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Molecular mechanisms of nonablative fractionated laser resurfacing

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Summary

Background Nonablative fractionated laser resurfacing improves the texture of treated skin, but little is known about the molecular mechanisms that underlie clinical improvements.

Objectives We sought to examine and quantify the time course and magnitude of dermal matrix changes that occur in response to nonablative fractionated laser resurfacing, with the dual goals of better understanding the molecular mechanisms that underlie clinical improvements and of gaining knowledge that will enable evidence-based treatment parameter optimization.

Methods Twenty patients (mean age 58 years) with photodamaged skin were focally treated on dorsal forearms with a nonablative fractionated laser. Serial skin samples were obtained at baseline and at various times after treatment. Biopsies were examined with real-time polymerase chain reaction technology and immunohistochemical techniques.

Results Laser treatment resulted in an initial inflammatory response as indicated by statistically significant induction of proinflammatory cytokines (interleukin-1β and tumour necrosis factor-α). This was followed by substantial increases in levels of several matrix metalloproteinases and later by significant induction of type I collagen. Dermal remodelling was noted with both low and high microbeam energy treatment parameters.

Conclusions Nonablative fractionated laser resurfacing induces a well-organized wound-healing response that leads to substantial dermal remodelling and collagen induction. Surprisingly, only minimal differences were observed between lower and higher microbeam energy settings. These data suggest that lower microbeam energy/higher microbeam density treatment parameters, which are generally better tolerated by patients, may yield dermal changes similar to those that result from higher microbeam energy/lower microbeam density treatment parameters.

Ablative laser resurfacing techniques provide marked clinical improvements in the signs of photoageing, but such procedures require substantial healing time and are fraught with the potential for significant complications.[1–3] Alternatively, traditional nonablative laser treatments have proven to be clinically useful for minimizing dyspigmentation and hypervascularity in photodamaged skin, but results with respect to textural improvements in rhytides and scars have generally been modest.[4–6]

To give potential enhancement of the efficacy of nonablative laser photorejuvenation, the concept of fractionated photothermolysis was introduced.[7] With fractionated photothermolysis, infrared laser irradiation is applied to the skin, generating microscopic columns of heated tissue with water acting as the primary energy-absorbing molecule. Epidermal integrity generally remains intact and untreated skin found between the laser-irradiated columns facilitates rapid healing, thus allowing for minimal social down time. Fractionated photothermolysis has revolutionized laser therapy for photodamaged skin and a variety of other conditions including striae and scars.[8–12] When nonablative lasers are applied in a fractionated format, clinically significant textural improvements in the skin are achievable with minimal risk. Several authors have examined the clinical effects of fractionated laser therapy and have demonstrated substantial efficacy.

In comparison, few studies have objectively examined the cellular and molecular effects of nonablative fractionated photothermolysis. Prior work that has included histological evaluation of treated skin has revealed rapid re-epithelializa-

tion associated with an inflammatory infiltrate and eventual reorganization of the dermal matrix.[11,13] There is also a pilot study that examines the histological effects of altering treatment settings of nonablative fractionated lasers in a pig model.[14] However, a detailed direct quantitative analysis of the cutaneous molecular and cellular alterations that result in humans from such treatment is lacking.

We hypothesize that induction of a cutaneous wound-healing response to the laser therapy underlies the clinical improvements in skin texture that have been reported by several authors. We thus sought to examine and quantify processes involved in dermal matrix remodelling that occur in response to nonablative fractionated laser resurfacing, with the dual goals of better understanding the mechanisms that underlie clinical improvements and of gaining knowledge that will enable evidence-based treatment parameter optimization.

Materials and methods

Volunteer recruitment and inclusion/exclusion criteria

This study was approved by the institutional review board of the University of Michigan Medical School, and written informed consent was obtained from all study subjects prior to entry into the study. Human volunteers included in the study were those of either gender of any racial/ethnic group who were at least 18 years of age with clinically evident photodamage of the forearm skin globally rated by investigators as at least moderate in severity. Included subjects were deemed to be in generally good health and willing and able to comprehend and comply with the requirements of the study protocol. Potential subjects were excluded for having used oral retinoids within 1 year of study entry, active infection or a history of herpetic infection of the forearm skin, a history of abnormal scarring, a history of allergy or sensitivity to lidocaine or topical anaesthetics, and a medical history or concurrent illness that investigators felt precluded safe participation in this study. Pregnant or breastfeeding women were also excluded. A minimum 2-week wash-out period was required for all subjects using topical agents on their forearm skin. In total, 20 subjects (two women and 18 men) were enrolled in the comparative study (15 vs. 70 mJ). Subjects' age ranged from 51 to 73 (mean 58) years.

Fractionated photothermolysis treatment and skin biopsies

For each patient, the specific site to be treated was marked by an investigator. Measurements from anatomical landmarks such as moles, scars or dyspigmented areas were documented to ensure that subsequent biopsies were obtained from laser-treated areas. Locally injected 1% lidocaine was used to achieve anaesthesia prior to laser treatment and biopsy. Fractionated photothermolysis treatment was performed on a localized area of photodamaged forearm skin using a 1550-nm erbium doped fibre laser (Fraxel® SR 1500 Laser/Fraxel® re:store; Reliant Technologies, Mountain View, CA, U.S.A.) utilizing the following treatment parameters: treatment level 8, eight passes, and energy of 7, 15, 30 or 70 mJ. Total energy (in kJ) applied to the skin with each set of treatment parameters was recorded. For laser energy comparison studies, both energy levels (15 and 70 mJ) were delivered to distinct sites on the same forearm. Treatment was well tolerated and there were no treatment-related adverse events in any subjects. Treated skin became immediately oedematous and erythematous, but these expected changes resolved within several days of treatment. Prior to laser treatment, a baseline punch biopsy (4 mm in diameter) from untreated forearm skin was obtained under sterile conditions from each subject. Additional full-thickness skin samples were obtained 1, 7, 14, 21 and 28 days after laser treatment. Biopsy sites were spaced a minimum of 2 cm apart. Immediately after biopsy, skin samples were embedded in Optimal Cutting Temperature embedding medium (Tissue-Tek OCT; Miles, Naperville, IL, U.S.A.), frozen in liquid nitrogen, and stored at −80 °C until processing.

Sirius red collagen staining

Frozen skin sections (7 μm thick) were fixed in 2% paraformaldehyde for 20 min at 4 °C, and stained for 1 h in Picro-Sirius solution, as described by Sweat *et al.*[15] (0·1% solution of Direct Red 80 in saturated aqueous picric acid, both from Sigma Chemical Co., St Louis, MO, U.S.A.). Skin sections were then washed in 0·01 mol L^{-1} HCl for 2 min, dehydrated, and mounted with organic mounting medium (Dako, Carpinteria, CA, U.S.A.). Digital images were taken of stained sections that were visualized by microscopy with bright light (where collagen appears dark pink/red), and with polarized light (where thick, mature collagen fibres fluoresce in red, and thin collagen fibres fluoresce in green).

