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PDGFR α Is a Key Regulator of T1 and T3's Differential Effect on SMA Expression in Human Corneal Fibroblasts

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PURPOSE. The goal of this study was to examine the mechanism behind the unique differential action of transforming growth factor β 3 (TGF- β 3) and TGF- β 1 on SMA expression. It was our hypothesis that platelet-derived growth factor receptor α (PDGFR α) played a key role in determining TGF- β 3's response to wounding.

METHODS. A stable cell line, human corneal fibroblast (HCF)-P, was created from HCFs by knocking down PDGFR α expression using a lentivirus-delivered shRNA sequence. A three-dimensional (3D) in vitro model was constructed by culturing HCF or HCF-P on poly-transwell membranes for 4 weeks in the presence and absence of 0.1 ng/mL TGF- β 1 or - β 3. At the end of 4 weeks, the constructs were processed for immunofluorescence and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In addition, HCF and HCF-P cell migration was evaluated.

RESULTS. In HCF, TGF- β 3 treatment resulted in significantly lower α -smooth muscle actin (SMA) mRNA expression and immunolocalization when compared to TGF- β 1, while in HCF-P, both TGF- β 1 and - β 3 treatment increased the SMA mRNA expression and immunolocalization compared to both the untreated HCF-P control and TGF- β 3-treated HCF. Human corneal fibroblast-P also had a lower migration rate and construct thickness when compared to HCF.

CONCLUSIONS. These results show that TGF- β 3 decreases SMA in HCF, while remarkably increasing SMA in HCF-P, thus indicating that the presence or absence of PDGFR α elicits contrasting responses to the same TGF- β 3 treatment. Understanding the role of PDGFR α in TGF- β 3's ability to stimulate SMA may potentially help in understanding the differential functions of TGF- β 1 and TGF- β 3 in corneal wound healing.

Keywords: TGF β 3, corneal scarring, PDGF, 3D cell culture

After a corneal injury, the cornea may scar (fibrosis) and become opaque, thus impairing vision and even leading to blindness. The process of corneal wound healing is complex and involves a variety of cytokines, growth factors, and proteases, which interact to regulate key phases of corneal healing. One such growth factor, transforming growth factor β (TGF β), has emerged as a key component of the corneal wound-healing response.¹ Transforming growth factor β has been found to regulate the transformation of quiescent keratocytes into activated fibroblasts, upregulate the secretion of extracellular matrix (ECM) materials, and enhance the formation of stress fibers.² Furthermore, TGF β directly stimulates the transformation of activated fibroblasts into myofibroblasts, which are filled with α smooth muscle actin (SMA) that forms microfilaments and are a major source of light scattering in corneal scars.^{3,4}

Transforming growth factor β isoforms, in particular, TGF- β 1 and - β 3 (T1 and T3, respectively), have been shown to play different roles that are key in determining the wound-healing outcome. For instance, T1 has been found to be a regulator of fibrosis and scarring, while T3 does not stimulate this scarring

effect in skin, lung, and kidney,⁵⁻⁷ and in some cases, even stimulates scar-free healing.^{8,9}

Like the TGF β s, there is also an extensive literature set associating platelet-derived growth factors (PDGFs) with fibrosis in different scarring models.¹⁰ The intrinsic affinity of each PDGF isoform for the two PDGFR subunits determines the type of receptor dimer that is assembled. Platelet-derived growth factor B is the universal ligand, whereas PDGFAB and PDGFC assemble and activate only PDGFR α homodimers and PDGFR $\alpha\beta$ heterodimers. Platelet-derived growth factor D activates PDGFR β homodimers, while PDGEA only activates PDGFR α homodimers.¹¹ Recently, it has been suggested that these PDGF receptors were activated through an autocrine loop leading to keratocyte proliferation and myofibroblast differentiation.¹² In addition, PDGFR α co-localizes with SMA, and PDGF-blocking antibodies inhibit the TGF β -induced differentiation of keratocytes to myofibroblasts.¹²

These studies led to the logical supposition that PDGFs contribute to SMA expression by differentially activating the PDGFRs. Therefore, in this study, we utilized our three-dimensional (3D) cell-culture model¹³ to study the relationship

TABLE. List of SYBR Green Primer Sequences Used for RT-qPCR

Growth Factor	Forward Sequences	Reverse Sequences
SMA	GGTGACGAAGCACAGAGCAA	CAGTTGGTGATGATGCCATGTT
PDGFA	GGTGGTCACAGGTGCTTTTT	AAACCACTTAAGGCTCTCAGGA
PDGFB	TGAGAAAAGATCGAGATTGTGCC	GGGCTTCGGGTCCACAGG
PDGFC	GAATGTCAATGTGTCCCAAGCA	TGCTCCCTCTGCACACACAG
PDGFD	ATTTGGCAGGCCATCATAGA	CCACAATTGACAACCTGGCTTC
GAPDH	GAAGGTGAAGGTCGGAGTCA	GACAAGCTTCCCGTCTCAG

between T1's and T3's interaction with PDGFR and determined if this interaction is responsible for their differential stimulation of SMA expression.

