

## Differential Effects of EGF and TGF- $\beta$ 1 on Fibroblast Activity in Fibrin-Based Tissue Equivalents\*

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### ABSTRACT

Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is commonly used to promote matrix production for engineered tissues *in vitro*, yet it also enhances fibroblast contractility. For applications where contraction is undesirable, we hypothesized that epidermal growth factor (EGF) would yield equivalent mechanical properties without enhancing contractility. In this study, the response of human dermal fibroblasts to EGF (5 ng/mL) and TGF- $\beta$ 1 (5 ng/mL) was determined within hemispheric fibrin-based gels by assessing matrix compaction and strength, cell number, collagen production, and contractility. After 3 weeks, both cytokines enhanced compaction relative to controls, and EGF roughly doubled matrix strength over controls and TGF- $\beta$ 1-treated samples. TGF- $\beta$ 1 induced  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) expression whereas EGF did not. TGF- $\beta$ 1 also increased retraction following substrate release while EGF reduced retraction. Treatment with cytochalasin D revealed that, regardless of growth factor, approximately 10% of the total retraction was due to residual matrix stress accumulated during cell-mediated remodeling. EGF increased the cell number by 17%, whereas TGF- $\beta$ 1 decreased the cell number by 63% relative to controls. EGF and TGF- $\beta$ 1 stimulated greater collagen content than controls by 49% and 33%, respectively. These data suggest that EGF may be an attractive alternative to TGF- $\beta$ 1 for engineering fibrin-based connective tissue substitutes with adequate strength and minimal tissue contractility.

### INTRODUCTION

**B**IOPOLYMER GELS ARE natural material choices for tissue engineering scaffolds due to their inherent biocompatibility and ability to be remodeled by cells without forming cytotoxic byproducts.<sup>1</sup> Collagen and fibrin, in particular, have been extensively utilized as biomaterials;<sup>2,3,4</sup> fibrin “glue” is used clinically as a surgical sealant and is being investigated as a cell delivery vehicle,<sup>5</sup> and a purified collagen gel seeded with human foreskin fibroblasts (HFF)

currently serves as the dermal equivalent in a commercially available living skin equivalent (Apligraf®; Organogenesis, Canton, MA).<sup>6</sup> Cell-populated collagen gels have also been investigated extensively as substitutes for other connective tissues such as blood vessels<sup>1,7</sup> and tendons and ligaments;<sup>8</sup> however, in load-bearing applications the strength of collagen gels is insufficient, even for topical applications.<sup>9,10</sup> As the overall goal of connective tissue engineering is to stimulate the production of collagen and alternative extracellular matrix (ECM) proteins that restore functionality to

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the tissue, it is necessary to utilize tissue analogs that either intrinsically retain or can be manipulated to confer comparable strength to the native tissue.

Due to the strength limitation of collagen gels, many researchers have adopted the fibrin gel as a tissue engineering scaffold, for example, for small-diameter blood vessels,<sup>3,11,12</sup> heart valves,<sup>4,13</sup> and tendon and ligament<sup>14</sup> applications. Fibrin is the major structural protein involved in hemostasis and coagulation cascades and, like collagen, is degraded and absorbed *in vivo*.<sup>4,11</sup> Although fibrin gels are generally mechanically less stable than collagen gels when first prepared,<sup>2,12</sup> fibrin gels do not suppress collagen synthesis by the embedded cells to the extent that occurs in collagen gels.<sup>12,15,16</sup> Thus, ECM proteins can be produced by the cells within the fibrin scaffold at a relatively high rate, eventually resulting in a construct with tissue-like composition and mechanical properties.<sup>3,11</sup>

To accelerate the rate of ECM protein synthesis and tissue development, the media used to culture the cells within fibrin gels are commonly supplemented with exogenous growth factors. These proteins provide chemotactic stimuli to dictate cell migration, contraction, proliferation, and differentiation and also guide the organization, degradation, and synthesis of new matrices.<sup>17,18</sup> Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is frequently used in fibroblast culture systems for stimulation of collagen production<sup>8,18,19</sup> and for accelerating development of fibrin-based tissue substitutes.<sup>11,12,16</sup> However, TGF- $\beta$ 1 has been shown to stimulate wound contraction and cellular differentiation into the highly synthetic and contractile myofibroblast phenotype, characterized by the expression of  $\alpha$ -smooth muscle actin ( $\alpha$ SMA).<sup>20,21</sup> While cellular contraction during early-phase wound healing is required for initial wound closure as well as the mechanical function of muscular and cardiovascular tissues, contracture-induced scarring is potentially motion-limiting and cosmetically undesirable.<sup>8,16,22</sup> Excessive fibroblast differentiation to contractile phenotypes is characteristic of pathological states *in vivo*<sup>23</sup> and therefore should be avoided in tissue substitutes cultured *in vitro*. In order to engineer tissue substitutes that parallel healthy tissue, it is necessary to employ a medium that promotes and maintains the native phenotypic state of the cells.

