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Enhancing structural support of the dermal microenvironment activates fibroblasts, endothelial cells and keratinocytes in aged human skin *in vivo*

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Abstract

The dermal extracellular matrix (ECM) provides strength and resiliency to skin. The ECM consists mostly of type I collagen fibrils, which are produced by fibroblasts. Binding of fibroblasts to collagen fibrils generates mechanical forces, which regulate cellular morphology and function. With aging, collagen fragmentation reduces fibroblast-ECM binding and mechanical forces, resulting in fibroblast shrinkage and reduced function including collagen production. Here, we report that these age-related alterations are largely reversed by enhancing structural support of the ECM. Injection of dermal filler, cross-linked hyaluronic acid, into the skin of persons over seventy years-old stimulates fibroblasts to produce type I collagen. This stimulation is associated with localized increased of mechanical forces, indicated by fibroblast elongation/spreading, and mediated by up-regulation of type II TGF-β receptor and connective tissue growth factor. Interestingly, enhanced mechanical support of the ECM also stimulates fibroblast proliferation, expands vasculature, and increases epidermal thickness. Consistent with our observations in human skin, injection of filler into dermal equivalent cultures causes elongation of fibroblasts, coupled with type I collagen synthesis, which is dependent on the TGF-β signaling pathway. Thus, fibroblasts in aged human skin retain their capacity for functional activation, which is restored by enhancing structural support of the ECM.

INTRODUCTION

The dermal extracellular matrix (ECM) plays vital roles in structural support, immunity, circulation, and sensory perception (Fisher *et al.*, 2008; Quan *et al.*, 2009; Uitto and Bernstein, 1998). Dermal ECM supports the epidermis and consists mostly of type I collagen fibrils, which are synthesized by fibroblasts. As the most abundant structural protein in the dermis, type I collagen provides strength and resiliency to skin (Fisher *et al.*, 2008).

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A wealth of evidence indicates that interactions between adherent cells, such as fibroblasts, and the ECM are critical for cellular function (Dupont *et al.*, 2011; Eckes *et al.*, 2006; Fisher *et al.*, 2008; Grinnell, 2003; Ingber, 1997; Kessler *et al.*, 2001; Lambert *et al.*, 1992; Ruoslahti, 1997; Silver *et al.*, 2003). In healthy young skin, dermal fibroblasts attach to collagen fibrils through transmembrane integrin receptors. Engagement of integrins with the ECM triggers formation of focal adhesion complexes, which couple the ECM to the intracellular actin cytoskeleton (Delon and Brown, 2007; Eckes *et al.*, 2006; Lambert Ch *et al.*, 1998; Olson and Nordheim, 2010). The actin cytoskeletal machinery generates mechanical forces that determine cell shape, which in turn greatly influences fibroblast function (Fisher *et al.*, 2008; Grinnell, 2003; Ingber, 2006; Olson and Nordheim, 2010; Silver *et al.*, 2003).

With aging, dermal collagen fibrils undergo enzyme-catalyzed cleavage (Fisher *et al.*, 2002; Fisher *et al.*, 2008; Fligiel *et al.*, 2003). This degenerative process compromises the mechanical microenvironment of the dermis and impairs fibroblast attachment to the ECM, resulting in reduced mechanical forces (Varani *et al.*, 2006). Consequently, fibroblasts in aged skin display a collapsed cytoplasm and rounded shape, which contrasts with the spread shape of fibroblasts in young skin. Importantly, fibroblasts with a collapsed morphology down-regulate production of type I collagen and up-regulate production of collagen-degrading matrix metalloproteinases (MMPs) (Fisher *et al.*, 2008; Fligiel *et al.*, 2003; Varani *et al.*, 2006; Varani *et al.*, 2001).

The TGF-β signaling pathway is influenced by mechanical force and pivotal to dermal fibroblast function (Eckes *et al.*, 2006; Fisher *et al.*, 2009; Rittie and Fisher, 2002; Varga and Jimenez, 1995). TGF-β is a multifunctional cytokine that acts through a receptor complex composed of type I, II, and III TGF-β receptors (TβR) (Massague and Gomis, 2006). TGF-β induces connective tissue growth factor (CTGF/CCN2), which in concert with TGF-β regulates fibroblast function, including synthesis of type I procollagen and other ECM proteins (Duncan *et al.*, 1999; Oliver *et al.*, 2010; Quan *et al.*, 2002a; Quan *et al.*, 2010; Rittie and Fisher, 2002). In fibroblasts in aged skin, reduced TGF-β-mediated signaling and CTGF/CCN2 expression contribute to decreased collagen production (Quan *et al.*, 2006; Quan *et al.*, 2010).

Clinically, impaired fibroblast function, coupled with reduced collagen synthesis, translates into atrophy, wrinkling, and fragility of aged skin (Fisher *et al.*, 2008; Lapiere, 1990). We hypothesized that fibroblast function in naturally aged skin could be stimulated by enhancing structural support of the ECM with an injectable space-filling material, cross-linked hyaluronic acid (Wang *et al.*, 2007). Hyaluronic acid (HA), which is a component of the extracellular matrix in all tissues, is a glycosaminoglycan disaccharide composed of repeating units of d-glucuronic acid and *N*-acetyl-d-glucosamine. Hyaluronic acid-based dermal fillers are composed of disacharide chains ranging in molecular weight from 500,000–6,000,000 that are cross-linked with butanediol diglycidyl ether (Kablik *et al.*, 2009). We report that injection of this filler induces fibroblast spreading, in turn stimulating type I collagen production. Our data, therefore, demonstrate that fibroblasts in aged skin retain their capacity for functional activation, highlighting the importance of the ECM microenvironment in regulating fibroblast behavior. Thus, aging of connective tissue in skin,

and perhaps other organs, is largely attributable to alterations in the extracellular microenvironment, in addition to inherent cellular changes.

