Clinical and Molecular Effects on Mature Burn Scars After Treatment With a Fractional CO_2 Laser

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Background and Objective: There have been several case reports of improvement in the appearance of mature burn scars following treatment with fractional CO_2 lasers. However, the biochemical mechanisms responsible for these improvements have not been elucidated.

Materials and Methods: Ten patients with mature, full-thickness, hypertrophic burn scars received initial treatment with a fractional CO_2 laser. Clinical improvement was measured with Vancouver Scar Scale as well as Patient and Observer Scar Assessment Scale. Fresh tissue samples were obtained before the initial treatment and 48 hours after the first treatment for TaqMan Real-time RT-PCR analyses. Expressions of several scar-related biological markers, including types I and III procollagen, matrix metalloproteinase (MMP)-1, -13, transforming growth factor (TGF)-β1, β2, β3, and basic fibroblast growth factor (bFGF), as well as microRNA miR-17-92 cluster, were investigated.

Results: There were significant improvements in both observer and subject ratings in all scales. Both types I and III procollagen mRNA levels were dramatically down-regulated after treatment, but the ratio of types I/III procollagen mRNA was not different. The expression of MMP-1 was significantly up-regulated after treatment, while TGF-β2, -β3, and bFGF levels were significantly down-regulated. Expression of miR-18a and miR-19a were dramatically up-regulated ($P < 0.05$) after treatment.

Conclusions: Our study indicated that fractional CO_2 resulted in clinical improvement of mature burn scar. Alteration of types I and III procollagen, MMP-1, TGF-β2, -β3, bFGF, as well as miRNAs miR-18a and miR-19a expression may be responsible for the clinical improvement after treatment. Our finding may have implications for novel treatments and further our understanding of fractional CO_2 laser treatment. Lasers Surg. Med. 44:517–524, 2012. © 2012 Wiley Periodicals, Inc.

Key words: fractional CO_2 laser; scar; MMP-1; TGF-β; bFGF; microRNAs; miR-17-92

INTRODUCTION

Hypertrophic scars are abnormal scars characterized by a benign proliferation in the dermis, with increased tissues fibrosis and types I and III collagen deposition [1]. Clinically, treatment is challenging despite a wide array of options including corticosteroids, radiation, lasers, surgery, and interferon. In order to improve our understanding and management of this condition, it is important to explore the mechanisms of action of these diverse forms of therapy. There have been several case reports of improvement in mature burn scars after treatment with fractional CO_2 lasers [2–5]. However, no prospective studies have been performed and the clinical improvement has not been correlated with biological markers. There are several factors frequently reported to be involved in scar remodeling.

Collagenases are categorized as matrix metalloproteinases (MMPs), which are endoproteinases involved in extracellular matrix (ECM) remodeling. MMP-1 preferentially degrades type III collagen. MMP-13 can degrade all fibrillar collagen subtypes with almost equal efficacy [6]. Previous studies indicate that MMPs and its inhibitors

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are involved in the remodeling of abnormal scars [7]. Transforming growth factor-β (TGF-βs) is considered to be the most potent growth factor involved in wound healing and is believed to be the key regulator in the pathogenesis of hypertrophic scars and other fibrotic conditions [8]. It was reported that three TGF-β isoforms (TGF-β1, β2, and β3) and their receptors had different expression in abnormal scars and normal skin [9,10]. This indicated that these isoforms played diverse roles in the development of different subtypes of abnormal scars. Additionally, recent evidence suggested that TGF-βs were involved in the mechanisms related to scar regression after laser treatment [11]. Basic fibroblast growth factor (bFGF) promotes the growth and differentiation of many cell types. It has both angiogenic and mitotic properties, influencing tissue remodeling, wound healing, and promoting tumor growth. It has been found to significantly inhibit the differentiation of mesodermal progenitor cells into myofibroblasts, which are the key mediators of tissue fibrosis and the primary producer of collagen. It also accelerates wound healing and improves scar quality by regulating the production and degradation of extracellular matrix, as seen in studies involving rabbit ear models [12].