Epidermal growth factor (EGF) also plays an important role in wound healing and has been utilized for its stimulation of ECM protein synthesis and cellular proliferation.<sup>24,25,26</sup> *In vivo*, sustained release of EGF has been shown to promote the formation of granulation tissue<sup>24,27,28</sup> and accelerate wound healing.<sup>27</sup> The overall increased collagen accumulation in the wound bed is generally believed to be caused by mitogenic effects of EGF rather than the activation of procollagen genes that occurs with TGF- $\beta$ 1 stimulation<sup>25</sup> as EGF has previously been shown to inhibit collagen synthesis<sup>29</sup> *in vitro*. However, unlike TGF- $\beta$ 1, EGF does not appear to induce fibroblast differentiation to the contractile myofibroblast phenotype in collagen gels and scaffolds, and has been shown to aid in reducing wound contraction.<sup>22</sup>

During *in vivo* wound healing, a reduction in the wound bed diameter is greatly enhanced through cellular contractility (i.e., contraction). Within *in vitro* cultured tissue equivalents such as fibrin gels, a reduction in tissue dimensions may occur due to compaction of the biopolymer as a consequence of cell migration through the matrix.<sup>30,31</sup> Rapid reduction of the matrix dimensions may also occur upon release of the matrix from the surrounding boundaries both via active cellular contractility (i.e., active retraction) as well as a release in passive residual stress imparted on the matrix during cell-mediated remodeling (i.e., passive retraction).<sup>30,31,32</sup> Thus, observations of both the relatively slow compaction and the quick retraction of cell-populated matrices have been utilized in many studies, investigating the extent to which various stimuli induce the resident cells to remodel the matrix and enhance or inhibit contraction.

Our objective in the current study is to develop culture conditions that facilitate the rapid development of a strong planar soft-tissue equivalent marked by an absence of active cell contractility. While the engineering of cardiovascular tissues requires vasoactivity, such contractile events would be deleterious to the function of ocular, mucosal, and other passive connective tissue analogs. Specifically, the goal of this study is to investigate EGF as an alternative medium supplement to TGF- $\beta$ 1 for the growth of fibroblast-populated fibrin gels. We hypothesize that EGF supplementation will enhance cell proliferation, which will lead to overall ECM protein production and matrix strength equivalent to TGF- $\beta$ 1 supplementation without increasing the contractility of the cells. We compare the effects of these two supplements on the dimensional, compositional, mechanical, and contractile properties of hemispheric fibrin-based tissue equivalents cultured for 3 weeks.

## MATERIALS AND METHODS

### Cell culture

HFF (ATCC, Manassas, VA) were cultured in a standard medium consisting of high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Mediatech, Herndon, VA) supplemented with 10% bovine calf serum (BCS; HyClone, Logan, UT), 100 U/mL penicillin G sodium, 100  $\mu$ g/mL streptomycin sulfate, and 250 pg/mL amphotericin B (Gibco, Grand Island, NY). Cells were maintained at 37°C in humidified 10% carbon dioxide (CO<sub>2</sub>) and used at passage six.

### Fibrin gel preparation

Fibrin gels were fabricated according to methods described by Tuan *et al.*<sup>16</sup> with final concentrations of 3.8 mg/mL bovine fibrinogen, 0.2 u/mL bovine thrombin (Sigma, St. Louis, MO), and  $5 \times 10^5$  HFF cells/mL. The fibrinogen concentration selected is close to the physiological concentration found in human plasma (approximately 3.3 mg/mL), and is within the range of concentrations commonly utilized in literature for

fibrin gel fabrication.<sup>2,33,34</sup> Hemispheric gels were prepared in 6-well tissue culture plates (Corning, Acton, MA) with 25 mm diameter circular score marks. Specifically, fibrinogen was combined with cells, and then thrombin was added to initiate the clotting process. After the solution was thoroughly mixed, 1.5 mL of gel solution was pipetted into the scored area. Gels were maintained in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS; HyClone), 100 U/mL penicillin G sodium, 100  $\mu$ g/mL streptomycin sulfate, 250 pg/mL amphotericin B (Gibco), 5  $\mu$ g/mL insulin (Sigma), 50  $\mu$ g/mL L-ascorbate (Wako, Richmond, VA), and 5 ng/mL of either EGF or TGF- $\beta$ 1 (EMD Biosciences, San Diego, CA); no supplementary growth factors were added to the control group.

Standard levels of serum were added to the media in all groups, since eliminating serum would inhibit growth of the cells and may inhibit synergism between growth factors, which would otherwise improve the characteristics of the cultured tissue substitute. For example, Lynch *et al.*<sup>35</sup> found EGF to be inactive under serum-depleted conditions, and Assoian *et al.*<sup>36</sup> demonstrated that TGF- $\beta$ 1 and EGF work in concert. The concentration of EGF chosen for this study was based on preliminary studies in our laboratory, in which we found maximal production of ECM proteins by human dermal fibroblasts when supplemented with 5 ng/mL EGF (unpublished data), and also based on the finding that concentrations of EGF similar to those found in human plasma (2 ng/mL)<sup>24</sup> inhibit collagen gel contraction.<sup>22</sup> The concentration of TGF- $\beta$ 1 utilized in this study (5 ng/mL) is commonly utilized in tissue engineering applications to maximize collagen production.<sup>3,11,12</sup>

Fourteen samples were used for each group, and the experiment was repeated. Subsets of the gels were used for each assay as indicated, and multiple assays were performed on each gel when possible (i.e., for nondestructive assays). Gels were incubated for 21 days at 37°C in humidified 10% CO<sub>2</sub>, and the culture medium was changed three times per week.