RESULTS

Expression of type I procollagen in aged human skin is restored by enhancing structural support of the dermal ECM

We injected vehicle (saline) or filler into buttock skin of aged individuals (81.4±1.0 years-old) and obtained biopsies at 1, 2, 4 and 12 weeks later. We initially evaluated whether injection caused an inflammatory response. Neither clinical nor histological inflammation (determined by immunostaining for immune/inflammatory monocytes, macrophages, neutrophils) was observed in any subject during the course of the study (data not shown). This lack of inflammation following injection of cross-linked hyaluronic acid has been previously reported (Flynn *et al.*, 2011). As reduced fibroblast function and ECM synthesis are prominent features of aged skin, we first analyzed the localization of type I procollagen protein expression by immunohistochemistry. Type I procollagen is the precursor of mature type I collagen fibrils. Compared with vehicle injection, filler injection induced intense immunostaining within the ECM and dermal fibroblasts (Fig. 1A). Staining was particularly strong adjacent to pockets of injected filler, which were present primarily in the mid to lower dermis. Interestingly, positively stained fibroblasts tended to align around pockets of injected filler and exhibited an enlarged, elongated morphology, indicating increased mechanical force and structural support within the dermal ECM (Fig. 1A, **inset**). Elongated fibroblasts were mostly embedded within ECM fibers surrounding pockets of injected filler, but not directly contacting the filler material. Overall, the amount of staining was increased 6-fold at 4 weeks post-filler injection and remained elevated at least 12 weeks (Fig. 1A). Quantitation by ELISA confirmed type I procollagen protein induction (Fig. 1B).

Additionally, we performed immunostaining for two proteins induced in fibroblasts actively producing type I procollagen. Prolyl-4-hydroxylase catalyzes the formation of hydroxyproline, which is required for stable assembly of the triple helical region of type I collagen, and heat shock protein 47 (HSP47) is an intracellular molecular chaperone required for shuttling type I procollagen through the endoplasmic reticulum during synthesis. Staining patterns for prolyl-4-hydroxylase (Fig. 1C) and HSP47 (Fig. 1D) were similar to that of type I procollagen, with elongated/spread fibroblasts surrounding filler displaying increased and intense staining at 4 and 12 weeks, compared with vehicle-treated skin (Fig. 1C,D).

To complement our immunostaining results, we measured gene expression of type I procollagen, prolyl-4-hydroxylase, HSP47, and type III procollagen, the precursor of type III collagen, which associates with type I collagen fibrils. Expression of these genes was significantly induced at 4 weeks post-filler injection, and with the exception of HSP47, their expression remained elevated at least 12 weeks (Supplemental Fig. 1). These data indicate that enhanced structural support of the dermal ECM induces fibroblast elongation and procollagen synthesis in aged human skin.

Enhanced structural support of the dermal ECM up-regulates type I procollagen expression and the TGF-β pathway specifically in elongated fibroblasts in aged human skin

Since procollagen-producing fibroblasts appeared elongated and aligned around pockets of deposited filler (Fig. 2A), we next used laser capture microscopy (LCM) to specifically isolate these cells and analyze their gene expression. Consistent with our immunostaining results, elongated fibroblasts surrounding injected filler demonstrated a 12-fold induction of type I procollagen gene expression, compared with an equivalent number of fibroblasts from the middle and deep dermis of vehicle-injected skin (Fig. 2B).

Additionally, we measured TβRII and CTGF/CCN2 gene expression in LCM-captured fibroblasts. Elongated fibroblasts adjacent to filler exhibited a 3-fold and 10-fold induction of TβRII and CTGF/CCN2, respectively, compared with cells from vehicle-injected skin (Fig. 2B). These data indicate that enhanced structural support of the dermal ECM up-regulates the TGF-β pathway through induction of TβRII and CTGF/CCN2 in elongated fibroblasts in aged human skin.

Deposition of mature collagen is increased by enhancing structural support of the dermal ECM in aged human skin

Having found that enhanced mechanical support of the ECM promotes type I procollagen synthesis, we next considered whether newly made procollagen is processed to form stable collagen fibrils. To address this question, we used atomic force microscopy (AFM) to assess the nanoscale structure of collagen fibrils. In vehicle-injected skin, collagen fibrils in the mid and deep dermis appeared disorganized and fragmented (Fig. 2C, **left panel**). However, in areas adjacent to injected filler, we observed highly organized, dense bundles of collagen fibrils, with characteristic banded structure (D-spacing) representing the staggered alignment of individual collagen molecules within fibrils (Fig. 2C, **right panel**). These highly-organized bundles extended from pockets of injected filler as far away as approximately 500 μm. More distantly, collagen fibrils appeared similar to those in vehicle-injected skin (Supplemental Fig. 2). Additionally, we performed a metabolic labeling assay to measure the rate of production of insoluble collagen fibrils. Skin samples obtained 4 weeks after vehicle or filler injection were incubated with [^{14}C]-proline, and insoluble collagen was extracted after 48 hours. The level of radioactivity was 90% greater in filler- versus vehicle-injected skin ($p<0.05$, data not shown). These findings indicate that enhanced structural support of the dermal ECM stimulates synthesis of procollagen, which is processed into mature collagen in aged human skin.

