

# Age-Associated Increase in Skin Fibroblast–Derived Prostaglandin E<sub>2</sub> Contributes to Reduced Collagen Levels in Elderly Human Skin

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Production of type I collagen declines during aging, leading to skin thinning and impaired function. Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is a pleiotropic lipid mediator that is synthesized from arachidonic acid by the sequential actions of cyclooxygenases (COX) and PGE synthases (PTGES). PGE<sub>2</sub> inhibits collagen production by fibroblasts *in vitro*. We report that *PTGES1* and *COX2* progressively increase with aging in sun-protected human skin. *PTGES1* and *COX2* mRNA were increased 3.4-fold and 2.7-fold, respectively, in the dermis of elderly (>80 years) versus young (21–30 years) individuals. Fibroblasts were the major cell source of both enzymes. PGE<sub>2</sub> levels were increased 70% in elderly skin. Fibroblasts in aged skin display reduced spreading due to collagen fibril fragmentation. To investigate the relationship between spreading and PGE<sub>2</sub> synthesis, fibroblasts were cultured on micropost arrays or hydrogels of varying mechanical compliance. Reduced spreading/mechanical force resulted in increased expression of both *PTGES1* and *COX2* and elevated levels of PGE<sub>2</sub>. Inhibition of PGE<sub>2</sub> synthesis by diclofenac enhanced collagen production in skin organ cultures. These data suggest that reduced spreading/mechanical force of fibroblasts in aged skin elevates PGE<sub>2</sub> production, contributing to reduced collagen production. Inhibition of PGE<sub>2</sub> production may be therapeutically beneficial for combating age-associated collagen deficit in human skin.

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## INTRODUCTION

Elderly skin is typically thin and fragile, with increased susceptibility to bruising and impaired wound healing (Fisher *et al.*, 2008). These alterations largely reflect fragmentation and reduction in type I collagen fibrils, which comprise the bulk of the dermal extracellular matrix (ECM) (Varani *et al.*, 2006). Dermal fibroblasts secrete type I procollagen, which is converted into collagen via proteolytic modifications. Mature collagen self-assembles into collagen fibrils, which are stabilized by inter- and intra-fibrillar cross-linking (Canty and Kadler, 2005). Although mature cross-linked collagen fibrils are very stable, with a half-life of approximately 15 years (Verzijl *et al.*, 2000), gradual cleavage, mediated by matrix metalloproteases, occurs during aging (Fisher *et al.*,

2009). This cleavage results in the accumulation of cross-linked fragments, owing to resistance of cross-links to proteolysis. Thus, during aging, the dermal ECM becomes progressively degraded and disorganized, which deleteriously alters the function of resident fibroblasts (Fisher *et al.*, 2008).

Fibroblasts attach to surrounding collagen fibrils to form adhesion complexes, which act through the cytoskeleton to exert contractile forces. Resistance to this contraction generates mechanical forces within fibroblasts, which largely determine morphology, cytoskeletal organization, signal transduction, and gene expression (Tarutani *et al.*, 2003; Hegedus *et al.*, 2008; Mammoto *et al.*, 2013). Collagen fragmentation causes loss of attachment sites, resulting in reduced fibroblast spreading, which is observed in aged human skin. This contracted state, with concomitant reduced mechanical force, is associated with downregulation of collagen fibril production in human skin (Varani *et al.*, 2006; Fisher *et al.*, 2008; Xia *et al.*, 2013).

PGE<sub>2</sub> is a pleiotropic lipid signaling molecule produced by multiple cell types (Humphrey *et al.*, 2014). PGE<sub>2</sub> is synthesized from arachidonic acid by the sequential actions of cyclooxygenases (COX1 and COX2) and prostaglandin E synthases (PTGES1, 2, and 3) (Liu *et al.*, 1995; Iskratsch *et al.*, 2014). Elevated COX2 often coincides with PTGES1 induction

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Abbreviations: COX2, cyclooxygenase 2; ECM, extracellular matrix; EIA, enzyme immunoassay; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; PTGES, prostaglandin E synthase

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in a wide variety of tumor lesions and in response to inflammatory stimuli (Verrecchia *et al.*, 2001; Pickup *et al.*, 2013; Quan and Fisher, 2015). COX1, PTGES2, and PTGES3 are not typically inducible or involved in excess PGE<sub>2</sub> production seen in abnormal conditions. PGE<sub>2</sub> is a major prostaglandin in human skin and is normally synthesized at low levels but is markedly increased in skin squamous cell carcinoma and inflammatory conditions, such as sunburn (Muller-Decker, 2011). Non-steroidal anti-inflammatory drugs are a class of commonly used drugs including aspirin, ibuprofen, indomethacin, and diclofenac, which inhibit the activities of both COX1 and COX2, thereby inhibiting PGE<sub>2</sub> production (Qin *et al.*, 2014). COX2 and PTGES1 induction in response to acute inflammatory stimuli has been extensively investigated (Verrecchia *et al.*, 2001; Samuelsson *et al.*, 2007; Pickup *et al.*, 2013; Quan and Fisher, 2015); however, the potential effects of cellular mechanical forces on COX2 or PTGES1 expression are unknown.