### Gel compaction

Gel thickness was measured according to methods described by Neidert *et al.*<sup>12</sup> Briefly said, a digital micrometer ( $\pm 3 \mu$ m; Mitutoyo, Aurora, IL) was attached to a light phase microscope to measure the gel thickness,  $h$ , by altering the focal plane from the edge of each score mark until the top focal plane of the gel could be observed. The thickness of each gel was recorded three times per sample and averaged on days 1, 7, 14, and 21. Gel compaction was calculated as percentage of original thickness,  $h/h_0 \times 100$ . The estimated initial thickness of the samples was 3.06 mm, assuming a cylindrical shape (1.5 cm<sup>3</sup> initial volume, 2.5 cm diameter).

### Mechanical characterization

After 3 weeks of culture, the strength of a subset of the samples was determined using a custom equibiaxial tissue

inflation system described in detail by Billiar *et al.*<sup>37</sup> In order to minimize active cellular retraction prior to testing, the cytoskeletons of the fibroblasts were disrupted with 6  $\mu$ M cytochalasin D (Sigma) treatment for 3 hours, prior to removal from the culture plates. The gels were first measured for thickness ( $n = 6$  per group) using a laser displacement system ( $\pm 10 \mu$ m, LK-081; Keyence Corporation, Woodcliff Lake, NJ). This method is more accurate than the traveling microscope used to assess compaction, yet it cannot be completed under sterile conditions. The samples were then circularly clamped (10 mm diameter orifice) and inflated with isotonic room temperature saline until failure at a rate of 1 mL/minute using a syringe pump. The center displacement was measured by the laser displacement system and the pressure monitored by a pressure transducer ( $\pm 0.4$  kPa, PM/4; Living Systems Instrumentation, Burlington, VT). The radius of curvature at failure was determined from the burst height, assuming a spherical cap geometry, as verified previously.<sup>37</sup> The tension at failure,  $T$  (force/length), was determined using the Law of Laplace,  $T = P \times R/2$ , where  $P$  is the burst pressure and  $R$  is the radius of curvature. The ultimate tensile strength, UTS (force/area) was defined as the tension at failure divided by the undeformed thickness of the gel, and the extensibility was defined as the average stretch ratio at failure along a given meridian; for example, if the gel fails when it reaches a hemispherical geometry, the stretch ratio equals  $\pi/2$  (i.e., 1.57).<sup>37</sup>

### Hydroxyproline assay

Collagen content was quantified using a hydroxyproline assay.<sup>38</sup> Specifically, tissue samples ( $n = 3$  per group) were hydrolyzed using 6 N HCl and lyophilized. A series of solutions of 70% perchloric acid, chloramine-T (Mallinckrodt, St. Louis, MO), and *p*-Dimethylaminobenzaldehyde (pDAB; J.T. Baker, Phillipsburg, NJ) were added to create a colorimetric reaction. The sample absorbencies were measured on a Spectramax plate reader (Molecular Devices, Sunnyvale, CA) at 561 nm and compared to a standard curve. Collagen content was calculated assuming 0.13 g hydroxyproline/g collagen.<sup>39</sup>

### Retraction

After the culture period of 3 weeks, the fibrin gels were gently released from the wells using a spatula to initiate rapid retraction. Digital images were acquired and analyzed for gel area,  $A$ , at times 0, 1, 2, 3, 4, 5, 6, 10, 15, 20, 25, 30 minutes and 24 hours using a Biorad Flour-S MultiImager system (Biorad, Hercules, CA). Total percent retraction,  $\{[1 - (A/A_0)] \times 100\}$  was calculated at each time point. To determine the extent of passive retraction due to residual stress built up in the matrix during the remodeling process, a separate subset of gels in the second experiment ( $n = 6$  per group) was treated with 6  $\mu$ M cytochalasin D for 3 hours prior to release from the culture plates. The passive retraction was subtracted from the total retraction to calculate the active retraction.

### Cell count

Following the retraction assay (i.e., at 24 hours), HFF were isolated from a subset of gels ( $n=6$  per group) through disruption with a 0.05% trypsin (Gibco) and 2 mg/mL collagenase solution (Sigma). The solution was centrifuged, the supernatant was aspirated, and the cells were resuspended in standard medium. Viable cells found using the trypan blue exclusion assay were counted using a hemocytometer.

### Immunohistochemistry

Representative samples from each group were fixed overnight in 10% buffered formalin (EM Science, Gibbstown, NJ) prior to release from the surface, paraffin embedded, cut into 5  $\mu$ m sections, and immunostained for  $\alpha$ SMA, the defining marker for the myofibroblast phenotype,<sup>20</sup> with 1A4 antibody (Dakocytomation, Carpinteria, CA) followed by biotinylated goat anti-mouse IgG2A (Vector Laboratories, Burlington, CA). Avidin and biotinylated horseradish peroxidase complex and diaminobenzidine tetrachloride (all from Vector Laboratories) were added for visualization. Harris hematoxylin counterstain (Richard-Allan Scientific, Kalamazoo, MI) was used to identify the nuclei in the tissue.