Enhanced structural support of the dermal ECM is associated with increased epidermal proliferation and thickening in aged human skin

Aged human skin is characterized by a thinned epidermis, caused in part by decreased proliferation of basal keratinocytes (Zouboulis and Makrantonaki, 2011). Interestingly, we noticed that epidermal thickness appeared greater following injection of filler, compared with vehicle. Indeed, quantitative morphometric analyses revealed that epidermal thickness was increased 19% and 14% at 4 and 12 weeks, respectively, after filler injection (Fig. 3A). Additionally, keratinocyte proliferation, assessed by Ki67 immunostaining, was significantly

increased within 1–2 weeks after filler injection (Fig. 3B). Thus, enhanced structural support of the dermal ECM is associated with increased keratinocyte proliferation and epidermal thickening.

Enhanced structural support of the dermal ECM is associated with proliferation of endothelial cells and fibroblasts in aged human skin

In addition to epidermal changes, we noticed increased prominence of blood vessels in the mid to deep dermis in filler-injected skin. Indeed, immunostaining for the endothelial cell marker CD31 revealed increased staining by 60% and 97% at 4 and 12 weeks, respectively, after filler injection, compared with vehicle injection (Fig. 4A).

Next, having observed increased proliferation of epidermal cells and increased prominence of endothelial cells, we examined proliferation of dermal cells. As early as 1–2 weeks after filler injection, dermal cell proliferation, assessed by Ki67 immunostaining, was readily evident in filler-injected skin, particularly in areas adjacent to the filler material (Fig. 4B). Dermal cell proliferation was rarely detected in vehicle-injected skin.

Positively stained cells in filler-injected skin appeared to include a fraction of endothelial cells and fibroblasts. Therefore, we performed double-label immunofluorescence staining to confirm the identity of proliferating cells. We found Ki67/CD31-positive endothelial cells in vessel structures near pockets of injected filler (Fig. 4C, **upper row**). Similarly, Ki67/HSP47-positive fibroblasts were localized to areas adjacent to injected filler (Fig. 4C, **bottom row**). Together, these data indicate enhanced structural support of the dermal ECM is associated with proliferation of endothelial cells and fibroblasts in aged human skin.

Enhanced structural support in three-dimensional collagen lattices induces fibroblast elongation and up-regulates collagen production via the TGF-β signaling pathway

To further elucidate mechanisms by which enhanced structural support stimulates fibroblasts, we employed dermal equivalent cultures, composed of human dermal fibroblasts embedded in three-dimensional (3-D) collagen lattices. After focal injection into collagen lattices, filler material remained confined to pockets at injection sites, where it caused localized expansion of lattices (Fig. 5A). Thus, the space filling property of injected filler in collagen lattices appeared similar to that observed in human skin. Injection of vehicle had no observable effect on lattices.

Consistent with our findings in human skin, we observed intense immunostaining of type I procollagen within elongated fibroblasts adjacent to pockets of injected filler (Fig. 5A). Additionally, protein levels of secreted type I procollagen were increased approximately 2-fold in filler-injected cultures, compared with vehicle injection (Fig. 5B). Filler injection also increased the gene expression of HSP47 and prolyl-4-hydroxylase (Supplemental Fig. 3).

As mentioned previously, expression of type I procollagen is dependent on the TGF-β/CCN2 axis (Quan *et al.*, 2006; Quan *et al.*, 2010). Similar to our data in human skin, we found that type I procollagen, TβRII, and CTGF/CCN2 gene expression were significantly induced following filler injection into dermal equivalent cultures (Fig. 5C). As noted above,

expansion of collagen lattices occurs after filler injection. To examine the role of this expansion, non cross-linked hyaluronic acid, which readily diffuses within the lattices and does not cause expansion, was injected. Similar to vehicle injection, injection of non cross-linked hyaluronic acid had no effect on type I procollagen, TβRII, and CTGF/CCN2 gene expression (Fig. 5C).

To further examine the role of lattice expansion in inducing procollagen production, filler material was dispersed into collagen solution prior to lattice formation. Under these conditions, fibroblast morphology appeared similar to that in untreated lattices or lattices injected with vehicle or non-cross-linked hyaluronic acid (data not shown). Furthermore, dispersal of filler, as opposed to injection into preformed lattices, failed to induce type I procollagen, TβRII, or CTGF/CCN2 (Fig. 5D). Thus, lattice deformation was required for up-regulation of fibroblast function.

Finally, we investigated the role of the TGF-β pathway in procollagen induction following filler injection. Addition of TβRI kinase inhibitor to collagen lattices prior to filler injection prevented up-regulation of type I procollagen and CTGF/CCN2 (Fig. 5E), indicating that collagen up-regulation following filler injection is dependent on the TGF-β signaling pathway.

DISCUSSION

We have proposed that accumulation of fragmented collagen during natural skin aging negatively impacts fibroblast function (Fisher *et al.*, 2002; Fisher *et al.*, 2008; Varani *et al.*, 2006; Varani *et al.*, 2004; Varani *et al.*, 2001). Collagen fragmentation alters the physical properties of the dermal microenvironment and reduces ECM binding by fibroblasts, which in turn lessens mechanical force. Under these conditions, fibroblasts down-regulate collagen production and up-regulate MMPs (Fisher *et al.*, 2009; Fisher *et al.*, 2008; Varani *et al.*, 2006). This cellular response promotes further loss and fragmentation of collagen, thereby promoting self-perpetuating progression of the aged phenotype in human skin. Inherent to our model is the concept that quality of the ECM, rather than chronologic age of dermal fibroblasts, is a key determinant of age-dependent decline of fibroblast function.