MATERIALS AND METHODS

Generation of Stable PDGFR α Knockdown Cell Line (HCF-P)

Human corneal fibroblasts (HCFs) were isolated as previously described¹⁴ from human corneas obtained from the National Disease Research Interchange (NDRI; Philadelphia, PA, USA). All research adhered to the tenets of the Declaration of Helsinki. Once isolated, HCFs were plated on six-well plates and grown to 75% confluency in media (Eagle's minimum essential media [EMEM]; American Type Culture Collection [ATCC], Manassas, VA, USA) with 10% serum (fetal bovine serum [FBS]; Atlanta Biologicals, Flowery Branch, GA, USA) and 1% Antibiotic-Antimycotic (ABAM; Thermo Fisher Scientific, Waltham, MA, USA).

To generate the HCF-P cell line, HCFs were infected with a PDGFR α shRNA lentivirus, as previously described.¹⁵ In brief, HCFs were grown to ~70% confluency in six-well cell culture plates. Media with lentivirus particles (pCMV-dR8.91, VSV-G/pMD2.G, hairpin-pLKO.1 vector containing the PDGFR α shRNA oligo [Dana Farber Cancer Institute/Harvard Medical School, Boston, MA, USA]), and Polybrene (4 μ g/mL; Sigma-Aldrich Corp., St. Louis, MO, USA), which was used to improve the transduction efficiency, was prepared. This media was applied to HCF, incubated overnight at 37°C with 5% CO₂, and then the cells were cultured with puromycin (2 μ g/mL; Sigma-Aldrich Corp.) for 1 month to exclusively select cells that expressed the PDGFR α shRNA. Samples from HCF-P and HCF were collected for Western blot¹⁶ and examined for PDGFR α knockdown. β -actin was used as a loading control. Four individual shRNA oligo sequences for PDGFR α were tested, and the cell line containing the shRNA with the maximum PDGFR α knockdown was selected and used in these studies (TRCN0000194855; Dana Farber Cancer Institute/Harvard Medical School).

3D Construct Assembly

Constructs were assembled as previously described.^{13,17,18} Briefly, the HCFs or HCF-Ps were plated at a density of 10⁶ cells/mL on six-well plates containing polycarbonate membrane inserts with 0.4- μ m pores (Transwell; Corning Costar, Charlotte, NC, USA). Human corneal fibroblasts or HCF-Ps were cultured for 4 weeks in construct medium (EMEM, 10% FBS, and a stable vitamin C [VitC] derivative [0.5 mM 2-O- α -D-glucopyranosyl-L-ascorbic acid; Wako Chemicals USA, Richmond, VA, USA]). Three different groups were tested per cell type: (1) control or C: construct medium; (2) T1: construct medium containing 0.1 ng/mL T1; and (3) T3: construct medium containing 0.1 ng/mL T3. Experiments using all groups were repeated at least three times, collected, and

processed for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and immunofluorescence.

Reverse Transcription-Quantitative Polymerase Chain Reaction

Total RNA was extracted (Trizol; Thermo Fisher Scientific) from the constructs, and cDNA was synthesized (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Carlsbad, CA, USA) according to manufacturer's protocols. The cDNA was combined with qPCR master mix (KAPA SYBR Fast qPCR master mix; KAPA Biosystems, Wilmington, MA, USA) and primers (Table: SMA,¹⁹ PDGFA, PDGFB, PDGFC, PDGFD, or glyceraldehyde 3-phosphate dehydrogenase [GAPDH]²⁰), and then amplified (Mastercycler ep realplex real-time PCR system; Eppendorf, Hauppauge, NY, USA) with the following thermal cycling conditions: 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C, and 1 minute at 60°C. GAPDH, an endogenous control, was used to normalize target genes. The primers for each gene were prepared by the CCIB DNA Core Facility at Massachusetts General Hospital (Cambridge, MA, USA), and the sequences are given in the Table below. The relative gene expression of the growth factors was calculated by using the comparative Ct method.²¹

Migration Assay

Human corneal fibroblasts or HCF-Ps were cultured in six-well cell culture plates. After growing to confluence, a scratch was made in the middle of the well using the end of a 200- μ L pipette tip. Three different groups were examined in serum-free construct media per cell type: (1) control: no growth factors; (2) T1: 0.1 ng/mL T1; and (3) T3: 0.1 ng/mL T3. The cells were imaged using a brightfield microscope (Nikon E8000; MicroVideo Instruments, Avon, MA, USA) at 0 and 24 hours, and were quantified using ImageJ (version 5.2; <https://imagej.nih.gov/ij/> provided in the public domain by National Institutes of Health, Bethesda, MD, USA). The percent migration was calculated by measuring the area without cells at 0 and 24 hours and then dividing by the corresponding 0-hour value, then multiplying by 100.

Immunofluorescence Staining

The constructs were collected and processed for immunofluorescence, as previously described.^{13,17,18} In brief, constructs were fixed in 4% paraformaldehyde for at least 24 hours, placed in blocking buffer (1% bovine serum albumin [BSA] and 0.1% Triton-X [Sigma-Aldrich Corp.]) for 1 hour, and incubated overnight at 4°C with primary antibody against SMA (Dako North America, Carpinteria, CA, USA) in blocking buffer. The next day, constructs were washed and incubated overnight at 4°C with secondary antibody (donkey anti-mouse IgG-FITC; Jackson ImmunoResearch, West Grove, PA, USA) in blocking buffer. TO-PRO-3 (Thermo Fisher Scientific) was used as a marker of all cell nuclei. Constructs were washed, mounted

