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Transcriptomic analysis of human skin wound healing and rejuvenation following ablative fractional laser treatment

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Abstract

Ablative fractional laser treatment is considered the gold standard for skin rejuvenation. In order to understand how fractional laser works to rejuvenate skin, we performed microarray profiling on skin biopsies to identify temporal and dose-response changes in gene expression following fractional laser treatment. The backs of 14 women were treated with ablative fractional laser (Fraxel®) and 4 mm punch biopsies were collected from an untreated site and at the treated sites 1, 3, 7, 14, 21 and 28 days after the single treatment. In addition, in order to understand the effect that multiple fractional laser treatments have on skin rejuvenation, several sites were treated sequentially with either 1, 2, 3, or 4 treatments (with 28 days between treatments) followed by the collection of 4 mm punch biopsies. RNA was extracted from the biopsies, analyzed using Affymetrix U219 chips and gene expression was compared between untreated and treated sites. We observed dramatic changes in gene expression as early as 1 day after fractional laser treatment with changes remaining elevated even after 1 month. Analysis of individual genes demonstrated significant and time related changes in inflammatory, epidermal, and dermal genes, with dermal genes linked to extracellular matrix formation changing at later time points following fractional laser treatment. When comparing the age-related changes in skin gene expression to those induced by fractional laser, it was observed that fractional laser treatment reverses many of the changes in the aging gene expression. Finally, multiple fractional laser treatments, which cover different regions of a treatment area, resulted in a sustained or increased dermal remodeling response, with many genes either differentially regulated or continuously upregulated, supporting previous observations that maximal skin rejuvenation requires multiple fractional laser treatments. In conclusion, fractional laser treatment of human skin activates a number of biological processes involved in wound healing and tissue regeneration.

Introduction

Aging of epithelial tissues, such as skin, is a complex biological phenomenon involving the epidermis, dermis and sub-dermis. Analysis of skin aging using systems biology approaches such transcriptomics, RNASeq, proteomics, etc., have demonstrated that skin aging is a dynamic condition with epidermal, dermal and subdermal aging progressing through different processes [1–13]. Multiple approaches including chemical peels, dermabrasion, laser treatment (e.g. Fraxel®), non-laser light treatments, needling/microneedling, photodynamic therapy, radiofrequency treatment (e.g. Thermage®), ultrasound (e.g. Ultherapy®) and combinations of the above have been utilized to rejuvenate aged skin by activating the innate wound repair processes in both the epidermis and dermis [14–21]. Of these approaches, the laser-based wound/repair modality appears to have the best efficacy in terms of rejuvenating both the epidermis and dermis, with fractional laser treatment having the least negative patient experience [22, 23]. Through the rejuvenation of the epidermal and dermal structures, laser resurfacing of aging skin leads to multiple visual benefits including skin wrinkling, skin tone and skin texture improvements [22].

Lasers have been used since the early 1980s to deliver skin rejuvenation benefits [22]. The first clinically used laser treatment was total ablative laser-based rejuvenation [22]. This treatment resulted in significant dermal remodeling and epidermal regeneration that improves both skin texture and tone; however, as a consequence of removal of most of the epidermis and significant amount of dermis, long healing times and significant side effect profiles were observed [23, 24]. Nonablative laser rejuvenation procedures were subsequently developed, and while these approaches reduced the damage to the epidermis, they had limited rejuvenation efficacy [22]. Next, fractional ablative laser rejuvenation procedures were developed, using a grid pattern of separate individual micrometer laser shots to ablate only a fraction of the epidermis and dermis, resulting in dermal remodeling and improved texture and tone with much faster healing times and a significantly improved side effect profile [22–25]. Of these three approaches, ablative laser rejuvenation is considered the best treatment in terms of efficacy for the treatment of photoaging, acne scars and rhytides, although it is associated with serious side effects including long healing times, scarring and delayed onset hypopigmentation [22, 25]. In contrast, because the laser does not injure the epidermis and just heats the dermis, non-ablative laser rejuvenation is very safe but not as effective as ablative lasers in skin rejuvenation [24, 27]. Finally, fractional ablative laser rejuvenation combines both the safety of non-ablative laser rejuvenation with the efficacy of ablative laser rejuvenation and has become the new gold standard for skin rejuvenation with long lasting rejuvenation effects in multiple ethnicities at multiple body sites [22–30].

Fractional laser skin rejuvenation works by creating microscopic thermal holes in a grid pattern through the epidermis and deep into the dermis, thus stimulating dermal and epidermal regeneration while leaving most (up to 95%) of the epidermis and dermis intact [31]. This allows the laser-induced damage to be more quickly repaired [25, 32–34]. To achieve maximum benefit, an average of at least four sessions are needed because with each treatment only a fraction (20–25%) of the treated area is covered [31]. The depth and width of the thermal damage and laser intensity can be altered to achieve maximal dermal rejuvenation, often with efficacy like ablative laser treatment, without lengthy re-epithelialization (2 days for fractional laser versus 2–3 weeks for ablative laser), fewer infections, fewer acneiform eruptions and greatly reduced erythema [25, 31, 35, 36]. Significant work has been performed to understand how ablative, non-ablative and fractional lasers work to rejuvenate skin. Histopathological studies have demonstrated that fractional laser treatment of skin results in columnar ablative zones surrounded by thermal coagulation zones containing denatured proteins in the

epidermis and dermis. Epidermal repair occurs within 1–3 days, the epidermal/dermal junction is repaired by 7 days while dermal repair takes weeks to months [31, 32, 33, 37, 38]. Finally, Fraxel laser resurfacing is FDA approved for use on all regions of the body and skin types [31]. Also observed was an increase in epidermal thickness, an increase in dermal collagens and a decrease in dermal elastin (elastosis) [39, 40]. These observations have been confirmed using the non-invasive techniques of confocal microscopy and two-photon microscopy [26, 41]. Histochemical studies looking at specific proteins demonstrated an increase in collagens (COL-1, COL-3), tropoelastin, vimentin, cytokines (EGF, bFGF, PDGF, VEGF, TGF-β, FGF9), heat shock proteins (HSP70, HSP47, HSP72), apoptosis markers (annexin-VII, Bcl-2, caspase-9), inflammatory cells (CD3+, CD20+, CD68+, CD31+), proteases (MMP1, MMP3, MMP9, neutrophil elastase) and a decrease in p53 [42–48]. Gene expression studies (typically using qPCR evaluating a limited number of genes) demonstrate an increase in proteases (MMP1, MMP2, MMP3, MMP9, MMP10, MMP11, MMP13), protease inhibitors (TIMP), hyaluronic acid synthesis enzymes (HAS, HYAL), collagens (COL1A1, COL3A1), extracellular matrix components (ELN, EMILIN1), cytokines (IL-1β, IL-8), and defensins (CAMP, HBD2) [23, 49–52]. Many of the observations of the effects of fractional laser treatment on human skin have been confirmed using animal and human skin organotypic models with the advantage that more detailed time courses and larger, more numerous sampling can be performed [33, 53–63]. These analyses have provided a more complete picture of fractional laser treatment; however, the human and/or *in vivo* relevance of these findings need to be confirmed by actual human studies. More recently, transcriptomic analyses have been performed using microarrays, gene chips and RNAseq approaches, and have identified several gene expression changes occurring following fractional laser treatment on human skin [54, 64, 65]. Because of the global nature of these approaches, many gene expression changes have been observed following fractional laser treatment, albeit the generation of a holistic understanding of how this treatment results in skin rejuvenation has been hampered by small and limited sampling. Thus, a study evaluating a larger base size of individuals over longer time periods, with frequent sampling using sensitive transcriptomics, is needed to better understand how fractional laser treatment rejuvenates skin. Here, we present our findings from a detailed time course transcriptomic analysis of in vivo fractional laser treated human skin with the goal of providing a holistic understanding of how fractional laser-induced wounding and healing rejuvenates aged human skin.

Materials and methods

Study design

This study was approved by the Schulman Associates Institutional Review Board (IRB# 201203768). All subjects provided written informed consent prior to undergoing any study procedures and were compensated for their participation. Fourteen women in good general health, aged 30–55, with Fitzpatrick skin type I-IV, were enrolled in this study. Fractional laser treatments were performed using a Fraxel® laser set to 12 mJ and 250 mtz with a 1 cm^2 treatment area, with all treatments and biopsy sampling performed by licensed medical personnel. For this study, the 14 study participants were treated on the back at 9 different equally spaced sites between the neck, upper buttocks and the left/right edges of the back with a minimum distance of 5 cm between each treatment site (plus one untreated site), and all sites received a 1 mm tattoo for site recognition. The treatment sites were also randomized to account for any potential variability between the lateral, superior or inferior portions of the back. The timing of the treatments was designed such that all biopsies were collected by a licensed dermatologist on the last day of the study. Sixty minutes prior to Fraxel® laser treatment, the treatment site

was dosed with a topical anesthetic (BLT Cream: 20% benzocaine, 6% lidocaine, 4% tetracaine) after which the hand-held Fraxel® laser was moved over the treatment site 8 times. The treatment groups were as follows: (1) single laser treatments at 1, 3, 7, 14, 21 and 28 days before biopsy and (2) multiple laser treatments at 28-day intervals with 1, 2, 3, and 4 treatments. Following the final treatment, a 4 mm full thickness biopsy was collected at each site by a licensed dermatologist (including a non-treated site), the biopsy site was sutured for closure and good healing, and the biopsy sample was placed in a cryotube and flash frozen in liquid nitrogen. A total of 140 biopsy samples were generated for analysis. The study participants returned to the test site 7–10 days following biopsy to have their biopsy sites assessed by a Physician's Assistant and to have sutures removed. Following completion of the healing assessment, the study was considered completed. No subject had issues with healing of their biopsy sites and no study related adverse events were reported. Study design, cohort description, and sample collection for the aged dermis study has been previously described [8].