Not only the above proteins-coding RNAs, but also non-coding RNAs, may be involved in the mechanism of the fractional CO_2 laser treatment on burn scar. Recently, there were several newly published documents that indicated that microRNAs might play an important role in the progress of scar development and regression [13–15]. MicroRNAs (miRNAs) were first discovered in 1993 by Victor Ambros. They are a class of 21–25 nucleotides single-stranded non-coding small RNAs often found in introns, and are increasingly being recognized as important regulators of gene expression by imperfect base pairing to the target mRNAs, initiating the degradation of target mRNAs, or inhibiting effective translation. Additional documents indicated that miRNAs play an important role in skin morphogenesis, cancer and wound healing by regulating cells proliferation, apoptosis and differentiation [16]. In the current study, we measured the expression levels of some protein coding RNAs, including types I and III procollagen, MMP-1 and -13, TGF-β1, β2, and β3, and bFGF mRNA, as well as non-protein coding miRNA miR-17-92 gene cluster in 10 patients with mature burn scars, before and 48 hours after the first treatment. Clinical improvement was seen as measured by the Vancouver Scar Scale as well as the Patient and Observer Scar Assessment Scale. Our study sheds light on the mechanism by which the fractional CO_2 laser affects scar remodeling and may lead to potential new therapeutic targets for scar treatment.

MATERIALS AND METHODS

Patients

Ten patients with mature burn scars from various causes were recruited to complete a series of 3–4 treatments with the fractional CO_2 laser. All of the patients had pre- and post-treatment biopsies of fresh tissue for RNA analysis of the biochemical changes following treatment. Patients ranged from 24 to 58 years of age, with an average age of 38 (Table 1). The causes of the burns included hot water (n = 1), acid (n = 1), grease (n = 1), and fire (n = 7). The average total body surface area of involvement was 28% and ranged from 4% to 60%. The average treated area was 225 cm^2. These patients had not received other treatments including intralesional corticosteroid injections, compression, skin grafts, z-plasty, and other surgical interventions within 6 months before the study began. The study was conducted in coordination with the Moy–Fincher Medical Group, Grossman Burn Center, and researchers from the Department of Dermatology, Henry Ford Hospital. This study was approved by the Western International Review Board. All subjects provided written informed consent.

Laser Treatment and Tissues Collection

Pre-treatment biopsies were collected from the area to be treated, which was outlined with a sterile marking pen, and photographs were taken. Specimens were immediately transferred to RNAlater (Qiagen, Valencia, CA) and shipped to Henry Ford Hospital in Detroit for analysis.

TABLE 1. Summary of Patient Characteristics*

Patient no.	Age (year)	Cause of burn	Gender	Ethnicity	BSA affected (%)	Treatment area	Treatment size (cm^2)
1	40	Acid	F	Asian	30	Chest/neck	200
2	24	Fire	M	Caucasian	26	Left arm	158
3	28	Fire	M	Latino	12	Chest/abdomen	455
4	29	Fire	F	Asian	35	Neck	126
5	39	Grease	F	Indian/black/white	20	Chest	120
6	35	Fire	M	Latino	60	Neck/clavicle	180
7	47	Fire	M	Latino	38	Neck	197
8	28	Hot water	M	Asian	10	Chest/abdomen	225
9	54	Fire	F	Caucasian	40	Chest	332
10	58	Fire	F	African American	4	Thighs	256

*Mature burn scars were defined as stable at least 1 year after thermal injury.

Patients were anesthetized with either topical BLT (Betacaine, lidocaine, tetracaine), intralesional lidocaine with epinephrine or tumescent anesthesia. All 10 subjects received an initial treatment with a fractional CO_2 laser (Lumenis, Santa Clara, CA). The laser settings were developed from clinical experience with prior scar and resurfacing treatments. No pilot study was performed. All areas selected for inclusion had predominantly hypertrophic scarring and were treated with Active FX, energy settings 80–100 mJ (53–79 μm ablation), 200 Hz, 75 MTZ/cm^2, Density 2–4 (68–100%), 1.3 mm spot size. The thicker, banded areas within these scars were treated with both Active FX (energy settings as above) and Deep FX, average energy settings 20 mJ (600 μm ablation), 300 Hz, 361 MTZ/cm^2 to 529 MTZ/cm^2 (density 10–15%), 0.12 mm spot size. Adjustments were made within the described parameters for patient comfort. A second biopsy was taken from an area adjacent to the initial biopsy site 48 hours after treatment and was immediately placed in RNAlater solution and shipped for analysis.