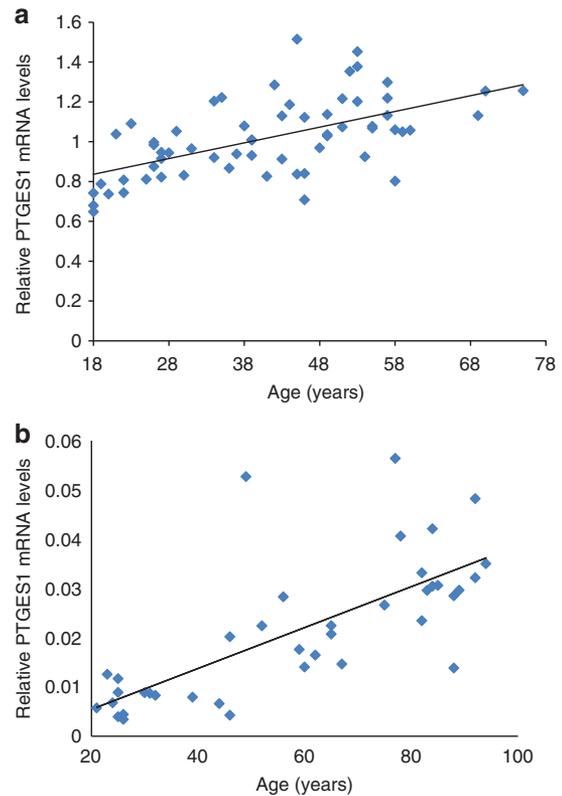
In cell cultures, PGE<sub>2</sub> inhibits fibroblast collagen production, in part through impeding actions of transforming growth factor- $\beta$  (Sandulache *et al.*, 2007). The inhibitory effect of PGE<sub>2</sub> on collagen synthesis has been shown to have a protective role against lung fibrosis, where impaired PGE<sub>2</sub> production and action are implicated in excessive fibroblast collagen deposition (Huang *et al.*, 2007).

This study reports that *PTGES1* mRNA expression progressively increases during aging and investigates the role of PGE<sub>2</sub> in the age-related decline of type I collagen production in human skin. Taken together, the data support the concept that the dermal microenvironment raises PTGES1 and PGE<sub>2</sub> levels, which contributes to reduced collagen in aged skin. Given that PGE<sub>2</sub> synthesis can be effectively inhibited by a wide range of agents (Qin *et al.*, 2014), targeting PGE<sub>2</sub> is an appealing strategy to combat age-associated skin collagen deficiency.

## RESULTS

### *PTGES1* mRNA expression progressively increases during aging in human skin *in vivo*

In order to investigate age-related alterations in gene expression, total RNA was extracted from full-thickness skin samples obtained from sun-protected buttock skin of persons between the ages of 18 and 75 ( $n=62$ ) (Swindell *et al.*, 2012). Global gene expression analysis was conducted using an *in situ* oligonucleotide array platform (Affymetrix Human Genome U133 Plus 2.0 array). Out of 19,851 human genes, 268 exhibited statistically significant age-associated changes in expression levels (false discovery rate  $<0.10$ ). *PTGES1* expression positively correlated with age, and this correlation was the most statistically significant. *PTGES1* expression was assessed by two probes targeting two different regions of the *PTGES1* transcript and yielded similar correlation coefficients and yearly rates of increase, as calculated by linear regression. The results obtained from one probe, 210367\_s\_at, are shown in Figure 1a. The linear correlation between increased *PTGES1* expression and increased age was highly statistically significant ( $P=2.6 \times 10^{-7}$ ,  $n=62$ ), and the degree of linearity, expressed as Pearson's correlation coefficient, was  $r=0.6$  ( $r$  can vary from 0 to 1, with 0



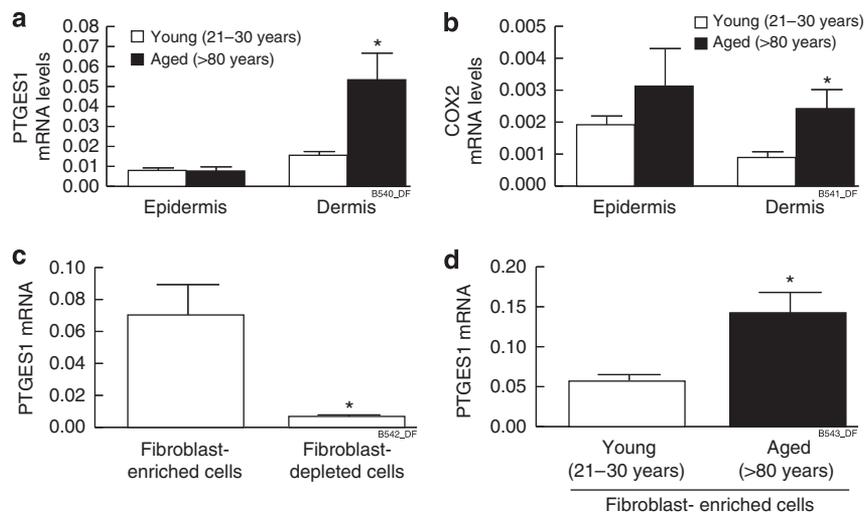
**Figure 1. *PTGES1* mRNA expression progressively increases in human skin during chronological aging.** (a) Relative *PTGES1* mRNA expression in human buttock skin was determined by cDNA microarray analysis. Results show positive correlation of the *PTGES1* mRNA level and age of donors ( $n=62$ ,  $P=2.6 \times 10^{-7}$ ,  $r=0.60$ ). (b) *PTGES1* mRNA expression in buttock skin was determined by qPCR. ( $n=40$ ,  $P=1.38 \times 10^{-7}$ ,  $r=0.73$ ). The subjects used in microarray analysis were different from the subjects used in qPCR analysis. cDNA, complementary DNA.

representing no correlation and 1 representing perfect linear correlation). When compared with young skin (18 years of age), elderly skin (75 years of age) had a 1.6-fold overall increase in *PTGES1* expression.

