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Transforming growth factor beta 3 regulates assembly of a non-fibrotic matrix in a 3D corneal model

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Abstract

Purpose—Corneal tissue engineering has attracted the attention of many researchers over the years in part due to the cornea's avascularity and relatively, straight forward structure. However, the highly organized and structured nature of this optically clear tissue has presented a great challenge. We have previously developed a model where human corneal fibroblasts (HCFs) are stimulated by a stable Vitamin C (VitC) derivative to self-assemble an extracellular matrix (ECM). Addition of TGF- β 1 enhanced the assembly of ECM; however, it was accompanied by the upregulation of specific fibrotic markers. In this study, we tested the effects of all three TGF- β isoforms ($-\beta$ 1, $-\beta$ 2 and $-\beta$ 3) on ECM production, as well as, expression of fibrotic markers.

Methods—HCFs were grown in four media conditions for 4 weeks: (Control) VitC only; (T1) VitC+TGF- β 1; (T2) VitC+TGF- β 2; or (T3) VitC+TGF- β 3. Cultures were analyzed with Western Blots, TEM and indirect-immunofluorescence (IF).

Results—Compared to controls, all TGF- β isoforms stimulated matrix production by \sim 3 times. IF showed the presence of type III collagen and smooth muscle actin (SMA) in T1 and T2; however, T3 showed little, to no, expression. In western blots, T3 stimulated a lower type III/type I collagen ratio when compared to the other conditions. In addition, TEM indicated that T3 stimulated a higher level of matrix alignment and organization.

Conclusions—HCFs stimulated by VitC and TGF- β 3 appear to generate a matrix that mimics the normal adult or developing human cornea; whereas, TGF- β 1 and $-\beta$ 2 are driving the constructs toward a more fibrotic path.

Keywords

Cornea; Corneal Fibroblasts; Extracellular Matrix; Growth Factors; TGF- β 3; fibrosis; Collagen; Vitamin C

1. Introduction

The cornea accounts for approximately two-thirds of the human eye's total optical power. It consists of three main layers: epithelium, stroma and endothelium. The stroma makes up \sim 90% of the corneal thickness and is composed of extracellular matrix (ECM) and cells. The ECM is made up of exquisitely aligned and organized collagen, with type I collagen being

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Conflicts of Interest: None (all authors)

Ethics: This research has been reviewed by the institute's internal review board (IRB) for the use of human tissue. The IRB approved the research and the research falls under exemption 4 for the NIH guidelines. All research adhered to the tenets of the Declaration of Helsinki.

the most abundant with its fibrillar structure conferring tensile strength to the cornea. The primary cell type that resides in the adult stroma is termed keratocytes. During development, these cells are known to secrete the precursors of all the ECM components, such as collagens, proteoglycans and other glycoproteins. Activated keratocytes or cells cultured in serum are generally termed corneal fibroblasts.

In the cornea, the corneal keratocytes or fibroblasts play a central role in mediating the corneal response to injury or refractive surgery (Jester *et al.* 1999; Moller-Pedersen *et al.* 1997; Petroll *et al.* 1992). In a normal unwounded cornea, the keratocytes are mainly quiescent. Following injury/wounding, the keratocytes adjacent to the wound undergo apoptosis (Helena *et al.* 1998; Zieske *et al.* 2001), while cells more distal to the wound differentiate into fibroblasts and/or myofibroblasts. The development of scar tissue is, in most cases, the end result of this healing process (Kubota and Fagerholm 1991; Suzuki *et al.* 2003).

The main regulators of corneal wound healing, and ultimately clarity, are growth factors, such as FGF-2 (fibroblast growth factor-2), PDGF (platelet derived growth factor), TGF- β (transforming growth factor- β), IL-1 α (interleukin-1 α), and IGF (insulin-like growth factor), which are known to be strongly involved in scarring and neovascularization (Guo *et al.* 2007; Jester and Chang 2003; Jester *et al.* 2002; Kane *et al.* 2009; Long *et al.* 2000; Musselmann *et al.* 2005; Musselmann *et al.* 2008). In the human eye, TGF- β is known to participate in fibrotic pathologies. TGF- β has been reported to modulate collagen synthesis and cause tissue fibrosis (Border and Noble 1994; Wahl *et al.* 1987; Younai *et al.* 1994). It is also known to increase granulation, tissue formation and breaking strength in healing dermal wounds (Mustoe *et al.* 1987; Sporn *et al.* 1983). There are three TGF- β isoforms identified, TGF- β 1, - β 2 and - β 3 (T1, T2 and T3, respectively), and their distinct and differential roles have been shown with studies involving mouse and human embryos (Gatherer *et al.* 1990; Millan *et al.* 1991; Pelton *et al.* 1990; Pelton *et al.* 1991; Schmid *et al.* 1991).

We have developed a stroma-like model that consists of primary human corneal fibroblasts (HCFs) and their self-assembled matrix, which we have shown to mimic the stroma seen during development (Guo *et al.* 2007; Karamichos *et al.* 2010; Ren *et al.* 2008). As many as 10–12 layers of flattened cells have been observed in this model. In addition, perpendicular orientation of adjacent fibril layers are present. In this study, we investigated the role of the different TGF- β isoforms on the cells, and the synthesis and assembly of ECM. Several studies have shown the contribution of these isoforms to tissue regeneration (O'Kane and Ferguson 1997), cell differentiation (Williams *et al.* 2003) and embryonic development (Roberts and Sporn 1992). However, their specific role is different. T1 and T2 are responsible for fibrosis and scar formation, whereas T3 acts as an inhibitor. In our studies, T1 and T2 have been found to stimulate ECM accumulation, along with markers of fibrosis, such as type III collagen and α -smooth muscle actin (SMA). In contrast, T3 stimulates ECM without fibrotic markers. These findings correlate with studies in skin, where T3 has been found to promote scar-free healing in cutaneous wounds (Shah *et al.* 1995).

In our model, corneal fibroblasts can be regulated by the means of growth factors to produce a better architectural ECM without expressing fibrotic markers. The novel system here and its development can help identify the factors involved in corneal scarring and how they may be regulated. This information will be vital for sight-threatening cases.

2. Materials and methods

2.1. Primary Culture of Human Corneal Fibroblasts (HCFs)

Human corneas were obtained from the National Disease Research Interchange (NDRI; Philadelphia, PA). HCFs were isolated as previously described (Guo *et al.* 2007). In brief, corneal epithelium and endothelium were removed from the stroma by scraping with a razor blade. The stromal tissue was cut into small pieces (~2×2mm) and put into 6-well plates (4 or 5 pieces per well). Explants were allowed to adhere to the bottom of the wells and Eagle's Minimum Essential Medium (EMEM: ATCC; Manassas, VA) containing 10% fetal bovine serum (FBS: Atlantic Biologicals; Lawrenceville, CA) was added. After 1–2 weeks of cultivation, the fibroblasts were passaged into a 100mm² cell culture plate. The cells were allowed to grow to 100% confluence before being used in the culture system. All research adhered to the tenets of the Declaration of Helsinki. Passages up to number three were used throughout these experiments.