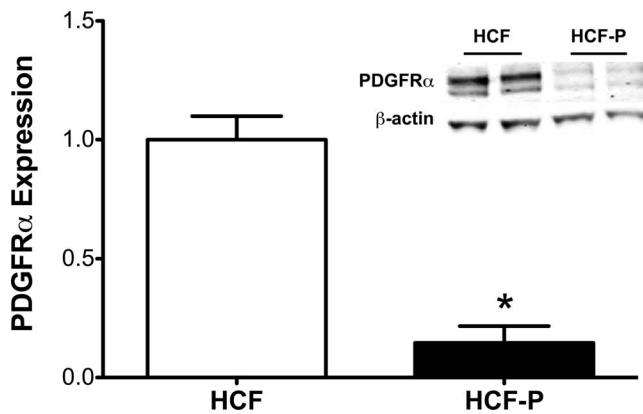


FIGURE 1. Graph and Western blot showing PDGFR α expression in HCF and HCF-P cell line. Platelet-derived growth factor receptor α expression significantly decreased in the HCF-P as compared with HCF (* $P < 0.05$, $n = 3$). Representative Western blot shows PDGFR α expression characterized by bands at 170 kDa (mature) and 160 kDa (immature). In the HCF, these bands are prominent; however, in HCF-P, these bands are less intense, indicating that the PDGFR α has been knocked down. β -actin (42 kDa) was used as the loading control.

(Vectashield; Vector Laboratories, Burlingame, CA, USA), observed, and photographed with either a fluorescent microscope (Nikon E8000; MicroVideo Instruments) for the 2 \times images or a confocal microscope (TCS-SP2; Leica Microsystems, Bannockburn, IL, USA) for the 40 \times images.

All images were acquired under identical photographic conditions for all treatment groups, and the brightness/contrast was kept constant. To quantify the amount of SMA in each slide, the area of staining in each slide was measured by ImageJ using the 2 \times images, which allowed for the complete view of the entire construct. The value obtained for the untreated control samples was considered to be background. The final amount of SMA staining for T1 and T3 was calculated by subtracting the background value from the T1 or T3 value. Three constructs per condition and cell type were examined. Construct thickness was measured with the confocal microscope (40 \times), beginning with the first cell visible at the top of the construct and the last cell visible at the bottom. Three constructs per condition and cell type were examined, and four readings from different areas of each construct were

recorded. The mean of these readings was then calculated and graphed.

Statistical Analysis

All experiments were performed in triplicate and analyzed for statistical significance ($P < 0.05$; GraphPad Prism version 5a; GraphPad Software, Inc., La Jolla, CA, USA) with either Student's t -test or ANOVA followed by Tukey's post hoc test.

RESULTS

Generation of a Stable PDGFR α Cell Line

To begin our examination of the effect of PDGF signaling on TGF β -mediated corneal SMA expression, we generated HCF that had PDGFR α expression permanently knocked down (HCF-P). As seen in Figure 1A, both the mature (170 kDa) and immature (160 kDa) bands of the dimeric form of PDGFR α ²² were visible for the control HCF, while it was greatly reduced in HCF-P. This result was further confirmed by image analysis (Fig. 1B), which showed that PDGFR α expression was significantly reduced ($P < 0.05$) in HCF-P when compared to HCF.

Comparison of SMA RNA Expression and Construct Thickness

In Figure 2A, SMA mRNA expression was examined by RT-qPCR in HCF and HCF-P constructs in response to T1 or T3. Upon T1 stimulation, SMA expression was significantly increased in both HCF (* $P < 0.05$) and HCF-P (** $P < 0.01$) when compared to their respective controls; however, with T3, a differential response was observed between the two cell types. In HCF, the SMA mRNA expression remained similar to its control, whereas, in HCF-P, as with T1 stimulation, T3 increased SMA mRNA expression significantly, as compared to its control (** $P < 0.01$). This suggests that PDGFR α plays a critical role in the stimulation of SMA by T3.

Figure 2B compares the mean construct thickness of both HCF and HCF-P in response to T1 or T3. As seen in the graph, HCF produced a significantly thicker construct than HCF-P overall (** $P < 0.01$). As expected, the thickness of T1- and T3-treated HCF constructs was significantly greater than that of