In order to substantiate the age-associated increase in *PTGES1*, we acquired buttock skin samples from an additional 40 subjects aged 21–94. Consistent with our microarray data, *PTGES1* expression progressively increased with age as determined by quantitative PCR (qPCR) and increased 2.92-fold overall in 94-year- versus 21-year-old skin ( $n=40$ ,  $P=1.38 \times 10^{-7}$ ,  $r=0.73$ ) (Figure 1b). Taken together, these data demonstrate an intriguing correlation between aging and skin *PTGES1* gene expression.

### Dermal fibroblasts are the primary source of increased expression for both *PTGES1* and *COX2* mRNA in aged human skin

Elevation of *COX2* expression often occurs concomitantly with *PTGES1* induction. However, *COX2* mRNA levels in whole skin preparations did not significantly differ between young and aged individuals (data not shown). We hypothesized that dermal expression of *PTGES1* and *COX2* mRNA



**Figure 2. Dermal fibroblasts are a major cell source for increased expression of *PTGES1* and *COX2* in aged (>80 years) versus young (21–30 years) skin.** The dermis and the epidermis of frozen skin sections were separated and collected using laser capture microscopy. Relative (a) *PTGES1* and (b) *COX2* mRNA levels in the epidermis and dermis of young and aged individuals were determined by qPCR normalized to 36B4 mRNA.  $n=6$ ,  $*P<0.05$ . (c and d) Cells were released from fresh sun-protected skin samples by collagenase digestion. Released cells were fractionated by immuno-affinity magnetic beads to separate fibroblasts for other cell types. Total RNA was extracted from isolated cells. (c) *PTGES1* mRNA levels were determined in fibroblast-enriched and fibroblast-depleted cells ( $n=4$ ,  $*P<0.05$ ). (d) *PTGES1* mRNA levels were determined in fibroblast-enriched cells isolated from skin samples from young and aged individuals. ( $n=5-7$ ,  $*P<0.05$ ).

may account for age-dependent differences. In order to determine dermal and epidermal *PTGES1* and *COX2* expression, we used laser capture microdissection of skin sections, followed by qPCR. Consistent with our hypothesis, dermal, but not epidermal, *PTGES1* and *COX2* gene expression were significantly different in young versus aged individuals. *PTGES1* and *COX2* levels were significantly increased 3.4-fold ( $P<0.05$ ) and 2.7-fold, respectively ( $P<0.05$ ), in aged dermis (Figure 2a and b). This finding suggests that aging dermal, but not epidermal, cells express more *PTGES1* and *COX2*.

In order to determine whether fibroblasts are responsible for elevated dermal *PTGES1* gene expression, we separated fibroblasts from other dermal cells in skin samples using anti-fibroblast antibody-coated magnetic microbeads. Fibroblast enrichment was validated by mRNA quantification of several cell markers (Supplementary Table S1 online). Isolated cells were directly analyzed without culturing. *PTGES1* mRNA levels were approximately 10-fold ( $P<0.05$ ) higher in fibroblast-enriched cells than in fibroblast-depleted dermal cells (Figure 2c). These data indicate that dermal fibroblasts are responsible for the majority of dermal *PTGES1* expression.

In order to determine whether dermal fibroblasts are responsible for the changes to *PTGES1* expression in aging, we isolated fibroblasts from young (21–30 years) and aged (>80 years) skin. Consistent with our data, *PTGES1* mRNA expression was 2.2-fold higher in fibroblasts from aged skin ( $P<0.05$ ) (Figure 2d). We also quantified *COX2* mRNA expression in isolated fibroblasts; however, *COX2* mRNA levels in freshly isolated fibroblasts were markedly elevated by the isolation process. Taken together, the data suggest that dermal fibroblasts are the primary cell source of elevated *PTGES1* and *COX2* mRNA expression in aged skin.

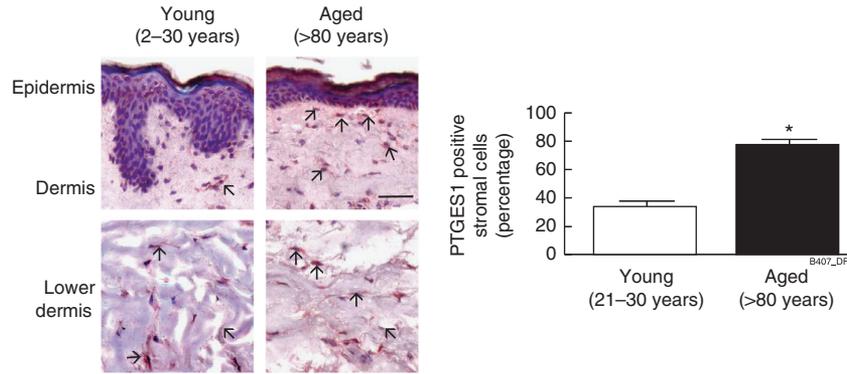
#### **PTGES1 protein expression is elevated in skin fibroblasts of elderly (>80 years) versus young (21–30 years) individuals**

In order to determine whether *PTGES1* protein levels were increased in aged dermal fibroblasts, we performed immunohistochemistry on both young and aged skin samples. Positive staining that overlapped with or was immediately adjacent to the nuclei was most prominently seen in dermal stromal cells in both young and aged skin. In addition, *PTGES1*-positive dermal cells displayed morphology characteristic of fibroblasts, including being embedded in collagenous ECM without direct contact with other cells or structures, elongated cell bodies, and oval shaped nuclei (Figure 3). Statistical analysis showed that the percentage of positively stained dermal stromal cells was increased 2.1-fold (75 vs. 35%,  $P<0.01$ ) in aged skin. These data suggest that *PTGES1* protein is preferentially expressed by fibroblasts, and fibroblast-derived *PTGES1* protein expression is increased in the aged dermis, consistent with increased *PTGES1* mRNA expression (Figure 2).