2.2. Fibroblast Assembled ECM

The HCFs were plated on transwell 6-well plates containing polycarbonate membrane inserts with 0.4µm pores (Costar; Charlotte, NC). One milliliter of the HCFs were plated per well (10⁶ cells/ml) and cultured for 4 weeks with EMEM plus 10% FBS and 0.5mM 2-O-α-D-glucopyranosyl-L-ascorbic acid (VitC: Wako Chemicals USA, Inc.; Richmond, VA), as well as, one of the following: No Growth Factor (Control), T1, T2, or T3. T1, T2 and T3 were used at a concentration of 0.1ng/ml. Initial concentration-dependent experiments investigated cell-ECM responses using all three isoforms. Contraction was shown for all T1, T2 and T3 conditions at concentrations greater than 0.5ng/ml. At week 4, samples of the resulting constructs were collected and processed for whole mount indirect-immunofluorescence (IF), light microscopy, transmission electron microscopy (TEM), and Western Blotting (WB). All experiments were repeated at least three times.

2.3. Whole mount indirect-immunofluorescence

Constructs were collected and fixed in 4% paraformaldehyde. IF was performed as previously described (Zieske *et al.* 2001). Following fixation, the constructs were incubated at 4°C overnight with the primary antibody—anti-type III collagen (1:40: Southern Biotech; Birmingham, AL), or anti-SMA (1:50: Dako North America; Carpinteria, CA)—diluted in 1%BSA + 0.1%Triton-X. The constructs were then washed and incubated overnight at 4°C with the corresponding secondary antibody—donkey anti-goat IgG (1:200, type III collagen), or donkey anti-mouse IgG (1:200, SMA)—diluted in 1%BSA + 0.1%Triton-X. Phalloidin-rhodamine (Invitrogen; Carlsbad, CA), which binds to the f-actin in all cells, was also used. Constructs were counterstained with TOPRO-3 iodide (1:1000, Invitrogen), a marker of all cell nuclei. Negative controls, where the primary antibody was omitted, were run with all experiments. Constructs were washed, mounted with Vectashield Mounting Media (Vector Laboratories; Burlingame, CA), observed and photographed using a confocal TCS-SP2 Leica microscope (Leica Microsystems; Bannockburn, IL). In addition, construct thicknesses were also measured with the confocal microscope, beginning with the first cell visible at the top of the construct and the last cell visible at the bottom. Data was averaged and analyzed.

2.4. Light and Transmission Electron Microscopy

Constructs collected for TEM were fixed in ½ strength Karnovsky's fixative (2% paraformaldehyde, 2.5% glutaraldehyde in cacodylate buffer, pH 7.4) and processed using standard procedures, as described previously (Gipson *et al.* 1983). Briefly, a diamond knife ultramicrotome (LKB ultramicrotome; Bromma, Sweden) was used to cut transverse to the

plane of the construct. Sections were collected for both light microscopy and TEM. For light microscopy, optical thick-sections of 1–2 μ m were obtained and stained with phenylenediamine. These thick-sections were viewed and documented with a Nikon Eclipse E800 equipped with a SPOT camera (Nikon Instruments, Inc.; Melville, NY). For TEM, 60–90Å-sections were obtained, viewed and photographed with a Philips 410 Transmission Electron Microscope (Philips Electronics N.V.; Eindhoven, The Netherlands)

2.5. Western Blot

Constructs were flash frozen with liquid nitrogen and processed for Western blotting, as previously described (Chung *et al.* 1999). Briefly, tissues were homogenized and lysed. The protein was then purified and assayed by the Bradford dye-binding method (Bio-Rad; Hercules, CA). Equal amounts of total protein were loaded on a reduced 6% Tris-Glycine gel (Invitrogen). After separation, the protein was transferred to a membrane (Immobilon-P; Millipore; Billerica, MA), which was then stained with Ponceau (Sigma; St.Louis, MO) to check for transfer efficiency. Membranes were blocked with Blotto (5% milk in TBS) and then probed, as previously described (Chung *et al.* 1999), with the following antibodies: type I (1:2000: Abcam; Cambridge, MA) and type III (1:2000: Novus Biologicals; Littleton, CO) collagen. Results were normalized to Control (VitC only) and plotted as the fold enhancement of type III/type I collagen ratio. In addition, culture media from all the conditions were collected every week and equal volumes were loaded. Identical analysis was performed as with the tissue (see above).

2.6. 3D Software – Intensity measurements

Image Pro Plus (Image Pro Plus v.7: Media Cybernetics; Bethesda, MD) is a 2 and 3D analysis program and was used to analyze and quantitate data from the confocal z-series and TEM photomicrographs. The analyses performed for this study were as follows:

Cell Counting and Integrated Optical Density (IOD)—Image Pro Plus was used to quantitate the number of cells/construct and the intensity of antibody binding in the constructs. We used the confocal z-series in order to calculate the total binding intensity of the antibody of interest (IOD-intensity value). We also counted each cell throughout the construct, as well as the diameter of the nuclei. At least three confocal z-series were used at each condition and their average was plotted and analyzed.

Fibril diameter—At least ten random TEM photomicrographs were used for each condition, and a total of 100 fibrils were measured for their diameter. The scale bar for the TEM was used to calibrate the measurements.

2.7. Statistical Analysis

All experiments were performed in triplicates and data was averaged and analyzed for significant variations ($p < 0.05$) using the Student's t-test and/or Dunnett's Multiple Comparison test.

3. Results

3.1. Cellular organization and growth factor effects

Previously, we demonstrated that cells incubated with VitC on transwell polycarbonate membranes lead to multi-layer 3D constructs. These constructs showed alternate orientations of both cells and ECM components (Guo *et al.* 2007; Karamichos *et al.* 2010). We have also demonstrated that TGF- β 1 stimulates accumulation of fibrotic matrix in a time and dose dependent manner (Karamichos *et al.* 2010). In this current study, we extend our

investigation to all three TGF- β isoforms in order to investigate their differential effect on our system. These experiments were stimulated by many reports that the three isoforms can have differing effects, even though they appear to bind and activate the same set of receptors. The three different growth factors were used at the same concentration (0.1ng/ml) to promote matrix deposition. All experiments were performed in triplicate.

Our constructs showed several cell layers and high cell alignment throughout, with the highest levels of organization observed at the top and bottom. Interestingly, cell nuclei diameters were found to vary significantly, with the nuclei of cells stimulated with any of the three isoforms being ~3-fold smaller than controls (Table 1, * $p < 0.001$). We postulated that the smaller nuclei may be present in fibrotic cells; however, no correlation between cell shape and nuclei size was observed.

Previously, we have shown that T1 stimulated the accumulation of a fibrotic matrix. In the current study, all three isoforms, T1, T2 and T3, stimulated cell stratification and matrix component production when compared to controls. We found no significant differences in the thickness of the constructs cultured with any of the three isoforms (Figure 1); however, they were all significantly different, * $p < 0.05$, when compared to controls (2.6-, 2.6- and 2.2-fold, respectively).

To determine if this increase in thickness was the result of an increase in the number of cells or an increase in the amount of matrix produced per cell, we examined the cell number. Results showed a significant increase in cell number following TGF- β stimulation (* $p < 0.001$) compared to controls (Table 1). However, the amount of ECM produced per cell remained relatively constant under all conditions.