In this study, we used a space-filling material, cross-linked hyaluronic acid, as a tool to test the hypothesis that enhanced structural support could stimulate fibroblast function in aged skin. We observed that the filler, when injected focally into skin, distributes in the dermis as large pools, filling space and pushing against the surrounding ECM. Adjacent to these pockets of filler, fibroblasts display an elongated morphology, indicating increased mechanical force and structural support within the dermal ECM. Importantly, fibroblast elongation is associated with up-regulation of the TGF-β signaling pathway, and its downstream targets CTGF/CCN2 and type I procollagen. Thus, we found that structural properties of the dermal ECM play a significant role in modulating fibroblast function during human skin aging. Furthermore, we conclude that impaired fibroblast function in aged human skin is not solely due to irreversible cellular alterations, but instead dynamically responsive and, in part, reversible via manipulation of the ECM microenvironment (Varani *et al.*, 2002).

We considered the possibility that fibroblast stimulation may occur by direct binding of filler to cellular receptors. Addition of exogenous, monomeric hyaluronic acid (HA) to cultured fibroblasts has been reported to trigger TGF-β signaling and collagen production (David-Raoudi *et al.*, 2008; Mast *et al.*, 1993). Some of these responses are mediated by binding of HA to CD44, a cell surface glycoprotein (David-Raoudi *et al.*, 2008). However, other studies have not reproduced these observations (Croce *et al.*, 2001; Huang *et al.*, 2009). Here, we observed that collagen-producing, elongated fibroblasts were embedded within the ECM adjacent to injected filler and did not appear to directly contact the filler. Moreover, uniform dispersion of filler in dermal equivalent cultures, unlike focal injection, failed to induce fibroblast elongation or procollagen synthesis. These data make it unlikely that injected filler acts through direct interactions with fibroblast receptors.

Our findings, in fact, suggest that collagen production following filler injection occurs by enhanced structural support within the dermal ECM. Supporting this interpretation is a wealth of evidence, derived from model systems indicating that morphology and function of adherent cells are linked by mechanical properties of the ECM (Dupont *et al.*, 2011; Eckes *et al.*, 2006; Fisher *et al.*, 2008; Grinnell, 2003; Ingber, 1997; Kessler *et al.*, 2001; Lambert *et al.*, 1992; Ruoslahti, 1997; Silver *et al.*, 2003; Varani *et al.*, 2006; Varani *et al.*, 2002). For instance, when cultured with fragmented collagen fibrils, fibroblasts display a collapsed appearance, indicative of low mechanical force. These cells lack direct attachment to the ECM and adopt a catabolic phenotype, with decreased collagen synthesis and up-regulation of MMP-1 (Eckes *et al.*, 2006; Kessler *et al.*, 2001; Varani *et al.*, 2006; Varani *et al.*, 2002; Varani *et al.*, 2004). This situation reflects aged human skin (Fisher *et al.*, 2009; Fisher *et al.*, 2008; Fligiel *et al.*, 2003). In contrast, immobilized 3-D matrices composed of intact collagen fibrils provide a stable framework for fibroblast adherence via integrins (Barczyk *et al.*, 2010). In this setting, fibroblasts display an elongated/spread morphology, coupled with procollagen synthesis. This scenario reflects healthy young human skin (Fisher *et al.*, 2008; Fligiel *et al.*, 2003; Quan *et al.*, 2010; Silver *et al.*, 2003; Varani *et al.*, 2006).

Interestingly, fibroblasts cultured in mechanically stiff ECM also up-regulate production of TGF-β and its effector CTGF/CCN2 (Eckes *et al.*, 2006; Garrett *et al.*, 2004; Kessler *et al.*, 2001; Skutek *et al.*, 2001). TGF-β-mediated signaling, in turn, modulates fibroblast responses to mechanical force by stimulating integrin expression and reorganization of the actin cytoskeleton, suggesting that TGF-β is a “mechanoregulatory” growth factor (Brown *et al.*, 2002; Grinnell and Ho, 2002). Additionally, expression of TβRII is reduced in dermal fibroblasts in aged human skin, thus decreasing cellular responsiveness to TGF-β (Quan *et al.*, 2006).

Given these observations, we propose that filler injection into aged skin stiffens the ECM, which induces fibroblast elongation and activation. The result is up-regulation of the TGF-β pathway leading to synthesis and deposition of collagen. Since mature collagen has an estimated half-life of 15 years (Verzijl *et al.*, 2000), it is likely that newly formed collagen fibrils facilitate additional elongation/spreading of fibroblasts and, hence, further activation of TGF-β signaling.

We found that filler injection stimulates localized proliferation of fibroblasts, many of which are synthetically active. Fibroblast proliferation can be driven by numerous mechanisms, including increased mechanical force (Eckes *et al.*, 2006; Varani *et al.*, 2002). It has been reported that substrate stiffness controls proliferation in a variety of cell types through integrin-dependent signaling to FAK, Rac, and cyclin D1 (Klein *et al.*, 2009). Similar mechanisms may be operative in human skin in response to enhanced structural support by injection of cross-linked hyaluronic acid. Increased fibroblast number would be expected to contribute to collagen production following filler injection.