Scar Rating Scales

Eight of the ten patients completed an additional two planned laser treatments to evaluate the clinical efficacy after three total treatment sessions. The two patients who did not complete the study reported pain with tumescent anesthesia as the reason for withdrawing from the study. Prior to treatment and 2 months after the final treatment, the patient and treating physician completed scar rating scales. Patients completed two scales: (1) The Vancouver Scar Scale [17], the most widely used assessment scale which measures vascularity, pigmentation, pliability, and height. (2) The Patient portion of the Patient and Observer Scar Assessment Scale [18], which asks more concrete questions regarding scar conditions such as pruritus, pain, and stiffness. The treating physician completed the Observer portion of the Observer Scar Assessment Scale.

TaqMan Real-Time RT-PCR

Total RNA was isolated from the scars before and after treatment using the mirVana miRNA isolation kit (Ambion, Austin, TX) according to the manufacturer's specifications. Reverse transcription reactions were performed using High-Capacity cDNA Reverse Transcription kits (Applied Biosystems, Foster City, CA). For each reaction, 200 ng RNA was added. PCR reaction was done in the system mixed with 0.5 ul 20 × TaqMan® Gene Expression assays, 5 ul TaqMan® Gene Expression Master Mix, 20 ng cDNA template and 2.5 ul RNase-free water. Results were obtained from the average measured in triplicate and normalized to a control gene GAPDH. Fold changes were generated by calculating $2^{-\Delta\Delta Ct}$.

MicroRNA Quantitation

Quantitation of miRNAs expression was performed by RT-PCR using TaqMan MicroRNA Assay human set (Applied Biosystems) in one subject, which was performed on 7900HT Fast Real-Time PCR System (Applied Biosystems), according to the manufacturer's recommended protocol. Raw cycle threshold (Ct) values were calculated using the SDS 2.3 and RQ manager 1.2 software (Applied Biosystems) applying automatic baseline and threshold settings. RNU6B was used as endogenous control. Relative quantitations using the ΔΔCt method were carried out and fold changes were calculated by calculating $2^{-\Delta\Delta Ct}$ for each miRNAs gene.

Statistics

Data were analyzed by the Paired Student t-test using GraphPad Prism 5.0. Differences with a $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

The appearance of mature burn scars markedly improved after a series of three treatments with fractional CO_2 laser (Fig. 1). Subjects had statistically significant improvement in scar quality after treatment as measured by both patient and observer validated scar scales. The mean initial Patient Score was 41.2 with a standard error of 2.7, while the mean post Patient Score was 24.1 with a standard *d* error of 2.2 ($P = 0.0006$; Fig. 2A). The mean initial Observer Score was 33.8 with a standard error of 1.9, while the mean post Observer Score was 19.4 with a standard error of 2.4 ($P = 0.00001$; Fig. 2B). The mean initial Vancouver Score was 8.6 with a standard error of 0.5, while the mean post treatment Vancouver Score was 5.0 with a standard error of 0.6 ($P = 0.0002$; Fig. 2C). The decrease was statistically significant.

Hypertrophic scars are characterized by increased tissue fibrosis and collagen deposition after injury. Collagen is a family of closely related, yet genetically distinct, proteins of the extracellular matrix. In human skin, collagen comprises 70–80% of the dry weight. Normal skin and normal scars, as well as hypertrophic scars and keloids (abnormal scars), produce mostly types I and III collagen. Type I collagen accounts for 80–90% and type III 10–15% of the total collagen present in the skin [19]. When the ten mature hypertrophic burn scars were treated with the fractional CO_2 laser, as the major component of abnormal scars, both types I and III collagen mRNA expression levels were significantly decreased (Fig. 3A,B, $^{**}P < 0.01$, $^{***}P < 0.001$). This indicated that fractional CO_2 laser treatment suppressed hypertrophic scar by decreasing the expression levels of both types I and III collagen. But the mRNA ratio of types I/III procollagen was not changed after treatment (Fig. 3C, $P = 0.438$). Interestingly, additional histology work to be published from our lab revealed an increase in type III collagen and a decrease in type I collagen in treated skin 2 months after a series of three fractional CO_2 laser treatments. This suggests that the initial suppression may be followed by gradual orderly remodeling with a ratio favoring normal skin.