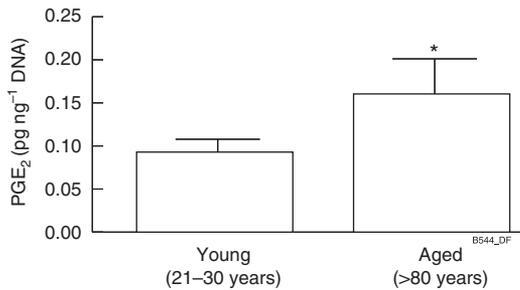
*COX2* protein expression was also examined by immunohistochemistry but did not yield specific staining, consistent with previous studies showing that *COX2* protein expression in normal human skin is below the limit of immunohistochemistry detection (Hoot *et al.*, 2010).

#### **PGE<sub>2</sub> levels are higher in elderly versus young skin**

Age-associated increases in *COX2* and *PTGES1* likely result in enhanced PGE<sub>2</sub> production. To examine this possibility, we incubated fresh samples of buttock skin from young (21–30 years) and aged (>80 years) persons in culture medium for 5 minutes and quantified PGE<sub>2</sub> levels in the conditioned media. PGE<sub>2</sub> levels were 1.7-fold higher ( $P<0.05$ ) in skin specimens from elderly individuals (Figure 4). Taken together,



**Figure 3. PTGES1 protein expression is increased in fibroblasts in aged human skin.** Immunohistochemistry of PTGES1 protein in young (21–30 years old) and aged (>80 years old) sun-protected buttocks human skin. Left panels: representative immunostaining in upper and lower dermis. PTGES1 protein staining is brownish, and nucleus counterstaining with hematoxylin is blue. Arrows point to PTGES1 positively stained cells. Right panel: Percentage of total dermal stromal cells that were PTGES1-positive. (*n* = 6, \**P* < 0.01) Scale bar = 50 μm.



**Figure 4. Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) levels are increased in the skin of elderly compared with young individuals.** Skin biopsies (2 mm) were obtained from young (21–30 years) and elderly (>80 years) sun-protected buttock skin. PGE<sub>2</sub> levels were quantified by substrate enzyme immunoassay and normalized to DNA content of corresponding skin samples. (*n* = 10, \**P* < 0.05).

these data indicate that increased *COX2* and *PTGES1* expression in aged dermal fibroblasts is associated with increased PGE<sub>2</sub> production in human skin *in vivo*.

**Enhanced ECM compliance elevates *COX2* and *PTGES1* expression and PGE<sub>2</sub> synthesis in primary adult human dermal fibroblasts**

Reduced fibroblast spreading/mechanical force is considered a driving force of fibroblast dysfunction in elderly skin (Fisher *et al.*, 2008). We therefore examined whether *PTGES1* and *COX2* expression were regulated by changes to ECM compliance. For these studies, we utilized micropost arrays and hydrogel assays. Fibroblasts were cultured on a micropost array with two different compliances—low compliance (15.3 kPa) and high compliance (1.3 kPa).

Electron micrographs (Figure 5a) of fibroblasts adhered to type I collagen-coated micropost arrays revealed bending of the microposts through cytoskeleton contractile forces. More compliant microposts (1.3 kPa) were more deformed, resulting in reduced fibroblast spreading, resembling fibroblasts seen in fragmented collagenous ECM of aged dermis *in vivo* (Fisher *et al.*, 2008). Less compliant microposts (15.3 kPa) resisted

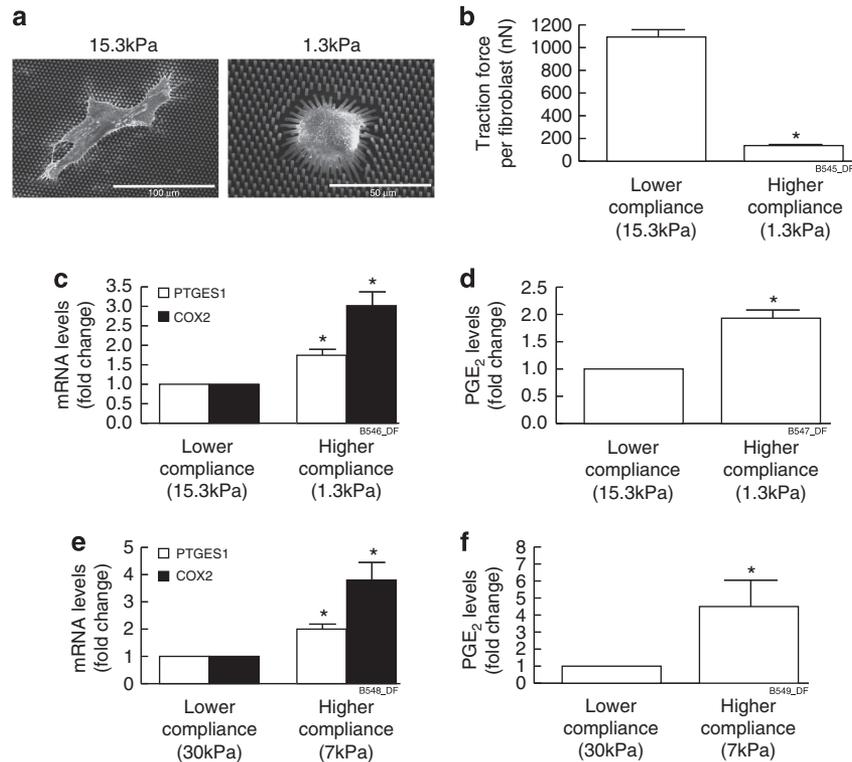
deformation and fibroblasts had a spread morphology, resembling the stretched fibroblasts seen in the intact collagenous ECM of young dermis *in vivo* (Figure 5a). On average, fibroblasts cultured on 1.3 kPa microposts displayed a 7.9-fold lower traction force compared with those cultured on 15.3-kPa microposts (Figure 5b), as measured by calculating the number of posts deformed by a cell, as well as the degree of deformation. These data confirm that fibroblasts grown on a less compliant ECM experience more mechanical force.