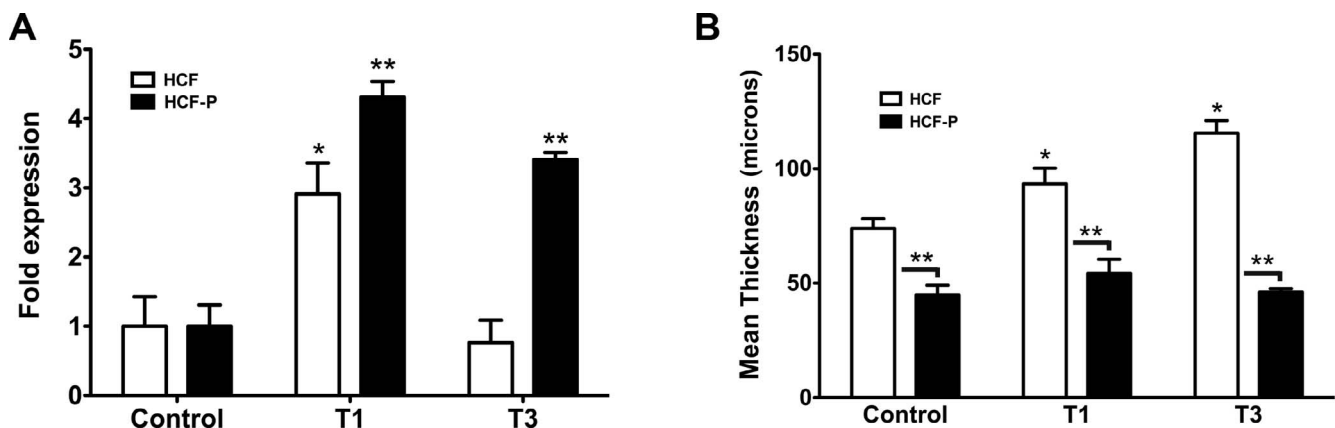


FIGURE 2. Comparison of SMA mRNA expression and mean thickness of constructs in HCF and HCF-P cell line treated with Control (no growth factors), TGF- β 1 (T1), or TGF- β 3 (T3). (A) Graph of RT-qPCR of SMA expression in HCF and HCF-P. Upon T1 stimulation, SMA expression increased in both cell types (* $P < 0.05$, ** $P < 0.01$) compared with their respective Control. With T3 stimulation, SMA expression for the HCF remained similar to Control level; however, it increased (** $P < 0.01$) in HCF-P compared to its Control. (B) Graph of the mean thickness of the HCF and HCF-P constructs. In HCF, T1 and T3 produced significantly thicker constructs (* $P < 0.05$) when compared to the untreated Control. The human corneal fibroblast-P in contrast had significantly thinner constructs (** $P < 0.01$) when compared to HCF cells even in the presence of T1 and T3.

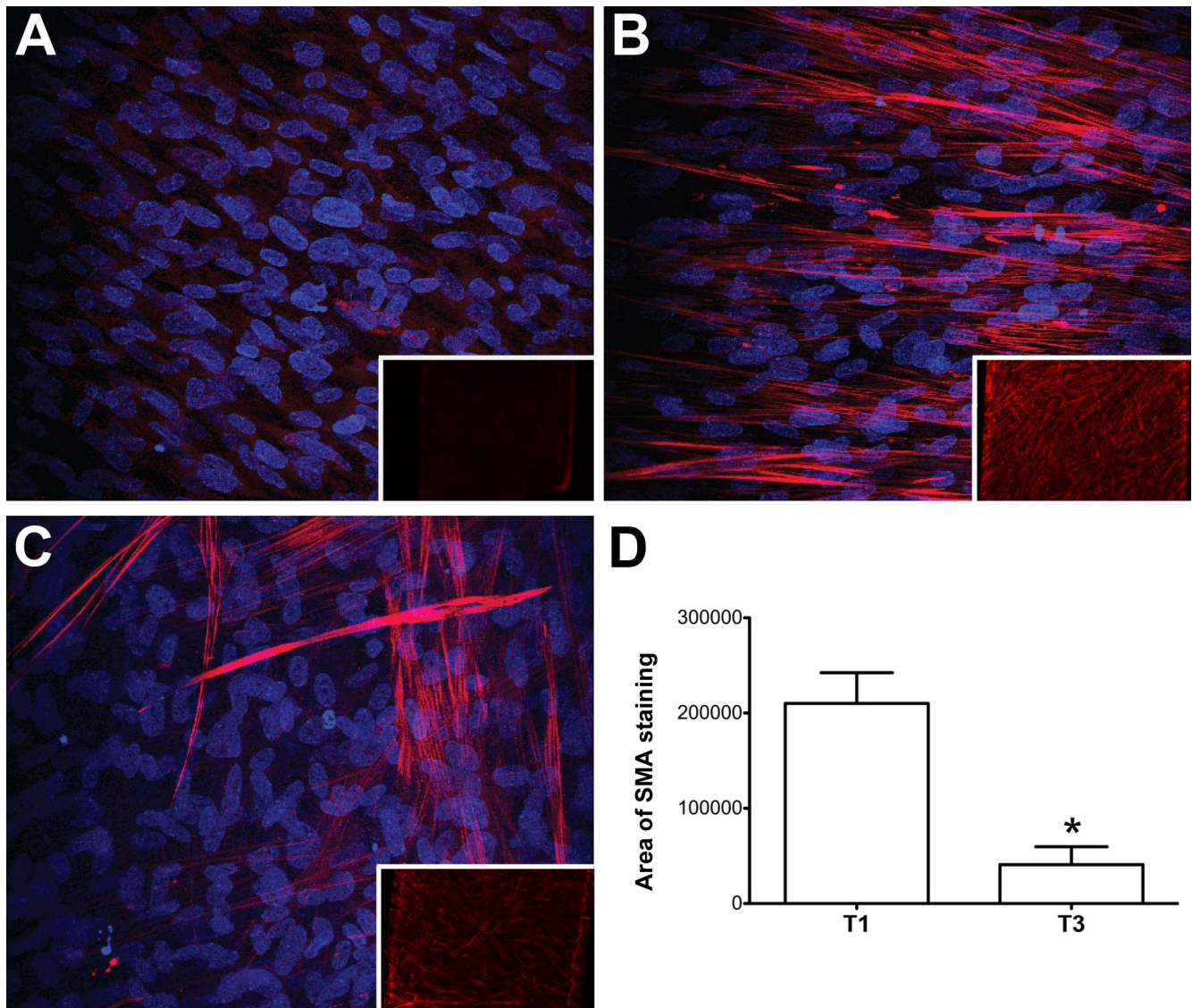


FIGURE 3. Smooth muscle actin immunofluorescence staining of HCF constructs: (A) Control: no growth factors, (B) T1: TGF- β 1, and (C) T3: TGF- β 3. Images were taken at 40 \times and 2 \times (*insets*). The 2 \times images were used for image analysis as it shows the entire surface area of the construct. (D) Graph of the area of SMA staining in square pixels measured using ImageJ, background corrected to Control. After 4 weeks, T3 resulted in significantly lower SMA expression than that of T1 (blue = DAPI; red = SMA; $n = 3$, $^*P < 0.05$).