3.2. ECM organization

Constructs were examined at the end of 4 weeks using TEM. Representative images for each condition are shown in Figure 2. Cell-ECM interactions were observed in each of the constructs. T1 and T2 (Figs. 2B and C) showed areas of organized ECM, as well as many cells with aligned actin-like filaments, indicative of myofibroblasts (Fig. 2E, white arrowhead). T3 (Fig. 2D) showed the highest density of collagen fibrils and ECM alignment, as compared to control (Fig. 2A) and the other two TGF- β isoforms (Figs. 2B and C). It was clear that fewer cells with actin stress fibers were present, suggesting a “quieter” and less fibrotic construct (Fig. 2D). Furthermore, TEM revealed an increased number of cells with vesicles (Fig. 2E, black arrows). The vesicles appeared to correlate with the presence of actin filaments and may be another marker for myofibroblasts. Presence of vesicles has not been previously suggested as a marker of myofibroblasts. Our previous publication (Guo *et al.* 2007) noted the presence of these structures.

Following TEM, photomicrographs (31 000 \times) of the constructs were used to measure and quantify the fibril diameter for each condition. A mature human corneal stroma is comprised primarily of hydrated type I/V collagen fibrils of uniform diameter, 30–35 nm. The scale bar for the photomicrographs was used to calibrate the measurements. One hundred random fibril diameters were measured for each condition. Controls (Fig. 3A) showed a fibril diameter range of 18–35nm with the peak at 25nm. Similar peak (25nm) was found with T2 (Fig. 3C), with a slightly larger range of fibril diameters (17–46nm). T1, on the other hand, showed a peak at 30nm (Fig. 3B), and T3’s peak increased even further, 37nm (Fig. 3D). T3 had 46% of its fibrils measuring at 35nm compared to 20–35% of controls and the other two TGF- β isoforms.

3.3. HCF response to growth factors – IF

To assess cellular responses to growth factors, media was supplemented with 10% FBS, VitC and one of three TGF- β isoforms. Controls were cultured in 10% FBS and VitC only, no growth factors. The final concentration of the growth factors was determined from a preliminary dose-dependent study (data not shown) and represents the common dose for all growth factors for maximum response without contraction of the construct.

We investigated the presence and expression of specific fibrotic markers and how these may be regulated when the cells were stimulated by the three isoforms. In agreement with our previous work (Karamichos *et al.* 2010), few, if any myofibroblasts were present in control constructs (Fig. 4A); however, the addition of both T1 and T2 led to a clear increase of positive SMA cells (Fig. 4B and C, respectively). These cells appeared throughout the construct with a high degree of alignment and a preference towards the top and bottom of the construct. T3 on the other hand, showed minimal, if any, expression of SMA-positive cells, indicating a less fibrotic effect (Fig. 4D).

Also, as seen in Figure 5, moderate amounts of type III collagen was found in control (Fig. 5A); whereas, T1 and T2 treated cells showed high amounts of type III collagen, with a high degree of alignment (Fig. 5B and C, respectively). Type III collagen is another fibrotic marker, and as with SMA, when T3 was used to stimulate the cells, its expression was down to a minimum, if any (Fig. 5D).

Our data shows that with the addition of T3, specific fibrotic markers were not upregulated, as seen with the other isoforms. This data is supported by the observations made using TEM, as well as the quantification of fibril diameters, shown above.

3.4. Evaluation of integrated optical density

In order to quantitate the amount of antibody binding present in our 3D constructs, we analyzed our IF data using the Image Pro Plus Software. With this software we were able to calculate and compare the integrated optical density (IOD), which is the total amount of antibody present within the area of interest, which, in our study, are the 3D constructs. Results were in agreement with our IF observations, showing significant increase of the IOD when cells were treated with T1 and T2 (* $p < 0.05$), and an IOD of almost zero when T3 was present. As shown in Figure 6, we plotted the percent (%) increase of IOD for all conditions (T1, T2 and T3) compared to controls for both SMA (Fig. 6A) and type III collagen (Fig. 6B). Both T1 and T2 showed enormous % IOD increase of SMA (500 and 1200, respectively) and type III collagen (800 and 700, respectively). This is in agreement with our data, as well as previous work, that these isoforms lead to myofibroblast formation and fibrotic ECM. In contrast, T3 was higher than controls; however, the % IOD was only 1 and 74% for type III collagen and SMA, respectively.

3.5. Protein analysis

The constructs were examined for the presence of types I and III collagens. Results were normalized to controls and plotted as the fold enhancement of type III/type I collagen ratio. As shown in Figure 7, cultures treated with T1 showed a significant increase of 2.1-fold ($p < 0.5$), as compared to controls. This indicated that the constructs treated with T1 were progressively becoming fibrotic, which agrees with our IF and TEM data, shown above. T3, on the other hand, was confirmed for its non-fibrotic effects on our model by resulting only in a 1.4-fold increase, which was not statistically significantly from controls. Furthermore, T3 was found to be statistically different from T1 ($p < 0.05$), supporting the data shown here about T3's non-fibrotic effects. Although IF and TEM data for constructs treated with T2 indicated that the constructs were becoming more fibrotic, the increase in the typeIII/type I

collagen ratio was not significant. Interestingly, when we analyzed the amounts of type I and III collagen secreted in the culture media, T3 showed a reduction of ~2-fold when compared to the other two isoforms (data not shown). This indicates that more of the collagen secreted by the cells treated with T3 gets deposited in the ECM compared to the other two isoforms.

4. Discussion

The concept of “curing” corneal opacity by replacement with a clear substitute has been discussed in published form for over 200 years, with the first suggestion being to transplant a piece of quartz to repair the cornea. In the ensuing years, many advances have been made in corneal transplantation, including the use of cadaver corneas. However, since there has been a constant shortage of donor corneas worldwide, and since certain types of injuries and diseases are not amenable to transplantation, many efforts have been made to find a corneal replacement. Over the past 50–60 years, several investigations have been made into the use of plastics to develop an artificial cornea, also termed keratoprosthesis. These keratoprosthesis have enjoyed some success, with the Boston keratoprosthesis being placed in over 1000 patients in 2009 (Gomaa *et al.* 2010). Another device that initially gave promising results is the AlphaCor (Bleckmann and Holak 2006; Hicks *et al.* 2006), although recent reports have been less encouraging (Chalam *et al.* 2007; Holak *et al.* 2009). As an alternative to the use of plastics, several investigations have been made to engineer an artificial cornea using natural compounds, such as collagens, cells and cell lines (Alaminos *et al.* 2006; Fagerholm *et al.* 2009; Gonzalez-Andrades *et al.* 2009; Huang and Li 2007; Mi *et al.* 2010; Schneider *et al.* 1999; Vrana *et al.* 2008). The goal of these studies is to develop a synthetic cornea that mimics the native cornea and also integrates into the human eye.

Minami *et al.* (Minami *et al.* 1993) were one of the initial studies trying to reconstruct a cornea *in vitro*. They developed all three layers using primary bovine corneal cells and tested their system in animals. Zieske *et al.* (Zieske *et al.* 1994) also developed a similar model using primary rabbit epithelial and stromal cells and immortalized mouse endothelial cells. This system was remarkable for developing a continuous basement membrane in culture. Griffith *et al.* (Griffith *et al.* 1999) has more recently published the development of a full three-layer cornea-like construct using human corneal epithelial, endothelial and keratocyte cell lines in a type I collagen/chondroitin sulphate substrate. Li *et al.* (Li *et al.* 2003) evaluated the use of hydrogels (a collagen-copolymer) and reported successful growth of epithelium and stromal cells. The constructs had good clarity and were strong enough to be sutured. Most recently, Proulx *et al.* (Proulx *et al.* 2010) reported a three-layer substitute, which used human cells and exhibited excellent epithelial and endothelial morphology, as well as an intact basement membrane. Other studies have also used human cells, combined with immortalized cell lines, in order to reconstruct an artificial three-layer construct (Reichl and Muller-Goymann 2003; Vrana *et al.* 2008) with promising results.