To determine the effect of mechanical force on PGE<sub>2</sub> production, *PTGES1* and *COX2* mRNA expression were measured in fibroblasts cultured on 15.3 kPa and 1.3 kPa microposts. *PTGES1* and *COX2* expression increased by 1.7-fold and 3.0-fold, respectively, when cultured on more compliant (1.3 kPa) compared with less compliant (15.3 kPa) microposts (Figure 5c). In addition, PGE<sub>2</sub> levels in the conditioned media increased by 1.9-fold (Figure 5d), indicating that increased ECM compliance results in increased PGE<sub>2</sub> production.

To further confirm the stimulatory effects of high ECM compliance on PGE<sub>2</sub> synthesis, we analyzed fibroblasts cultured on type I collagen-coated polyacrylamide hydrogels. Fibroblasts cultured on a more compliant hydrogel (7 kPa) exhibited increased *PTGES1* (2.0-fold) and *COX-2* (3.8-fold) mRNA expression and enhanced PGE<sub>2</sub> levels (4.5-fold) compared with fibroblasts cultured on a less compliant 30 kPa hydrogel (Figure 5e and f). In concert with changes of PGE<sub>2</sub>, but in an opposite direction, procollagen mRNA and protein expression were increased by 2.2- and 2.8-fold, respectively, in 30 kPa hydrogels compared with 7 kPa hydrogels (Supplementary Figure S1 online). Taken together, these data indicate that increased ECM compliance results in reduced fibroblast spreading/mechanical force, which leads to enhanced PGE<sub>2</sub> production and reduction in procollagen expression.

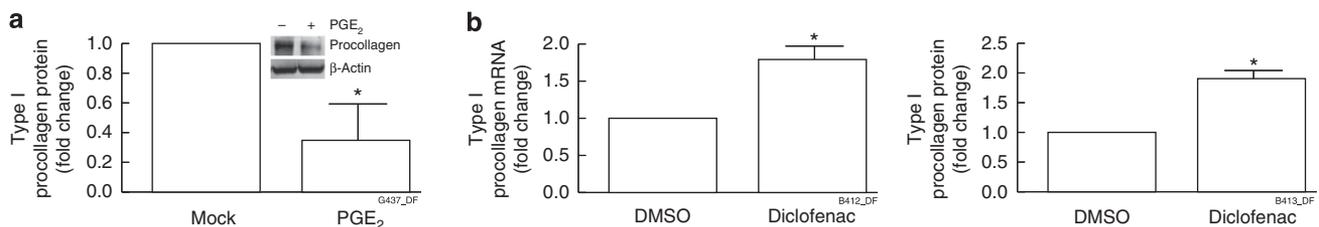
**PGE<sub>2</sub> inhibits procollagen production in human dermal fibroblasts**

We next assessed the effects of PGE<sub>2</sub> on collagen production of fibroblasts from young (21–30 years) skin. Fibroblasts were



**Figure 5. Reduced spreading/mechanical force elevates COX2 and PTGES1 expression and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) levels in human skin fibroblasts.**

(a–d) Fibroblasts obtained from young (21–30 years) individuals seeded on micropost arrays. (a) Scanning electron microscopy of fibroblasts. Micrographs are representative of three experiments examining >60 fibroblasts. (b) Quantification of traction force (nanonewton, nN) for 10 fibroblasts/condition ( $n=3$ ,  $*P<0.001$ ). (c) COX2 and PTGES1 mRNA levels were determined by qPCR ( $n=3$ ,  $*P<0.05$ ). (d) PGE<sub>2</sub> levels in conditioned medium were quantified by EIA and normalized to cell number ( $n=3$ ,  $*P<0.05$ ). (e and f) Fibroblasts cultured from young skin were seeded on type I collagen-coated hydrogels with low or high compliance. (e) PTGES1 and COX2 mRNA levels were quantified by qPCR. ( $n=3$ ,  $*P<0.05$ ). (f) PGE<sub>2</sub> levels in conditioned media quantified by EIA. ( $n=3$ ,  $*P<0.05$ ). EIA, enzyme immunoassay.



**Figure 6. Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) inhibits procollagen production in adult human skin fibroblasts.** (a) Fibroblasts were cultured with the addition of PGE<sub>2</sub> (10 nM) or vehicle (DMSO) for 24 hours. Type I procollagen protein levels in cell lysates were determined by western blot, normalized to  $\beta$ -actin. Inset shows representative western blots. ( $n=5$ ,  $*P<0.05$ ). (b) Skin samples were incubated in serum-free  $\alpha$ -MEM media with the addition of diclofenac (10  $\mu$ M) or vehicle (DMSO) for 16 hours. Type I collagen mRNA levels were quantified by qPCR and normalized to 36B4 mRNA levels ( $n=6$ ). Type I procollagen protein levels in conditioned media were quantified by EIA. ( $n=3$ ,  $*P<0.05$ ).

cultured in serum-free medium in the presence of PGE<sub>2</sub> or vehicle (DMSO) for 24 hours. Type I procollagen protein was examined by western blot. PGE<sub>2</sub> treatment reduced procollagen protein levels by approximately 66% ( $P<0.05$ ) (Figure 6a).