RNA extraction, reverse transcription, and quantitative real-time polymerase chain reaction

RNA extraction from whole skin biopsy samples, reverse transcription, and quantification by quantitative real-time polymerase chain reaction was performed as previously described.[16] Custom primers and probes were used for collagen I (COL1A1), collagen III (COL3A1), matrix metalloproteinase (MMP)-1, MMP-3, MMP-9 and 36B4 (primer sequences available upon request). All other primer-probe sets were validated gene expression assays (TaqMan; Applied Biosystems, Foster City, CA, U.S.A.) (assay references available upon request). Results are presented as fold change in treated vs. untreated skin sample, normalized to transcript levels of housekeeping gene 36B4 (RPLP0, ribosomal protein, large, P0).

Immunohistochemistry

Frozen skin sections (7 μm thick) were immunostained using antibodies directed to laminin γ2 (kalicrin; AbCam, Cambridge, MA, U.S.A.), heat shock protein 70 kDa (HSP70; Biogenex, San Ramon, CA, U.S.A.), neutrophil elastase (Dako),

MMP-1, MMP-3, MMP-9 or procollagen I (all from Chemicon/Millipore, Billerica, MA, U.S.A.). Tissue-bound primary antibody was visualized with a secondary antibody–peroxidase–AEC system (Biogenex) as previously described.[16]

Estimation of laser beam number and depth of dermal penetration

Frozen skin sections were obtained 24 h after laser treatment and were immunostained for procollagen I as described above. Areas where laser treatment caused denaturation of collagen were clearly demarcated by a lack of staining. Unstained areas ('columns') were quantified using the image analysis software component of a laser capture microscope (Leica AS LMD; Leica, Allendale, NJ, U.S.A.). Three parameters were measured for each section: column number, column width, and column dermal depth (in μm, from location of basement membrane to deepest dermal point). Measurements were made in duplicate on consecutive sections for each skin sample.

Procollagen I protein measurement

Procollagen I protein levels were quantified in skin samples by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Panvera, Madison, WI, U.S.A.), and normalized to sample dermal volume, as previously described.[17]

Statistical analysis

Fold induction of biomarkers over time was compared with baseline levels with paired sample t-tests. Differences were considered statistically significant when $P < 0{\cdot}05$, using a two-tailed test. When necessary, logarithmic transformations of the data were made before analysis to achieve normality. Data were analysed with SPSS statistical software (SPSS, Chicago, IL, U.S.A.). Data are presented as mean ± SEM.

Results

Skin damage induced by fractional photothermolysis

Histologically, focal areas of intercellular oedema ('microvesicles') were observed in the epidermis 24 h after laser treatment (Fig. 1a, b). Column-like areas of altered dermal collagen fibrils were observed in the papillary dermis 24 h after laser treatment. These areas were evidenced both by Sirius red staining, which specifically stains collagen fibrils (Fig. 1c, d; damaged collagen demarcated by thin dotted lines), and procollagen I immunostaining, which reveals cell-associated and extracellular matrix-associated procollagen I protein (Fig. 1e, f).

Fractional photothermolysis induces focal heat shock response, but does not destroy the basement membrane

In the epidermis, fractional photothermolysis induced a rapid and transient expression of HSP70 protein, a heat shock protein that protects against misfolding of proteins during heat stress[18] (Fig. 2a–g). In addition, laminin γ2 immunostaining indicated that the basement membrane remained essentially intact 1 day following the procedure, and revealed partial detachment of basal keratinocytes from the basement membrane beneath the 'microvesicles' (Fig. 2h, i).

Characterization of damaged dermal areas induced by fractional photothermolysis

Based on the laser's software design, increasing energy levels decreases the density of individual microbeams for a given selected 'treatment level'. This reciprocal relationship causes the 'percentage surface area coverage' displayed on the device's touch screen to remain constant. Subjects were treated with the laser on photoaged forearms with energy settings of 7, 15, 30 or 70 mJ. Skin samples obtained 24 h after treatment were immunostained for procollagen I and used to measure column number and dimensions as described in Materials and methods. Representative staining for each treatment setting and mean measurements are presented in Figure 3. As expected, the dermal depth of laser microbeam penetration was increased with increasing energy, averaging 134 ± 24, 207 ± 16, 253 ± 18 and 413 ± 32 μm after 7, 15, 30 and 70 mJ, respectively ($n = 4$ for 7 and 30 mJ, $n = 13$ for 15 and 70 mJ; Fig. 3f). A similar dose-dependent increase in mean dermal column width with increased energy settings was observed in the same samples (ranging from 87·4 ± 5·3 μm at 15 mJ to 213 ± 14 μm at 70 mJ, $n = 13$, $P < 0{\cdot}001$; Fig. 3g). However, the number of columns per section was decreased with increased microbeam energy (mean of 6·5, 5·7, 2·8 and 2·8 columns/section for 7, 15, 30 and 70 mJ, respectively ($n = 4$ for 7 and 30 mJ, $n = 13$ for 15 and 70 mJ; Fig. 3h). The total volume of thermal damage at varying settings was mathematically estimated to be similar at all energy settings, given that microbeam density decreases with increasing microbeam energy. Based on these results, we selected 15 and 70 mJ microbeam energy settings as representative of lower and higher energy settings, respectively, for subsequent comparative purposes.

Fractional photothermolysis induces a rapid inflammatory reaction in photodamaged human skin *in vivo*

Fractionated photothermolysis quickly triggered an acute inflammatory response, as evidenced by neutrophil infiltration (Fig. 4a–c) and acute elevation of the two key proinflammatory cytokines tumour necrosis factor (TNF)-α (Fig. 4d) and interleukin (IL)-1β (Fig. 4e), 1 day after treatment. Interestingly, the neutrophil infiltrate appeared denser in samples treated with the 70 mJ vs. the 15 mJ energy setting (Fig. 4c vs. b). Consistent with an acute inflammatory response, TNF-α transcript levels were found to be elevated in laser-treated skin as compared with baseline levels 24 h post-treatment (115- and 45-fold for 70 and 15 mJ, respectively, $n = 10$,