HCF controls ($^*P < 0.05$); however, neither T1 nor T3 had any effect on the HCF-P construct thickness, compared to HCF-P controls.

Visualization of SMA Using Immunofluorescence

In order to visualize the presence of SMA in HCF (Fig. 3) and HCF-P (Fig. 4) constructs, as well as to quantify the amount of SMA staining, immunofluorescence was performed on each of the replicates. T1-treated samples had higher amounts of SMA staining (Fig. 3B) when compared to both the control (Fig. 3A) and T3-treated samples (Fig. 3C). When the area of SMA staining in each of the replicates was measured using ImageJ (Figs. 3A–C insets, 3D), SMA staining in T3-treated HCF constructs was found to be $\sim 95\%$ ($^*P < 0.05$) lower than in T1-treated samples (Fig. 3D).

However, in HCF-P constructs (Fig. 4), both T1 (Fig. 4B) and T3 (Fig. 4C) showed an increase in the amount of SMA staining when compared to their control (Fig. 4A). No

significant difference was noted between T1 and T3 treatment when the amount of SMA staining was quantified (Figs. 4A–C, insets, 4D). Therefore, in these samples, T3 increased the amount of SMA-staining in HCF-P cells, while it failed to do so in regular HCF.

Comparison of RNA Expression of PDGF Isoforms

Since our results suggested that PDGFR α plays an important role in the differential effect of T1 and T3, we then examined the expression of various PDGF ligands (PDGFA, PDGFB, PDGFC, and PDGFD) in both cell types in response to T1 or T3, in the hopes that these ligands may help in determining the dramatic difference between treatments. In both HCF and HCF-P, no change was noted in the mRNA expression of PDGFA (Fig. 5A) and PDGFB (Fig. 5B), except for a slight decrease in PDGFB when cells were stimulated with T3. In both cell types, T1 increased the expression of PDGFC (Fig. 5C) and PDGFD (Fig. 5D); however, T3 failed to stimulate the