Even though these approaches obtain clear strong constructs, they are still somewhat limited by the lack of a cornea-like ECM. The partial corneal construct approach has become of great interest since it has the advantage of 1) targeting the area or layer that has been damaged or injured, and 2) can concentrate more on the optimum growth conditions of this layer.

This current study focuses on the stromal region and using a cell-based approach involving the stimulation of corneal cells to secrete and assemble their own ECM. Guo *et al.* (Guo *et al.* 2007) used primary human corneal fibroblasts to self-assemble a stromal substitute by stimulating them with a stable form of Vitamin C (VitC). Specific ECM macromolecules were identified, and collagen fibril alignment, along with changes in orientation of the fibrils, similar to that seen *in vivo*, was documented (Guo *et al.* 2007). More recently, our

group showed the effect of T1 on the development of these 3D constructs (Karamichos *et al.* 2010). T1 stimulated the accumulation of enhanced amounts of matrix; however, the limitation and potential problem for this model is the accompanying enhanced presence of myofibroblasts and fibrotic markers. The presence of fibrotic markers is associated with corneal opacity, making it unclear if this model would be useful for corneal transplantation. In an unpublished study, the constructs without T1 have been transplanted in vivo into the corneas of Balb/C mice, and have been accepted by the host cornea. However, the long-term survival, as well as mechanical integrity, has yet to be tested.

The cell-based stromal constructs developed here were stimulated with one of three TGF- β isoforms, and the matrix secretion increased dramatically. We showed striking differences between the three isoforms using a variety of techniques, such as IF, TEM and 3D analysis. Our data shows a novel way to control specific fibrotic markers using T3, as well as the improvement on collagen density, cell proliferation and collagen fibril diameter, which mimic corneal development. The main conclusion from this current study is that stimulation with T3 leads to a construct that has non-fibrotic characteristics, as well as ECM properties that closely mimic in vivo corneal stroma. Type III collagen and SMA were elevated in cultures stimulated by T1 or T2, and suppressed by T3, while ECM deposition remained unchanged (ie. construct thickness). In an adult cornea Type III collagen levels are very low; however, following wounding, these levels are elevated. Our findings correlate well with studies in skin, where T3 was found to promote scar-free healing in cutaneous wounds. Occlleston *et al.* (Occlleston *et al.* 2008) have demonstrated the benefits of exogenous addition of TGF- β 3 to cutaneous wounds. The study showed not only improvement on the neodermis architecture, but also safe use in humans. This could potentially lead to the treatment of cornea wounds as well.

In both healthy and injured human corneas, all three TGF- β isoforms have been observed (Carrington *et al.* 2006; Tseng *et al.* 1999), indicating that their regulation is vital for both the development and wound healing of the cornea. In a cutaneous wound model, Shah and coworkers (Shah *et al.* 1995) showed that an increase in T3 levels relative to T1/T2 results in a scarfree phenotype. The ability of all the three isoforms to stimulate cells to synthesize type I collagen was shown; however, only T3 inhibited the secretion and deposition of type III collagen, which is well known for its fibrotic appearance. This agrees with our findings reported here, suggesting that T3 has similar effects in the cornea.

One of the puzzling questions regarding TGF- β signaling is how do the three isoforms stimulate different responses? TGF- β functions by signaling through an intracellular Smad pathway (Yao *et al.* 2010) or a non-Smad pathway. Despite the fact that the three isoforms have 70–80% sequence homology (Hao *et al.* 2008) and share most cell-surface receptors, they have been shown to possess distinct roles in wound healing. Propagation of TGF- β signals is vital for understanding differences and similarities of these isoforms. Briefly, phosphorylation of intracellular Smad proteins follows activation of Type I and II receptors. The active form of TGF- β engages to TGF- β RII. T1 and T3 bind to TGF- β RII with high affinity; however, T2 may only bind to this receptor in the presence of TGF- β RIII, a membrane bound betaglycan (Li *et al.* 2006). It has been speculated that T3 stimulates the activation of different signaling molecules than T1 or T2; however, this is yet to be fully documented. In our current study, we have demonstrated the non-fibrotic effects of T3, which suggests a differential T3 function stimulating a signal transduction pathway to inhibit the synthesis of type III collagen. Examining these pathways will be the subject of future experimentation.

In tissue engineering, the development of this model holds great potential benefits. Firstly, T3 may be a therapeutic agent of corneal repair, especially when no harmful effect is known

to be caused by it on corneal re-epithelialization (Carrington *et al.* 2006). Secondly, the use of this natural cell-assembled construct means that from one cadaver eye we can get enough cells for the equivalent of over 100 transplanted corneas. It is also conceivable that the patient's own cells could be used to generate the replacement matrix.

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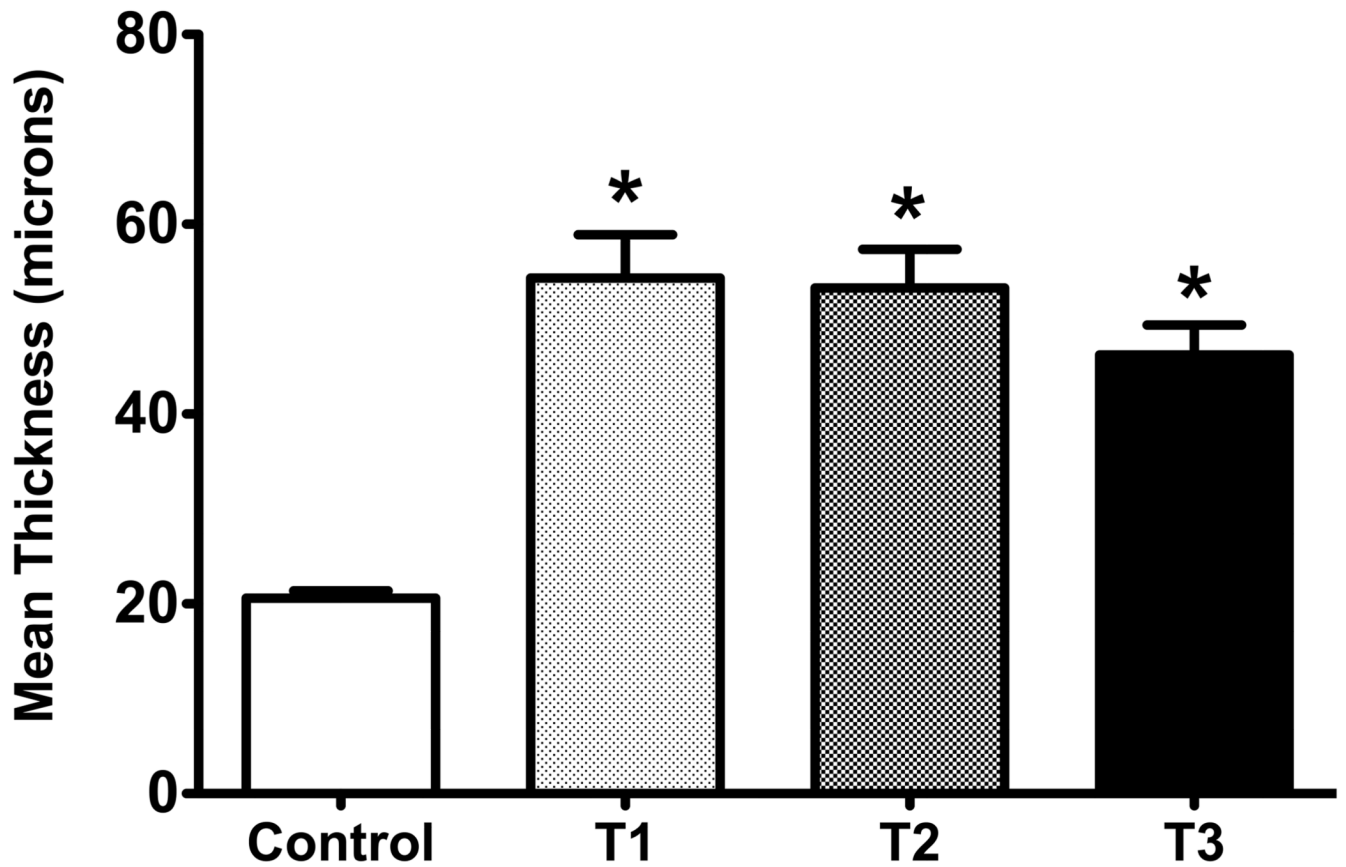