Along with decreased fibroblast function and proliferation, reduced vasculature and epidermal thinning contribute to fragility and impaired wound healing in aged skin (Chung and Eun, 2007; Holt *et al.*, 1992; Zouboulis and Makrantonaki, 2011). Here, we observed that enhanced structural support of the dermal ECM is associated with proliferation of endothelial cells and keratinocytes. Based on previous studies, proliferation of these cell types might result from increased mechanical force (Chen *et al.*, 1997; Reichelt, 2007), or production of diffusible mediators, such as vascular endothelial growth factor (VEGF), TGF-β, CTGF/CCN2, and/or cysteine-rich angiogenic inducer 61 (Chen *et al.*, 2001; Chen and Du, 2007; Kessler *et al.*, 2001; Shirakata, 2010). Additional studies may clarify which of these mechanisms are involved in stimulating endothelial and keratinocyte proliferation following enhancement of structural support.

Together, our findings extend current knowledge of mechanisms of skin aging beyond intrinsic cellular processes to include the dermal ECM microenvironment. Our data indicate that collagen production in aged skin can be substantially restored. Restoration of this synthetic capacity is intimately linked with structural integrity/support of the dermal ECM, which dynamically interacts with fibroblasts and modulates their function and proliferation. Our data also suggest that proliferation and function of other cell types, including endothelial cells and keratinocytes, can be enhanced in aged skin. These findings provide a rationale for maintaining and/or enhancing the structural integrity of dermal ECM, which in turn may improve the health, function, and wound healing capacity of aged human skin.

METHODS

Procurement of skin specimens

This study was approved by our Institutional Review Board, and conducted according to the Declaration of Helsinki principles. Healthy volunteers (74–95 years-old, 18 females, 10 males) provided written informed consent and underwent 2 injections of filler (Restylane®, cross-linked hyaluronic acid, Medicis, Scottsdale, AZ) and 2 injections of vehicle (0.9% NaCl in sterile water) into buttock skin. Injections were each 0.5ml and spaced 2–4 cm apart. Later, sites were located reliably, and punch biopsies (4 mm) were obtained under local anesthesia (lidocaine) at 4 and 12 weeks (n=22), or 1 and 2 weeks (n=6). Specimens were stored in OCT at −80°C, or immediately placed into cell culture medium for collagen-labeling studies.

Type I procollagen ELISA

Using whole cell extracts from cryosections (1000μm), type I procollagen levels were measured, according to manufacturer protocol (Takara Bio, Japan), as previously described (Orringer *et al.*, 2011).

Immunohistochemical/immunofluorescence staining

Staining was performed as described previously (Fisher *et al.*, 2009; Quan *et al.*, 2002b; Quan *et al.*, 2006; Wang *et al.*, 2007) using antibodies against HSP47 (Stressgen Biotechnologies, Victoria, Canada), prolyl-4-hydroxylase (Acris, Hiddenhausen, Germany), type I procollagen (Takara Bio; used in human skin studies), type I procollagen (obtained as described previously (Kang *et al.*, 2005); used in organ culture studies), Ki67 (BioGenex, San Ramon, CA) and CD31 (BD Pharmingen, San Diego, CA). Sections were analyzed with Image-Pro Plus v4.1 (Media Cybernetics, Silver Spring, MD). Matching subtype, non-immune antibodies were used as controls to determine non-specific signal. In all cases, immune antibodies were used at concentrations and fixation conditions that yielded no observable non-specific staining.

Measurement of gene expression

After total RNA extraction (RNeasy Micro kit, Qiagen, Valencia, CA), real-time PCR was performed, as previously described (Quan *et al.*, 2002a, 2004).

Laser capture microdissection

As previously described (Quan *et al.*, 2002a, 2004), approximately 200 fibroblasts from each cryosection (14μm) were collected in lysis buffer (RNeasy Micro kit, Qiagen), followed by RNA extraction and RT-PCR, as described above.

Collagen synthesis in skin organ culture

For each subject, 2 punch biopsies (2mm) of vehicle-injected skin and 2 punch biopsies (2mm) of filler-injected skin were cultured in labeling medium (DMEM/Ham-F12 1:1, v/v, 1% FBS, 50μg/ml L-ascorbic acid, 2 μCi/ml [^{14}C]-proline, and 46 μg/ml L-proline). After incubation at 37°C under 5% CO_2 for 2 days, samples were rinsed 3 times in PBS, frozen in liquid nitrogen, powdered, and weighed. Soluble proteins were extracted under rotation for 24 hours at 4°C in 10mM Tris, pH 7.5, 0.15M NaCl, 5mM EDTA, and protease inhibitors (Complete Mini, Roche, Indianapolis, IN), followed by centrifugation at 16,000g at 4°C for 30 minutes. Mature, extractable collagens were released from the resulting pellet by adding 1mg/ml pepsin (Sigma-Aldrich, St. Louis, MO) in 0.5M acetic acid at 4°C for 16 hours, repeated 5 times. Remaining insoluble, cross-linked material was used for radioactivity counting. Counts per minute were normalized to mg of tissue.

Atomic force microscopy

Cryosections (10μm) were mounted on microscope cover glass (1.2mm diameter, Fisher Scientific, Pittsburgh, PA), allowed to air dry at least 24 hours, and examined using a Dimension Icon AFM system (Bruker AXS, Santa Barbara, CA) in tapping mode, with a silicon-etched cantilever (NSC15/AIBS, MikroMasch, San Jose, CA) with a full tip cone

angle ~40° and tip radius of curvature ~10 nm. Images were acquired at a scan rate of 1.0Hz at 512×512 pixel resolution, with integral and proportional gain settings of 0.4 and 0.6, respectively. Image quality was optimized by dynamically lowering the scan rate and setpoint, and increasing the gains and drive amplitude. Images were analyzed using NanoScope Analysis software v1.20 (BrukerAXS).

