Published in final edited form as:

Biomaterials. 2011 November ; 32(33): 8684–8693. doi:10.1016/j.biomaterials.2011.07.079.

Engineering fibrin-binding TGF- β 1 for sustained signaling and contractile function of MSC based vascular constructs

Mao-Shih Liang¹ and Stelios T. Andreadis^{1,2,*}

¹Bioengineering Laboratory, Department of Chemical and Biological Engineering, University at Buffalo, State University of New York, Amherst, NY 14260-4200

²Center of Excellence in Bioinformatics and Life Sciences, Buffalo, NY 14203

Abstract

We present a strategy to conjugate TGF- β 1 into fibrin hydrogels to mimic the in vivo presentation of the growth factor in a 3D context. To this end, we engineered fusion proteins between TGF- β 1 and a bi-functional peptide composed of a Factor XIII domain and a plasmin cleavage site. In another version the protease cleavage site was omitted to examine whether the growth factor that could not be released from the scaffold by cells had different effects on tissue constructs. The optimal insertion site which yielded correctly processed, functional protein was found between the latency associated peptide and mature TGF- β 1 domains. In solution the fusion proteins exhibited similar biological activity as native TGF- β 1 as evidenced by inhibition of cell proliferation and promoter activity assays. Immunoprecipitation experiments demonstrated that the fusion TGF- β 1 protein bound to fibrinogen in a Factor XIII dependent manner and could be released from the peptide by the action of plasmin. In contrast to bolus delivery, immobilized TGF- β 1 induced sustained signaling in fibrin-embedded cells for several days as evidenced by Smad2 phosphorylation. Prolonged pathway activation correlated with enhanced contractile function of vascular constructs prepared from hair follicle mesenchymal stem cells or bone marrow derived smooth muscle cells. Our results suggest that fibrin-immobilized TGF- β 1 may be used to enhance the local microenvironment and improve the function of engineered tissues in vitro and potentially also after implantation in vivo where growth factor delivery faces overwhelming challenges.

1. INTRODUCTION

Transforming growth factor- β 1 (TGF- β 1) is a member of the TGF- β superfamily that is involved in many physiological processes from inhibition of cell proliferation to stem cell differentiation. TGF- β 1 has been shown to promote differentiation of mesenchymal stem cells (MSC) to the myogenic lineage [1–5] and enhance contractility and mechanical properties of vascular constructs in culture [2, 6–10]. At higher concentrations, TGF- β 1 was shown to promote chondrogenic differentiation in the 3D context of cellular aggregates [11–13]. Interestingly, TGF- β 1 was shown to induce immunosuppression and afford immune privilege by suppressing the function of CD4(+)CD25(+) regulatory T cells [14–19].

© 2011 Elsevier Ltd. All rights reserved

*Address for all Correspondence: Stelios T. Andreadis, Bioengineering Laboratory, 908 Furnas Hall, Department of Chemical and Biological Engineering, University at Buffalo, State University of New York, Amherst, NY 14260-4200, tel: (716) 645-1202, fax: (716) 645-3822, sandread@buffalo.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Therefore, release of TGF- β 1 from implanted bioengineered tissues may protect the grafts by reducing the immune reaction of the host.

However, supplementing 3D bioengineered tissues with growth factors presents challenges as diffusion limitations may necessitate use of high concentrations and generate growth factor gradients leading to non-uniform tissue properties. Growth factor delivery to the site of the graft presents additional challenges, as injection to the blood stream is inefficient and site-specific delivery onto the graft is hampered by protein instability and clearance, necessitating multiple injections for sustained effect [20]. These limitations may be overcome by development of strategies to immobilize the growth factor(s) onto scaffolds and control their release by physical/chemical signals or through the action of the cells that are co-delivered within the same scaffold.

Synthetic biomaterials have been used to deliver TGF- β 1 by exploiting its affinity with poly(ethylene glycol) (PEG). For example, PEG-modified poly(lactic-co-glycolic acid) (PLGA) improved TGF- β 1 release kinetics [21] and enhanced bone healing [22], osteocyte proliferation and osteoblastic differentiation [23]. Prolonged release kinetics was also observed with TGF- β 1 containing PLGA microspheres that were embedded within PEG hydrogels [24]. PEGylated fibrin gels also slowed down the release of TGF- β 1 without affecting the release kinetics of platelet derived growth factor BB (PDGF-BB), thereby resulting in sequential release of the two factors [25]. Others reported increased chondrogenic potential of adipose-derived mesenchymal stem cells when TGF- β 1 was loaded into heparin functionalized poly(lactide-co-caprolactone) nanoparticles to exploit the natural affinity of TGF- β 1 for heparin [26]. Finally, TGF- β 1 that was released from freeze-dried collagen sponges improved skull bone healing in vivo to a greater extent than the combined administration of the collagen sponge and free TGF- β 1 [27].