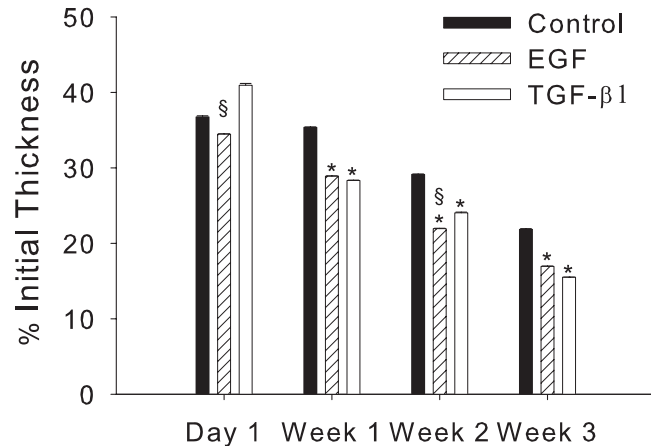
### Statistical analysis

The data from all treatment groups were compared at each time point using one-way analysis of variance (ANOVA) with  $p < 0.05$  considered significant (SigmaStat, Systat, San Jose, CA). If significant differences were found, *post hoc* analysis was performed using Tukey's honest significant difference (HSD) to identify significant differences between individual treatment groups. If the power of a statistical test was below 0.8 for an individual experiment (as indicated in the results), the data from both runs were normalized to the mean of the control group and the analysis was repeated for the pooled data.

## RESULTS

### EGF and TGF- $\beta$ 1 stimulate equivalent cell-mediated matrix compaction, matrix strength, and collagen production

Long-term cellular remodeling of the fibrin matrix was quantified by measuring compaction of the hemispheric gels at day 1 and weeks 1, 2, and 3 (Fig. 1). The growth factor-treated gels were compacted by the cells to a significantly greater extent compared to the controls at all time points (maximum  $p < 0.001$ ) after day 1 ( $p = 0.476$ ). The measured gel thicknesses were approximately 1.12 mm, 1.05 mm, and 1.25 mm at day 1; 1.08 mm, 0.88 mm, and 0.87 mm at week 1; 0.89 mm, 0.67 mm, and 0.73 mm at week 2; and 0.67 mm, 0.52 mm, and 0.47 mm at week 3 in the control, EGF, and TGF- $\beta$ 1 treatment groups, respectively. The EGF- and TGF-



**FIG. 1.** Fibrin gel compaction during the 3-week culture period. While treated groups significantly differed from controls at all time points except day 1, EGF- and TGF- $\beta$ 1-treated groups differed from each other only at day 1 and week 2 ( $p < 0.05$ ). Mean percentages of gel original thickness ( $3.06 \text{ mm}$ )  $\pm$  standard deviation (SD) are plotted for data from the first experiment ( $n = 14$ ); \* indicates different from control; § indicates EGF differs from TGF- $\beta$ 1.

$\beta$ 1-treated groups differed from each other statistically only at day 1 ( $p = 0.006$ ) and week 2 ( $p = 0.039$ ).

After 3 weeks in culture, the tensile failure properties of a subset of fibrin gels were measured. The EGF-treated gels were significantly stronger than controls in both experiments and stronger than the TGF- $\beta$ 1-treated samples in the second experiment, although the power of this statistical test was below the desired value due to sample variability (Table 1). When normalized and pooled, the EGF-treated gels were significantly stronger than the controls and TGF- $\beta$ 1-treated samples ( $p < 0.001$ , Fig. 2) and acceptable power was achieved. No significant difference was detected between the TGF- $\beta$ 1-treated samples and the controls in any case ( $p = 0.24$ ). To determine if the augmentation of gel strength was associated with enhanced ECM production, collagen production was quantified in a subset of samples (Fig. 3). The EGF- and TGF- $\beta$ 1-treated groups contained 48% ( $p = 0.007$ ) and 33% ( $p = 0.038$ ) more collagen than controls, respectively; no significant difference was observed between the EGF- and TGF- $\beta$ 1-treated groups after 3 weeks of culture ( $p = 0.31$ ). The UTS increased with collagen content indicating a possible role of collagen in strengthening, although the correlation was not strong ( $r^2 = 0.25$ ).

Unlike the trends in the strength of the samples, the extensibility was inconsistent between experiments. In the first experiment, the stretch ratio at failure of the TGF- $\beta$ 1-treated group was greater than that of both the control and EGF-treated groups, yet in the second run it was lower (Table 1). Further, the extensibility was much greater in the second experiment for all treatment groups.

As differences in the cell number could have affected all parameters tested in this study, cell number was analyzed.



**TABLE 1. ULTIMATE TENSILE STRENGTH (UTS), STRETCH RATIO AT FAILURE, TOTAL RETRACTION (RETR.) OF THE GELS AT 10 MINUTES AND 24 HOURS, AND PASSIVE RETRACTION OF THE FIBRIN GELS FOR EACH GROUP (MEAN  $\pm$  SD). DATA FROM TWO INDEPENDENT EXPERIMENTS (EXPT 1 AND 2) ARE SHOWN.**