Microarray profiling

Microarray profiling was performed on 140 untreated or laser-treated samples using the HG-U219 array (Affymetrix) as similarly described for the aged dermis samples [8]. Flash-frozen, bisected 4 mm full-thickness skin punch biopsies were homogenized in TRIzol reagent (ThermoFisher, Waltham, MA) and total RNA was extracted according to the manufacturer's protocol. RNA was further purified using RNEasy spin columns (QIAGEN, Hilden, Germany). Biotinylated cRNA libraries were synthesized from 500 ng of total RNA using the Affymetrix HT 3' IVT Express kit (Affymetrix, Santa Clara, CA) and the Beckman Biomek FXp Laboratory Automation Workstation (Beckman Coulter, Brea, CA). Biotinylated cRNA was subsequently fragmented by limited alkaline hydrolysis and then hybridized overnight to the Affymetrix Human Genome U219-96 GeneTitan array. Plates were scanned using the Affymetrix GeneTitan Instrument utilizing AGCC GeneTitan Instrument Control software version 4.2.0.1566. Image data was summarized using Affymetrix PLIER algorithm with quantile normalization. Microarray data from this study as well as the aged dermis study have been deposited in the Gene Expression Omnibus (GEO) under GSE168760 and GSE139300, respectively.

Statistical analyses

After rigorous quality check using methods and metrics described by Canales, et al [66], a linear mixed ANOVA model was fitted to the microarray data using SAS software PROC GLIMMIX, version 9.4 of the SAS System for Windows. Gene expression (in log scale) of each single treatment site was compared to that of the baseline (day 0) untreated site, and each of the multiple treatment sites was compared to the single treatment site at day 28. Subject, Affymetrix plate (samples from 14 subjects were processed on two plates with seven subjects per plate) and Site were class variables. Subject (nested within Plate) was the random effect in the model. No multiple comparison adjustment was used due to the exploratory nature of the study.

Bioinformatic analyses

Gene signature analyses were performed using GeneSpring version 14.8 (Agilent Technologies, Santa Clara, CA). In brief, low-expressed probe sets were removed based on establishing a minimum expression threshold (>20th percentile across all samples within any one treatment group) and condensed to a single Entrez gene ID using the arithmetic mean. $\log_2$ fold changes were calculated for the single treatment sites relative to untreated sites (Day 0) or, for the multiple treatment sites, relative to the single treatment site (Day 28). Hierarchical clustering of the $\log_2$ fold change values using Euclidean distance was performed to create the single

treatment and multiple treatment dendrograms. Biological pathway enrichment was performed on the filtered single treatment signatures ($p < 0.01$, absolute fold change > 1.1) and multiple treatment signatures ($p < 0.05$) using Ingenuity Pathway Analysis version 01-07 (QIAGEN). Fisher's exact t test was used to assess significantly associated pathways, and z-scores were calculated to predict pathway activation states (activated pathways, z-score >0; inhibited pathways z-score <0).

Results

In order to understand how fractional laser treatment works to rejuvenate human skin, an Affymetrix gene chip based transcriptomic analysis was performed on human skin biopsies from fractional laser treated skin at various time points after a single and multiple treatments. The experimental design was two-fold: (1) to understand the biological process that occurs during healing following one fractional laser treatment, 6 different sites on the backs of 14 individuals were treated with a single fractional laser treatment followed by biopsy sampling of those sites at 1, 3, 7, 14, 21 and 28 days after treatment; and (2) to understand if multiple treatments sustain the transcriptional response, 3 different sites on the backs of the same 14 individuals received either 2, 3 or 4 fractional laser treatments at the same site (with the treatments 28 days apart) followed by biopsy sampling. All the single treatments were compared to an untreated biopsy sample, allowing the use of the same individual as their own control (see Materials and Methods for a detailed description). For single treatment sites, the number of differentially expressed probe sets was highest 1 day after treatment and continued to drop throughout the 28-day treatment period (Table 1). Interestingly, multiple treatments of the same site, spaced 28 days apart, showed an increase in differential gene expression when compared to a single treatment after 28 days (Table 1). Investigating the nature of the gene expression changes with time after fractional laser treatment demonstrated both increases and decreases in gene expression with a gradual normalization over time (Fig 1A). Clustering analysis showed several interesting patterns including a sharp increase in gene expression at 1 day post treatment followed by a sharp decline between 3–7 days post treatment–this cluster contains a number of keratin genes, S100 family alarmin genes and metalloproteinase genes–indicative of early epidermal and dermal wound healing responses. Also observed was a sharp decrease in gene expression at 1 day post treatment followed by an increase in expression 3 days post treatment and normalization to background levels by 7 days post treatment of a wide variety of genes. Finally, a sharp decrease in gene expression at 1 day post treatment was

Table 1. Kinetic effects of laser resurfacing treatment(s) on gene expression in human skin.

Days post-treatment	Number of treatments	Comparison	No. differentially regulated probesets[1]
1	1	vs Day 0 (untreated)	24,358
3	1		16,754
7	1		11,473
14	1		10,693
21	1		5,456
28	1		6,555
56	2	vs Day 28 (1 treatment)	4,750
84	3		2,017
112	4		4,211

[1] Number of statistically significant probe sets (ANOVA p-value < 0.05) expressed above the 30th percentile in $\geq$ 50% samples in a treatment group
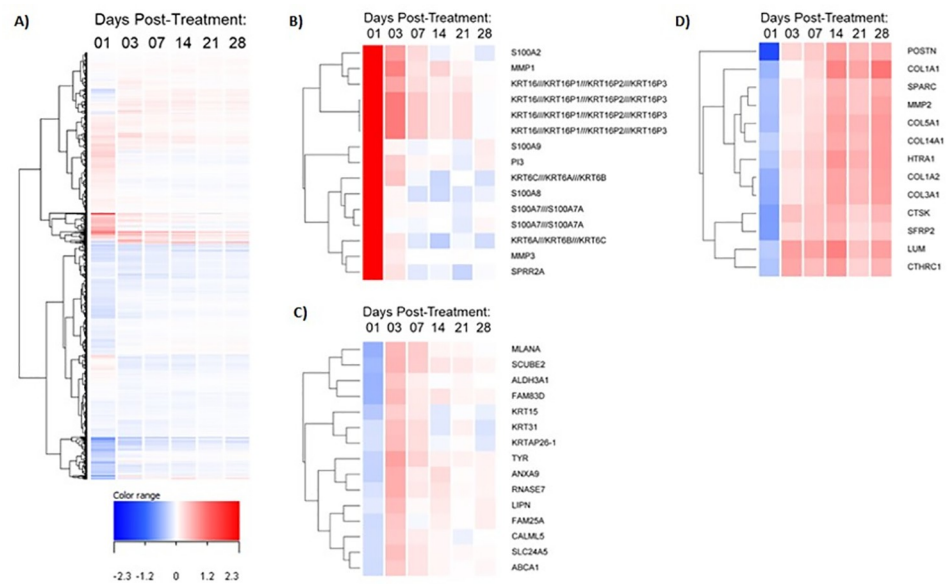
Fig 1. Kinetic effects of single laser resurfacing treatment on gene expression. A) Dendrogram displaying hierarchical clustering of genes by average $\log_2$ fold change that were significantly changed at any one time point compared to baseline untreated samples (n = 15,067 total). **B-D)** Dendrograms of select gene clusters from A) showing kinetic dysregulation from early (**B**, days 1–3), early-to-mid (**C**, days 3–7), and mid-to-late (**D**, days 14–28) time points following single laser resurfacing treatment.

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observed followed by a gradual increase in expression over the 28 day treatment period–this cluster contains a number of dermal extracellular matrix genes (Fig 1B–1D).

Skin wound healing involves inflammatory, epidermal and dermal processes

A bioinformatic analysis of the top differentially regulated genes demonstrated a collection of activated inflammation related pathways was differentially regulated as early as 1 day after fractional laser treatment with most inflammatory processes normalized by 14 days post treatment (Table 2). Epidermal related biological pathways involving proliferation and migration were similarly activated 1 day after treatment with many of the processes normalized by 7 days post treatment (Table 3). Interestingly, in addition to proliferation and movement of epidermal cells, an epidermal-mesenchymal transition pathway was activated 3 to 7 days post treatment (Table 3), which returned to baseline levels by day 14. This suggests the epidermis as a potential source for the mesenchymal cells participating in tissue repair at this early stage. Evaluation of dermal related biological pathways were complex with some processes initiating 1 day after fractional laser treatment such as proliferation and differentiation of connective tissue cells while other processes beginning much later such as the formation and maturation of collagen fibrils (Table 4). An analysis of the top 20 molecular functions 28 days post treatment supports the observations that cell death and removal, cell proliferation/movement and extracellular matrix maturation are the key processes during the wound healing process (Table 5). A more granular display of the top 20 molecular functions over time and with multiple treatments is shown in S1–S9 Tables.