MMP-1 and MMP-13 are recognized for their unique ability to cleave the triple helical domain of fibrillar collagen types I, II, and III. However, each one differs in the extent to which it cleaves these fibrillar collagen subtypes in vitro. MMP-1 preferentially degrades type III collagen, but appears to have no significant activity against types

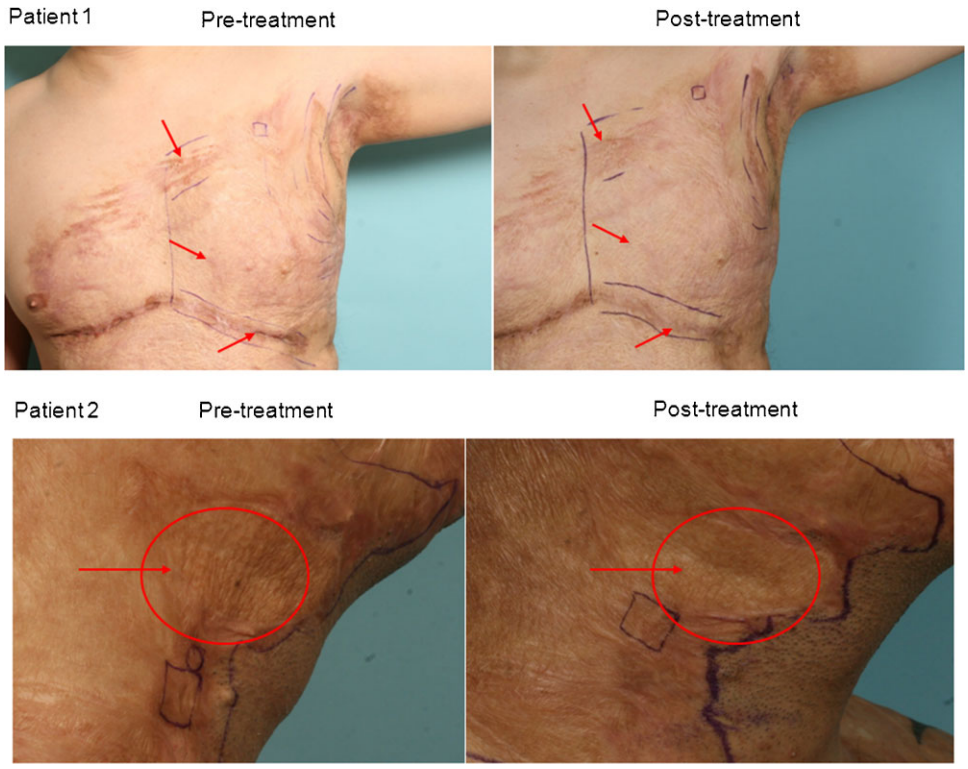

Fig. 1. Pre- and post-treatment photographs following three sessions with the fractional CO_2 laser. Arrows directs improvement in hypertrophic portion of scars. The outlined area is treatment area with thicker areas emphasized. The square area (1 cm^2) is marked for biopsy site.