Group	UTS (kPa)		Stretch ratio at failure		Total Retr. at 10 min (%)		Total Retr. at 24 h (%)		Passive Retr. (%)
	expt 1	expt 2	expt 1	expt 2	expt 1	expt 2	expt 1	expt 2	expt 2
Control	93 $\pm$ 19	140 $\pm$ 10	1.39 $\pm$ 0.08	3.48 $\pm$ 0.21	52.4 $\pm$ 2.5	64.6 $\pm$ 3.1	79.7 $\pm$ 1.2	81.5 $\pm$ 1.9	10.7 $\pm$ 2.2
EGF	160 $\pm$ 52 <sup>a</sup>	291 $\pm$ 70 <sup>a,b</sup>	1.36 $\pm$ 0.05	3.24 $\pm$ 0.38 <sup>b</sup>	45.8 $\pm$ 2.5 <sup>a,b</sup>	55.2 $\pm$ 2.7 <sup>a,b</sup>	78.3 $\pm$ 2.8	78.3 $\pm$ 2.4 <sup>b</sup>	9.8 $\pm$ 0.9
TGF- $\beta$ 1	128 $\pm$ 53	140 $\pm$ 49	1.70 $\pm$ 0.31 <sup>a</sup>	2.27 $\pm$ 0.02 <sup>a</sup>	64.1 $\pm$ 3.0 <sup>a</sup>	70.4 $\pm$ 2.6 <sup>a</sup>	78.6 $\pm$ 1.9	84.8 $\pm$ 3.3	8.4 $\pm$ 1.1

<sup>a</sup>indicates statistical difference from control ( $p < 0.05$ )

<sup>b</sup>indicates statistical difference between EGF and TGF- $\beta$ 1 ( $p < 0.05$ )

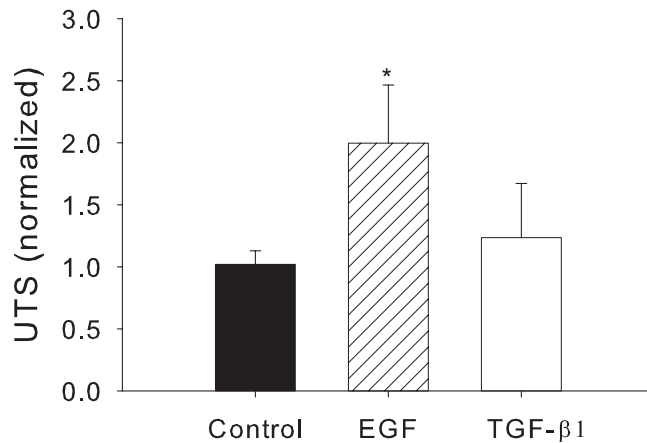
The number of viable cells was significantly lower in the TGF- $\beta$ 1 group ( $0.9 \times 10^6 \pm 0.09 \times 10^6$  cells) than in the control group ( $2.4 \times 10^6 \pm 0.16 \times 10^6$  cells,  $p < 0.001$ ) and the EGF group ( $2.8 \times 10^6 \pm 0.26 \times 10^6$  cells,  $p < 0.001$ ). The EGF and control groups were not significantly different ( $p = 0.14$ ). The viability was between 89% and 94% for all groups. From a technical standpoint, it is important to note that retrieval of all cells from the gels requires complete digestion of the gels, which involves different digestion times for each group. This was not accomplished in the first experiment, thus the data are not shown.

*TGF- $\beta$ 1 and EGF treatments promote rapid retraction of fibroblast-populated fibrin gels, yet only TGF- $\beta$ 1 induces the expression of  $\alpha$ SMA*

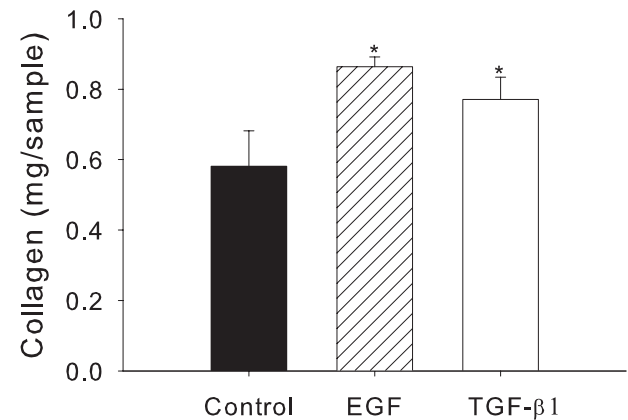
The gels retracted quickly to 50–70% of their initial area following release from the culture substrate, achieved steady state decrease in diameter after about 10 minutes, and then retracted somewhat more overnight as illustrated in Figures 4 and 5. The magnitude of total retraction was slightly different

between experimental runs (Table 1), yet the general trend observed was that the EGF-treated gels retracted to a lesser extent than the controls, and the controls retracted less than the TGF- $\beta$ 1-treated gels; that is, EGF < control < TGF- $\beta$ 1 at all time points up to 30 minutes. At 24 hours there was no difference between groups ( $p = 0.47$ ) in the first run, and there was only a small, but statistically significant ( $p = 0.005$ ), enhancement in retraction in the TGF- $\beta$ 1 group in the second run. Cytochalasin D-treated samples immediately retracted to approximately 90% of their initial area, which was not significantly different between treatment groups ( $p = 0.28$ , Table 1), indicating that the residual strain in the samples is not affected by the treatment and that the majority of the total retraction was active (i.e., due to contraction of the cells). The chosen time points of 10 minutes and 24 hours in Figure 4 and Table 1 provide a representative sample of short- and long-term retraction, respectively.