Figure 1. Graph of the mean thickness of the constructs treated with one of three TGF- β isoforms and control. Four conditions were examined and analyzed: (Control) VitC only, (T1) VitC + TGF- β 1, (T2) VitC + TGF- β 2, and (T3) VitC + TGF- β 3. All three isoforms led to a significant increase in thickness as compared to the control (* p <0.05).

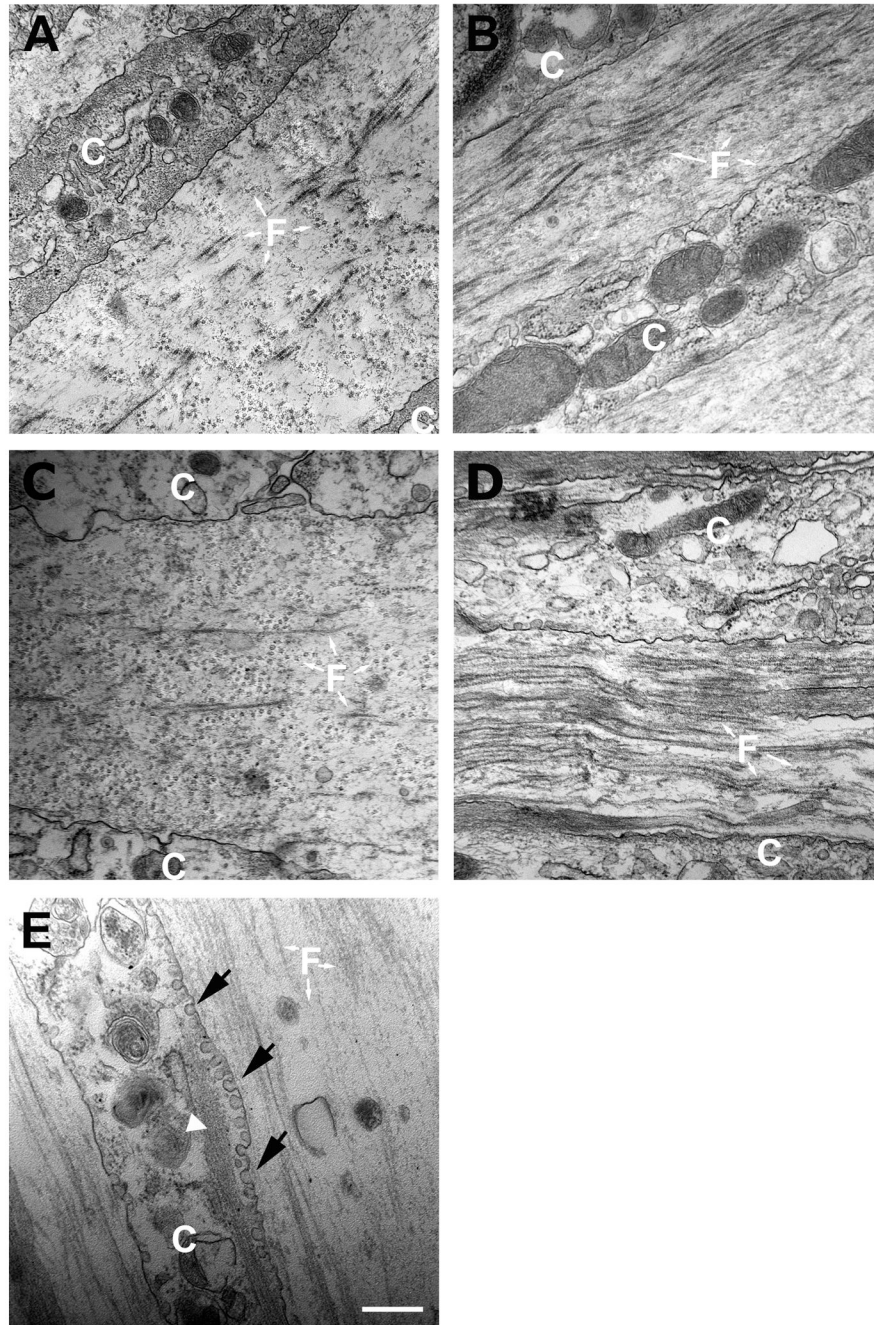


Figure 2.

TEM (31 000 \times) showing cell-matrix interaction and matrix condition. A) Control, B) TGF- β 1 treated (T1), C) TGF- β 2 treated (T2), and D) TGF- β 3 treated (T3) constructs. The fibril orientation changes direction more than once in all conditions. T3 treated construct (D) shows higher density of collagen fibril and ECM alignment compared to controls, T1 and T2. T3 also appeared to show fewer cells with actin stress fibers (data not shown). E) Example of cell with vesicles (arrows) correlating with actin filaments (arrowhead). White “C” = cell and “F” = fibrils. Bars = 500nm.