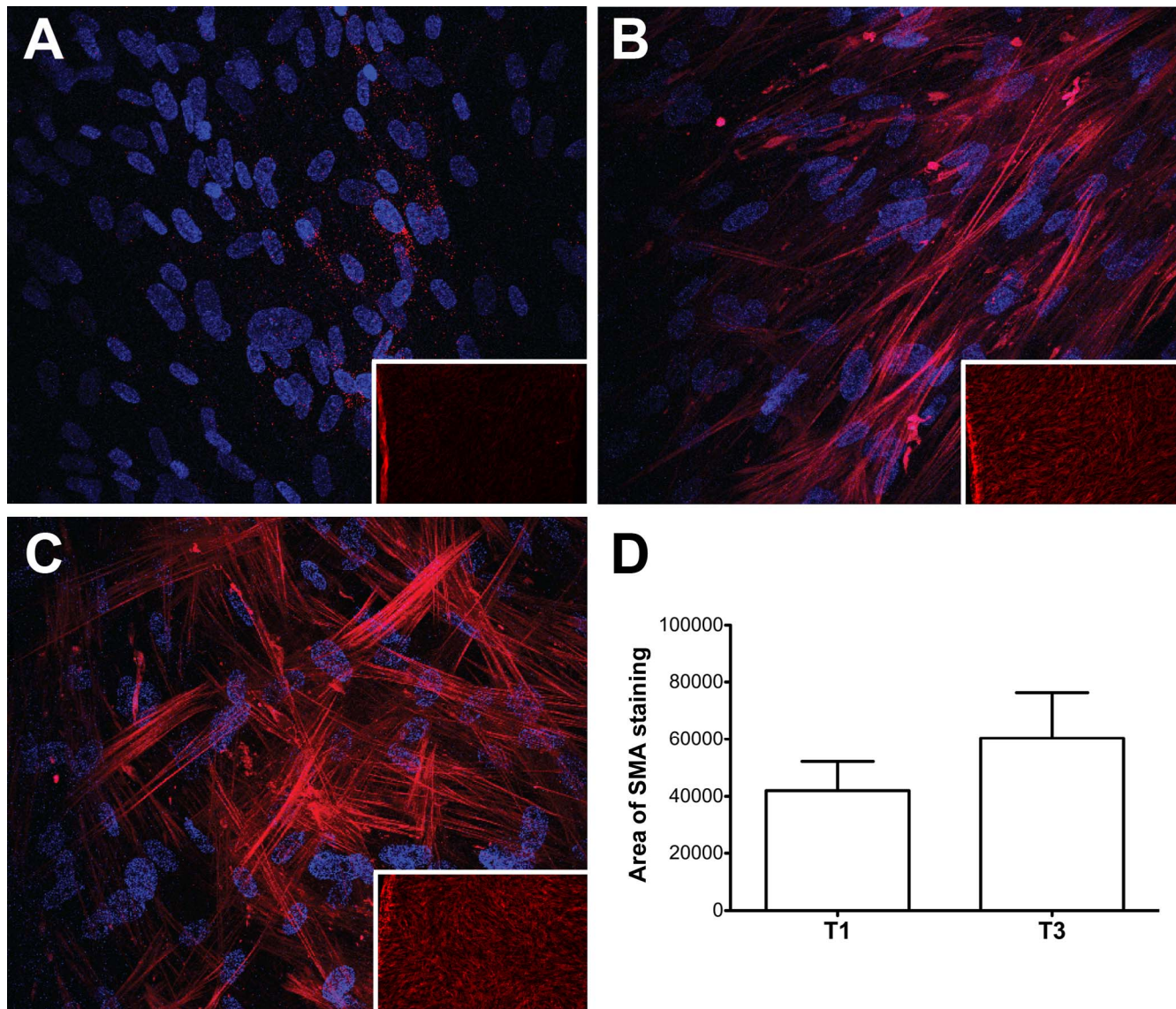


FIGURE 4. Smooth muscle actin immunofluorescence staining of HCF-P constructs: (A) Control: no growth factors, (B) T1: TGF- β 1, and (C) T3: TGF- β 3. Images were taken at 40 \times and 2 \times (*insets*). The 2 \times images were used for image analysis as it shows the entire surface area of the construct. (D) Graph of the area of SMA staining in square pixels measured using ImageJ, background corrected to Control. After PDGFR α knockdown in HCF, T3 stimulated SMA at a level comparable to T1 (*blue* = DAPI; *red* = SMA; *n* = 3).

expression of PDGFC and only slightly decreased the expression of PDGFD. For comparing the treatment groups within the same cell line, ANOVA followed by Tukey's post hoc test was used. Student's *t*-test was used for comparing individual gene expression between HCF and HCF-P (Fig. 5E). If $P < 0.05$, then "y," and if no significance, then "n."

PDGFR α Knockdown Inhibited Cell Migration

A migration assay was performed to assess the functional effect of knocking down PDGFR α . Figure 6 shows the images of HCF (Fig. 6A) and HCF-P (Fig. 6B) in response to T1 or T3 at day 0, immediately after a pipette scratch (Figs. 6A, 6B, 6Ba, 6Bc, 6Be), and 24 hours after the scratch (Figs. 6A, 6B, 6Bb, 6Bd, 6Bf). For control samples (Figs. 6Aa-b, 6Ba-b, 6C), the percent migration for the HCF-P was significantly lower than that of HCF ($*P < 0.05$). In the HCF samples, significantly faster migration ($*P < 0.05$) was observed with T1 (Figs. 6Ac-d, 6C) or T3 (Figs. 6Ae-f, 6C) treatment compared to control (Figs.

6Aa-b, 6C). Interestingly, T1- and T3-treated HCF-P (Figs. 6Bc-d, 6Be-f, 6C) had no significant difference when compared to the control (Figs. 6Ba-b, 6C), suggesting that T1 and T3's effect on migration is dependent on PDGFR α .

DISCUSSION

In this study, we hypothesized that T1 and T3 induced diametrically opposed responses in HCF by differentially activating PDGFR α . To test this hypothesis, PDGFR α was permanently knocked down in HCF-P. These cells were grown either on plates or in our 3D cell-culture model, stimulated in response to T1 or T3, and examined for SMA mRNA expression and protein localization, as well as cell migration. Our data showed that in HCF, T1 significantly stimulated SMA mRNA expression, while T3 had little, if any, effect compared with control (Figs. 2A), which is in agreement with our previous papers^{14,23}; however, in HCF-P, both T1 and T3 significantly