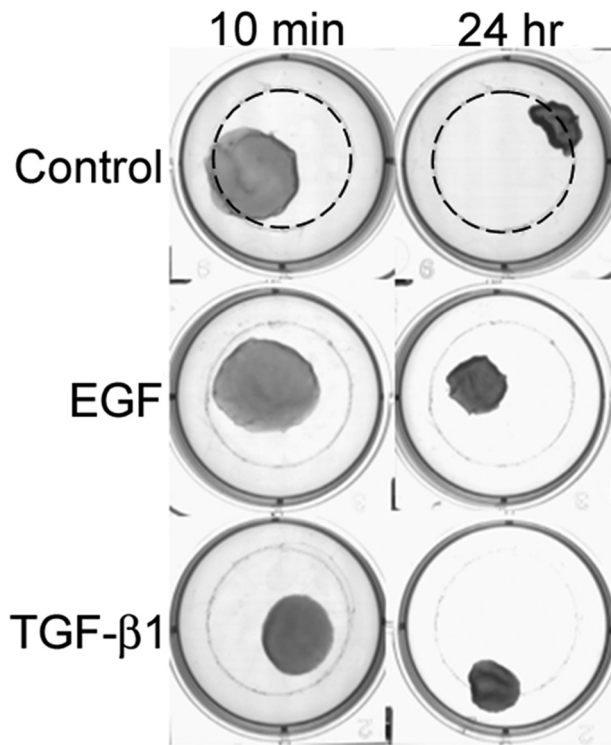
To determine if enhanced retraction was due in part to phenotypic changes in the cell populations, samples from each treatment group were stained for  $\alpha$ SMA. As shown in Figure 6, while neither the control nor the EGF-treated



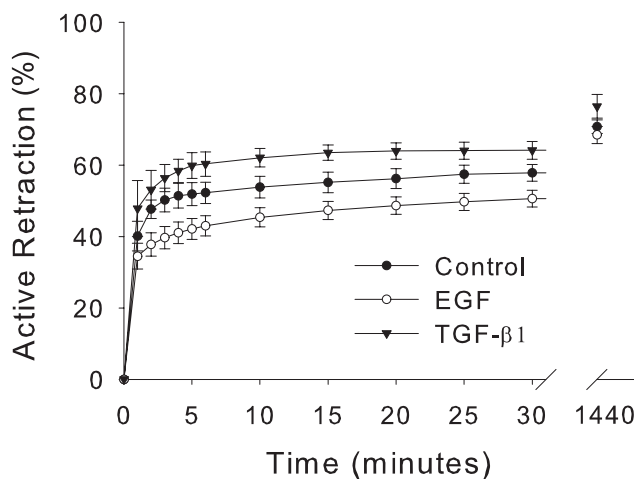
**FIG. 2.** Ultimate tensile strength (UTS) of fibroblast-populated fibrin gels at 3 weeks, normalized to the mean of the control group. EGF-treated gels were approximately two-fold stronger than control and TGF- $\beta$ 1-treated gels (\* $p < 0.001$ ). Mean values  $\pm$  SD are plotted ( $n = 12$  pooled from two separate experiments).



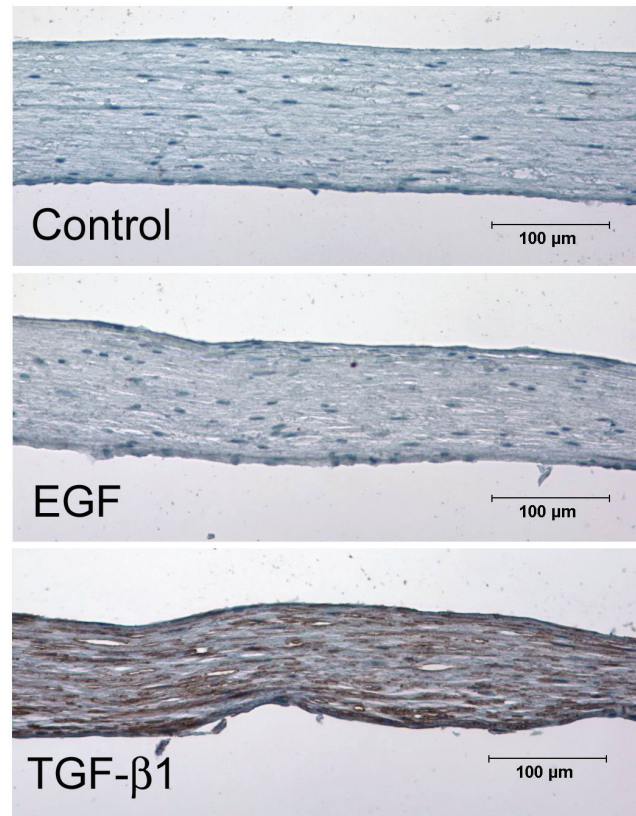
**FIG. 3.** Collagen accumulation in fibrin gels at 3 weeks of growth factor treatment. Both growth factors significantly increased collagen content relative to controls (\* $p < 0.05$ ), but EGF and TGF- $\beta$ 1 groups were not significantly different from each other. Mean values  $\pm$  SD are plotted from the first experiment ( $n = 3$ ).



**FIG. 4.** Visual representation of retraction of cultured gels over time. Pictures are representative of each group at the selected time points following release from the culture plate. Time points at 10 minutes and 24 hours were selected to provide visual portrayals of early and late phases of retraction. Score marks are highlighted with a dotted line in control pictures to show the original diameter of 25 mm prior to retraction.