An evaluation of the canonical signaling pathways associated with fractional laser treatment demonstrated that apoptosis/proliferation, stress/inflammation and cell adhesion were the key pathway groupings activated (Table 6). In these 3 key pathway groupings, individual pathways

Table 2. Stress and immune response biological pathways associated with laser resurfacing kinetics.

| Annotation | z-score[1] | | | | | | -log10 p-value[2] | | | | | |
| | Days Post-Treatment | | | | | | Days Post-Treatment | | | | | |
	01	03	07	14	21	28	01	03	07	14	21	28
Activation of leukocytes	N/A	3.59	3.96	4.85	N/A	2.45	0.00	8.22	8.22	23.62	0.00	3.92
Adhesion of immune cells	N/A	5.04	4.27	4.81	N/A	N/A	0.00	8.18	9.59	21.44	0.00	0.00
Antimicrobial response	N/A	N/A	2.21	2.71	1.55	1.73	0.00	0.00	10.48	14.79	12.35	4.22
Cell movement of lymphocytes	N/A	N/A	4.46	4.34	N/A	N/A	0.00	0.00	7.62	18.89	0.00	0.00
Cell movement of neutrophils	N/A	3.32	2.69	3.62	N/A	N/A	0.00	12.13	10.66	15.95	0.00	0.00
Degranulation of granulocytes	2.11	3.06	N/A	2.54	N/A	N/A	18.10	14.10	0.00	14.25	0.00	0.00
DNA damage	-1.72	N/A	N/A	N/A	N/A	N/A	6.83	0.00	0.00	0.00	0.00	0.00
Endocytosis	3.04	3.71	3.02	3.34	N/A	N/A	10.13	9.86	10.50	11.32	0.00	0.00
Engulfment of cells	2.74	3.79	3.42	3.54	N/A	N/A	9.71	8.41	9.84	11.34	0.00	0.00
Immune response of cells	1.76	3.83	3.87	4.95	N/A	N/A	9.45	10.96	7.40	13.65	0.00	0.00
Metabolism of reactive oxygen species	3.71	1.84	N/A	3.10	N/A	N/A	7.53	9.38	0.00	8.68	0.00	0.00
Proliferation of immune cells	1.62	2.06	2.96	1.74	1.86	1.37	8.57	11.22	9.27	26.51	3.57	3.49
Recruitment of granulocytes	N/A	N/A	N/A	3.02	N/A	N/A	0.00	0.00	0.00	8.76	0.00	0.00
Response of phagocytes	N/A	3.17	2.88	4.30	N/A	N/A	0.00	8.64	7.93	16.92	0.00	0.00
Synthesis of reactive oxygen species	3.66	1.87	N/A	3.10	N/A	N/A	8.18	9.23	0.00	8.35	0.00	0.00
T cell migration	N/A	N/A	N/A	5.39	N/A	N/A	0.00	0.00	0.00	13.09	0.00	0.00
T cell response	N/A	N/A	N/A	3.27	N/A	N/A	0.00	0.00	0.00	8.11	0.00	0.00

[1] Activation z-score (see Methods); n/a, activation z-score not calculated.

[2] Fisher's exact p-value.

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demonstrated unique kinetics with peak activation of some pathways such as remodeling of epithelial adherens junctions, ephrin receptor signaling, cell cycle control, and inflammation (IL-6 and IL-8 signaling) occurring at early time points (days 1–7). Other pathways including activated interferon signaling and negative inhibition of MMP signaling remained elevated throughout the time points. Finally, the HOTAIR regulatory pathway remained active the entire 28 days of observation (Table 6 and Fig 2).

Fractional laser treatment is very effective in reversing skin aging changes. Thus, the transcriptional changes induced by fractional laser resurfacing were compared with the gene expression changes that occur during facial epidermis and dermis aging. The overlap of genes in common between the fractional laser treatment differentially expressed genes at 28 days post treatment and the differentially regulated genes in 50 versus 20-year-old epidermis (Fig

Table 3. Epidermal-related biological pathways associated with laser resurfacing kinetics.

| | | z-score[1] | | | | | | -log10 p-value[2] | | | | | |
| | | Days Post-Treatment | | | | | | Days Post-Treatment | | | | | |
	Annotation	01	03	07	14	21	28	01	03	07	14	21	28
Growth & Differentiation	Cell movement of epithelial cells	1.39	0.44	-0.44	0.54	1.51	0.84	7.32	7.81	8.11	7.84	3.38	3.97
	Epithelial-mesenchymal transition	N/A	1.35	1.89	N/A	N/A	N/A	0.00	8.99	8.61	0.00	0.00	0.00
	Proliferation of epithelial cells	2.18	-1.28	0.56	N/A	N/A	N/A	8.26	10.88	7.54	0.00	0.00	0.00
	Necrosis of epithelial tissue	0.06	0.56	N/A	N/A	N/A	N/A	13.48	14.79	0.00	0.00	0.00	0.00

[1] Activation z-score (see Methods); n/a, activation z-score not calculated.

[2] Fisher's exact p-value.

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Table 4. Dermal-related biological pathways associated with laser resurfacing kinetics.

| | Annotation | z-score[1] | | | | | | -log10 p-value[2] | | | | | |
| | | Days Post-Treatment | | | | | | Days Post-Treatment | | | | | |
		01	03	07	14	21	28	01	03	07	14	21	28
Growth & Differentiation	Angiogenesis	1.24	2.14	3.28	2.36	0.94	0.07	20.35	22.51	16.15	23.10	9.49	8.08
	Cell death of fibroblasts	-0.64	N/A	N/A	N/A	N/A	N/A	6.70	0.00	0.00	0.00	0.00	0.00
	Cell movement of connective tissue cells	0.42	1.99	0.57	N/A	N/A	N/A	10.09	8.88	7.49	0.00	0.00	0.00
	Cell movement of dermal cells	N/A	N/A	N/A	N/A	N/A	1.41	0.00	0.00	0.00	0.00	0.00	3.65
	Cell movement of vascular smooth muscle cells	N/A	N/A	N/A	N/A	1.54	2.76	0.00	0.00	0.00	0.00	4.59	4.32
	Differentiation of connective tissue cells	N/A	0.82	0.02	0.56	0.79	0.41	0.00	13.23	10.00	12.88	10.50	4.96
	Growth of connective tissue	0.99	1.11	2.05	0.35	0.44	0.33	13.71	15.73	17.89	13.03	4.29	4.93
	Proliferation of connective tissue cells	1.43	1.33	1.98	0.41	0.26	-0.04	14.33	14.57	16.63	10.83	4.61	4.87
	Quantity of connective tissue	N/A	N/A	N/A	N/A	0.16	2.17	0.00	0.00	0.00	0.00	3.57	3.87
	Vasculogenesis	0.41	1.58	2.83	1.91	0.66	-0.35	16.85	17.46	9.48	16.18	6.47	5.28
Matrix Development	Adhesion of connective tissue cells	N/A	N/A	N/A	N/A	N/A	1.87	0.00	0.00	0.00	0.00	0.00	3.30
	Attachment of cells	N/A	N/A	N/A	1.53	1.04	1.39	0.00	0.00	0.00	10.67	3.28	5.53
	Cell spreading of fibroblasts	N/A	N/A	N/A	N/A	0.90	N/A	0.00	0.00	0.00	0.00	5.02	0.00
	Fibrogenesis	-0.27	1.00	0.92	2.05	2.08	2.07	14.62	16.76	17.43	11.87	5.36	6.73
	Formation of collagen fibrils	N/A	N/A	N/A	N/A	1.07	1.07	0.00	0.00	0.00	0.00	4.19	4.58
	Tensile strength of skin	N/A	N/A	N/A	N/A	2.20	2.60	0.00	0.00	0.00	0.00	5.54	7.90

[1] Activation z-score (see Methods); n/a, activation z-score not calculated.

[2] Fisher's exact *p*-value.

3A and 3B) and the 50 versus 20-year-old dermis were compared (Fig 3C and 3D). A large number of genes in common was observed with several genes being expressed in opposite directions in the fractional laser samples compared to the 50 versus 20-year-old epidermis and dermis samples (Fig 2B and 2D). A closer analysis revealed several extracellular matrix-related genes; in particular, collagen genes were the most highly upregulated with fractional laser treatment at day 28 yet showed the greatest decrease in expression in aged versus young skin (Figs 3B, 3D, 4A and 4B). Notably, many of the genes shared across multiple pathways displayed an increasing expression pattern over time following fractional laser treatment while their expression was decreased in 50-year-old individuals (Fig 4C). This indicates one of the major effects of fractional laser treatment is the reversal of the loss of extracellular matrix with skin aging.

Finally, based on the clinical observation that multiple fractional laser treatments result in the best skin rejuvenation outcome, the gene expression changes that occur following 2, 3 or 4 sequential fractional laser treatments were examined (Fig 5). When compared to a single fractional laser treatment, increased numbers of differential gene expression changes were observed following 2 treatments (Fig 5A), many of which were maintained following 3 and 4 treatments and some which were only observed following 3 treatments (Fig 5B and 5C). An evaluation of two specific genes illustrated that multiple treatments are required for maximal effect for COL1A1 expression (Fig 5D) and NR4A1 (Fig 5E), albeit with different trajectories.

Discussion

In this report, temporal transcriptomic analysis has been utilized to better understand how fractional laser treatment functions to rejuvenate aged skin. This temporal analysis was conducted on back skin because it allowed for the extensive investigative transcriptional analysis

Table 5. Top 20 molecular functions and system development process annotations associated with day 28 post-laser resurfacing treatment (by Fisher's *p*-value).