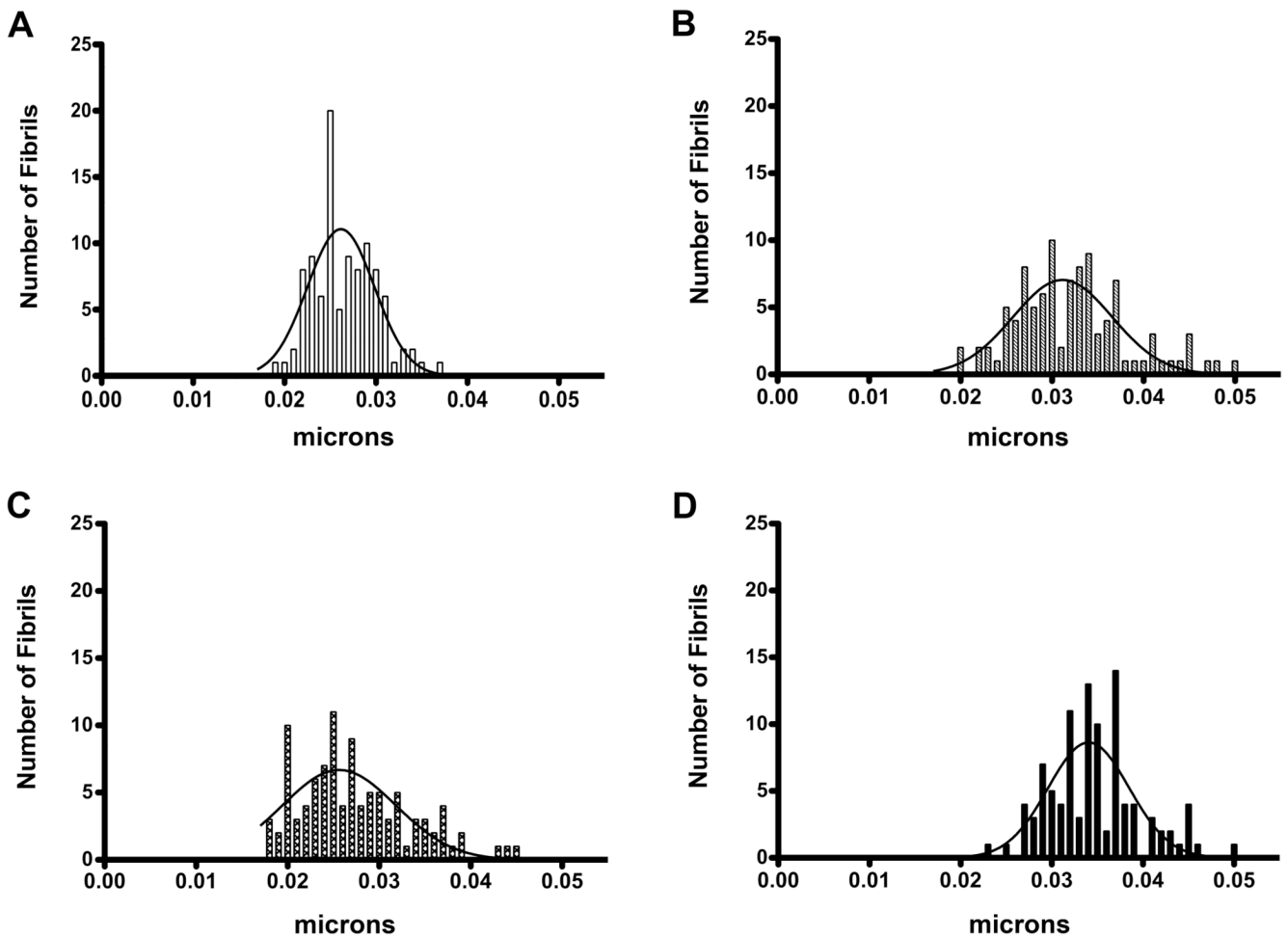


Figure 3.

Histograms demonstrating the distribution of fibril diameters. A) Control, B) TGF- β 1 treated (T1), C) TGF- β 2 treated (T2), and D) TGF- β 3 treated (T3) constructs. Control fibril diameters ranged from 18 to 35nm with a peak at 25nm. T2 also peaked at 25nm, but had a slightly larger range (17–46nm). T1's range was similar to T2 (20–50nm), with a peak of 30nm. T3, however, peaked at 37nm with 46% of its fibrils measuring 30–35nm in diameter, compared to 20–35% for Control, T1 and T2. A mature human corneal stroma has fibrils of 30–35nm.

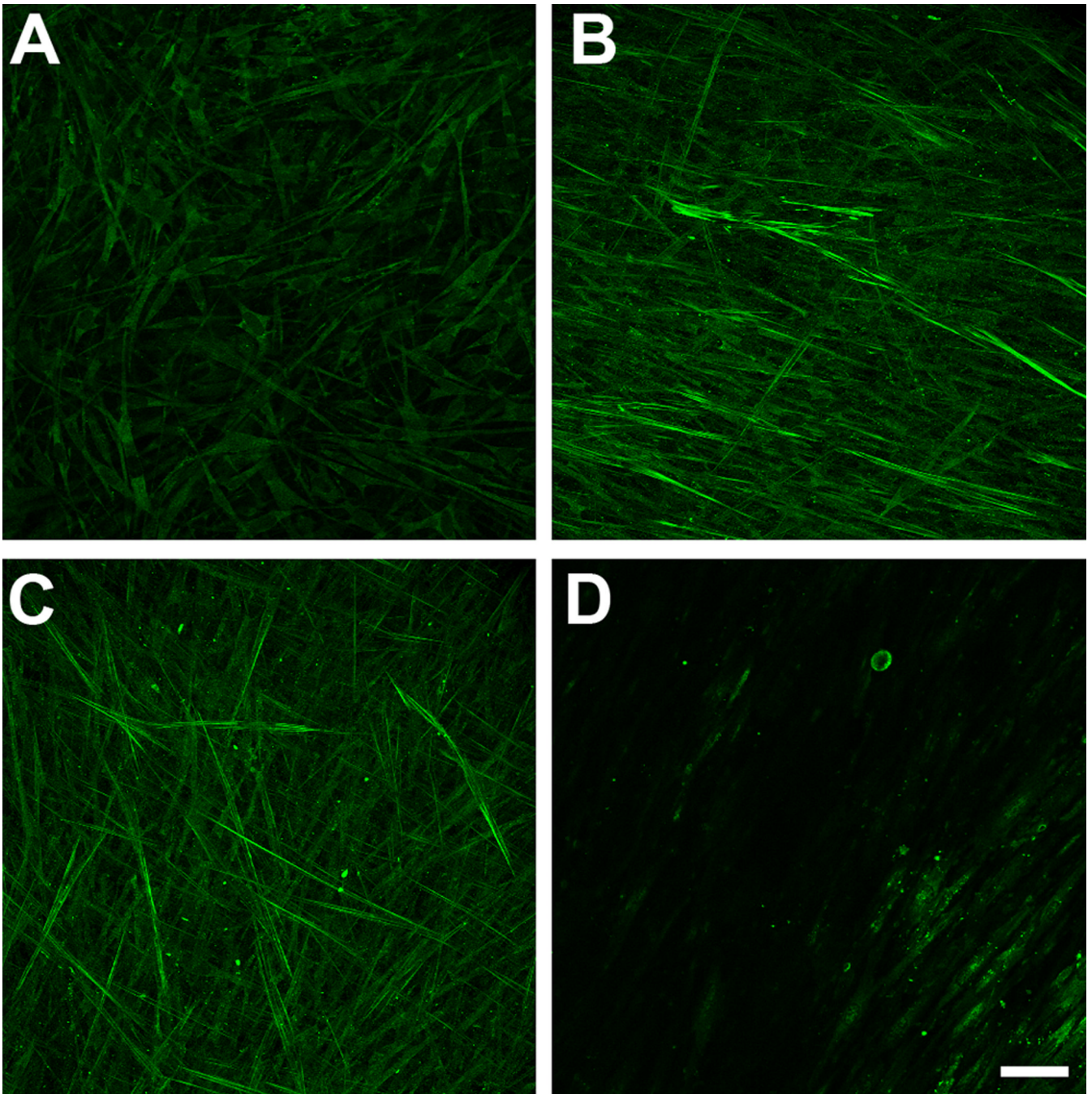


Figure 4. Maximum projection confocal images of indirect immunofluorescence with α -smooth muscle-actin (SMA) on full-thickness constructs at 4 weeks. A) Control, B) TGF- β 1 treated (T1), C) TGF- β 2 treated (T2), and D) TGF- β 3 treated (T3) constructs. Compared to controls, the number of cells expressing SMA stress fibrils increased considerably when T1 or T2 were introduced to the system. T3 treated constructs, however, showed minimal, if any expression of SMA. T1 and T2 appeared to stimulate fibrosis, while T3 did not. Bar = 50 microns.

