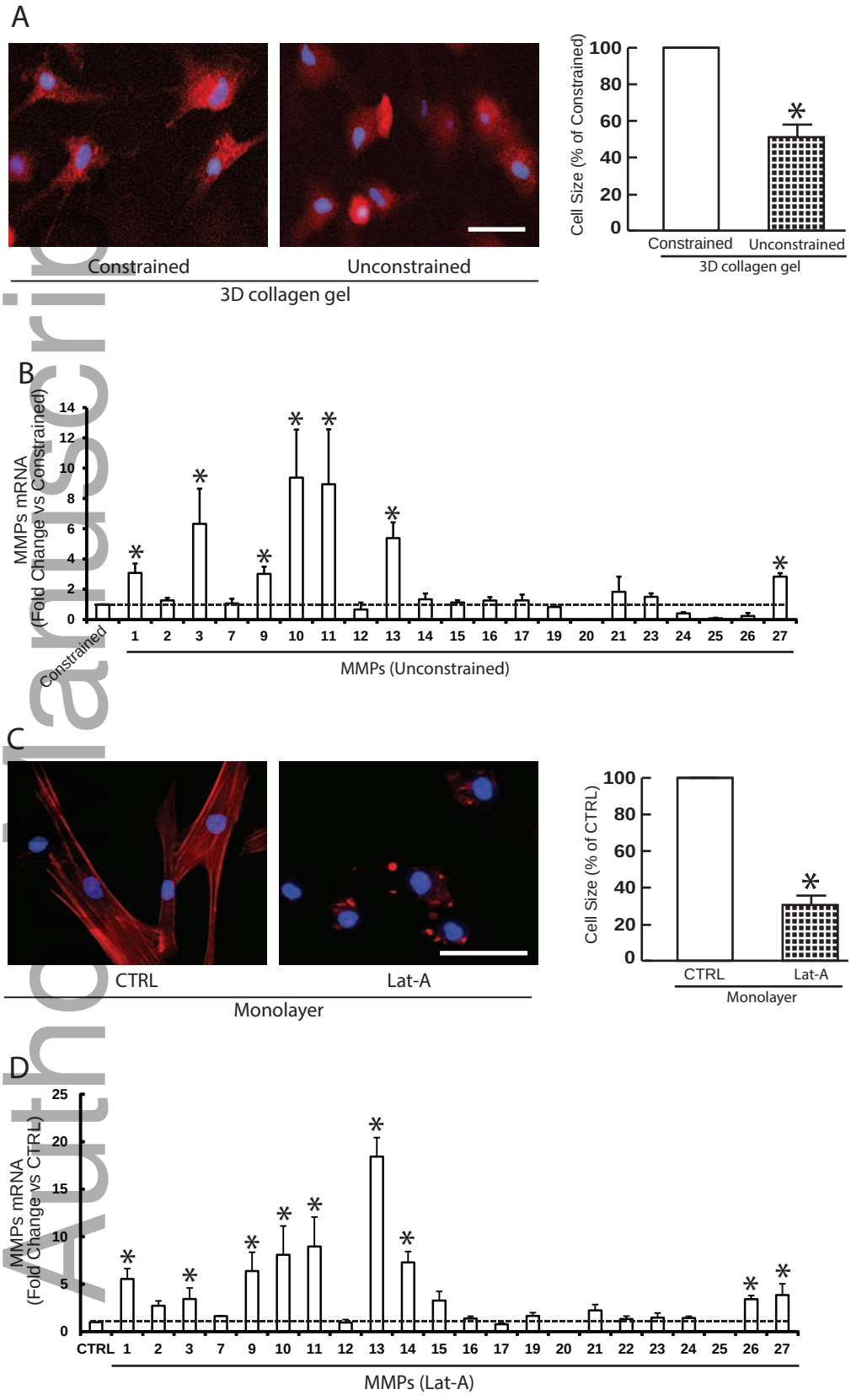
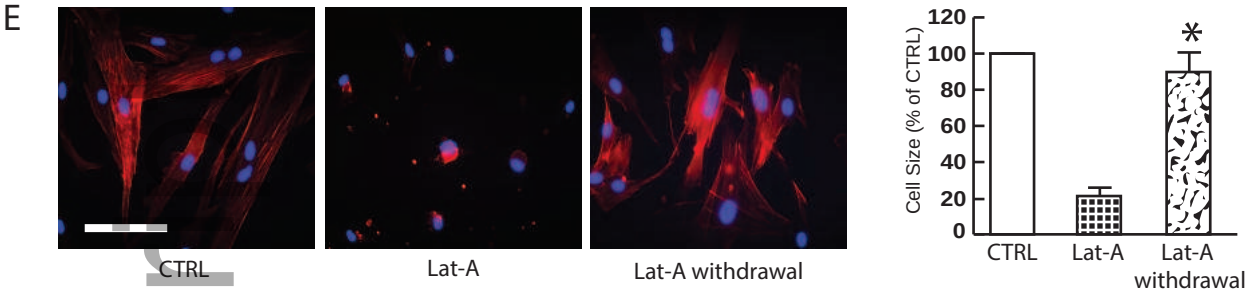