Annotation	z-score[1]	p-Value[2]	# Molecules[3]
Cell movement	2.38	2.07E-18	242
Organization of extracellular matrix	n/a	2.41E-17	48
Organization of collagen fibrils	n/a	1.83E-14	19
Migration of cells	2.25	7.78E-14	208
Necrosis	-0.18	9.00E-13	250
Apoptosis	-0.04	2.60E-12	244
Morphology of collagen fibrils	n/a	6.14E-12	11
Morphology of fibrils	n/a	8.09E-11	12
Metabolism of protein	1.13	4.20E-10	125
Abnormal morphology of collagen fibrils	n/a	1.47E-09	9
Organization of filaments	1.90	1.79E-09	35
Angiogenesis	0.07	8.33E-09	106
Tensile strength of skin	2.60	1.27E-08	9
Development of vasculature	0.07	2.20E-08	113
Initiation of translation of protein	n/a	2.63E-08	23
Cell death of tumor cell lines	-0.74	2.87E-08	152
Quantity of cells	3.49	7.38E-08	168
Formation of filaments	2.19	1.61E-07	54
Fibrogenesis	2.07	1.87E-07	58
Invasion of cells	0.73	4.32E-07	101

[1] Activation z-score (see Methods); n/a, activation z-score not calculated.

[2] Fisher's exact *p*-value.

[3] Number of genes differentially regulated at day 28 within the annotated pathway.

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into the mechanism of Fraxel-induced skin rejuvenation. Because Fraxel laser resurfacing has been successfully performed on multiple skin sites, such as face, lips, neck, and hands [22, 25, 28], it is believed that the treatment of back skin is still relevant for yielding meaningful mechanistic insights. In addition, no long-term clinical measures of skin rejuvenation were measured in this study, given the large amount of published evidence (22–30) that supports the diverse skin benefits obtained from Fraxel laser resurfacing.

Thus, this study focused on the transcriptional response of the skin to Fraxel treatment and demonstrated that fractional laser treatment induces many biological processes related to wound healing and tissue regeneration, in a highly orchestrated manner, resulting in rejuvenation of long-lived skin macromolecules like components of the extracellular matrix. These data greatly expand the previously published temporal analysis of the effects of laser treatment on skin by providing a much more detailed analysis of the changes in gene expression that occur (whole genome) compared to the approximately 20 genes previously analyzed [23, 49–52]. In addition, this study greatly extends previous transcriptomic analyses by including more sampling times, larger number of study participants and increased number of laser treatments [54, 64, 65].

As part of the wound healing process following fractional laser-induced treatment, immediate gene expression changes were observed indicative of epidermal and dermal repair mediated by keratinocytes, fibroblasts and immune cells which were followed by longer term repair of the dermis mediated by fibroblasts and immune cells. Activation of keratinocytes as early as 1 day post fractional laser treatment is exemplified by the large changes in keratin gene

Table 6. Canonical signaling pathways associated with laser resurfacing kinetics.

| | | z-score[1] | | | | | | -log10 p-value[2] | | | | | |
| | | Days Post-Treatment | | | | | | Days Post-Treatment | | | | | |
	Annotation	01	03	07	14	21	28	01	03	07	14	21	28
Apoptosis & Proliferation	14-3-3-mediated Signaling	2.45	2.48	0.71	0.00	N/A	N/A	2.74	2.77	0.00	0.00	0.00	0.00
	Cell Cycle Control of Chromosomal Replication	3.55	3.71	2.53	N/A	N/A	N/A	3.17	2.78	1.48	0.00	0.00	0.00
	Cyclins and Cell Cycle Regulation	1.72	2.45	2.00	-0.38	N/A	N/A	2.54	4.13	5.02	0.61	0.23	0.00
	HOTAIR Regulatory Pathway	-0.96	0.46	0.39	1.71	2.89	2.18	1.51	2.65	3.32	2.48	3.59	3.86
	HGF Signaling	0.27	0.56	-0.28	-1.00	N/A	-2.00	3.22	2.92	1.85	1.33	0.48	0.64
	Kinetochore Metaphase Signaling Pathway	1.76	4.13	3.29	1.67	N/A	N/A	1.21	14.39	11.91	0.47	0.36	0.00
Stress & Inflammation	EIF2 Signaling	-2.35	-1.23	-2.40	-2.52	0.00	-1.90	17.24	5.42	5.71	5.03	0.42	5.32
	ERK/MAPK Signaling	-0.11	-0.43	-0.39	0.00	N/A	0.00	3.05	2.47	2.46	1.46	0.27	0.00
	IL-6 Signaling	1.21	1.41	0.54	0.28	N/A	0.71	3.13	1.30	0.54	0.73	0.44	0.80
	IL-8 Signaling	1.12	2.02	0.96	2.27	0.00	0.58	4.70	3.16	1.58	2.59	0.28	0.92
	Inhibition of ARE-Mediated mRNA Degradation Pathway	1.00	3.02	0.28	1.27	N/A	N/A	2.57	0.70	0.48	0.00	0.00	0.00
	Interferon Signaling	3.58	3.64	2.89	3.46	2.83	2.45	2.02	4.30	4.93	5.36	6.84	3.21
	NF-κB Signaling	0.97	2.38	0.66	1.15	N/A	1.00	2.48	2.93	0.93	1.03	0.00	0.54
Cell Adhesion	Actin Cytoskeleton Signaling	1.99	1.36	-0.35	-0.43	N/A	-0.71	2.21	3.38	4.11	1.87	0.00	0.35
	Ephrin Receptor Signaling	1.38	3.09	1.34	0.69	N/A	-0.38	3.98	2.27	3.25	4.31	1.06	2.28
	Inhibition of Matrix Metalloproteases	-0.94	0.00	-0.38	-0.91	-1.89	-1.00	1.35	0.93	1.23	4.32	4.50	4.70
	Integrin Signaling	1.55	2.18	0.85	1.89	0.00	0.28	6.05	2.43	4.15	2.59	0.63	1.31
	Rac Signaling	2.21	1.52	0.47	-0.54	N/A	-2.45	3.97	4.13	3.57	2.20	0.25	0.89
	Remodeling of Epithelial Adherens Junctions	3.30	1.00	0.00	N/A	N/A	N/A	3.95	1.28	1.07	0.00	0.00	0.00

[1] Activation z-score (see Methods); n/a, activation z-score not calculated.

[2] Fisher's exact p-value.

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expression and the activation of epithelial cell pathways (necrosis of epithelial tissues, proliferation and cell movement of epithelial cells) and is consistent with current knowledge concerning the speed of epidermal repair following fractional laser treatment [32, 33, 37, 38]. Two pathways important in the re-epithelization of a wound (ephrin receptor signaling pathway and HOTAIR regulatory pathway) were analyzed in more detail. The ephrin receptor signaling pathway, which is important in wound closure and healing [67], is strongly activated as early as 1 day post treatment followed by a gradual decrease over the 28-day treatment period. The long non-coding RNA HOTAIR regulatory pathway was also activated following fractional laser treatment as would be required for proper wound healing [68]. Additionally, the large changes in S100 gene expression at 1 day post fractional laser treatment, along with the rapid induction of gene expression of several inflammatory pathways, confirms a quick immune response to the damage and greatly expands previous histochemical study findings [42–48].

Interestingly, other immune-related pathways showed prolonged (e.g., antimicrobial response) or even bi-phasic (e.g., activation of leukocytes) waves of activation over the course of 28 days post-laser resurfacing treatment. These observations are consistent with the published reports demonstrating unique roles of both innate and adaptive immune cells during distinct phases of the wound healing response in skin. For instance, in mouse models of acute wound healing, macrophages have been shown to have critical roles in the early and mid-phase inflammation and tissue repair responses, respectively, but are dispensable for tissue maturation in the late phase [69]. Conversely, γδ T cells within the epidermis display pleiotropic functions and maintain close, continuous interaction with keratinocytes to provide an immediate response to cellular damage as well as on-going surveillance for damage [70].
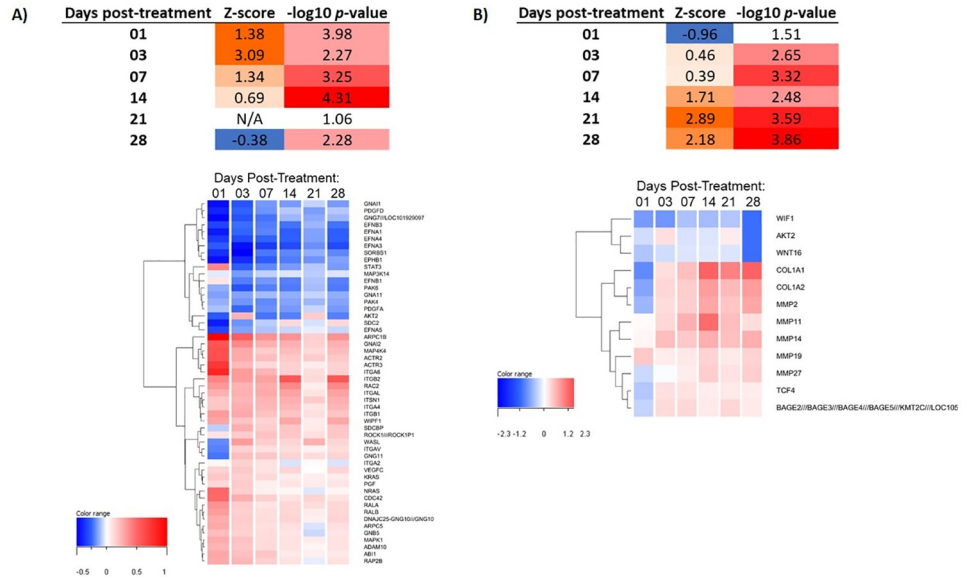
Fig 2. Single laser resurfacing treatment stimulates signaling pathways associated with wound repair. A) Activation of ephrin receptor signaling at early (days 1–3) time points following single laser resurfacing treatment. Dendrogram shows clustering of genes (n = 52) involved in the ephrin receptor signaling pathway by average log_2 fold change compared to baseline untreated samples. **B)** Activation of the HOTAIR regulatory pathway at mid-to-late (days 14–28) time points following single laser resurfacing treatment. Dendrogram shows clustering of genes (n = 12) involved in the HOTAIR regulatory pathway by average log_2 fold change compared to baseline untreated samples. Color scales for z-scores indicate predicted low/inhibition (blue) to high/activation (orange) states for each pathway at a given time point. Color scale for $-log_{10}$ p-value reflect low (white) to high (red) significance for each pathway at a given time point.