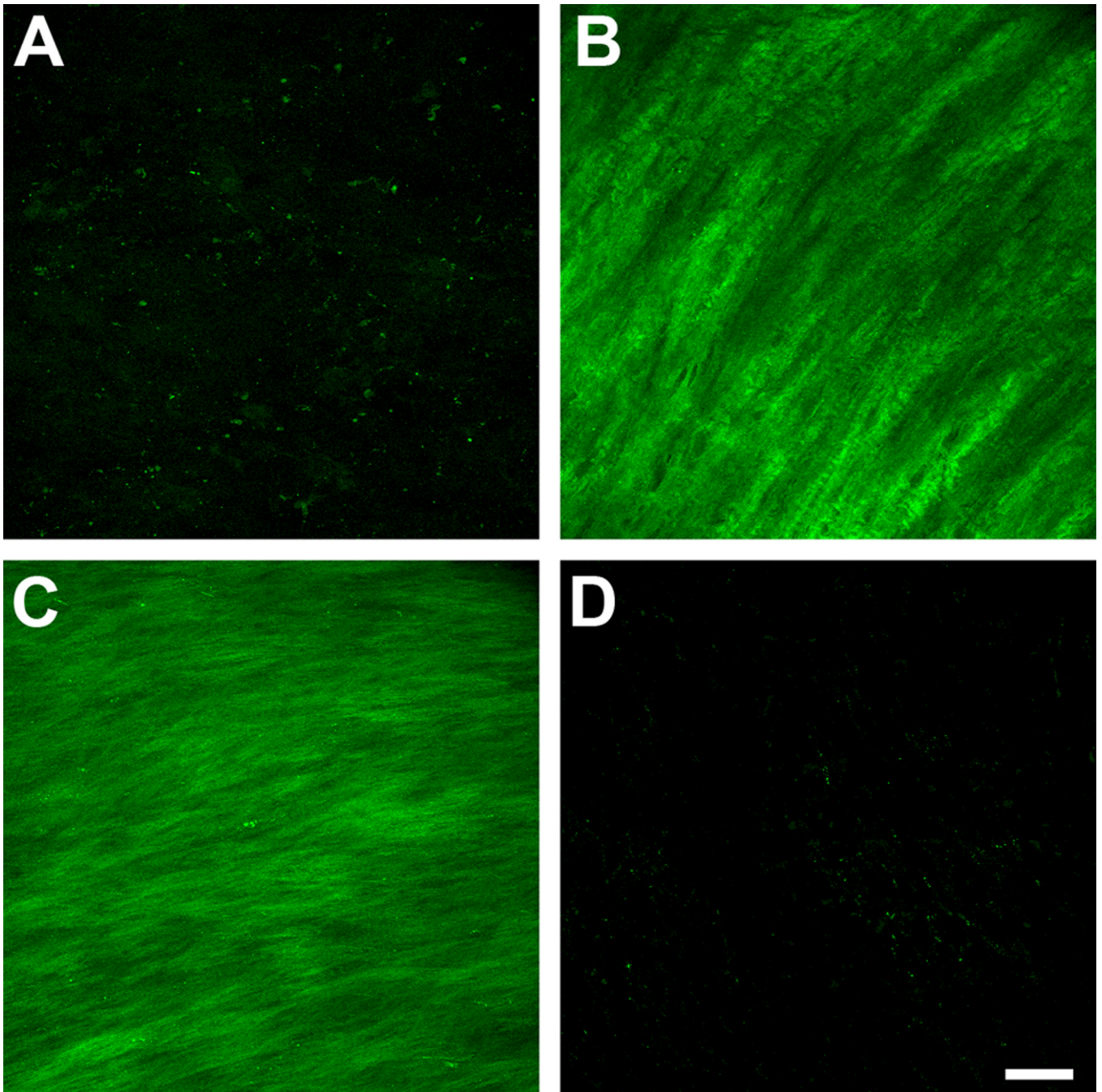


Figure 5. Maximum projection confocal images of indirect immunofluorescence with type III collagen on full-thickness constructs at 4 weeks. A) Control, B) TGF- β 1 treated (T1), C) TGF- β 2 treated (T2), and D) TGF- β 3 treated (T3) constructs. Compared to controls, the expression of type III collagen was increased considerably when T1 or T2 were introduced to the system. T3 treated constructs, however, showed minimal, if any expression of type III collagen. T1 and T2 appeared to stimulate fibrosis, while the T3 did not. Bar = 50 microns.