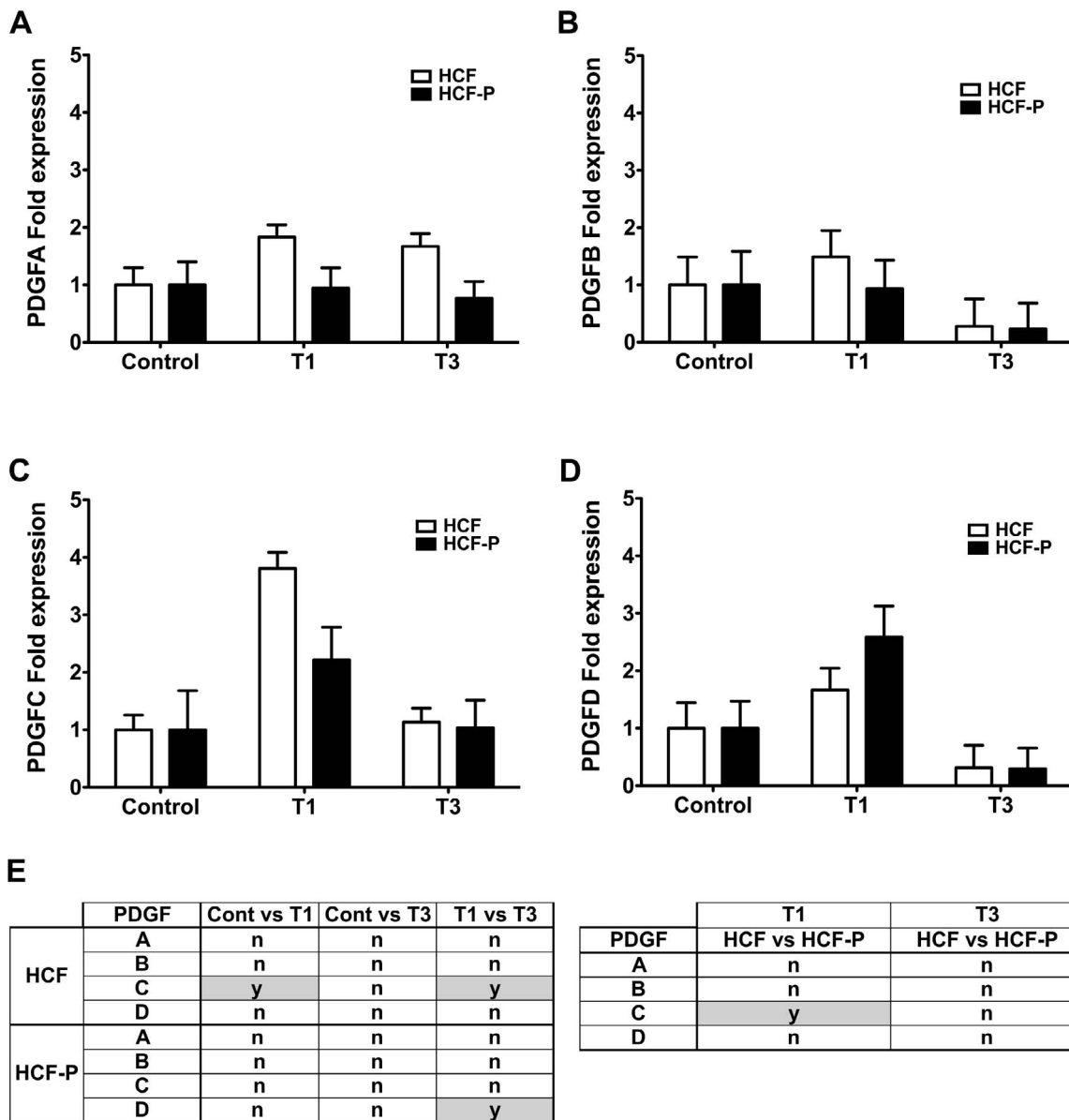


FIGURE 5. Comparison of RNA expression of PDGF isoforms in HCF and HCF-P cell line treated with Control (no growth factors), TGF- β 1 (T1), or TGF- β 3 (T3): (A) PDGFA, (B) PDGFB, (C) PDGFC, and (D) PDGFD. GAPDH was used as the housekeeping gene. Expressions for each cell line were normalized to that of their respective untreated control. (E) Table showing the results of *t*-tests performed by comparing all the treatment groups with each other ($n = 6$, “y” = $P < 0.05$, “n” = $P > 0.05$).

increased mRNA SMA expression to ~ 3 times that of control HCF-P (Fig. 2A). These results were confirmed by immunofluorescence results (Figs. 3, 4).

To determine if the difference in T1 and T3's effect on SMA levels was due to the stimulation of PDGF isoforms, we examined the expression of all four PDGF isoforms (A, B, C, and D) in both cell types (Fig. 5). Interestingly, PDGFC (Fig. 5C), which has been shown to play an important role in the regulation of fibrosis in different organs,^{24,25} notably increased in both HCF and HCF-P upon T1 stimulation. However, no significant difference was apparent with any of the PDGFs upon T3 stimulation when compared to controls for both cell types. Therefore, since PDGFC has been found to regulate fibrosis in various organs,^{24,25} T1 may be involved in fibrosis through PDGFC in HCF. Platelet-derived growth factor C also has been shown to activate both PDGFR α and PDGFR $\alpha\beta$,²⁶ which may explain the residual increase in PDGFC expression

in HCF-P. Therefore, our results suggest that in normal HCF, T1's high SMA expression may be through its high PDGFC stimulation, while T3 fails to stimulate PDGFC, thus leading to low SMA expression. However, this fails to explain T3's role in stimulating SMA in HCF-P, which leads us to believe that T3 may be acting through a different mechanism. Further studies are required to understand these relationships; however, we feel these results are quite exciting, in that they are the first data we have obtained that may begin to explain the disparate effects of T1 and T3.

Finally, to look at the functional differences between the two cell types, we looked at construct thickness and cell migration. In previous papers, we have used the thickness of the construct as a measure of the amount of ECM produced and showed that our 3D cell culture model produced a matrix that had a similar structure to the human stroma and contained Collagen type I and V.²³ Measuring the thickness of the