https://doi.org/10.1371/journal.pone.0260095.g002
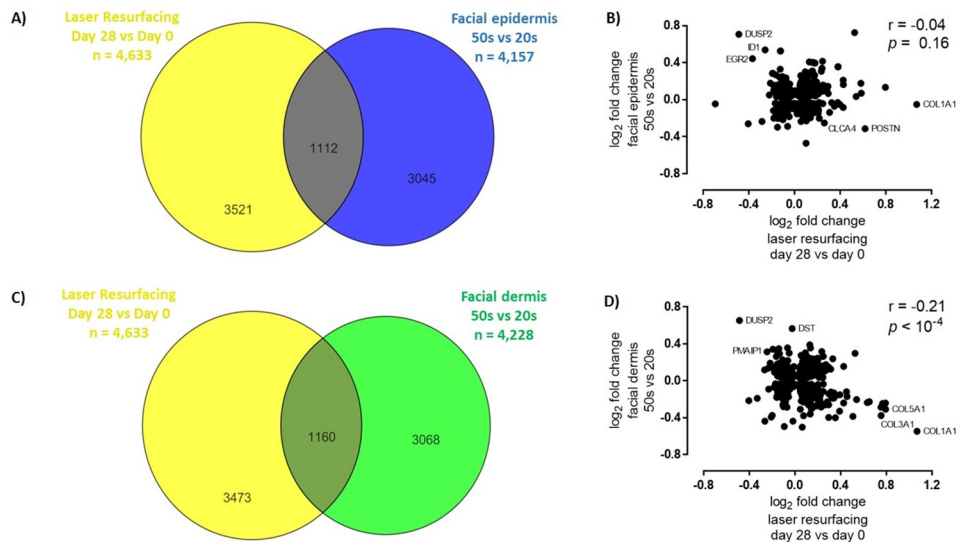

Fig 3. Laser resurfacing treatment reverses age-related gene expression changes. **A)** Venn diagram depicting the overlap in statistically significant genes altered at 28 days post-laser resurfacing and in facial epidermis between 50-year-olds and 20-year-olds described in Kimball et al., 2018. **B)** Log_2 fold change (FC) plot and Pearson correlation for the overlapping genes from A). **C)** Venn diagram depicting the overlap in statistically significant genes in the laser resurfacing single-treatment kinetic signature and in facial epidermis between 50-year-olds and 20-year-olds described in Kimball et al., 2018. **D)** Log_2 fold change (FC) plot and Pearson correlation for the overlapping genes from C).
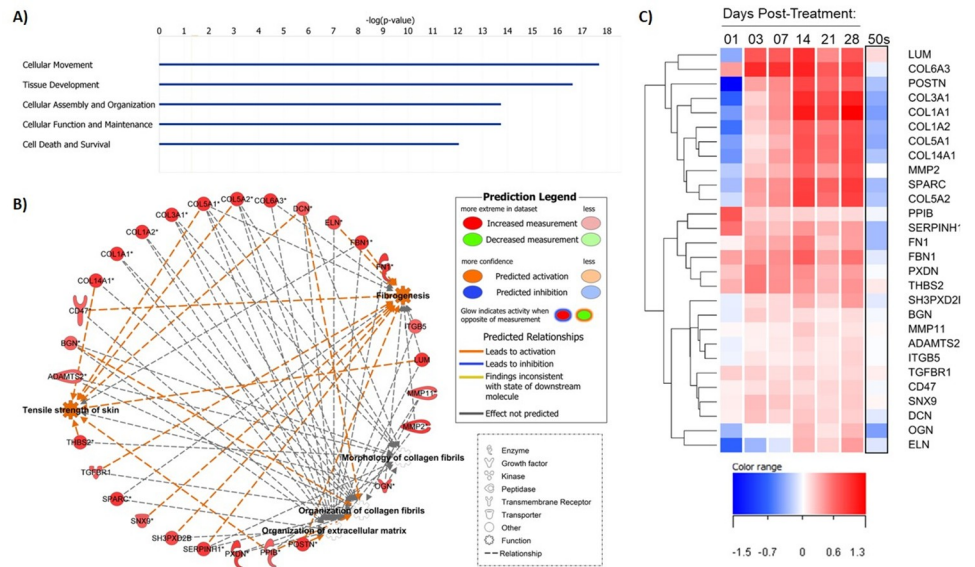
https://doi.org/10.1371/journal.pone.0260095.g003

Fig 4. Activation of multiple collagen-related pathways by day 28 post-treatment. A) Bar chart showing top 5 molecular functions and system development processes associated with the day 28 gene signature (by–$\log_{10}$ Fisher's *p*-value). **B)** Network of shared genes across select collagen-related pathways associated with day 28 gene signature (from Table 6). **C)** Dendrogram displaying hierarchical clustering of shared collagen pathways across single laser resurfacing treatments (vs. baseline untreated samples) and in facial dermis of 50-year-olds vs. 20-year-olds described in Kimball et al., 2018, by $\log_2$ fold change.

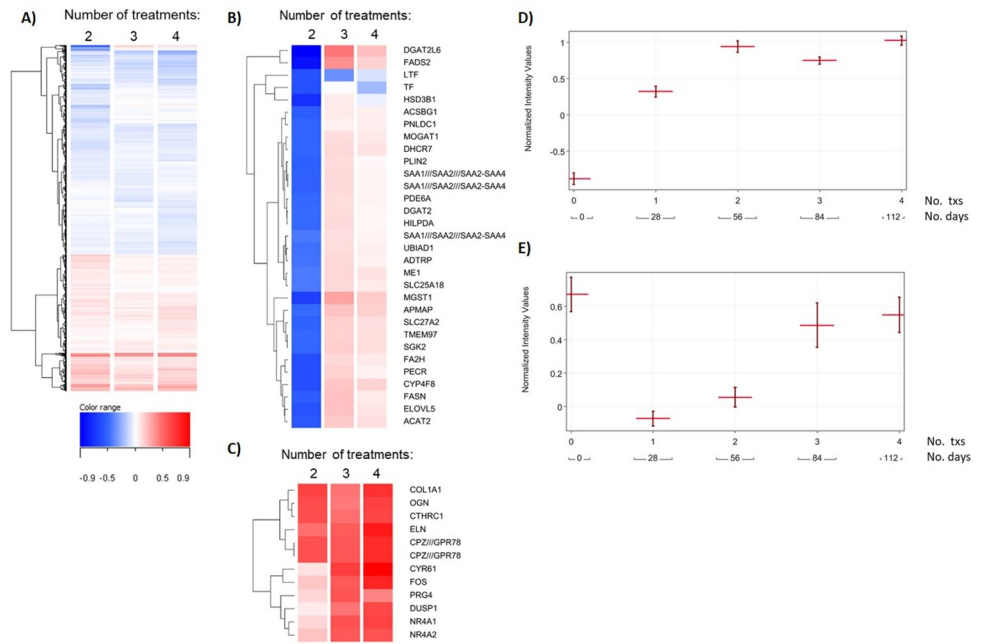https://doi.org/10.1371/journal.pone.0260095.g004

Fig 5. Effects of multiple laser resurfacing treatments over time on gene expression. A) Dendrogram displaying hierarchical clustering of genes by average $\log_2$ fold change that were significantly changed at any multiple-treatment sites compared to the single-treatment site (n = 5,795 total). **B-C)** Dendrograms of select gene clusters differentially regulated from 2–4 laser resurfacing treatments (**B**) or continuously upregulated with increasing number of treatments (**C**). **D-E)** Profile plots showing normalized expression values (±SEM) of *COL1A1* (**D**) and *NR4A1* (**E**) across the different number of treatments over time.

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Additional studies are needed to tease apart the precise components of the immune system that are involved in the resolution and repair of epidermal and dermal wound healing following laser resurfacing treatment.