**FIG. 5.** Active retraction (percentage of initial area minus passive retraction) of the fibrin gels following release from the culture substrate. The passive retraction due to residual stress in the matrix was determined by inhibition of active cell contraction by cytochalasin D treatment in a separate group of samples. The EGF-treated gels consistently retracted to a lesser extent than either the controls or the TGF-β1-treated gels during active retraction. Data presented are from the second experiment ( $n = 6$ ) as cytochalasin D was not used in the first experiment.



**FIG. 6.** Immunohistochemical staining for  $\alpha$ SMA. The presence of  $\alpha$ SMA was detected in cells throughout the entire TGF-β1 sample but not in the control or EGF samples. Color images available online at [www.liebertpub.com/ten](http://www.liebertpub.com/ten).

samples stained positive for  $\alpha$ SMA (brown staining), the TGF-β1-treated samples displayed  $\alpha$ SMA staining in the cells throughout the entire gel. This positive staining qualitatively correlates with the augmented retraction observed within the TGF-β1-treated group (Fig. 5).

## DISCUSSION

In this study we provide evidence that EGF can be used as an alternative supplement to TGF-β1 for the engineering of fibrin-based connective tissue substitutes. Fibrin gels are representative of the provisional matrix in wound healing, and EGF is known to be an essential cytokine throughout the wound-healing and repair process.<sup>26</sup> In our experiments, EGF treatment stimulated cell-mediated matrix remodeling and enhanced the mechanical strength of fibroblast-populated fibrin gels to an equivalent or greater extent compared to TGF-β1 treatment. Further, unlike TGF-β1 treatment, EGF reduced active retraction and did not induce the fibroblasts to express  $\alpha$ SMA, a marker for the contractile myofibroblast phenotype.

The essential goal of growth factor supplementation of cell-populated tissue engineering scaffolds is to effectively

maximize the strength and thickness of the resulting tissue substitute. In the case of connective tissues such as skin and heart valves, it is also important to minimize contractility of the cells within the tissue. In this study, EGF induced an approximate two-fold increase in strength compared to controls, whereas the increase in the TGF- $\beta$ 1-treated gels was not significant. Quantitatively, the gels in this study appear somewhat stronger than previously reported for uniaxially tested gels ( $\sim 90$  kPa), especially those cultured without TGF- $\beta$ 1 and plasmin supplementation ( $\sim 35$  kPa).<sup>12</sup> The greater strength may be attributed to the slightly higher initial concentration of fibrin (3.8 vs. 3.3 mg/mL), the difference in cell source, or possibly the method of testing (uniaxial vs. biaxial). Fibrin concentration has a strong influence on the mechanical properties of fibrin gels<sup>2,40</sup> as does the purity of the fibrin, which can result in large lot-to-lot variability (unpublished data). Further, dermal fibroblasts appear to degrade fibrin relatively slowly, thus we did not add aprotinin or aminocaproic acid to decrease the fibrinolytic rate in this study. In contrast, we have observed that embryonic lung fibroblasts degrade fibrin gels very quickly (unpublished data) similar to that reported for vascular smooth muscle cells.<sup>11</sup> Finally, uniaxial testing of these small fragile gels is extremely difficult and often leads to failure at the point of attachment (i.e., grip failure). To accurately measure the failure properties of these and other engineered tissues, we developed a custom biaxial inflation device that limits premature failure at the grips due to stress concentrations.<sup>37</sup> Using this device, we have also shown that control gels in this study are approximately four to six times stronger than gels made without insulin and ascorbate (vitamin C) supplementation, indicating that ECM production stimulated by these supplements plays a role in the mechanics of the matrix.<sup>37</sup>

The effects of growth factor supplementation on the extensibility of the tissue are unclear due to inconsistent findings in separate experiments. The TGF- $\beta$ 1-treated group had the highest extensibility in one run and the lowest in the other, and the stretch ratio at failure was much greater in the second experiment for all treatment groups. The reasons for these discrepancies are not clear; however, they may be due to a difference in fibrin lots as we have found high variability between lots in the past.

The strength of cultured fibrin gels can also be enhanced by synthesis of matrix molecules, most notably, collagen.<sup>41</sup> Similar to previous findings in fibrin gels, monolayer cultures, and *in vivo*,<sup>12,19</sup> TGF- $\beta$ 1 supplementation led to a significant increase in collagen content in this study. Surprisingly, EGF supplementation, which has previously been shown to inhibit collagen synthesis<sup>29</sup> and antagonize the effects of TGF- $\beta$ 1 on collagen formation by cultured fibroblasts,<sup>19</sup> led to a similar increase in collagen content. In a rat dermal wound model, the increase in tensile strength upon EGF treatment has been attributed to increased collagen content secondary to EGF-enhanced cell proliferation.<sup>27</sup> *In vitro*, correlations between tensile strength and collagen have been shown by others using fibrin gel model systems exposed to TGF- $\beta$ 1 treatment,<sup>11,12</sup>

and we have shown this trend with mechanical stimulation.<sup>41</sup> The weak correlation between collagen amount and matrix strength in this study is likely due to the small collagen concentration; it also indicates that other strengthening mechanisms play a role in enhancing the strength of the EGF-treated gels. Additional studies are necessary to further characterize the effects of EGF on noncollagenous protein synthesis, including, but not limited to, fibronectin and proteoglycans such as decorin, each of which may play a role in the mechanics of the resulting matrix.