In order to determine whether inhibition of PGE<sub>2</sub> production can enhance collagen production, we examined the effect of the general COX inhibitor, diclofenac, and the COX2-specific inhibitor, celecoxib, in organ culture of skin

samples obtained from buttock skin. Fresh skin biopsies were cultured in the presence of inhibitor or vehicle (DMSO) for 16 hours. Type I procollagen protein in conditioned medium was quantified by enzyme immunoassay (EIA). Biopsies also underwent RNA extraction for quantification of type I procollagen expression. Diclofenac significantly reduced PGE<sub>2</sub> levels by  $92 \pm 7\%$  ( $n=3$ ,  $P<0.05$ , data not shown) and enhanced procollagen mRNA and protein expression by 2.3-fold and 1.9-fold, respectively ( $P<0.05$ ) (Figure 6b).

Celecoxib reduced PGE<sub>2</sub> levels by approximately 80%, and enhanced procollagen mRNA expression by 1.9-fold (Supplementary Figure S2 online), suggesting that PGE<sub>2</sub> derived from COX2 is largely responsible for inhibition of procollagen expression in human skin organ cultures. The results are consistent with the notion that increased COX-2 expression in aged dermis can lead to increased PGE<sub>2</sub> production and consequently reduced collagen expression.

## DISCUSSION

Chronological aging is a progressive process driven by the accumulation and synergy of subtle alterations, resulting in declining organ structure and function. Because of its accessibility, the skin is an ideal organ for investigating the cumulative nature of aging and its underlying causalities in humans (Fisher *et al.*, 2008). Age-related increase in PTGES1 mRNA and protein expression was consistently revealed by examining whole skin, isolated dermis, and freshly isolated fibroblasts, using different methods and different groups of donors. Fibroblasts in aged human skin displayed comparable increases in mRNA (2.2-fold) and protein (2.1-fold) levels, although direct quantitative comparisons between results obtained from two different techniques should be interpreted with caution.

Although we found that epidermal COX2 and PTGES1 mRNA expression do not differ between young and aged, the results do not exclude the possibility that aged epidermal cells may also contribute to enhanced PGE<sub>2</sub> levels observed in aged skin. The relative contribution of dermal and epidermal cells to enhanced PGE<sub>2</sub> levels observed in whole skin specimens remains to be determined by future investigations.

We have proposed that age-associated molecular and cellular alterations in the dermis create a self-perpetuating cycle that drives the progression of ECM degeneration (Fisher *et al.*, 2014). Age-associated fragmentation of collagen fibrils impairs interactions of fibroblasts with the collagenous ECM, leading to suppression of collagen production and enhanced production of matrix metalloproteases, which collectively cause further deterioration of the ECM. Impaired ECM causes further fibroblast dysfunction in a self-sustaining loop (Varani *et al.*, 2006; Fisher *et al.*, 2008; Xia *et al.*, 2013). This study implicates PGE<sub>2</sub> in this self-perpetuating cycle. Impaired ECM causes reduced mechanical force within fibroblasts, which in turn elevates COX2 and PTGES1 gene expression, likely through activations of transcription factors such as NF-κB, which are known to regulate expression of these two genes (Bage *et al.*, 2010).

Several age-associated alterations found in skin fibroblasts *in vivo* may conspire to cause reduced collagen production in the context of collagen fibril fragmentation. In addition to PGE<sub>2</sub>, previous studies have found reduced expression of connective tissue growth factor/CCN2 and enhanced expression of CCN1 (cysteine-rich protein 61) in aged human skin fibroblasts. These alterations inhibit collagen production, at least in part, through blockade of transforming growth factor-β signaling (Quan *et al.*, 2006; Quan *et al.*, 2010; He *et al.*, 2014). Therapeutics targeting these pathways may retard or reverse the decline of skin collagen production during aging,

thereby improving skin health. Elevated PGE<sub>2</sub> is an appealing target for intervention, as PGE<sub>2</sub> production in human skin can be effectively reduced by topical non-steroidal anti-inflammatory drugs (Qin *et al.*, 2014). This study provides rationale for investigation into the potential beneficial effects of non-steroidal anti-inflammatory drugs for collagen deficiency in elderly skin. Mice models that display elevated skin PGE<sub>2</sub> levels and resultant collagen reduction would provide opportunity for both further mechanistic studies and evaluation of agents that could retard age-related collagen deficiency via the inhibition of PGE<sub>2</sub> synthesis and actions.

In addition to a possible contribution to collagen deficiency, fibroblast-derived PGE<sub>2</sub> may be a risk factor for other prevalent age-associated skin disorders. PGE<sub>2</sub> may impair functions of adjacent immune cells and epidermal keratinocytes via a paracrine mechanism. Given the critical role of PGE<sub>2</sub> in promoting cutaneous squamous cell carcinoma and inhibiting functions of antigen-presenting cells required for allergenic contact dermatitis, elevated fibroblast-derived PGE<sub>2</sub> may contribute to the observed increase in skin cancer and reduced skin immunity in the elderly (Muller-Decker, 2011; Scott *et al.*, 2014). In addition to local effects, skin-derived PGE<sub>2</sub> has been demonstrated to have a systemic impact. This effect is mediated by transit of PGE<sub>2</sub> and PGE<sub>2</sub>-induced cytokines into the circulation (Soontrapa *et al.*, 2011; Ullrich and Byrne, 2012; Scott *et al.*, 2014). Whether constitutively elevated PGE<sub>2</sub> in chronologically aged, sun-protected, skin impacts other organ systems is an intriguing question for future investigations.

## MATERIALS AND METHODS

### Human tissue procurement

Full-thickness skin biopsies (2, 4, and 6 mm in diameter) were taken from buttocks of healthy human subjects as previously described (Li *et al.*, 2013). All procedures involving human subjects were approved by the University of Michigan Institutional Review Board. Informed written consent was obtained from all human subjects.