In the dermis, fibroblast mediated gene expression changes occurred immediately after treatment and continued throughout the four-month observation period. An interesting observation was the early (1 day after treatment) coordinated decrease in gene expression of fibroblast cell death and fibrogenesis pathways with an increase in gene expression of connective tissue growth, proliferation of connective tissue and cell movement of connective tissue pathways. These results indicate a complex fibroblast response to damage which appears to be focused on increasing fibroblast numbers and movement to damaged dermal areas. In contrast, later in the dermal wound healing process we observed an increase in the formation of collagen fibrils, tensile strength of skin, and attachment of cells pathways, all of which indicate a maturation of the wound healing response by synthesis and maturation of the extracellular matrix. Interestingly, negative inhibition of MMP signaling and MMP expression levels also remained elevated throughout the time points The observation that MMPs are induced with fractional laser treatment is consistent with previous studies demonstrating MMP expression upon injury in a variety of cell types, including mesenchymal, epithelial and immune cells [71]. It is furthermore well established that MMPs are necessary for normal regulation and remodeling of the ECM, and that MMP activity is tightly regulated at multiple levels, beyond regulation of transcription [71]. The observation that the later dermal gene expression changes following fractional laser treatment are associated with activated tensile strength of skin, in particular, supports a direct relationship between the molecular changes induced by fractional laser treatment and the biomechanical properties of the skin. Activation of this pathway 28 days after treatment is consistent with the delayed onset of the skin appearance benefits following fractional laser treatment, and further supports that the late-stage dermal remodeling processes are crucial to the ultimate cosmetic benefits delivered by this gold standard anti-aging procedure. Together, the temporal analysis of gene expression changes following fractional laser wounding demonstrate a complex, yet orderly, repair process and provides significant insight into which biological processes are required for skin repair and rejuvenation.

Given that each treatment covers only a fraction (20–25%) of the treatment area [31], the effects of multiple fractional laser treatments on the transcriptional response in the epidermis and dermis were evaluated. The results showed that an overall strong wound healing response is maintained or increased after multiple laser treatments, which is best exemplified by the increase in COL1A1 and other extracellular matrix genes, with at least 2–3 treatments needed for maximal increase in gene expression and this drives remodeling of the extracellular matrix. An evaluation of NR4A1, a nuclear receptor that functions as a transcriptional repressor of TGF-β targeted genes whose expression is decreased during wound healing [72], demonstrated at least 3 fractional laser treatments were required before NR4A1 levels increased to pretreatment levels. These observations fit well with the clinical experience in which maximal skin rejuvenation is observed following multiple fractional laser treatments [73].

Multiple cosmetic procedures work to rejuvenate skin by activating the wound repair process (e.g., Fraxel® Thermage®, Ultherapy®); however, fractional laser is considered the gold standard treatment for aging related skin rejuvenation. The analysis of the gene expression changes observed following fractional laser treatment identified many biological and molecular pathways that are activated following treatment. These pathways were compared to the biological pathways that are modulated during skin aging using our previously described aging transcriptomics dataset [8], in order to better understand how this treatment works for skin rejuvenation. A large number of genes in common between fractional laser treatment and aging of both the epidermis and dermis of human skin were observed, with treatment leading

to reversal of many aging associated gene expression changes. These findings are notable given the differences in body site sampled (back vs. face), tissue matrices (full-thickness biopsies vs. separated tissue) and individual variability, suggesting that fractional laser treatment acts holistically across both epidermal and in dermal compartments to restore a more youthful skin structure. Many of the key biological processes affected by fractional laser treatment that are instrumental in skin rejuvenation are the processes involved in the regeneration of the dermal extracellular matrix. Reversal of the age associated loss of extracellular matrix gene expression (most notably collagen genes) was observed along with changes in gene expression indicative of significant remodeling of the extracellular matrix. These observations demonstrate that approaches to rejuvenate aged skin should ideally provide these two functions. Interestingly, several material-based approaches that achieve similar increases in extracellular matrix production to rejuvenate skin include peptides (such as matrikines) and combinations of peptides [74–76] and retinoids [77, 78]. Together these observations provide evidence that maximal skin rejuvenation requires a multi-faceted approach addressing both epidermal and dermal aging, with a key driver being the modulation of the long-lived dermal extracellular matrix. The transcriptome-wide effects of fractional laser induced skin repair and rejuvenation, analyzed in the present study with both a high degree of temporal precision and across multiple treatments, provides many new potential intervention targets to improve the structure, function and appearance of aging skin.

Supporting information

S1 Table. Top 20 molecular functions and system development process annotations associated with day 1 post-laser resurfacing treatments (by Fisher's p-value).
(XLSX)

S2 Table. Top 20 molecular functions and system development process annotations associated with day 3 post-laser resurfacing treatments (by Fisher's p-value).
(XLSX)

S3 Table. Top 20 molecular functions and system development process annotations associated with day 7 post-laser resurfacing treatments (by Fisher's p-value).
(XLSX)

S4 Table. Top 20 molecular functions and system development process annotations associated with day 14 post-laser resurfacing treatments (by Fisher's p-value).
(XLSX)

S5 Table. Top 20 molecular functions and system development process annotations associated with day 21 post-laser resurfacing treatments (by Fisher's p-value).
(XLSX)

S6 Table. Top 20 molecular functions and system development process annotations associated with day 28 post-laser resurfacing treatment (by Fisher's p-value).
(XLSX)

S7 Table. Top 20 molecular functions and system development process annotations associated with 2 laser resurfacing treatments (by Fisher's p-value).
(XLSX)

S8 Table. Top 20 molecular functions and system development process annotations associated with 3 laser resurfacing treatments (by Fisher's p-value).
(XLSX)

S9 Table. Top 20 molecular functions and system development process annotations associated with 4 laser resurfacing treatments (by Fisher's p-value).
(XLSX)

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References

1. Lener T, Moll PR, Rinnerthaler M, Bauer J, Aberger F, Richter K. Expression profiling of aging in the human skin. Exp Gerontol. 2006; 41:387–397. https://doi.org/10.1016/j.exger.2006.01.012 PMID: 16530368

2. Makrantonaki E, Brink TC, Zempeli V, Elewa RM, Mlody B, Hossini AM, et al. Identification of biomarkers of human skin ageing in both genders. Wnt signaling–a label of skin ageing? PLOS ONE. 2012; 7 (11):e50393. https://doi.org/10.1371/journal.pone.0050393 PMID: 23226273

3. Sextius P, Marionnet C, Tacheau C, Bon F-X, Bastien P, Mauviel A, et al. Analysis of gene expression dynamics revealed delayed and abnormal epidermal repair process in aged compared to young skin. Arch Dermatol Res. 2015; 307:351–364. https://doi.org/10.1007/s00403-015-1551-5 PMID: 25740152

4. Holzscheck N, Sohle J, Kristof B, Gronniger E, Gallinat S, Wenck H, et al. Multi-omics network analysis reveals distinct stages in the human aging progression in epidermal tissue. Aging. 2020; 12 (12):12393–12409. https://doi.org/10.18632/aging.103499 PMID: 32554863

5. Yan W, Zhang L-L, Yan L, Zhang F, Yin N-B, Lin H-B, et al. Transcriptome analysis of skin photoaging in Chinese females reveals the involvement of skin homeostasis and metabolic changes. PLOS ONE. 2013; 8(4):e61946. https://doi.org/10.1371/journal.pone.0061946 PMID: 23637934

6. Blumenberg M. Skinomics, transcriptional profiling approaches to molecular and structural biology of epiderms. Semin Cutan Med Surg. 2019; 38(1):E12–E18. https://doi.org/10.12788/j.sder.2019.004 PMID: 31051018

7. Kuehne A, Hildebrand J, Soehle J, Wenck H, Terstgen L, Gallinat S, et al. An integrative metabolomics and transcriptomics study to identify metabolic alterations in aged skin of humans in vivo. BMC Genomics. 2017; 18:169. https://doi.org/10.1186/s12864-017-3547-3 PMID: 28201987

8. Kimball AB, Alora-Palli MB, Tamura M, Mullins LA, Soh C, Binder RL, Houston NA, et al. Age-induced and photoinduced changes in gene expression profiles in facial skin of Caucasian females across 6 decades of age. J Am Acad Dermatol. 2017; 78(1):29–39. https://doi.org/10.1016/j.jaad.2017.09.012 PMID: 29146147

9. Xu J, Spitale RC, Guan L, Flynn RA, Torre EA, Li R, et al. Novel gene expression profile of women with intrinsic skin youthfulness by whole transcriptome sequencing. PLOS ONE. 2016; 11(11):e0165913. https://doi.org/10.1371/journal.pone.0165913 PMID: 27829007

10. Sole-Boldo L, Raddatz G, Schutz S, Mallm J-P, Rippe K, Lonsdorf AS, et al. Single-cell transcriptomes of the human skin reveal age-related loss of fibroblast priming. Commun Bio. 2020; 3:188. https://doi.org/10.1038/s42003-020-0922-4 PMID: 32327715

11. Cho BA, Yoo S-K, Seo J-S. Signature of photo-aging and intrinsic aging in skin were revealed by transcriptome network analysis. Aging. 2018; 10(7):1609–1626. https://doi.org/10.18632/aging.101496 PMID: 30021930

12. Zou Z, Long X, Zhao Q, Zheng Y, Song M, Ma S, et al. A single-cell transcriptomic atlas of human skin aging. Dev Cell. 2020; S1534- 5807(20):30877–7. https://doi.org/10.1016/j.devcel.2020.11.002 PMID: 33238152

13. Haustead DJ, Stevenson A, Saxena V, Marriage F, Firth M, Silla R, Martin L, et al. Transcriptome analysis of human ageing in male skin shows mid-life period of variability and central role of NFkB. Sci Rep. 2016; 6:26846. https://doi.org/10.1038/srep26846 PMID: 27229172

14. Humphrey S, Beleznay K, Fitzgerald R. Combination therapy in midfacial rejuvenation. Dermatol Surg. 2016; 42 (Suppl 2):S83–88. https://doi.org/10.1097/DSS.0000000000000747 PMID: 27128249