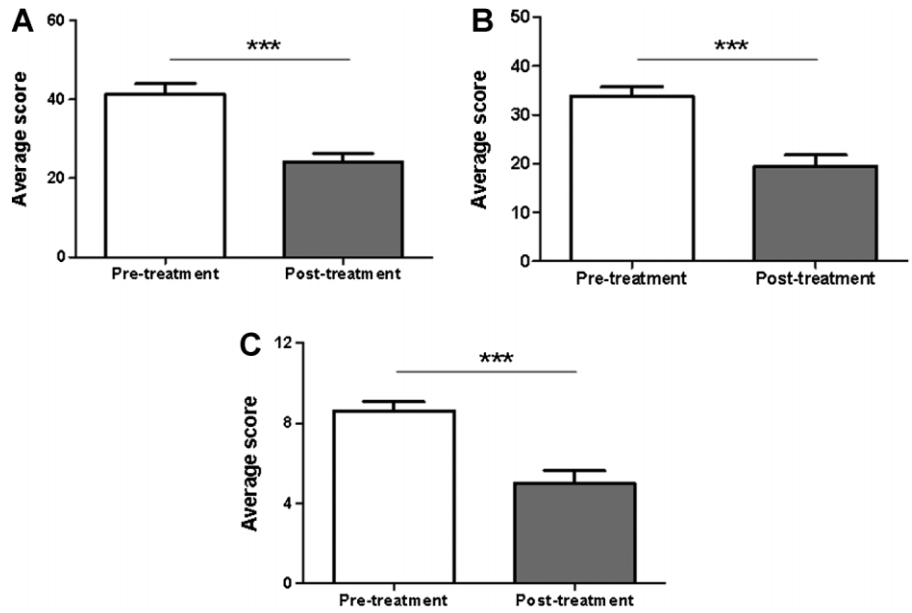

Fig. 2. Pre- and post-treatment scores for the Observer and Patient Scar Assessment Scale and the Vancouver Scar Scale. The Patient (**A**) and Observer (**B**) assessment scores were significantly decreased after treatment. The Vancouver Scar score (**C**) post-treatment was also dramatically decreased compared to pre-treatment. Error bar indicates mean ± SE, $^{***}P < 0.001$.

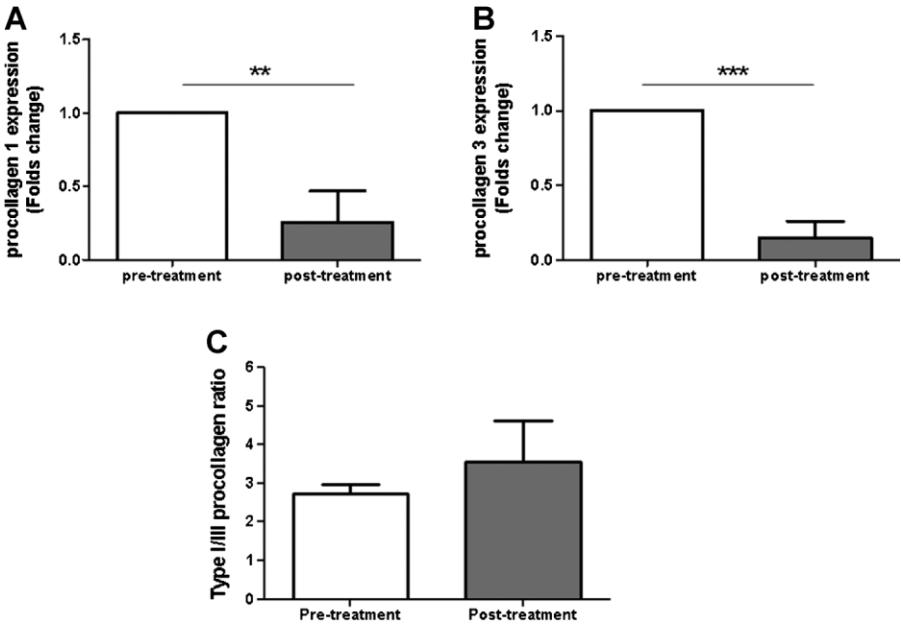

Fig. 3. Types I and III procollagen mRNA expression were detected. TaqMan Real-time RT-PCR showed that fractional CO_2 laser treatment significantly decreased the expression of type I (**A**) and type III (**B**) procollagen in scar tissue. But the ratio of type I/III procollagen (**C**) was not changed after treatment. Error bar indicates Mean ± SE, $^{**}P < 0.01$, $^{***}P < 0.001$.