### Complementary DNA microarray analysis

Affymetrix Human Genome U133 Plus 2.0 arrays (54,675 probe-sets of 19,851 genes) were used for expression profiling (Santa Clara, CA). Normalized expression values were calculated using the robust multichip average algorithm (Irizarry *et al.*, 2003). One probe-set was analyzed for each gene. The representative probe-set was chosen by the highest average robust multichip average expression intensity among all 62 samples. Raw robust multichip average intensities were adjusted to remove gender and batch variation. Simple linear regression was then used to identify genes significantly changed with age. Raw *P*-values were adjusted using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). False discovery rate -adjusted *P*-value threshold of 0.10 was used.

### Laser-capture microdissection

Laser capture microdissection was performed as previously described (Li *et al.*, 2013). Briefly, 30 tissue sections were prepared from each skin biopsy embedded in optimal cutting temperature. Appendage-free dermis and interfollicular-epidermis were separately

collected using laser capture microdissection (Leica ASLMD System; Leica Microsystems, Wetzlar, Germany).

### Fibroblast isolation

Skin biopsies were digested with collagenase (5 mg ml<sup>-1</sup> in DMEM) for 16 hours at 37 °C as described previously (Li et al., 2013). Fibroblasts that were released from the dermis were separated from other cell types using anti-fibroblast Microbeads (Miltenyi Biotec, San Diego, CA) according to the manufacturer's instruction.

### Cell culture

Fibroblasts were cultured from punch biopsies (6 mm) from buttock skin (aged 21–30 years) and maintained as previously described (Fisher et al., 2009). Fibroblasts between passages 3 and 8 were used in this study.

### RNA extraction and qPCR

RNA was extracted using an RNA extraction kit (Qiagen, Chatsworth, CA). qPCR reagents were from Applied Biosystems (Foster City, CA). Ribosomal protein 36B4 was used to normalize gene expression (Li et al., 2013).

### Micropost arrays and hydrogels

Polydimethylsiloxane micropost arrays were fabricated, analyzed by scanning electron microscopy, and used for fibroblast traction force measurements, as previously described (Fu et al., 2010; Bonnans et al., 2014). Hydrogels were fabricated on glass coverslips as previously described (Caulin et al., 2007).

### Protein extraction and western blot analysis

Protein extraction from primary fibroblasts and western analysis were performed as previously described (Li et al., 2013).

### Immunohistochemistry

Immunohistochemistry was performed with anti-PTGES1 mouse monoclonal antibody (Santa Cruz Biotech, Santa Cruz, CA) on frozen skin sections as previously described (Rittie et al., 2009). Fifteen microscopic fields of each section were used for quantitation of cell staining.

### PGE<sub>2</sub> enzyme immunoassay (EIA)

After excision, skin buttock biopsies (2 mm) were immediately incubated in 200 µl α-MEM medium for 5 minutes. PGE<sub>2</sub> levels in the conditioned media were quantified using the EIA assay (Cayman, Ann Arbor, MI). DNA was extracted from skin biopsies using the Micro-DNA Extraction Kit (Qiagen) and quantified by ultraviolet spectrometry. PGE<sub>2</sub> levels were normalized to DNA content in corresponding biopsies. This method was validated by quantifying increased PGE<sub>2</sub> levels in skin biopsies from acute ultraviolet-irradiated skin, compared with non-irradiated skin.

### Type I procollagen EIA from organ cultures

Fresh skin biopsies were cultured in the presence of diclofenac or vehicle (DMSO) for 16 hours. Type I procollagen in organ culture conditioned medium was determined by an EIA kit (Takara Bio, Otsu Shiga, Japan) and normalized to total protein based on the manufacturer's instruction.

### Statistical analysis

The correlations between *PTGES1* levels and age were analyzed using linear regression. Differences between two groups of samples were analyzed using the two-tailed *t*-test. Differences were considered significant when *P* < 0.05. Data are presented as mean ± standard error of mean. *N* numbers represent sample size of each group.

### CONFLICT OF INTEREST

The authors state no conflict of interest.

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### SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

### REFERENCES

- Bage T, Lindberg J, Lundeberg J et al. (2010) Signal pathways JNK and NF-kappaB, identified by global gene expression profiling, are involved in regulation of TNFalpha-induced mPGES-1 and COX-2 expression in gingival fibroblasts. *BMC Genomics* 11:241
- Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Series B Methodol* 57:289–300
- Bonnans C, Chou J, Werb Z (2014) Remodelling the extracellular matrix in development and disease. *Nat Rev Mol Cell Biol* 15:786–801
- Canty EG, Kadler KE (2005) Procollagen trafficking, processing and fibrillogenesis. *J Cell Sci* 118:1341–53
- Caulin C, Nguyen T, Lang GA et al. (2007) An inducible mouse model for skin cancer reveals distinct roles for gain- and loss-of-function p53 mutations. *J Clin Invest* 117:1893–901
- Fisher G, Sachs D, Voorhees J (2014) Ageing: collagenase-mediated collagen fragmentation as a rejuvenation target. *Brit J Dermatol* 171:446–9
- Fisher GJ, Quan T, Purohit T et al. (2009) Collagen fragmentation promotes oxidative stress and elevates matrix metalloproteinase-1 in fibroblasts in aged human skin. *Am J Pathol* 174:101–4
- Fisher GJ, Varani J, Voorhees JJ (2008) Looking older: fibroblast collapse and therapeutic implications. *Arch Dermatol* 144:666–72
- Fu J, Wang YK, Yang MT et al. (2010) Mechanical regulation of cell function with geometrically modulated elastomeric substrates. *Nat Methods* 7: 733–36
- He T, Quan T, Shao Y et al. (2014) Oxidative exposure impairs TGF-beta pathway via reduction of type II receptor and SMAD3 in human skin fibroblasts. *Age (Dordr)* 36:9623
- Hegedus L, Cho H, Xie X et al. (2008) Additional MDA-MB-231 breast cancer cell matrix metalloproteinases promote invasiveness. *J Cell Physiol* 216: 480–85
- Hoot KE, Oka M, Han G et al. (2010) HGF upregulation contributes to angiogenesis in mice with keratinocyte-specific Smad2 deletion. *J Clin Invest* 120:3606–16
- Huang S, Wettlaufer SH, Hogaboam C et al. (2007) Prostaglandin E(2) inhibits collagen expression and proliferation in patient-derived normal lung fibroblasts via E prostanoid 2 receptor and cAMP signaling. *Am J Physiol Lung Cell Mol Physiol* 292:L405–13
- Humphrey JD, Dufresne ER, Schwartz MA (2014) Mechanotransduction and extracellular matrix homeostasis. *Nat Rev Mol Cell Biol* 15: 802–12