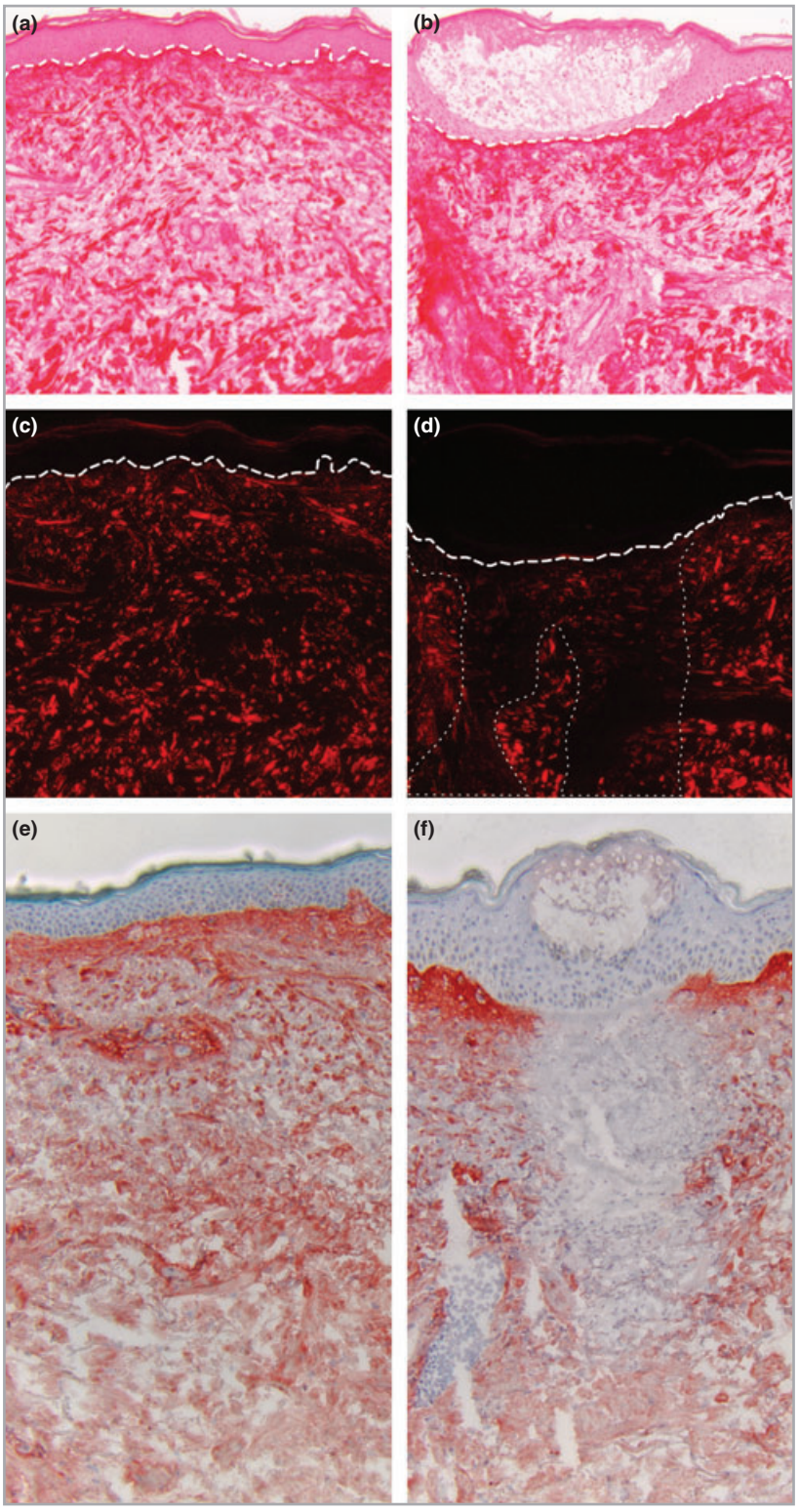

Fig 1. Epidermal and dermal damage induced by fractional photothermolysis treatment of photodamaged human skin. Photodamaged forearm skin was untreated (a, c, e) or treated with fractional photothermolysis at 70 mJ energy setting (b, d, f), as described in Materials and methods. Skin damage was assessed 24 h post-treatment. (a, b) Sirius red staining, viewed with nonpolarized light, reveals focal areas of intercellular oedema ('microvesicles') in the epidermis (whitish appearance) of treated skin. (c, d) Sirius red staining, viewed with polarized light, reveals areas of altered collagen fibrils in the papillary dermis (demarcated by dotted lines). Heavy dashed line indicates dermoepidermal junction. (e, f) Procollagen I immunostaining reveals areas of reduced staining in the papillary dermis of treated skin. Images are representative of 10 subjects. Original magnification × 60.