II and IV collagen. MMP-13 is the most recently discovered human collagenase, which can degrade all fibrillar collagen subtypes with almost equal efficacy, and is the only collagenase with significant activity against types II and IV collagen [6]. In our study, MMP-1 expression in mature hypertrophic burn scar tissues was prominently increased after treatment (Fig. 4A, $^{***}P < 0.001$). In contrast, there was no significant difference for the expression of MMP-13 before and after treatment (Fig. 4B, $P = 0.56$). These findings indicated that fractional CO_2 laser treatment performed its function through selectively increasing expression of MMP-1, but not MMP-13, in mature hypertrophic burn scars. It was also reported that flash lamp pulsed-dye laser (PDL) treatment suppressed TGF-ß1 expression in keloids, which was related with up-regulation of MMP-13 [20]. That implied that the TGF-ß signal pathway was related with MMP expression and/or function.

The mechanism by which fractional CO_2 laser treatment improves the appearance of mature hypertrophic burn scars is presently unknown. Transforming growth factor-ß (TGF-ßs), which can be released by degranulating platelets and macrophages at the site of injury, influence the inflammatory response, angiogenesis, re-epithelialization, ECM deposition, and remodeling. Three isoforms, TGF-β1, -β2, and -β3 exist. TGF-β1 and 2 are thought to be profibrotic, whereas TGF-β3 may have anti-fibrotic functions, depending on the study [21,22]. We had postulated that TGF-β3 expression would increase after treatment and that TGF-β1 and β2 would decrease. In our study, there was no significant difference of TGF-β1 expression before and after treatment (Fig. 4C, $P = 0.153$). However, the expressions of TGF-β2 and β3 were significantly decreased after treatment (Fig. 4D,E, $^{*}P < 0.05$). This suggests that both TGF-β2 and β3 were profibrotic in mature hypertrophic burn scars. Additional data points would have been helpful to determine if TGF-β3 levels rebound or increase during the healing or scar maturation phase. It has been documented that flash lamp pulsed dye laser (PDL) treatment suppressed keloid fibroblast proliferation through down-regulation of TGF-β1 expression [23]. It is possible that diverse therapies may function through differing isoforms, especially in different scar types.

Basic fibroblast growth factor (bFGF) has been found to significantly inhibit the differentiation of mesodermal progenitor cells into myofibroblasts, which are the key mediators of tissue fibrosis and the primary producer of collagen. It also accelerates wound healing and improves scar quality by regulating the extracellular matrix production and degradation, as seen in studies involving rabbit ear models [12]. In our study, expression of bFGF was significantly decreased after treatment (Fig. 4F, $^{*}P < 0.05$). This suggested that fractional CO_2 laser treatment also affects mature burn scar by regulating bFGF levels.

It was reported that there was alteration of miRNAs expression, such as miR-203, miR-205, and miR-200c in keloids tissue compared to normal skin tissue [15]. Recent published document indicated that over-expression or