15. Fathi R, Pfeiffer ML, Tsoukas M. Minimally invasive eyelid care in dermatology: medical, laser and cosmetic therapies. Clinics Dermatol. 2015; 33:207–216. https://doi.org/10.1016/j.clindermatol.2014.10.012 PMID: 25704940

16. Orringer JS, Hammerberg C, Hamilton T, Johnson TM, Kang S, Sachs DL, et al. Molecular effects of photodynamic therapy for photoaging. Arch Dermatol. 2008; 144(10):1296–1302. https://doi.org/10.1001/archderm.144.10.1296 PMID: 18936392

17. Fernandes D, Signorini MD. Combating photoaging with percutaneous collagen induction. Clinics Dermatol. 2008; 26:192–199. https://doi.org/10.1016/j.clindermatol.2007.09.006 PMID: 18472060

18. Fitzpatrick RE, Tope WD, Goldman MP, Satur NM2. Pulsed carbon dioxide laser, trichloroacetic acid, Baker-Gordon phenol and dermabrasion: a comparative clinical and histological study of cutaneous resurfacing in a procine model. Arch Dermatol. 1996; 132(4):469–471. PMID: 8629856

19. Helbig D, Simon JC, Paasch U. Epidermal and dermal changes in response to various skin rejuvenation methods. Int J Cosmetic Sci. 2010; 32(6):458–469. https://doi.org/10.1111/j.1468-2494.2010.00573.x PMID: 20572891

20. Pryor L, Gordon CR, Swanson EW, Reish RG, Horton-Beeman K, Cohen SR. Dermaplaning, topical oxygen and photodynamic therapy: a system review of the literature. Aesth Plast Surg. 2011; 35 (6):1151–1159. https://doi.org/10.1007/s00266-011-9730-z PMID: 21533984

21. Goldman A, Wollina U. Facial rejuvenation for middle-aged women: a combined approach with minimally invasive procedures. Clin Interventions in Aging. 2010; 5:293–299. https://doi.org/10.2147/cia.s13215 PMID: 20924438

22. Brightman LA, Brauer JA, Anolik R, Weiss E, Karen J, Chapas A, et al. Ablative and fractional ablative lasers. Dermatol Clin. 2009; 27(4):479–489. https://doi.org/10.1016/j.det.2009.08.009 PMID: 19850197

23. Helbig D, Paasch U. Molecular changes during skin aging and wound healing after fractional ablative photothermolysis. Skin Res Technol. 2011; 17:119–128. https://doi.org/10.1111/j.1600-0846.2010.00477.x PMID: 21226879

24. Papadavid E, Katsambas A. Lasers for facial rejuvenation: a review. Int J Dermatol. 2003; 42:480–487. https://doi.org/10.1046/j.1365-4362.2003.01784.x PMID: 12786881

25. Tierney EP, Eisen RF, Hanke CW. Fractionated CO_2 laser skin rejuvenation. Dermatol Ther. 2011; 24:41–53. https://doi.org/10.1111/j.1529-8019.2010.01377.x PMID: 21276157

26. Tan J, Lei Y, Ouyang HW, Gold MH. The use of fractional CO2 laser resurfacing in the treatment of photoaging in Asians: five-year long-term results. Lasers Surg Med. 2014; 46(10):750–756. https://doi.org/10.1002/lsm.22304 PMID: 25400224

27. Ortiz AE, Tremaine AM, Zachary CB. Long-term efficacy of a fractional resurfacing device. Lasers Surg Med. 2010; 42(2):168–170. https://doi.org/10.1002/lsm.20885 PMID: 20166157

28. Sadick NS, Smoller B. A study examining the safety and efficacy of a fractional laser in the treatment of photodamage on the hands. J Cosmet Laser Ther. 2009; 11:29–33. https://doi.org/10.1080/14764170802612992 PMID: 19177257

29. Kohl E, Meierhofer J, Koller M, Zeman F, Klein A, Hohenleutner U, et al. Fractional carbon dioxide laser resurfacing of rhytides and photoageing: a prospective study using profilometric analysis. Br J Dermatol. 2014; 170:858–865. https://doi.org/10.1111/bjd.12807 PMID: 24372002

30. Grunewald S, Bodendorf M, Illes M, Kendler M, Simon JC, Paasch U. In vivo wound healing and dermal matrix remodeling in response to fractional CO2 laser intervention: clinicopathological correlation in non-facial skin. Int J Hyperthermia. 2011; 27:1–8. https://doi.org/10.3109/02656736.2010.516305 PMID: 21070137

31. Buis J and Mazur J.-M. Fractional photo-thermolysis by laser Fraxel ® as an adjuvant for facial surgical rejuvenation. Annals of Aesthetic Plastic Surgery. 2007; 52 (3):222–233.

32. Hantash BM, Bedi VP, Kapadia B, Rahman Z, Jiang K, Tanner H, et al. In vivo histological evaluation of a novel ablative fractional resurfacing device. Lasers Surg Med. 2007; 39:96–107. https://doi.org/10.1002/lsm.20468 PMID: 17311274

33. Bedi VP, Chan KF, Sink RK, Hantash BM, Herron GS, Rahman Z, et al. The effects of pulse energy variations on the dimensions of microscopic thermal treatment zones in nonablative fractional resurfacing. Laser Surgery Med. 2007; 39:145–155. https://doi.org/10.1002/lsm.20406 PMID: 17096412

34. Rahman Z, MacFalls H, Jiang K, Chan KF, Kelly K, Tournas J, et al. Fractional deep dermal ablation induces tissue tightening. Lasers Surg Med. 2009; 41:78–86. https://doi.org/10.1002/lsm.20715 PMID: 19226572

35. Cohen SR, Goodacre A, Lim S, Johnston J, Henssler C, Jeffers B, et al. Clinical outcomes and complications associated with fractional lasers: a review of 730 patients. Aesth Plast Surg. 2017; 41:171–178. https://doi.org/10.1007/s00266-016-0767-x PMID: 28032173

36. Robati RM, Asadi E. Efficacy and safety of fractional CO2 laser versus fractional Er:YAG laser in the treatment of facial skin wrinkles. Lasers Med Sci. 2017; 32:283–289. https://doi.org/10.1007/s10103-016-2111-8 PMID: 27885522

37. Laubach H-J, Tannous Z, Anderson RR, Manstein D. Skin responses to fractional photothermolysis. Lasers Surg Med. 2006; 38:142–149. https://doi.org/10.1002/lsm.20254 PMID: 16392146

38. Trelles MA, Velez M, Mordon S. Correlation of histological findings of single session Er:YAG skin fractional resurfacing with various passes and energies and the possible clinical implications. Laser Surg Med. 2008; 40(3):171–177. https://doi.org/10.1002/lsm.20607 PMID: 18366083

39. El-Domyati M, Abd-El-Raheem T, Abdel-Wahab H, Medhat W, Hosam W, El-Fakahany H, et al. Fractional versus ablative eerbium:yttrium-aluminum-garnet laser resurfacing for facial rejuvenation: an objective evaluation. J Am Acad Dermatol. 2013; 68(1):103–112. https://doi.org/10.1016/j.jaad.2012.09.014 PMID: 23110966

40. El-Domyati M, Abd-El-Raheem T, Medhat W, Abdel-Wahab H, Anwer MA. Multiple fractional erbium:yttrium-aluminum-garnet laser sessions for upper facial rejuvenation: clinical and histological implications and expectations. J Cosmetic Dermatol. 2014; 13:30–37. https://doi.org/10.1111/jocd.12079 PMID: 24641603

41. Longo C, Galimberti M, De Pace B, Pellacani G, Bencini PL. Laser skin rejuvenation: epidermal changes and collagen remodeling by in vivo confocal microscopy. Lasers Med Sci. 2013; 28:769–776. https://doi.org/10.1007/s10103-012-1145-9 PMID: 22767322

42. Prignano F, Campolmi P, Bonan P, Ricceri F, Cannarozzo G, Troiano M, et al. Fractional CO$_2$ laser: a novel therapeutic device upon photobiomodulation of tissue remodeling and cytokine pathway of tissue repair. Dermatol Ther. 2009; 22:S8–15. https://doi.org/10.1111/j.1529-8019.2009.01265.x PMID: 19891690

43. Helbig D, Mobius A, Simon JC, Paasch U. Heat shock protein 70 expression patterns in dermal explants in response to ablative fractional photothermolysis, microneedle or scalpel wounding. Wounds. 2011; 23(3):59–67. PMID: 25881332

44. Xu XG, Luo YJ, Wu Y, Chen JZ, Xu TH, Gao XH, et al. Immunohistological evaluation of skin responses after treatment using a fractional ultrapulse carbon dioxide laser on back skin. Dermatol Surg. 2011; 37 (8):1141–1149. https://doi.org/10.1111/j.1524-4725.2011.02062.x PMID: 21649788

45. Prignano F, Ricceri F, Bonan P, Cannarozzo G, Campolmi P. Induction of apoptosis by fractional CO2 laser treatment. J Cosmetic Laser Ther. 2012; 14:267–271. https://doi.org/10.3109/14764172.2012.738820 PMID: 23058108

46. Helbig D, Bodendorf MO, Grunewald S, Kendler M, Simon JC, Paasch U. Immunohistochemical investigation of wound healing in response to fractional photothermolysis. J Biomed Opt. 2009; 14(6):064044. https://doi.org/10.1117/1.3275479 PMID: 20059282

47. El-Domyati MM, Attia SK, Esmat AM, Ahmad HM, Abdel Wahap HM, Badr BM. Effect of laser resurfacing on p53 expression in photoaged facial skin. Dermatol Surg. 2007; 33:668–675. https://doi.org/10.1111/j.1524-4725.2007.33141.x PMID: 17550442