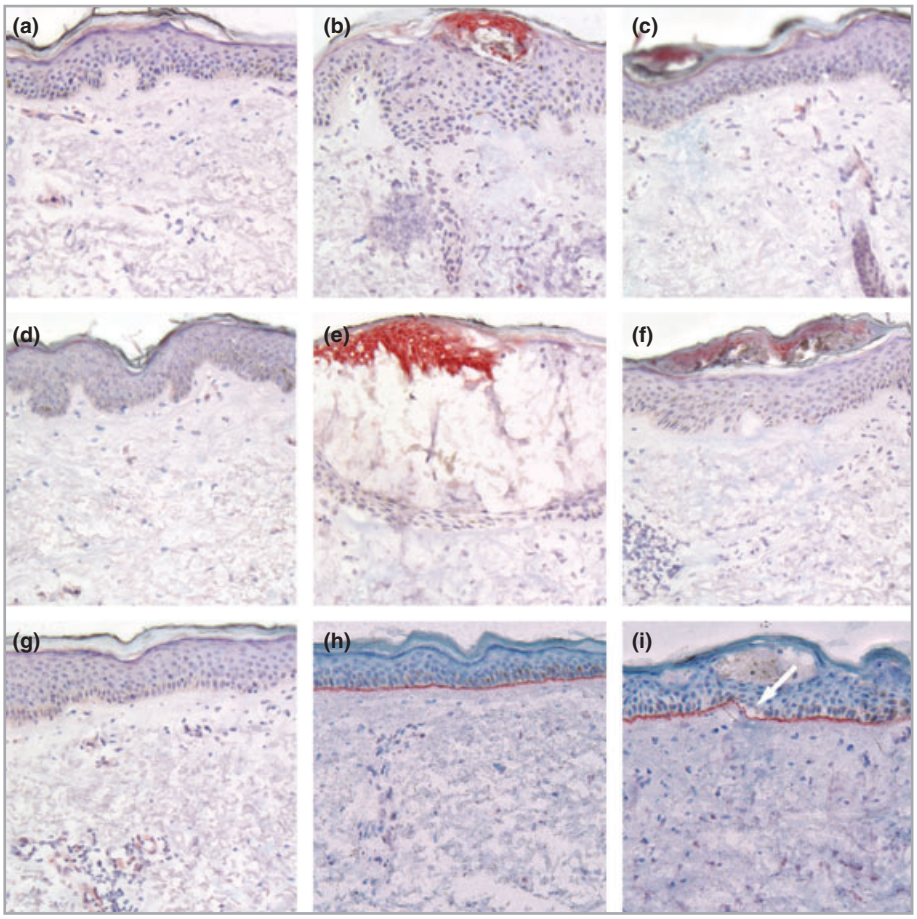

Fig 2. Fractional photothermolysis induces focal heat shock response, but does not destroy the basement membrane in photodamaged human skin *in vivo*. Photodamaged forearm skin was treated with fractional photothermolysis with 15 or 70 mJ energy settings, and analysed for heat shock markers. (a–g) Heat shock protein 70 immunostaining indicates rapid and transient thermal response in epidermal keratinocytes located above epidermal microvesicles. (a) Untreated skin; (b–d) 15 mJ; (e–g) 70 mJ; (b, e) day 1; (c, f) day 7; (d, g) day 14. (h, i) Laminin γ2 immunostaining indicates that the basement membrane remains intact. (h) Untreated skin; (i) 70 mJ at day 1. Note the partial detachment of basal keratinocytes from the basement membrane beneath the 'microvesicles' (white arrow). Laminin γ2 staining is red, counterstaining is blue. Images are representative of 10 subjects. Original magnification × 120.

$P < 0{\cdot}05$). The difference between the two treatment energy settings was statistically significant ($P < 0{\cdot}01$). Levels of TNF-α rapidly declined over the next 2 weeks after treatment (Fig. 4d). IL-1β mRNA levels were also found to be elevated 1 day post-treatment in the majority of treated individuals (Fig. 4e) (seven of 10 for 15 mJ treatment setting, ranging from 1·6- to 13·0-fold greater than baseline levels, and eight of 10 individuals for 70 mJ treatment setting, ranging from 2·0- to 18·9-fold greater than baseline levels; $P < 0{\cdot}05$).

Fractional photothermolysis increases matrix metalloproteinase expression in photodamaged human skin *in vivo*

Proinflammatory cytokines are known to induce several MMPs, and previous work has demonstrated the key role played by these MMPs in remodelling of dermal proteins following a variety of light-based treatments.[17] We thus measured MMP-1, MMP-3 and MMP-9 mRNA levels following fractionated photothermolysis treatment with 15 and 70 mJ settings. As shown in Figure 5a, MMP-1 mRNA levels were significantly increased over baseline 1 day after nonablative fractionated laser treatment (153- and 276-fold using 15 and 70 mJ energy treatment parameters, respectively, $n = 10$, $P < 0{\cdot}01$). The difference in magnitude of induction was significant between the two treatment levels ($P < 0{\cdot}01$). Levels of MMP-1 mRNA returned to baseline 1 week following laser exposure. Similar results were obtained for MMP-3 mRNA levels, which were acutely and significantly elevated 69-fold ($n = 10$, $P < 0{\cdot}01$) and 90-fold ($n = 10$, $P < 0{\cdot}01$) 1 day after 15 and 70 mJ treatments, respectively (Fig. 5b). However, the difference in MMP-3 induction between the two laser settings 1 day after treatment was not statistically significant ($P = 0{\cdot}249$). As observed with MMP-1, levels of MMP-3 quickly returned to baseline levels within 1 week. These results were consistent with immunohistochemical data, which