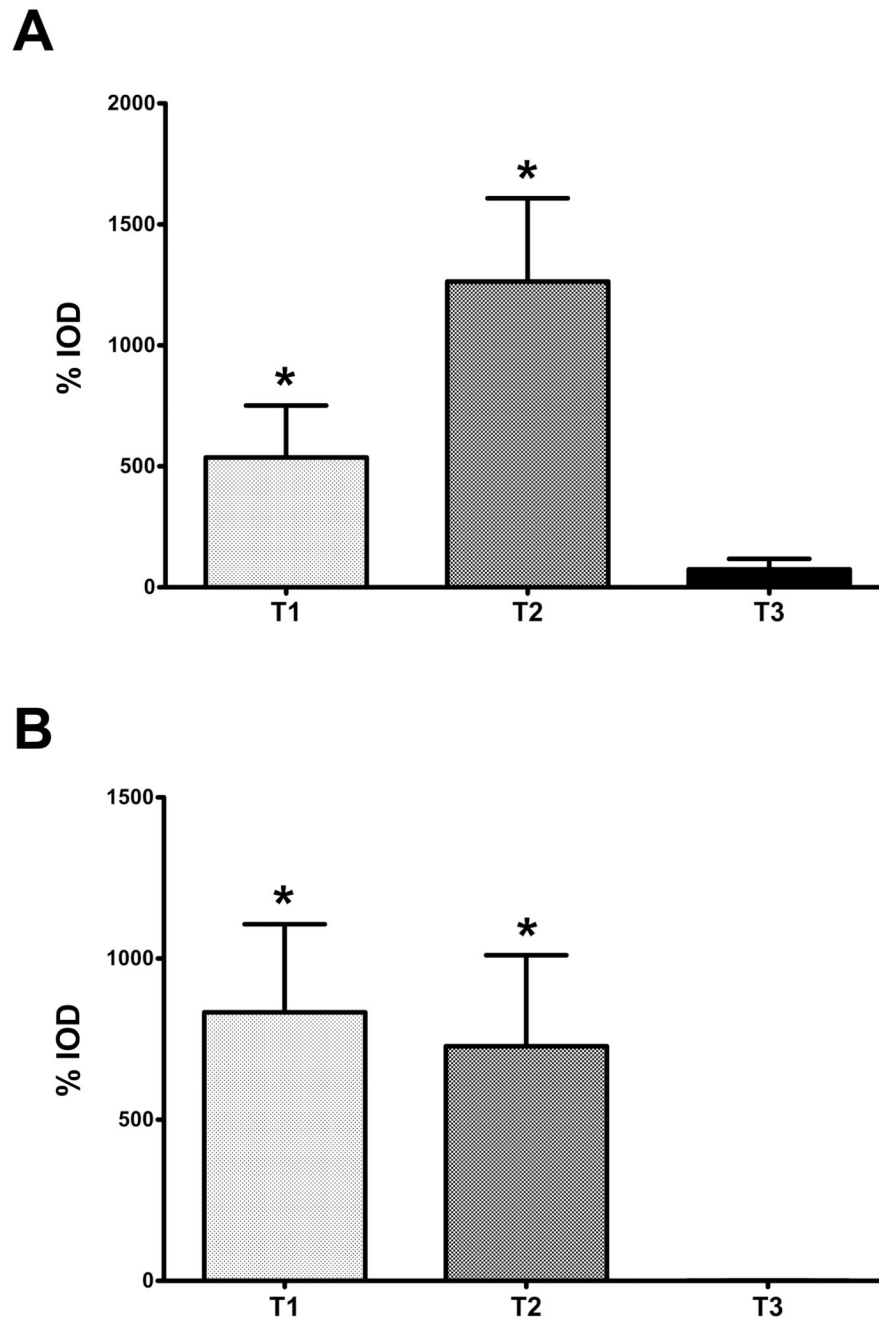


Figure 6.

Graph of the percent (%) integrated optical density (IOD) of SMA and type III collagen in the constructs. Four conditions were examined and analyzed after indirect immunofluorescence was performed with either SMA or type III collagen: (Control) VitC only, (T1) VitC + TGF- β 1, (T2) VitC + TGF- β 2, and (T3) VitC + TGF- β 3. Data was plotted as the % increase of IOD for all conditions (T1, T2 and T3) compared to controls for both smooth muscle actin (A) and type III collagen (B). (A) %IOD for SMA expression significantly increased (* p <0.05) when cells were treated with T1 and T2. (B) Similar results were shown for type III collagen, with * p <0.05 for both T1 and T2. T3, however, had a %IOD of almost zero for both SMA (A) and type III collagen (B).

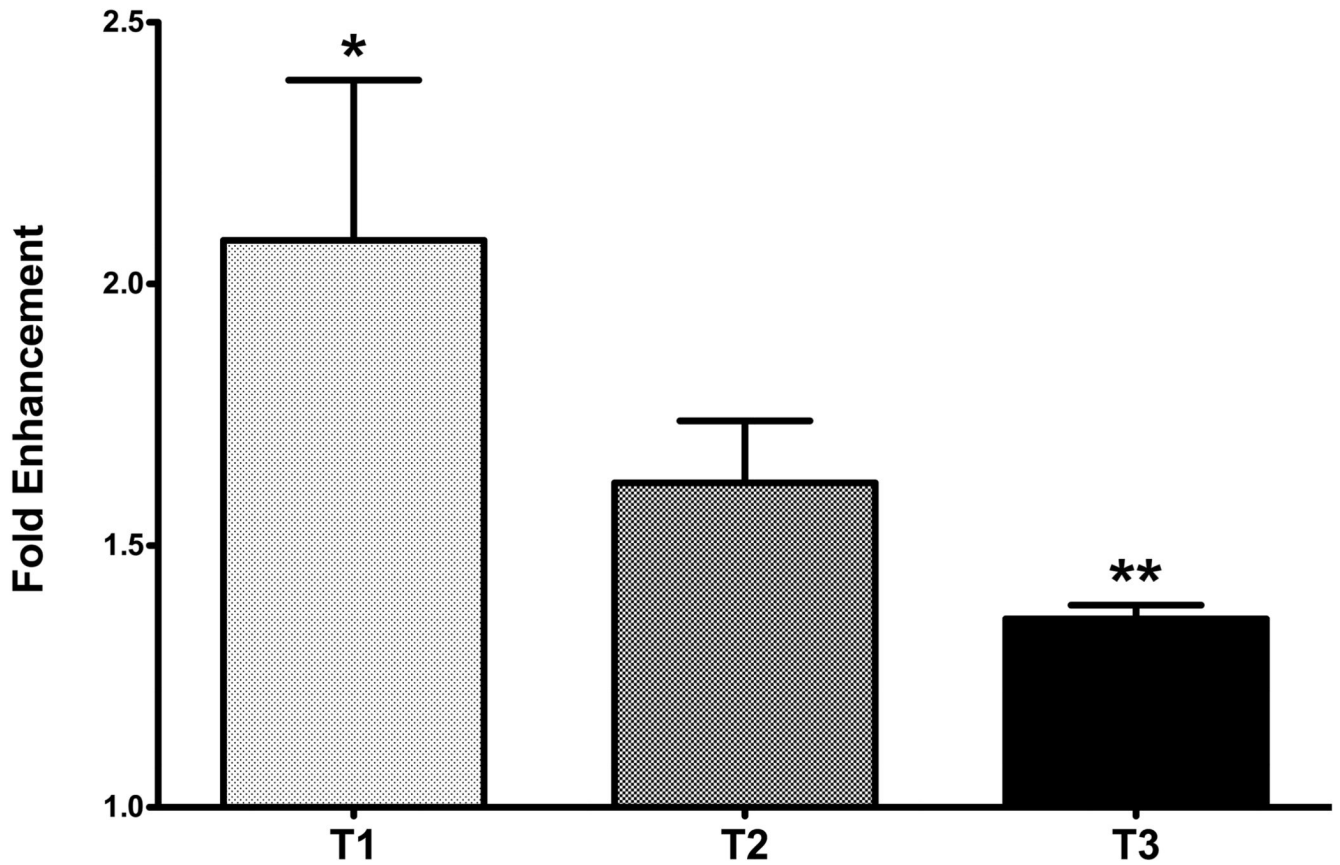


Figure 7.

Graph of the fold enhancement of the regulation of type III/type I collagen ratio following TGF-β1 (T1), -β2 (T2) or -β3 (T3) stimulation. Data is normalized to control. T1 showed a significant increase as compared with control (* $p < 0.05$). T3 showed a slight, but not significant, increase as compared to control; however, T3 decreased significantly as compared with T1 (** $p < 0.05$).

Table

The nuclear diameter and number of cells in the constructs were measured and analyzed.

	Control	T1	T2	T3
Nuclear Diameter (μm)	31.9 \pm 0.3	*11.3 \pm 0.2	*11.5 \pm 0.2	*13.2 \pm 0.1
Cell Number ($\times 10^6$)	2.48 \pm 0.2	*6.63 \pm 0.4	*6.99 \pm 0.5	*5.14 \pm 0.4

The nuclear diameters of the cells in the constructs of all three TGF- β isoforms (T1, T2, and T3) were significantly smaller than controls (* $p < 0.001$). The cell number in the constructs of all three TGF- β isoforms was significantly increased compared to controls (* $p < 0.001$).