Dermal equivalent cultures

Collagen lattices were prepared using early passage (<10 passages) primary adult dermal fibroblasts (2×10^5), obtained as previously described (Fisher *et al.*, 1991), mixed with type I collagen from calf skin (6mg/ml, Elastin Products Company, Owensville, MO) and medium (pH7.2, DMEM, 44mM $NaHCO_3$, 4mM L-glutamine, 9mM folic acid, and 1N NaOH). After formation of lattices (0.5ml/well) (Fisher *et al.*, 2009), 10–15 μl of filler, non cross-linked hyaluronic acid (0.2mg.ml, Sigma Chemical Co., St Louis), or vehicle (PBS) was injected into lattice centers and incubated for 48 hours at 37°C under 5% CO_2. Viable cells were recovered, as previously described (Fisher *et al.*, 2009). For certain wells, filler was dispersed/mixed throughout the medium before lattice formation. For TβRI kinase inhibition, lattices were pretreated overnight with SB431542 (10μM, Sigma-Aldrich) or an equivalent volume of vehicle (DMSO), prior to filler injection.

Statistics

Data are presented as means±SEM. When appropriate, logarithmic transformation of data was performed to achieve normality. For data with a small sample size ($n<9$), normality was assumed. Comparisons between treatment groups were assessed at each time point using the paired t-test. An overall α-level of 0.05 was used to determine statistical significance, and all tests were two-sided. Data were analyzed using SAS v9.2 (SAS Institute, Cary, NC).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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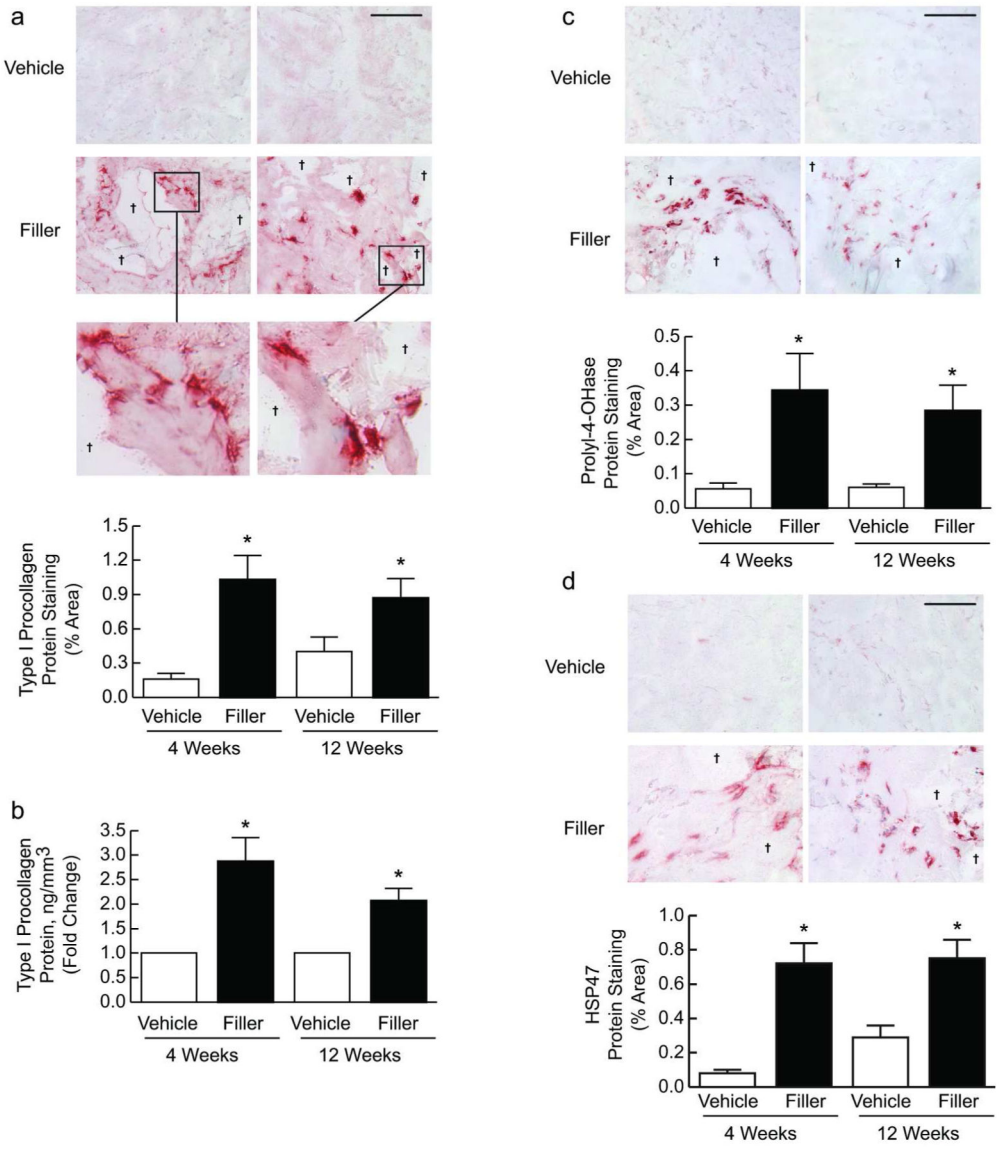

Figure 1. Type I procollagen expression in aged human skin is restored by enhancing structural support of the dermal microenvironment

Aged human skin was biopsied 4 and 12 weeks after injection of vehicle or filler. (**A**) Immunostaining of type I procollagen protein at 4 weeks (left panels) and 12 weeks (right panels) (n=21). Insets display elongated morphology of immunostained fibroblasts adjacent to pools of filler (†). Graphs display staining quantification. (**B**) The level of type I procollagen protein was determined by ELISA (n=22). Immunostaining localization of (**C**) prolyl-4-hydroxylase (n=22) and (**D**) heat shock protein 47 (HSP47, n=10) to fibroblasts adjacent to pools of filler (†) at 4 weeks (left panels) and 12 weeks (right panels), with quantification of staining. All images, bars=100μm; means+SEM. *p<0.05.