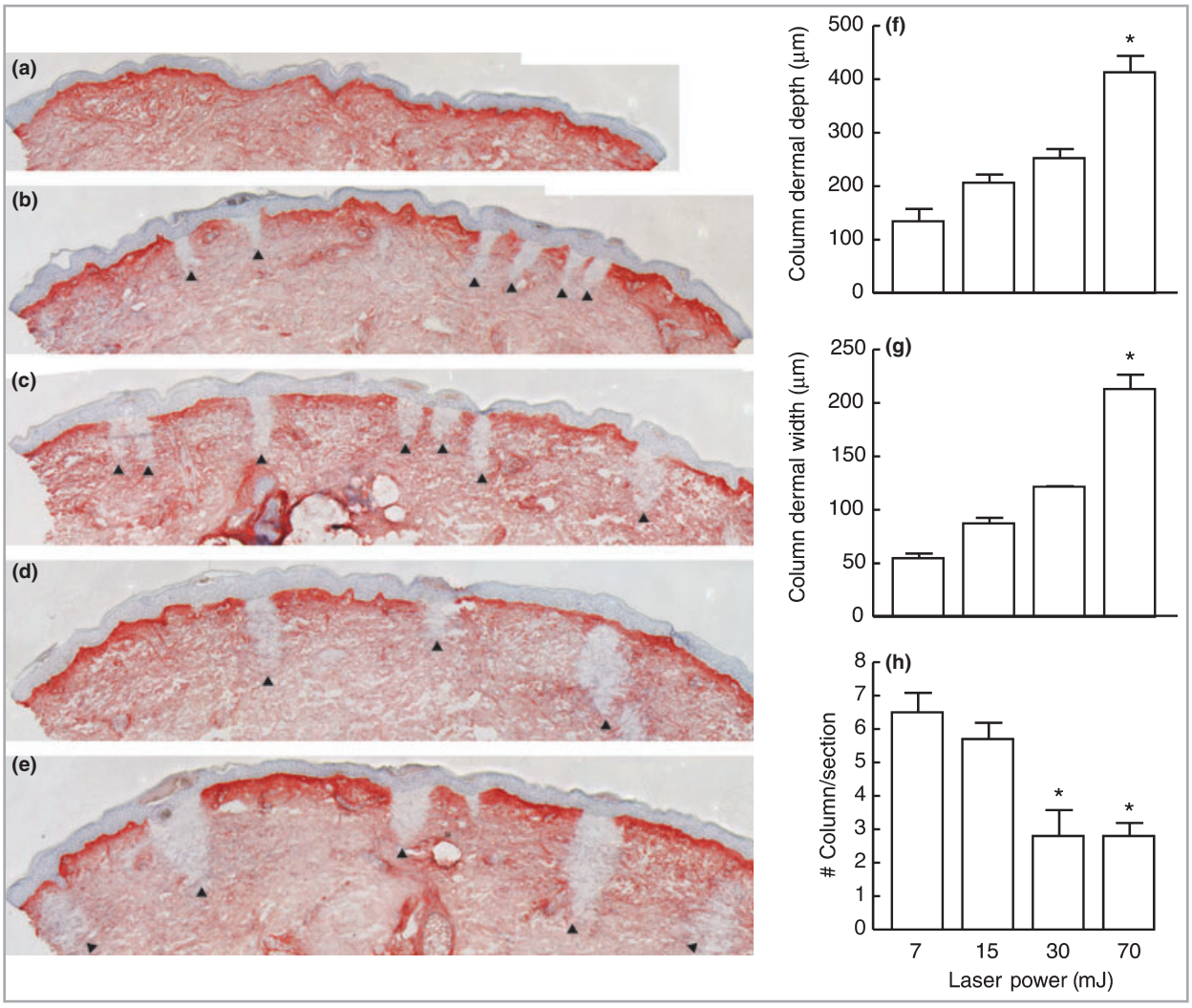

Fig 3. Characterization of damaged dermal areas induced by fractional photothermolysis treatment. Photodamaged forearm skin was treated with fractional photothermolysis with increasing laser microbeam energy (7, 15, 30 or 70 mJ energy settings), and dermal reduction of collagen I immunostaining was quantified 24 h after treatment. Procollagen I immunostaining in (a) untreated skin, and after fractional photothermolysis with (b) 7 mJ, (c) 15 mJ, (d) 30 mJ and (e) 70 mJ energy settings. Black arrowheads indicate bottom of photothermolysis column. Computerized image analysis of procollagen I immunostaining was performed to quantify (f) mean ± SEM depth of damaged dermal areas; (g) mean ± SEM width of damaged dermal areas, and (h) mean ± SEM number of columns per section. n = 4 for 7 and 30 mJ, n = 10 for 15 and 70 mJ. *$P < 0{\cdot}05$ vs. 15 mJ.

indicated that MMP-3 protein was produced primarily around thermally damaged zones 1 day post-treatment, but was not detectable 1 week after treatment (Fig. 5c–i). Similarly, MMP-9 transcript levels were found to be elevated 24 h after treatment, with both energy settings ($P < 0{\cdot}01$; Fig. 5j). The increase in MMP-9 mRNA levels was relatively persistent after 70 mJ energy treatment and remained 36 ± 14-fold increased at 2 weeks after treatment (n = 10, $P < 0{\cdot}01$). Increased MMP-9 mRNA persisted for at least 4 weeks after treatment in nine of 10 individuals tested (17·2 ± 4·4 the baseline levels, n = 10, $P < 0{\cdot}01$; Fig. 5j). However, MMP-9 mRNA levels quickly tapered back to baseline levels within 1 week after the 15 mJ energy treatment (Fig. 5j). These results were confirmed at the protein level: MMP-9 protein was detected by immunohistochemistry in the dermis and epidermis surrounding the damaged skin at 24 h after treatment (Fig. 5k–q). MMP-9 protein expression was rapidly reduced following 15 mJ energy treatment, but persisted in the dermis of skin areas treated with the 70 mJ energy setting for at least 2 weeks after treatment (Fig. 5q).

Fractional photothermolysis increases collagen I and III gene expression in photodamaged human skin *in vivo*

Consistent with the known sequence of events that takes place during the cutaneous wound-healing reaction,[19] we measured an acute initial decline in collagen I mRNA levels 24 h after treatment (0·36 ± 0·07 and 0·41 ± 0·08-fold vs. baseline levels with 15 and 70 mJ, respectively, n = 10, $P < 0{\cdot}01$; Fig. 6a). However, as the initial inflammatory response

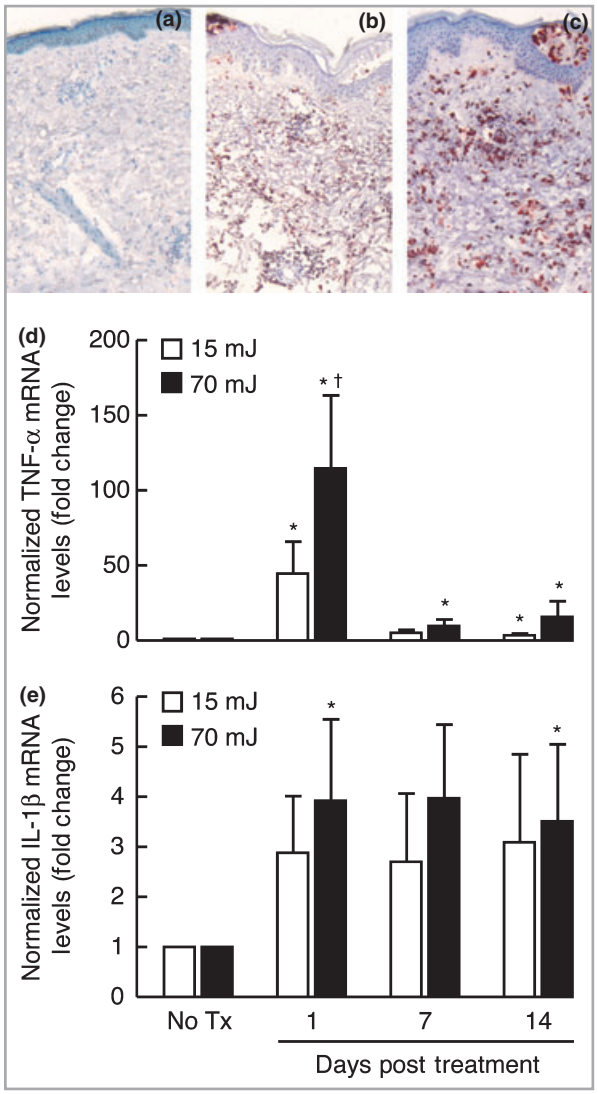

Fig 4. Fractional photothermolysis treatment induces a rapid inflammatory reaction in photodamaged human skin. Photodamaged forearm skin was treated with fractional photothermolysis with 15 or 70 mJ energy settings and analysed for markers of inflammation. Neutrophil elastase immunostaining 24 h after treatment in (a) untreated skin, or skin treated with (b) 15 mJ or (c) 70 mJ energy setting reveals intense neutrophil infiltrate in the dermis and epidermal vesicles of treated skin. Original magnification × 60. (d) mRNA levels of tumour necrosis factor (TNF)-α and (e) interleukin (IL)-1β were quantified by real-time reverse transcription–polymerase chain reaction. n = 10; *$P < 0{\cdot}05$ vs. no treatment (No Tx); †$P < 0{\cdot}01$ vs. 15 mJ. Results are shown as mean ± SEM.

waned, new collagen production was observed in treated skin, after both the 15 and 70 mJ energy setting treatments: collagen I mRNA levels rose steadily over the subsequent 2 weeks, peaking at week 2 with 3·1- and 5·0-fold vs. baseline levels ($n = 20$, $P < 0{\cdot}01$) with 15 and 70 mJ energy settings, respectively (Fig. 6a). These levels were maintained at least until week 4 post-treatment, both with the 15 mJ setting (2·9 ± 0·6 and 3·1 ± 0·6-fold vs. baseline at week 3 and 4, respectively, $n = 10$, $P < 0{\cdot}01$) and the 70 mJ setting (4·0 ± 0·8 and 5·0 ± 1·4-fold vs. baseline at week 3 and 4, respectively, $n = 10$, $P < 0{\cdot}01$). Interestingly, despite a trend towards relatively greater collagen I mRNA induction with the 70 mJ setting, the difference between collagen I mRNA induction in response to 15 or 70 mJ energy settings was not statistically different at 2 or 3 weeks after treatment ($P = 0{\cdot}086$, $P = 0{\cdot}072$, respectively). However, the amount of induction in response to 70 mJ energy treatment was greater than in response to 15 mJ energy treatment at 4 weeks post-treatment ($n = 10$, $P < 0{\cdot}05$).