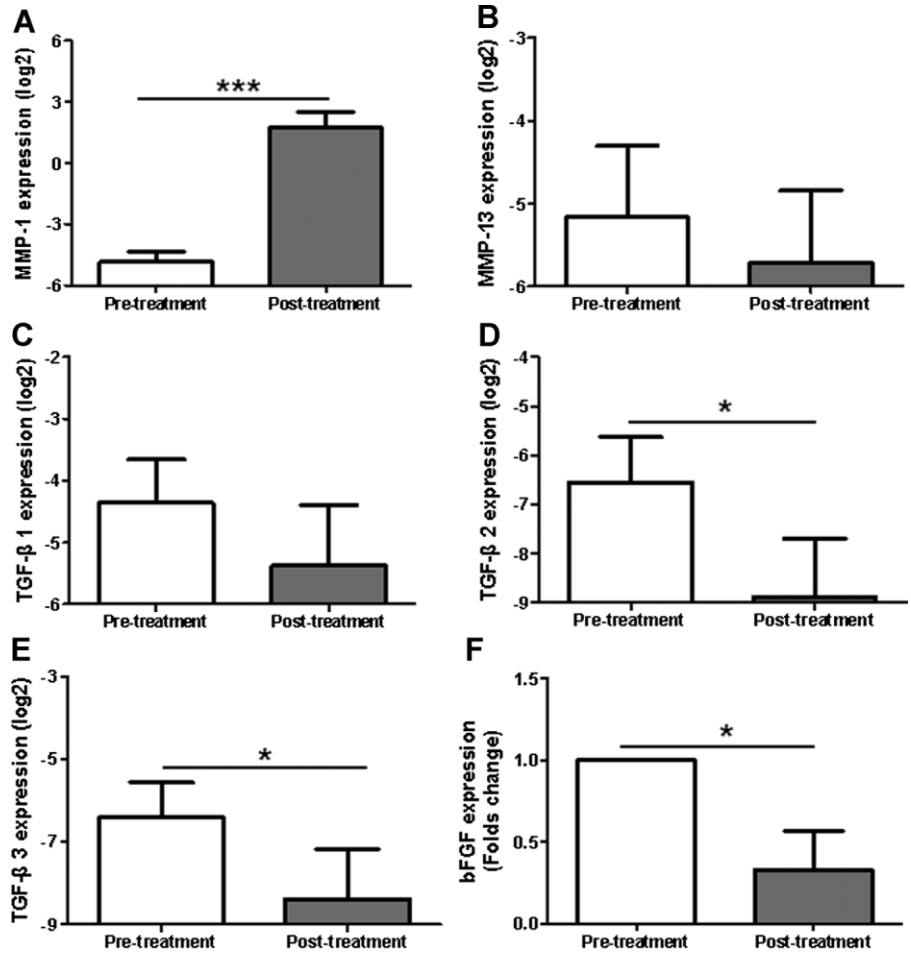

Fig. 4. MMP-1, -13, TGF-β1, -β2, -β3, and bFGF expression levels were assayed. TaqMan Real-time RT-PCR showed that MMP-1 expression (**A**) was dramatically increased, whereas the MMP-13 expression (**B**) was not different. TGF-β1 expression did not change after treatment (**C**). TGF-β -β2 (**D**), -β3 (**E**) expression decreased after treatment. Expression of bFGF (**F**) was down-regulated after treatment. Error bar indicates Mean ± SE. $^{*}P < 0.05$, $^{***}P < 0.001$.

knockdown of miR-196a led to a decreased or increased level of secreted types I/III collagens in keloid-derived fibroblasts (KFs) compared to normal fibroblasts (NFs) [14].

microRNA miR-17-92 cluster is involved in the TGF-β signaling pathway by targeting of several regulatory components in this signaling pathway. Specifically, miR-17-5p and miR-20 reduce the expression of the type II TGFbeta receptor and miR-18 limits the expression of Smad4 [24–26], which play important roles in the development of scar [22,27,28]. Single miRNAs RT-PCR showed significantly increased of miR-18a and miR-19a expression in hypertrophic burn scar after treatment (Fig. 5B,C). There was no detectable difference in the expression of miR-17, miR-19b, miR-20a, and miR-92 (Fig. 5A,D–F). But, there is a trend that other miRNAs expressions were up-regulated. It was also reported that miR-1792 regulated age-related heart failure by targeting ECM proteins connective tissue growth factor (CTGF), which was involved in the scar development [29–31]. Together, our data support a role of miR-18a and miR19a during the burn scar regression after fractional CO_2 treatment.

CONCLUSION

Our results indicated that fractional CO_2 laser treatment induced mature hypertrophic burn scar regression by suppressing both types I and III collagen deposition through decreasing TGF-β2, -β3, and bFGF expression and increasing MMP-1 expression. Meanwhile, a group of miRNAs, especially miR-18a and miR-19a were also involved in this clinical improvement. This is the first published correlation between clinical mature burn scar improvement from fractional CO_2 laser treatment and miRNAs. Our findings indicated that these factors may