- Irizarry RA, Hobbs B, Collin F et al. (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* 4:249–64
- Iskratsch T, Wolfenson H, Sheetz MP (2014) Appreciating force and shape—the rise of mechanotransduction in cell biology. *Nat Rev Mol Cell Biol* 15: 825–33
- Li Y, Xia W, Liu Y et al. (2013) Solar ultraviolet irradiation induces decorin degradation in human skin likely via neutrophil elastase. *PLoS One* 8:e72563
- Liu X, Wu H, Byrne M et al. (1995) A targeted mutation at the known collagenase cleavage site in mouse type I collagen impairs tissue remodeling. *J Cell Biol* 130:227–37
- Mammoto T, Mammoto A, Ingber DE (2013) Mechanobiology and developmental control. *Annu Rev Cell Dev Biol* 29:27–61
- Muller-Decker K (2011) Cyclooxygenase-dependent signaling is causally linked to non-melanoma skin carcinogenesis: pharmacological, genetic, and clinical evidence. *Cancer Metastasis Rev* 30:343–61
- Pickup M, Novitskiy S, Moses HL (2013) The roles of TGFβ in the tumour microenvironment. *Nat Rev Cancer* 13:788–99
- Qin Z, Voorhees JJ, Fisher GJ et al. (2014) Age-associated reduction of cellular spreading/mechanical force up-regulates matrix metalloproteinase-1 expression and collagen fibril fragmentation via c-Jun/AP-1 in human dermal fibroblasts. *Aging Cell* 13:1028–37
- Quan T, Fisher G (2015) Role of age-associated alterations of the dermal extracellular matrix microenvironment in human skin aging. *Gerontology*. e-pub ahead of print 4 February 2015
- Quan T, He T, Shao Y et al. (2006) Elevated cysteine-rich 61 mediates aberrant collagen homeostasis in chronologically aged and photoaged human skin. *Am J Pathol* 169:482–90
- Quan T, Shao Y, He T et al. (2010) Reduced expression of connective tissue growth factor (CTGF/CCN2) mediates collagen loss in chronologically aged human skin. *J Invest Dermatol* 130:415–24
- Rittie L, Stoll SW, Kang S et al. (2009) Hedgehog signaling maintains hair follicle stem cell phenotype in young and aged human skin. *Aging Cell* 8:738–51
- Samuelsson B, Morgenstern R, Jakobsson PJ (2007) Membrane prostaglandin synthase-1: a novel therapeutic target. *Pharmacol Rev* 59:207–4
- Sandulache VC, Parekh A, Li-Korotky H et al. (2007) Prostaglandin E2 inhibition of keloid fibroblast migration, contraction, and transforming growth factor (TGF)-β1-induced collagen synthesis. *Wound Repair Regen* 15:122–33
- Scott NM, Ng RL, Gorman S et al. (2014) Prostaglandin E2 imprints a long-lasting effect on dendritic cell progenitors in the bone marrow. *J Leukoc Biol* 95:225–32
- Soontrapa K, Honda T, Sakata D et al. (2011) Prostaglandin E2-prostaglandin E receptor subtype 4 (EP4) signaling mediates UV irradiation-induced systemic immunosuppression. *Proc Natl Acad Sci USA* 108:6668–73
- Swindell WR, Johnston A, Sun L et al. (2012) Meta-profiles of gene expression during aging: limited similarities between mouse and human and an unexpectedly decreased inflammatory signature. *PLoS One* 7: e33204
- Tarutani M, Cai T, Dajee M et al. (2003) Inducible activation of Ras and Raf in adult epidermis. *Cancer Res* 63:319–23
- Ullrich SE, Byrne SN (2012) The immunologic revolution: photoimmunology. *J Invest Dermatol* 132:896–905
- Varani J, Dame MK, Rittie L et al. (2006) Decreased collagen production in chronologically aged skin: roles of age-dependent alteration in fibroblast function and defective mechanical stimulation. *Am J Pathol* 168: 1861–68
- Verecchia F, Chu ML, Mauviel A (2001) Identification of novel TGF-β/Smad gene targets in dermal fibroblasts using a combined cDNA microarray/promoter transactivation approach. *J Biol Chem* 276:17058–62
- Verzijl N, DeGroot J, Thorpe SR et al. (2000) Effect of collagen turnover on the accumulation of advanced glycation end products. *J Biol Chem* 275: 39027–1
- Xia W, Hammerberg C, Li Y et al. (2013) Expression of catalytically active matrix metalloproteinase-1 in dermal fibroblasts induces collagen fragmentation and functional alterations that resemble aged human skin. *Aging Cell* 12:661–71