Similar to collagen I, we observed a transient decrease in collagen III mRNA levels 1 day post-treatment (0·29 ± 0·05 and 0·33 ± 0·06-fold vs. baseline levels with 15 and 70 mJ, respectively, $n = 10$, $P < 0{\cdot}01$; Fig. 6b). Collagen III mRNA levels were maximal 2 weeks after treatment with 2·6- and 3·9-fold vs. baseline levels ($n = 20$, $P < 0{\cdot}01$) with 15 and 70 mJ energy settings, respectively (Fig. 6b). Substantial induction of collagen III mRNA was noted to persist until at least 28 days after laser exposure with both 15 mJ (2·4 ± 0·6 and 3·0 ± 0·6-fold vs. baseline at weeks 3 and 4, respectively, $n = 10$, $P < 0{\cdot}01$) and 70 mJ (4·0 ± 0·7 and 3·5 ± 0·5-fold vs. baseline at weeks 3 and 4, respectively, $n = 10$, $P < 0{\cdot}01$) laser energy treatment parameters. Collagen III mRNA induction at weeks 2 and 3 was statistically different between the two energy treatment settings ($P < 0{\cdot}05$ and $P < 0{\cdot}01$, respectively), but was not statistically different at week 4 ($n = 10$ per group, $P = 0{\cdot}152$).

Fractional photothermolysis increases procollagen I protein expression in photodamaged human skin *in vivo*

Immunohistochemistry indicated that increased procollagen I production occurred throughout the dermis (Fig. 7a–i), albeit greater in the papillary dermis compared with the deep reticular dermis (Fig. 7d–i). Quantification of procollagen I protein by specific ELISA (Fig. 7j) was consistent with observed induction of collagen I mRNA levels. After a transient decrease in procollagen I protein levels 1 day post-treatment (0·57 ± 0·09 and 0·51 ± 0·05-fold vs. baseline levels with 15 and 70 mJ, respectively, $n = 10$, $P < 0{\cdot}01$), procollagen I protein levels were induced at 2 weeks after treatment (4·0- and 2·9-fold vs. baseline levels with 15 and 70 mJ energy settings, respectively; $n = 20$, $P < 0{\cdot}01$; Fig. 7j). Procollagen I protein induction was maximal at weeks 3 and 4, reaching 6·4 ± 2·4 and 6·3 ± 2·1-fold vs. baseline at week 4, with 15 and 70 mJ energy settings, respectively; $n = 10$, $P < 0{\cdot}01$). Procollagen I protein induction was not statistically different between treatment with 15 and 70 mJ energy settings ($P = 0{\cdot}391$, 0·939 and 0·489, for weeks 2, 3 and 4, respectively).

Discussion

In the present study, we evaluated the effects of nonablative fractionated photothermolysis on collagen remodelling in