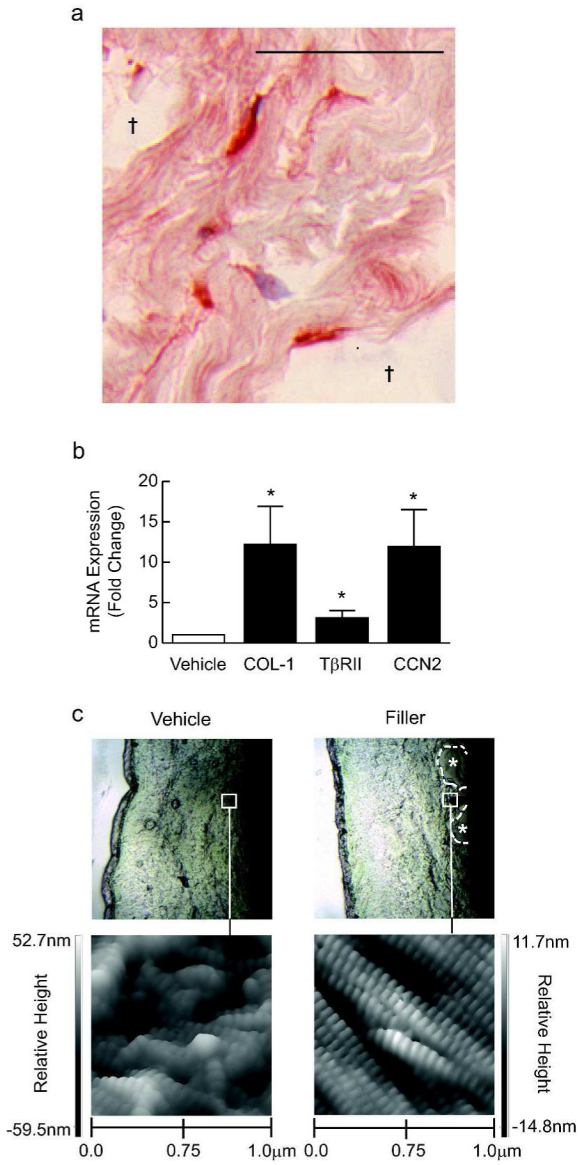

Figure 2. Enhanced structural support up-regulates TGF-β pathway and collagen deposition in aged human skin fibroblasts

Skin was obtained 4 weeks after injection of vehicle or filler. (**A**) Image of pools of injected filler (†), with adjacent elongated fibroblasts immunostained for type I procollagen (bar=50μm). (**B**) Fibroblasts from vehicle- and filler-injected skin were isolated by laser capture microdissection, and analyzed for type I procollagen (COL-1, n=9), type II TGF-β receptor (TβRII, n=9) and connective tissue growth factor (CCN2, n=7). Means+SEM, *p<0.05. (**C**) Nanoscale structure of collagen fibrils imaged by atomic force microscopy (n=3). Upper panels display probe location in mid dermis. Collagen fibrils with characteristic banded pattern appear intact, tightly packed, and spatially organized in filler-injected skin, but fragmented and disorganized in vehicle-injected skin.

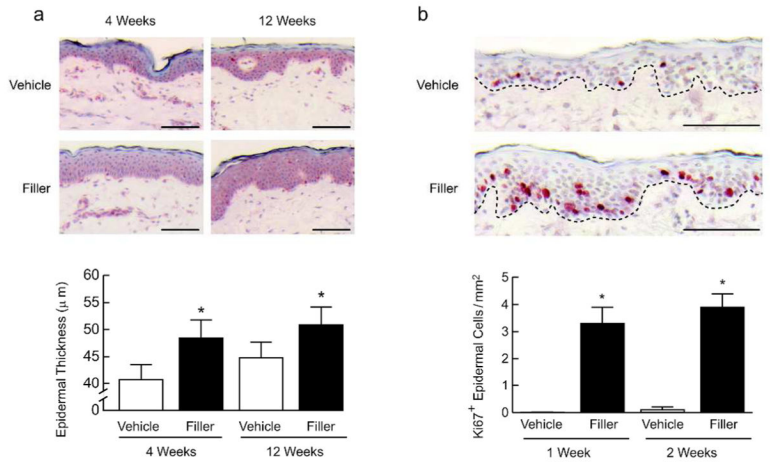

Figure 3. Increased epidermal thickening and keratinocyte proliferation is associated with enhanced structural support of the dermal microenvironment in aged human skin

Aged human skin was injected with vehicle or filler. Skin samples were obtained between 1 and 12 weeks later. (**A**) Epidermal thickness of skin samples was measured by computerized image analysis (n=21, bars=100μm). (**B**) Immunostaining of the proliferation marker Ki67, with positive cell staining in the epidermis quantified by image analysis (n=6, bars=100μm), and representative images at 1 week post-injection shown. Bar graphs display means+SEM. *p<0.05.