48. Zheng Z, Kang H-Y, Lee S, Kang S-W, Goo B, Cho SB. Up-regulation of fibroblast growth factor (FGF) 9 expression and FGF-WNT/β-catenin signaling in laser-induced wound healing. Wound Rep Reg. 2014; 22:660–665. https://doi.org/10.1111/wrr.12212 PMID: 25041895

49. Reilly MJ, Cohen M, Hokugo A, Keller GS. Molecular effect of fractional carbon dioxide laser resurfacing on photodamaged human skin. Arch Facial Plast Surg. 2010; 12(5):321–325. https://doi.org/10.1001/archfacial.2010.38 PMID: 20855774

50. Orringer JS, Sachs DL, Shao Y, Hammerberg C, Cui Y, Voorhees JJ, et al. Direct quantitative comparison of molecular responses in photodamaged human skin to fractionated and fully ablative carbon dioxide laser resurfacing. Dermatol Surg. 2012; 38(10):1668–1677. https://doi.org/10.1111/j.1524-4725.2012.02518.x PMID: 22805255

51. Starnes AM, Jou PC, Molitoris JK, Lam M, Baron ED, Garcia-Zuazaga J. Acute effects of fractional laser on photo-aged skin. Dermatol Surg. 2012; 38(1):51–57. https://doi.org/10.1111/j.1524-4725.2011.02136.x PMID: 22093071

52. Kislevitz M, Lu KB, Wamsley C, Hoopman J, Kenkel J, Akgul Y. Novel use of non-invasive devices and microbiopsies to assess facial skin rejuvenation following laser treatment. Lasers Surg Med. 2020; 52:822–830. https://doi.org/10.1002/lsm.23233 PMID: 32173884

53. Park S-H, Kim D-W, Jeong T. Skin tightening effect of fractional laser: comparison of non-ablative and ablative fractional lasers in animal models. J Plast Reconstr Aesthet Surg. 2012; 65:1305–1311. https://doi.org/10.1016/j.bjps.2012.04.028 PMID: 22633871

54. Jiang X, Ge H, Zhou C, Chai X, Ren QS. The role of vascular endothelial growth factor in fractional laser resurfacing with the carbon dioxide laser. Lasers Med Sci. 2012; 27:599–606. https://doi.org/10.1007/s10103-011-0996-9 PMID: 21960121

55. Jiang X, Ge H, Zhou C, Chai Z, Deng H. The role of transforming growth factor B1 in fractional laser resurfacing with a carbon dioxide laser. Lasers Med Sci. 2014; 29:681–687. https://doi.org/10.1007/s10103-013-1383-5 PMID: 23820975

56. Natari S, Kim KE, Ryu SI, Park JH, Kim I-H. Device induced neocollagenesis: profibrotic response or true neocollagenesis? Lasers Surg Med. 2020; 52:1010–1019. https://doi.org/10.1002/lsm.23258 PMID: 32383204

57. Qu Y, Wang Y, Zhang Y, Han C, Gao D, Jin W, et al. The comparison of skin rejuvenation effects of vitamin A, fractional laser, and their combination on rat. J Cosmet Laser Ther. 2019; 21:19–27. https://doi.org/10.1080/14764172.2018.1439967 PMID: 29543550

58. Amann PM, Marquardt Y, Steiner T, Holze F, Skazik-Voogt C, Heise R, et al. Effects of non-ablative fractional erbium glasss laser treatment on gene regulation in human three-dimensional skin models. Lasers Med Sci. 2016; 31:397–404. https://doi.org/10.1007/s10103-015-1863-x PMID: 26796701

59. Schmitt L, Huth S, Amann PM, Marquardt Y, Heise R, Fietkau K, et al. Direct biological effects of fractional ultrapulsed CO2 laser irradiation on keratinocytes and fibroblasts in human organotypic full-thickness 3D skin models. Lasers Med Sci. 2018; 33:765–772. https://doi.org/10.1007/s10103-017-2409-1 PMID: 29218493

60. Huth L, Marquardt Y, Heise R, Fietkau K, Baron JM, Huth S. Biological effects of hyaluronic acid-based dermal fillers and laser therapy on human skin models. J Drugs Dermatol. 2020; 19(9):897–899. https://doi.org/10.36849/JDD.2020.10.36849/JDD.2020.4856 PMID: 33026754

61. Schmitt L, Amann PM, Marquardt Y, Heise R, Czaja K, Gerber PA, et al. Molecular effects of fractional ablative erbium:YAG laser treatment with multiple stacked pulses on standardized human three-dimensional organotypic skin models. Lasers Med Sci. 2017; 32:805–814. https://doi.org/10.1007/s10103-017-2175-0 PMID: 28299490

62. Marquardt Y, Amann PM, Heise R, Czaja K, Steiner T, Merk HF, et al. Characterization of a novel standardized human three-dimensional skin wound healing model using non-sequential fractional ultrapulsed CO2 laser treatments. Lasers Surg Med. 2015; 47:257–265. https://doi.org/10.1002/lsm.22341 PMID: 25771913

63. Nogita T, Maeda T, Shimokata T, Tsuboi R. Cytokine profiling analysis following a non-ablative fractional laser on rat skin. J Dermatol. 2017; 44:207–208. https://doi.org/10.1111/1346-8138.13526 PMID: 28497906

64. Kim JE, Won CH, Bak H, Kositratna G, Manstein D, Dotto GP, et al. Gene profiling analysis of the early effects of ablative fractional carbon dioxide laser treatment on human skin. Dermatol Surg. 2013; 39(7):1033–1043. https://doi.org/10.1111/dsu.12170 PMID: 23489371

65. Kim D, Chen R, Sheu M, Kim N, Kim S, et al. Noncoding dsRNA induces retinoic acid synthesis to stimulate hair follicle regeneration via TLR3. Nat Commun. 2019; 10(1):2811. https://doi.org/10.1038/s41467-019-10811-y PMID: 31243280

66. Canales RD, Luo Y, Willey JC, Austermiller B, Barbacioru CC, Boysen C, et al. Evaluation of DNA microarray results with quantitative gene expression platforms. Nat Biotechnol. 2006; 24(9): 1115–1122. https://doi.org/10.1038/nbt1236 PMID: 16964225

67. Nunan R, Campbell J, Mori R, Piteluscu ME, Jiang WG, Harding KG, et al. Ephrin-Bs drive junctional downregulation and actin stress fiber disassembly to enable wound re-epithelization. Cell Rep. 2015; 13(7):1380–1395. https://doi.org/10.1016/j.celrep.2015.09.085 PMID: 26549443

68. Shi Y, Yang R, Tu L, Liu D. Long non-coding RNA HOTAIR promotes burn wound healing by regulating epidermal stem cells. Mol Med Rep. 2020; 22(3):1811–1820. https://doi.org/10.3892/mmr.2020.11268 PMID: 32582996

69. Lucas T, Waisman A, Ranjan R, Roes J, Krieg T, Müller W, et al. Differential roles of macrophages in diverse phases of skin repair. J Immunol. 2010; 184(7):3964–77. https://doi.org/10.4049/jimmunol.0903356 PMID: 20176743

70. Strbo N, Yin N,2 Stojadinovic O. Innate and Adaptive Immune Responses in Wound Epithelialization. Adv Wound Care (New Rochelle). 2014; 3(7): 492–501 https://doi.org/10.1089/wound.2012.0435 PMID: 25032069

71. Loffe k, et al. Biological Role of Matrix Metalloproteinases: A Critical Balance. Eur Respir J. 2011; 38:191–208. https://doi.org/10.1183/09031936.00146510 PMID: 21177845

72. Palumbo-Zerr K, Zerr P, Distler A, Fliehr J, Mancuso R, et al. Orphan nuclear receptor NR4A1 regulates transforming growth factor–β signaling and fibrosis. Nat Med. 2015; 21(2):150–158. https://doi.org/10.1038/nm.3777 PMID: 25581517

73. Allemann IB, Kaufman J. Fractional photothermolysis–an update. Lasers Med Sci. 2010; 25:137–144. https://doi.org/10.1007/s10103-009-0734-8 PMID: 19787413

74. Schagen SK. Topical peptide treatments with effective anti-aging results. Cosmetics. 2017: 4(16)1–14.

75. Katayama K, Seyer JM, Raghow R, Kang AH. Regulation of extracellular matrix production by chemically synthesized subfragments of type I collagen carboxy propeptide. Biochemistry. 1991; 30:7097–7104. https://doi.org/10.1021/bi00243a009 PMID: 1854722

76. Katayama K, Armendariz-Borunda J, Raghow R, Kang AH, Seyer JM. A pentapeptide from type I collagen promotes extracellular matrix production. J Biol Chem. 1993; 268(14):9941–9944. PMID: 8486721

77. Shao Y, He T, Fisher GJ, Voorhees JJ, Quan T. Molecular basis of retinol anti-ageing properties in naturally aged human skin in vivo. Int J Cosmet Sci. 2017; 39(1):56–65. https://doi.org/10.1111/ics.12348 PMID: 27261203

78. Kong R, Cui Y, Fisher GJ, Wang X, Chen Y, Schneider LM, et al. A comparative study of the effects of retinol and retinoic acid on histological, molecular and clinical properties of human skin. J Cosmet Dermatol. 2016; 15(1):49–57. https://doi.org/10.1111/jocd.12193 PMID: 26578346