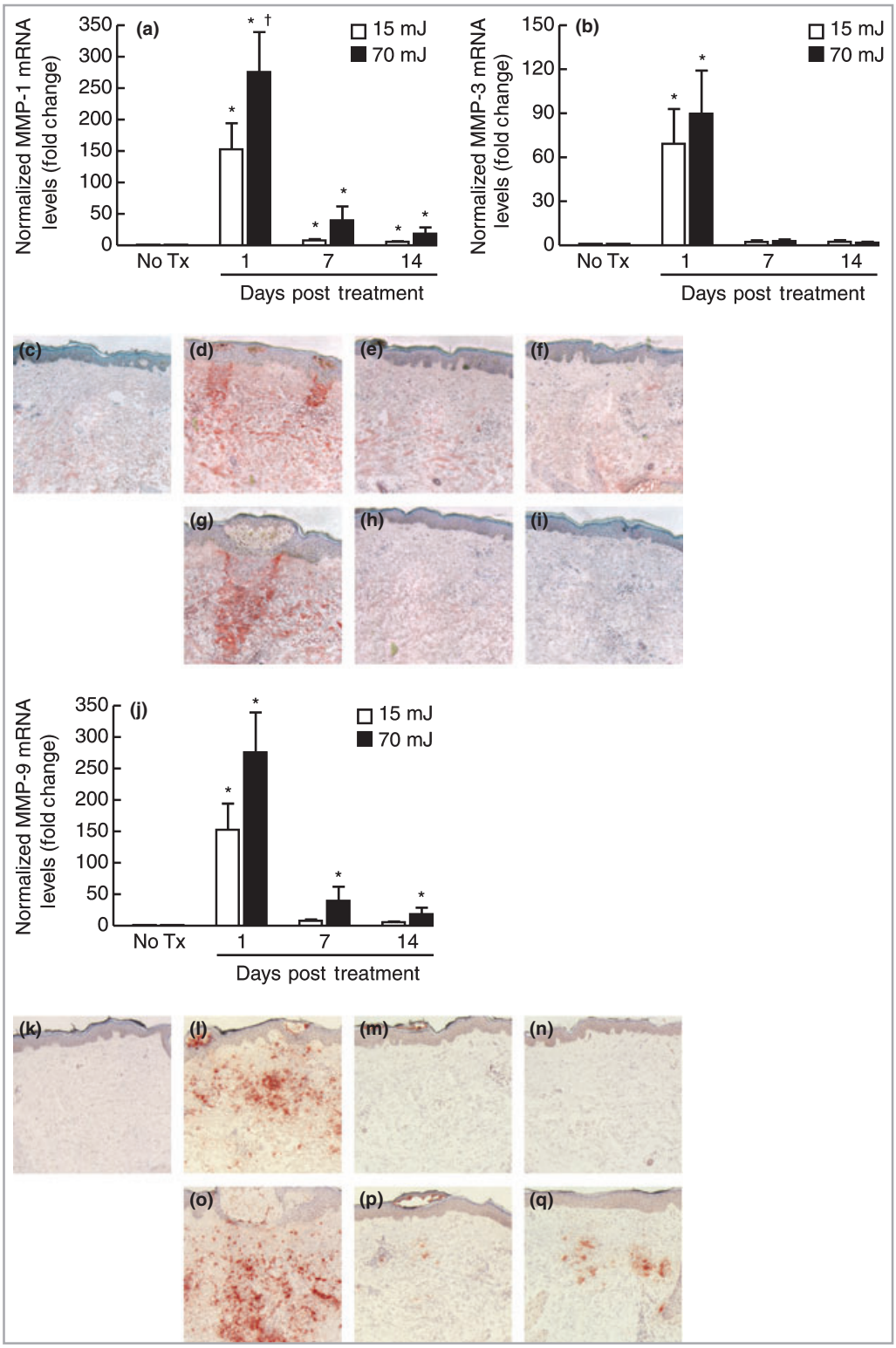

Fig 5. Fractional photothermolysis treatment increases matrix metalloproteinase (MMP) expression in photodamaged human skin *in vivo*. Photodamaged forearm skin was treated with fractional photothermolysis with 15 or 70 mJ energy settings and analysed for MMP expression. Normalized mRNA levels for (a) MMP-1, (b) MMP-3 and (j) MMP-9 were quantified by real-time reverse transcription–polymerase chain reaction. $n = 20$ for no treatment (No Tx) and day 14, $n = 10$ for day 1 and 7; *$P < 0{\cdot}01$ vs. day 0; †$P < 0{\cdot}01$ vs. 15 mJ. Results are shown as mean ± SEM. Immunohistochemical localization of protein expression for (c–i) MMP-3 and (k–q) MMP-9. (c, k) Untreated skin; (d–f, l–n) 15 mJ; (g–i, o–q) 70 mJ; (d, g, l, o) day 1; (e, h, m, p) day 7; (f, i, n, q) day 14. Images are representative of 10 individuals. Original magnification × 60.

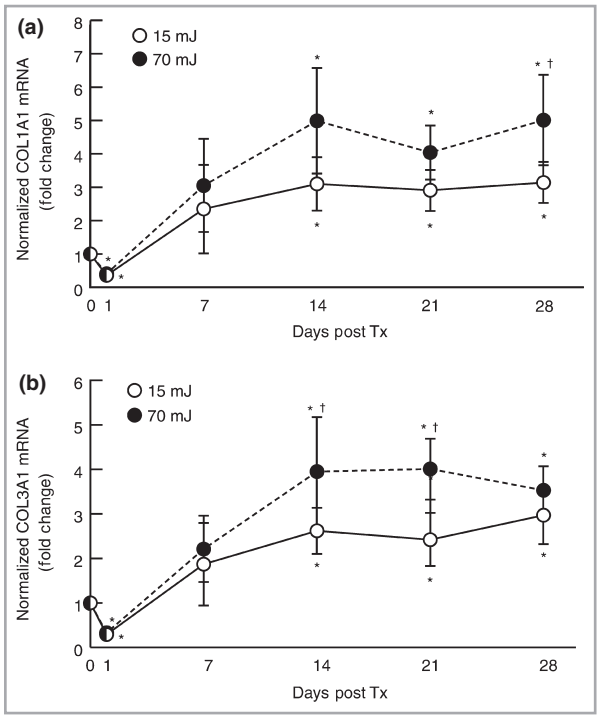

Fig 6. Fractional photothermolysis treatment increases collagen I and III gene expression in photodamaged human skin. Normalized mRNA levels of (a) COL1A1 and (b) COL3A1 were quantified by real-time reverse transcription–polymerase chain reaction after fractional photothermolysis with 15 mJ or 70 mJ energy settings. $n = 20$ for no treatment (Tx) and day 14, $n = 10$ for day 1 and 7; *$P < 0{\cdot}01$ vs. day 0; †$P < 0{\cdot}05$ vs. 15 mJ. Results are shown as mean ± SEM.

photoaged human skin *in vivo*. We first demonstrated the presence of thermally altered skin columns in the epidermis and dermis of treated skin, visible 24 h after treatment. The depths, widths and densities of columns were dependent upon laser energy settings. In a recently reported *in vitro* study, Thongsima *et al.*[20] also reported on the patterns of histological damage seen after nonablative fractionated photothermolysis. While slightly deeper and wider areas of thermal alteration were reported in that study, differences from the data presented here are likely to be based on methodological nuances such as measuring the widest breadth of damage vs. our measurements of the average width of damage. The focus of that study was on a comparison of two different laser systems, but in general, the principle of being able to manipulate the pattern of thermal damage with changes in laser treatment parameters held true between that study and the current work.

In addition, we demonstrated that a single fractional photothermolysis treatment induces a wound-healing reaction characterized by early and acute induction of an inflammatory reaction, followed by matrix remodelling as evidenced by new procollagen production. The wound-healing process detailed here offers substantial evidence as to the efficacy of nonablative fractionated photothermolysis and begins the process of revealing the molecular mechanisms that are involved. Notably, the cutaneous response to nonablative fractionated photothermolysis appears to mirror that produced by more invasive treatments with traditional ablative resurfacing.[21]

In examining a variety of therapeutic interventions often used to improve the appearance of photodamaged skin such as tretinoin applications, microdermabrasion, filler injections, photodynamic therapy, and both ablative and nonablative laser therapy, our group and others have developed a paradigm by which the efficacy of a treatment may be predicted by quantitative molecular measurements.[17,21–29] In keeping with the findings of a growing body of clinically oriented literature that suggests fractionated photothermolysis to be an effective means by which to improve the skin's appearance, our molecular data suggest substantial dermal remodelling that compares favourably with that seen with a variety of other treatments, despite the nonablative nature of this approach. Indeed, we have demonstrated reproducible and significant dermal remodelling including marked induction of procollagen I in treated skin. More than any other factor examined to date, collagen induction appears to correlate with eventual clinical efficacy.

Beyond determining that a given procedure is effective in improving skin texture, the process of developing rational, evidence-based treatment parameters is a daunting task facing cosmetic dermatologists. With respect to nonablative fractionated laser therapy, we have attempted to begin the process of optimizing laser settings. Here, it is noteworthy that both shallow and more deeply penetrating fractionated infrared laser treatments were capable of producing substantial collagen induction. In fact, at the time points examined, low microbeam energy treatments generally induced rather similar molecular changes to those that resulted from high microbeam energy treatments. These data suggest that lower microbeam energy/higher microbeam density treatments may even be preferable given the relatively lower level of pain experienced by patients at these settings. However, it is important to note that the current data alone are insufficient to necessarily recommend a change in treatment strategies. Evaluations of molecular changes at later time points in more subjects and at varying treatment parameters are required, more definitely to define optimal laser settings.