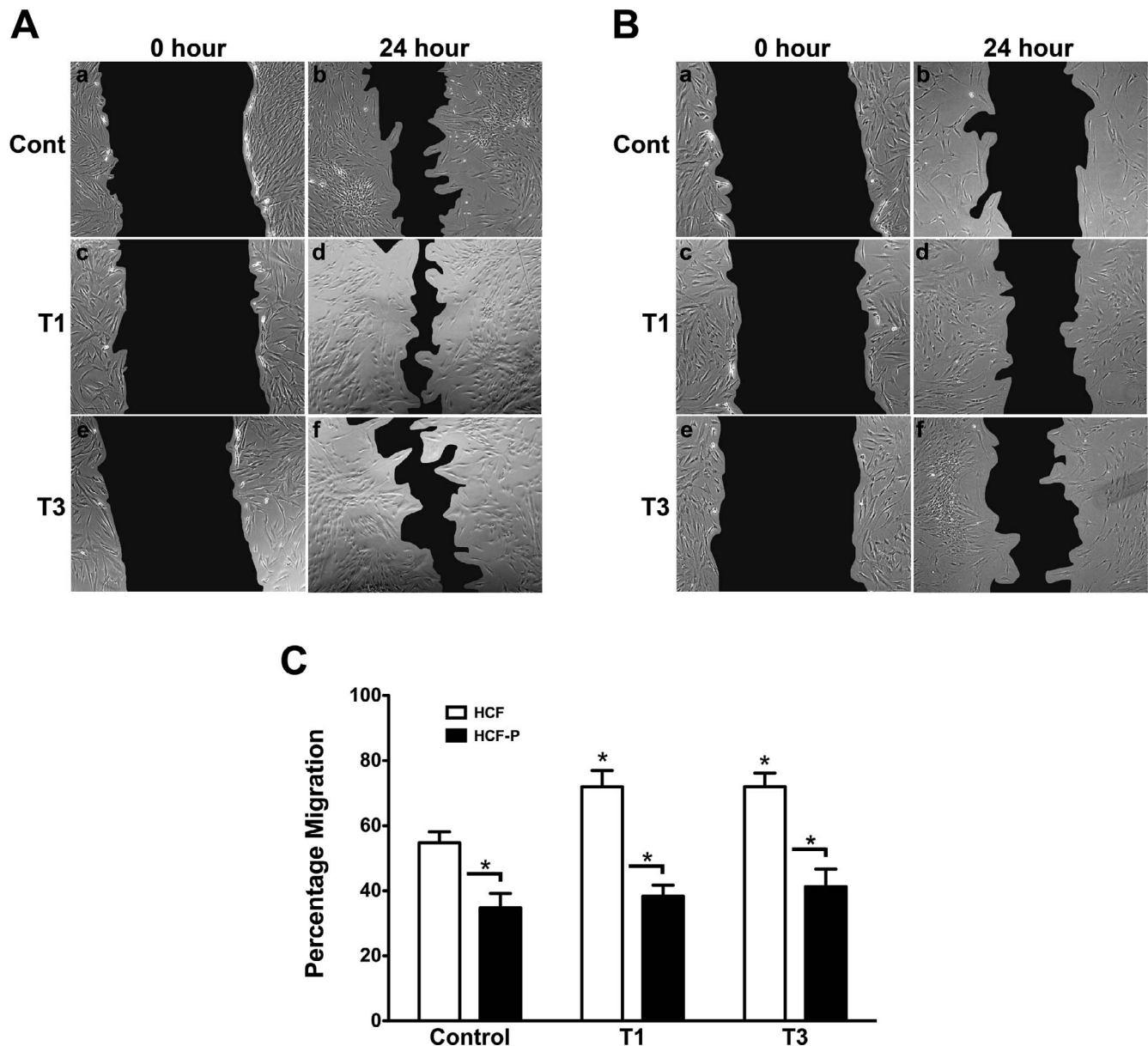


FIGURE 6. Image analysis of migration rates of (A) HCF and (B) HCF-P treated with Control (no growth factors; a–b), T1 (c–d), or T3 (e–f). Human corneal fibroblast and HCF-P were cultured in six-well plates and grown to confluence. At 0 hour (a, c, e), a scratch was made in the middle of the well using the end of a 200- μ L pipette tip. The cells were then cultured for an additional 24 hours in their respective media (b, d, f). The boundary of cells in A and B were traced manually and the area without cells was measured using ImageJ. (C) Graph showing the percentage of migration for HCF and HCF-P. T1 and T3 treatment significantly increased migration in HCF as compared with control ($*P < 0.05$); however, they had no effect on the HCF-P. The percent migration of HCF-P was also significantly slower than that of HCF cells ($*P < 0.05$).

constructs in this paper, we found that both T1- and T3-treated cells produced a thicker construct than their untreated control (Fig. 2B). This may be due to the effects of TGF β 's mitogenic properties.¹ The human corneal fibroblast-P constructs, in contrast, were significantly thinner than HCF constructs (Fig. 2B) and also had a slower migration rate than HCF (Fig. 5B). We believe this might be due to the fact that PDGFR α is strongly associated with the proliferation and migration of cells.^{27,28} Interestingly, neither T1 nor T3 had an effect on HCF-P migration or construct thickness. According to the literature,²⁸ both PDGFR α and PDGFR β stimulate fibroblast migration; however, it appears that PDGFR β does not compensate for the loss of PDGFR α in our model. These results are consistent with our biochemical results that indicated that PDGFR α played a

major role in determining T3's mechanism of action in stimulating SMA (Figs. 2, 3).

To summarize, in this present study we have captured the difference in function of T1 and T3 using our 3D cell culture model. We also showed that the presence or absence of PDGFR α affects T3's ability to stimulate SMA. We believe understanding this relationship between PDGFR α and T3 may be the first step toward developing T3 as an antiscarring therapy for the cornea.

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