Figure 3_2



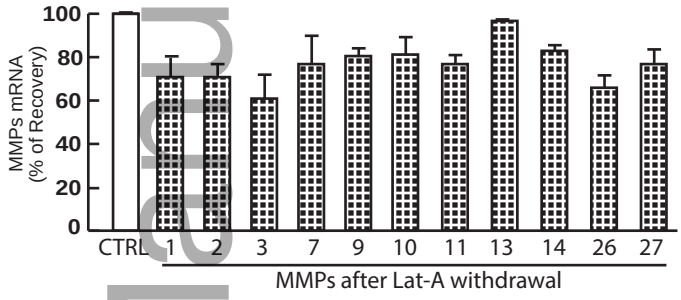


Figure 4

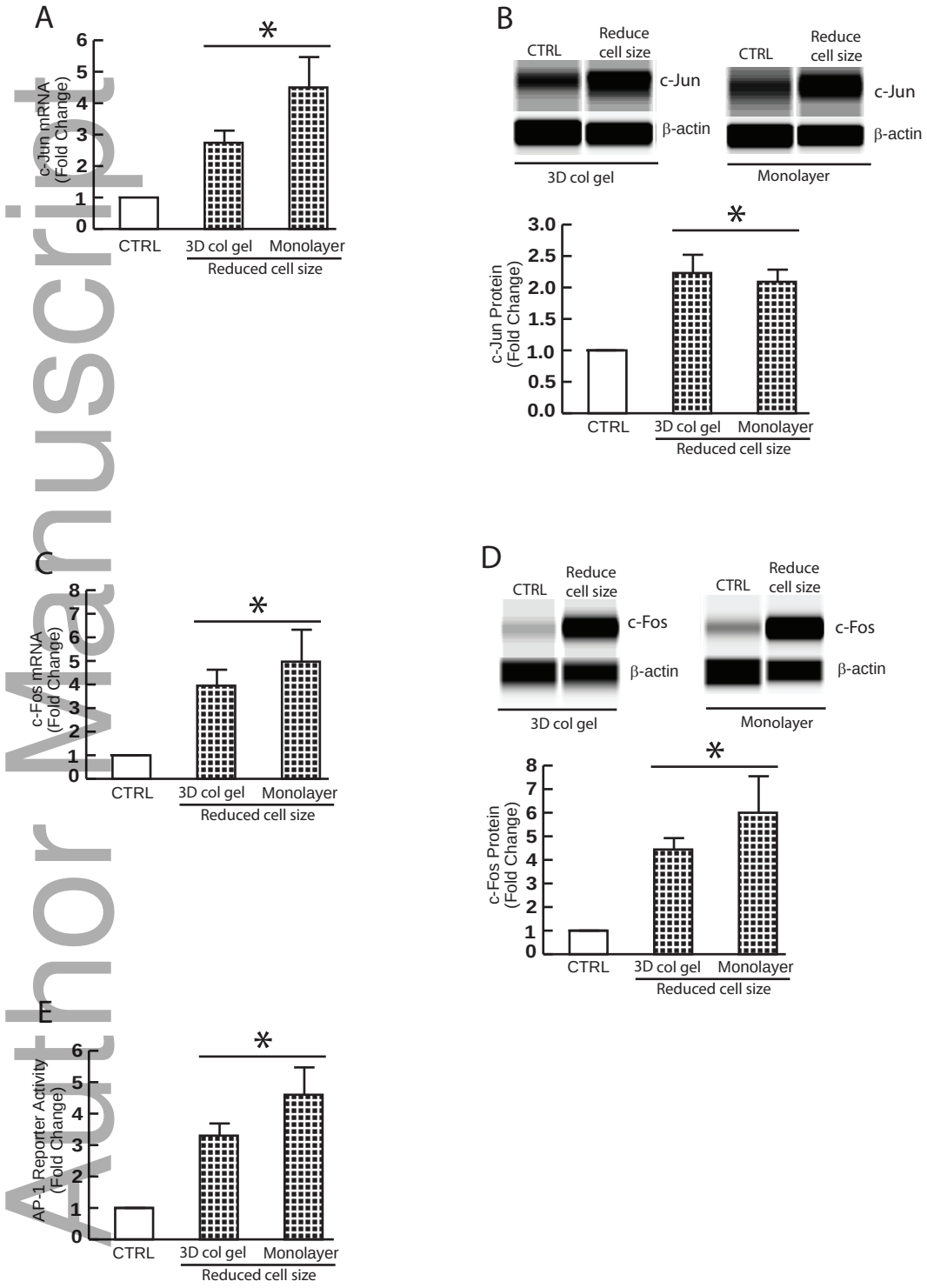


Figure 5