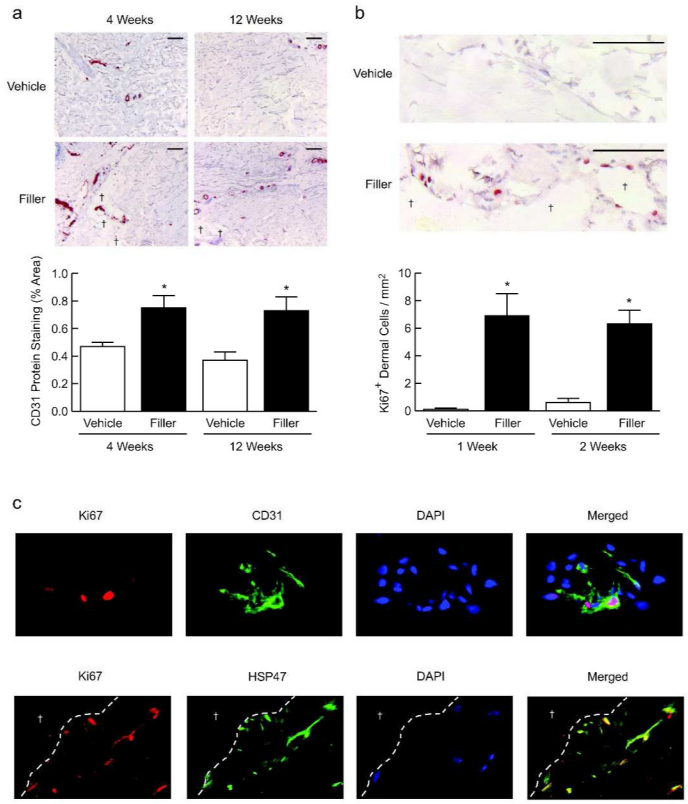

Figure 4. Proliferation of endothelial cells and fibroblasts is associated with enhanced structural support of the dermal microenvironment in aged human skin

Aged human skin was injected with vehicle or filler, and biopsied between 1 and 12 weeks later. Immunostaining of (**A**) Endothelial cell marker CD31 (n=10, bar=100μm) and (**B**) Ki67 (n=6, bar=100μm) near pockets of injected filler (†), with quantification of staining. (**C**) Double-label immunofluorescence staining of the dermis near injected filler, with Ki67 (red) plus CD31 (green, top panels) or HSP47 (green, bottom panels) (n=6). Nuclei are stained with DAPI (blue). Dashed lines separate dermal ECM from pools containing injected filler (†). All representative images are from 2 weeks post-filler injection. Bar graphs, means +SEM. *p<0.05.

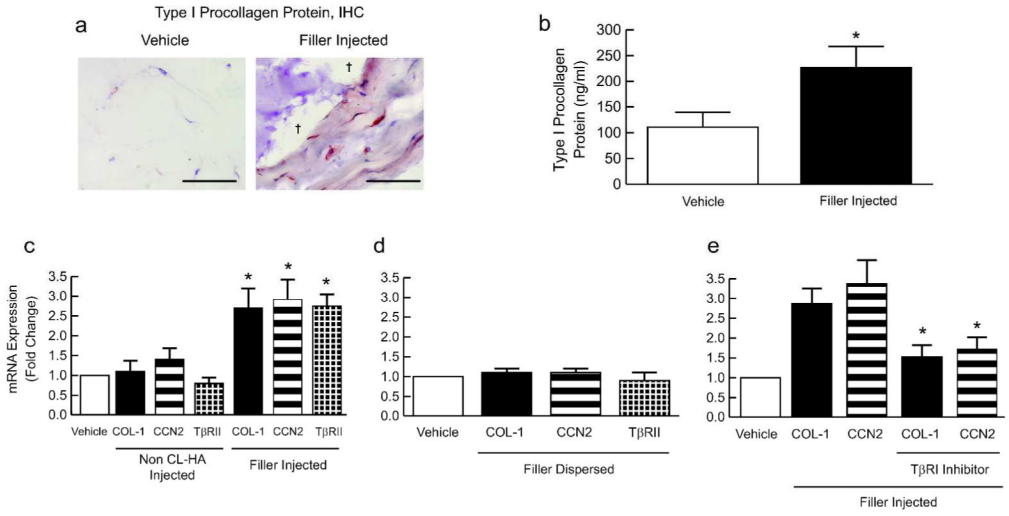

Figure 5. Enhanced structural support of dermal equivalent cultures induces fibroblast elongation and up-regulates TGF-β pathway and collagen production

Dermal equivalent cultures were analyzed 2 days after treatment. (**A**) Immunostaining of type I procollagen within elongated fibroblasts adjacent to pools of injected filler (†, purple) (n=3, bar=100μm). (**B**) Type I procollagen protein secreted into culture media was quantified by ELISA (n=3). Type I procollagen (COL-1), CCN2, and type II TGF-β receptor (TβRII) gene expression following : (**C**) vehicle, non cross-linked hyaluronic acid (CL-HA), or filler injection into preformed lattices (n=4, *p≤0.05 vs. vehicle), (**D**) vehicle or filler dispersed in collagen solution prior to lattice formation (n=4), (**E**) addition of type I TGF-β receptor (TβRI) kinase inhibitor prior to injection of vehicle or filler (n=4,*p<0.05 vs. filler injection without inhibitor).