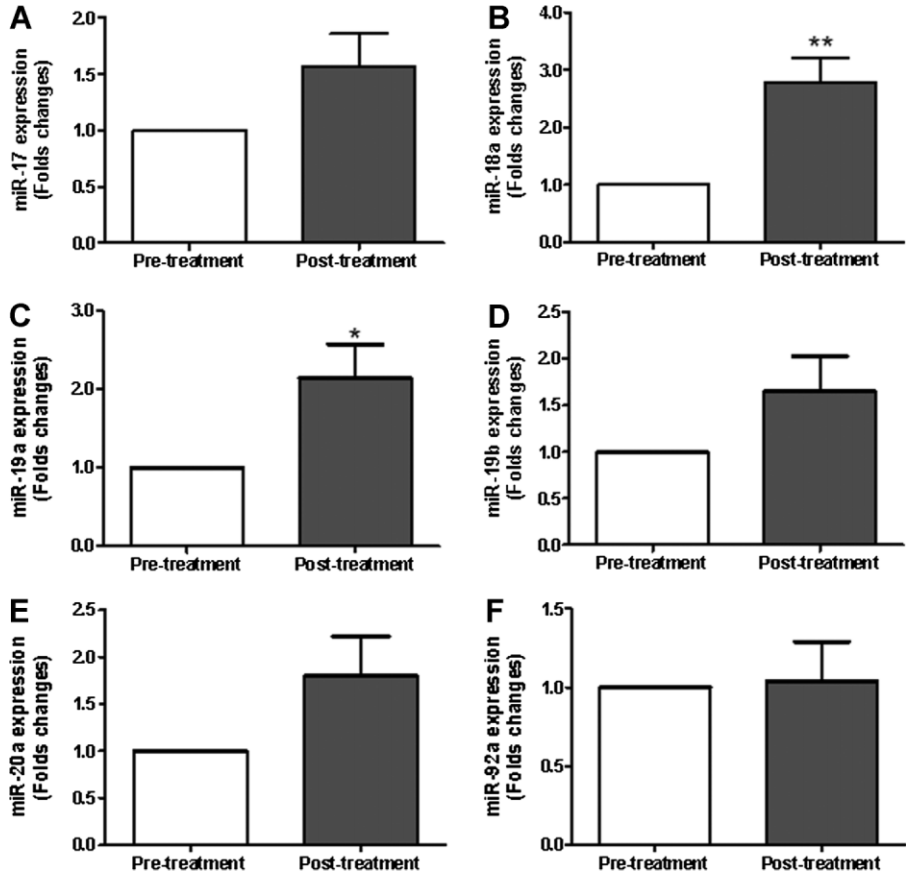

Fig. 5. Alteration of miR-17∼92 cluster miRNAs expression of after fraction CO_2 treatment. TaqMan Real-time RT-PCR showed significantly increased expression of miR-18a and miR-19a (**B**,**C**) in hypertrophic burn scar after fractional CO_2 treatment. There was no significant difference in the expression of miR-17, miR-19b, miR-20a, and miR-92 (**A**,**D**–**F**). Results were the average in triplicate and normalized to a control gene RNU6B. Student's *t*-test was employed for statistics. $P < 0.05$ was considered significant difference (error bars were mean ± SE, $^{*}P < 0.05$, $^{**}P < 0.01$).

play an important role in the regression of mature hypertrophic burn scars following fractional CO_2 laser treatment.

Limitations of this study include the single 48 hour post-treatment measurement of mRNA, the subjective nature of patient rating scar scales, and the single physician observer rating the clinical improvement. It would have been optimal to obtain multiple time points to further elucidate the dynamic changes in gene expression. This was not practical for some subjects who were traveling from out of state to participate in the trial. The subjective nature of scar rating scales is always an issue as patients have expectation bias toward improvement. This is somewhat mitigated by the significant physical involvement of the laser treatment. If the subjects did not feel that their scars were improving, it is not likely that 8 of 10 would have traveled for multiple visits, endured painful injections, and spent on average 1 week recovering from each treatment. The results of the observer ratings would have been strengthened if additional physicians had participated. However, during planning we could not be certain that multiple physician raters would be available to physically examine patients at both time points.

Further studies are needed to determine how fractional CO_2 laser treatment induces biosignals that suppress hypertrophic scar formation.

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