The laser used in our study automatically adjusts microbeam density when energy per microbeam level is changed.[20] Thus, it was not possible to make a strict comparison of the effects of microbeam energy settings, while maintaining a constant number of microbeams per area. Therefore, we compared the effects of lower microbeam energy/higher microbeam density with the effects of higher microbeam energy/lower microbeam density treatments. The observed similarity of skin responses to low and high energy settings may reflect the fact that, under each set of conditions, the measured *total* energy applied to the skin and estimated *total* volume of skin photothermolysis were very similar. Thus, it is possible that the total energy (in kJ) applied to the skin and the extent of thermal damage may be more predictive of clinical efficacy than the energy settings for individual microbeams with this device. Clearly, large well-designed clinical trials of patients treated at

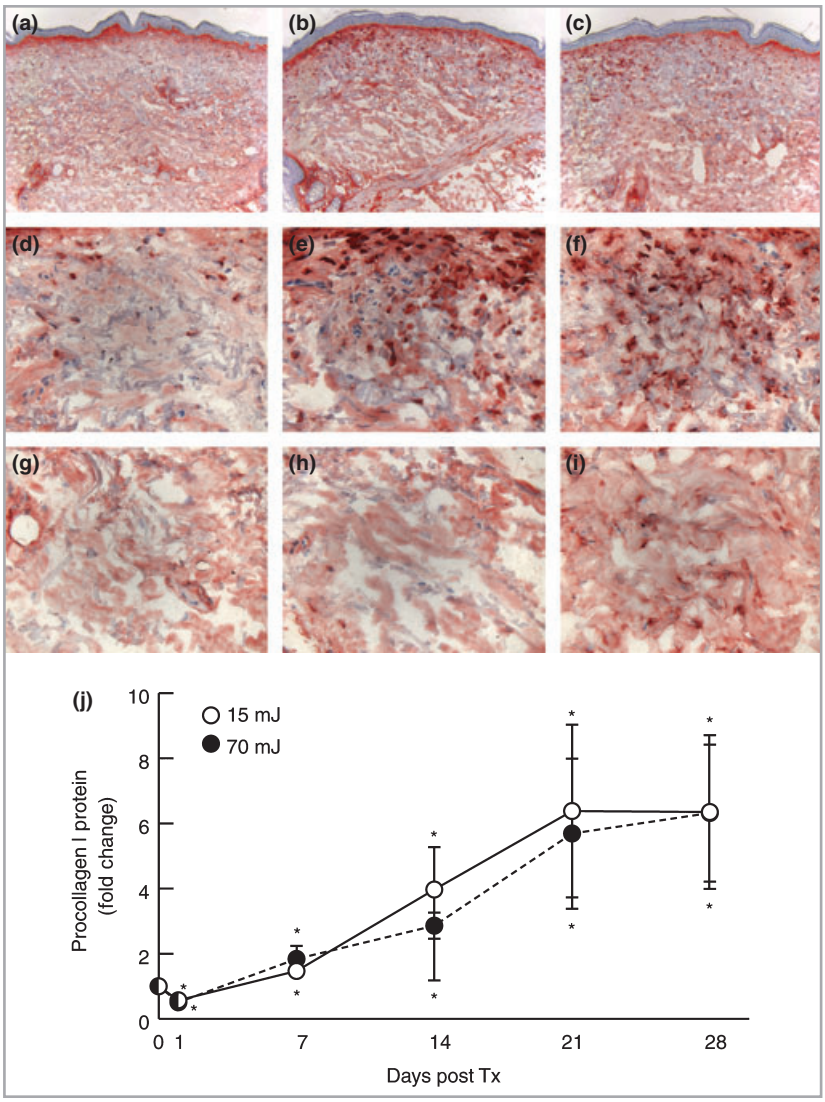

Fig 7. Fractional photothermolysis treatment increases procollagen I protein expression in photodamaged human skin *in vivo*. Photodamaged forearm skin was treated with fractional photothermolysis using 15 or 70 mJ energy settings and analysed for procollagen I protein synthesis by immunohistochemistry and enzyme-linked immunosorbent assay (ELISA). Immunostaining for procollagen I at 28 days post-treatment indicates increased procollagen I protein expression throughout the dermis. (a, d, g) Untreated skin; (b, e, h) 15 mJ; (c, f, i) 70 mJ. (a–c) Original magnification × 60; (d–f) upper dermis, original magnification × 240; (g–i) lower dermis, original magnification × 240. (j) Procollagen I protein quantification by ELISA in skin extracts. $n = 20$ for no treatment (Tx) and day 14, $n = 10$ for day 1, 7, 21 and 28; *$P < 0{\cdot}01$ vs. no treatment. Differences between 15 and 70 mJ were not statistically significant at any time points. Results are shown as mean ± SEM.

varying settings will be required to clarify the issue of optimal treatment parameters.

Our study examined molecular changes after a single nonablative fractionated photothermolysis treatment, whereas treatment is sometimes performed on a serial basis in the clinical setting. The cumulative molecular effects of multiple treatments on dermal remodelling are speculative at this point. It should also be noted that the final time point observed in our study was 4 weeks following treatment, a time at which collagen induction had yet to return to baseline levels. It thus remains possible that greater differences in dermal remodelling might be observed between the high and low energy settings at later time points, although the similarities in the observed kinetics argue against that possibility. In addition, due to the practical limitations of obtaining multiple skin samples from the face, treatments were applied to sun-damaged forearm skin. Although one of the advantages of nonablative fractionated photothermolysis is the fact that it may be used at a variety of anatomical locations off the face, the most common uses for this laser therapy involve treatment of facial skin. However, we have used photodamaged forearm skin as a model to study cutaneous molecular alterations in a variety of previous studies and have found that the skin's fundamental wound-healing responses are similar at various anatomical locations, and we

believe that the current study is a valid predictor of cutaneous responses to the treatment in general, including on the face.

Clinical applications for fractionated photothermolysis continue to increase and such laser therapy seems destined to play a central role in the treatment of wrinkles, scars and other unwanted cutaneous textural changes in the coming years. An evolving understanding of the molecular mechanisms related to these treatments will be vital for ultimate optimization of treatment protocols. As therapeutic options to improve the skin's appearance continue to expand, a combination of clinical and translational research will be vital in ensuring that these new treatments are being utilized to the maximal advantage of our patients.

What's already known about this topic?

- Nonablative fractionated lasers are clinically effective and offer brief down time and minimal risk. Scars, wrinkles and other textural issues may be improved with such laser treatments.
- Columns of thermally altered skin are seen microscopically.
- Histology of treated skin shows inflammation and reorganization of the dermal matrix.

What does this study add?

- A detailed quantitative analysis of the cutaneous molecular and cellular alterations that result from nonablative fractionated laser resurfacing.
- A better understanding the mechanisms involved in such treatment.
- A rationale for evidence-based treatment parameter optimization.

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