Compared to controls, fibrin gel compaction was enhanced by EGF and TGF- $\beta$ 1 treatment to a comparable extent over time (Fig. 1) except on the first day when the TGF- $\beta$ 1 group appears to lag behind the others. Similar enhancement of fibrin gel compaction by TGF- $\beta$ 1 supplementation has been observed previously.<sup>12,14,16</sup> As the TGF- $\beta$ 1-treated gels had a lower number of cells than the other groups, it appears that TGF- $\beta$ 1 may enhance the remodeling activity of the cells relative to EGF. Further, the EGF-treated cells compacted the matrix to a greater extent than the control group even though there were similar numbers of cells in these groups. The lower number of cells in the TGF- $\beta$ 1-treated group is consistent with findings that TGF- $\beta$ 1 suppresses fibroblast proliferation in biopolymer gels.<sup>14,22</sup> The lack of difference in cell number between the EGF and control groups was somewhat unexpected as EGF is a known mitogen,<sup>18,25,28,29</sup> although culture within biopolymer gels is known to inhibit cell proliferation relative to monolayer culture.<sup>2</sup>

Despite its mitogenic effects, EGF has been shown to reduce wound contraction in animal models<sup>22</sup> and to inhibit TGF- $\beta$ 1-induced collagen gel contraction by fibroblasts.<sup>42</sup> Unlike TGF- $\beta$ 1, which is well known to induce fibroblast-to-myofibroblast differentiation,<sup>23,32</sup> EGF has not been shown to induce differentiation to this contractile phenotype. In our study, both the rapid retraction upon release from the culture surface and the staining for  $\alpha$ SMA were reduced in the EGF-treated group. The rapid retraction over 30 minutes is reduced by EGF and enhanced by TGF- $\beta$ 1 relative to the control group. The time course and extent of retraction of the control group (with serum) and the enhancement with TGF- $\beta$ 1 treatment agree with previous reports of retraction of fibroblast-populated collagen gels.<sup>30,31,32</sup> In previous studies, this assay is often termed "stress relaxation" or "release of tension," and the extent of retraction is attributed to the relative ability of the resident cells to actively contract the surrounding ECM. Thus, the retraction assay is a relatively easy technique for measuring the functional state of a population of cells; however, interpretation of the total retraction is complex as it can be affected by many parameters. First, immediate retraction of the gel following release from the constraint of the culture dish can be caused by the residual stress in the matrix built up during cellular remodeling. We term this very rapid phenomenon passive retraction and, by depolymerizing the actin cytoskeleton of a group of samples with cytochalasin D, demonstrate that approximately 10% of the retraction is not dependent upon active cell contraction in



this study. The active portion of the retraction is believed to be representative of active contraction of the cells.<sup>30,31</sup> Thus, it appears that TGF- $\beta$ 1 increases the contractile ability of the cells whereas EGF does not. It has been previously suggested that TGF- $\beta$ 1 may increase synthesis of vasoactive peptides, for example, endothelin,<sup>3</sup> whereas the effects of EGF may be due, in part, to attenuating autogenic TGF- $\beta$ 1 signaling.<sup>42</sup> Alternatively, the enhanced or reduced active retraction may simply be due to the number of cells being greater or lesser in the TGF- $\beta$ 1 and EGF groups, respectively. However, in our study, we found the opposite trend in cell count data, that is, TGF- $\beta$ 1 < control < EGF. Further, even with an equivalent cell number, enhanced active retraction may be due to lower matrix stiffness of the TGF- $\beta$ 1 samples, and reduced retraction could be due to higher matrix stiffness of the EGF samples. Analysis of our biaxial inflation data indicates that the stiffness of the samples in the low stress region does not appear to follow this trend (data not shown); however, the inflation method was developed to characterize the failure properties of these tissues and is not well suited for determining the stiffness that the cells would "see" during contraction. We are currently pursuing indentation methods including atomic force microscopy to characterize the stiffness of the matrix at the cell level to answer this question more definitively. Thus, it appears that the difference in short-term active retraction between the treatment groups is due to the greater contractile ability of the TGF- $\beta$ 1-treated cells compared to the EGF-treated cells. Interestingly, the slow retraction of the matrix samples at 24 hours following release, attributed to traction forces generated as the cells spread and elongate during migration in the collagen gel literature,<sup>30,31</sup> appears to be relatively insensitive to the growth factor treatment (Table 1 and Fig. 5).

In summary, we provide evidence that EGF, an essential cytokine throughout the wound-healing and repair process,<sup>26</sup> can be used as an alternative supplement to TGF- $\beta$ 1 for the engineering of fibrin-based connective tissue substitutes. Specifically, EGF treatment stimulates cell-mediated matrix remodeling and enhances the mechanical strength of fibroblast-populated fibrin gels compared to TGF- $\beta$ 1 treatment. Further, unlike TGF- $\beta$ 1, EGF does not increase active retraction of the matrix by the cells and does not enhance the expression of  $\alpha$ SMA. The specific mechanisms of EGF and its receptor are left for future studies. For tissue engineering of certain tissues such as small-diameter vessel substitutes, vasoactivity of the embedded smooth muscle cells is desirable. However, for ocular, mucosal, dermal, valvular, and other passive connective tissue substitutes, contraction of the implant is generally detrimental to its function. This study advances efforts in developing optimal medium formulation for culturing tissue analogs with adequate strength and minimal cell contractility.

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