Fibrin has been used extensively as scaffold for tissue regeneration or for control delivery of growth factors to accelerate wound healing [28–30], repair articular cartilage [31] or promote vascularization [32]. Fibrin has also been used in tissue engineering of small diameter vascular grafts [8, 33–35], heart valves [36, 37] or myocardium [38]. In addition, previous studies developed methods to conjugate growth factors into fibrin hydrogels through the action of FXIIIa [32, 39, 40]. Using a similar approach our group showed that fibrin conjugated keratinocyte growth factor (KGF) was released in a cell-controlled manner resulting in a twofold enhancement of the wound healing rate of human bioengineered epidermis that was grafted on athymic mice [41].

In this communication we explored the hypothesis that conjugation of TGF- β 1 in a 3D matrix might affect the response of cells to this growth factor. To this end, we developed a biomimetic strategy to conjugate TGF- β 1 into fibrin hydrogels and evaluated its effects on embedded MSC. Our work provides a useful strategy to enhance the function of tissue engineered grafts in vitro and after implantation in vivo.

2. MATERIALS AND METHODS

2.1. Cloning for Fusion TGF-beta1Containing Plasmid

The TGF- β 1 encoding plasmid (pcDNA-GS-TGF- β 1) was kindly provided by Dr. P. D. Sun (National Institutes of Health) [42]. In order to improve TGF- β 1 production, several modifications were introduced to pro-TGF- β 1 cDNA [42]. First, Cys 33 in the latency associated peptide (LAP) was mutated to Ser (S33) to prevent binding of latent TGF- β 1 to the latent TGF- β 1 binding protein (Fig. 1a). In addition, the original TGF- β 1 leader sequence was replaced by the leader sequence of the rat serum albumin (RSA) to increase

protein expression and an 8x His tag sequence was introduced upstream of LAP to facilitate protein purification using nickel columns.

The modified TGF- β 1 was subcloned into the pCSCIG lentiviral vector downstream of the cytomegalovirus (CMV) promoter and followed by the internal ribosome entry site (IRES) and green fluorescent protein (GFP). To this end, TGF- β 1 was PCR amplified using pcDNA-GS-TGF- β 1 as template with primers For_1 and Rev_1 (Table 1) containing NheI (at 5'-terminus) and AgeI (at 3'-terminus) restriction sites, respectively, and the product was ligated into the lentivirus vector. In addition, primers For_1 and Rev_3 were used to introduce the Factor XIII substrate, PSVQEQN, and primers For_1 and Rev_2 were used to introduce the plasmin cleavage site, RSRYIG, followed by the Factor XIII substrate, PSVQEQN, at the 3'-terminus of TGF- β 1 sequence.

To introduce the Factor XIII substrate and plasmin cleavage sites between LAP and mature TGF- β 1, a two-step cloning approach was employed. The first primer pair, For_1 and Rev_LNG was used to amplify part of the LAP sequence and introduced NheI restriction sites at N-terminus and NQEQVSP-GIYRSR (NG) at 3'-terminus (LAP-NG). The second primer pair, For_LNG and Rev_1 was used to amplify part of the mature TGF- β 1 sequence and introduced NQEQVSP-GIYRSR at N-terminus and AgeI at 3'-terminus (NG-TGF β). PCR products LAP-NG and NG-TGF- β 1 were annealed at the common region (NG) and elongated in the presence of polymerase. Subsequently, the sequence was amplified by PCR with primers For_1 and Rev_1 and ligated between the AgeI and NheI sites of pCSCIG. The same approach was used to introduce the NQEQVSP sequence alone except that For_LNG and Rev_LNG primers were replaced with the For_LN and Rev_LN pair.

Although the IRES-GFP was useful to test transduction efficiency by flow cytometry, it was removed during production to avoid any potential negative effects of GFP accumulation in the producer cells. To this end, LNG-TGF- β 1 and LN-TGF- β 1 was PCR-amplified by PCR using For_1 and Rev_4 which replaced the AgeI by the XhoI site. Digestion of pCSVCG-IRES-GFP by NheI and XhoI removed IRES-GFP sequences. After ligation of the GFP free vector into the lentiviral vector, the two new plasmids were named pCSC-LNG-TGF- β 1 and pCSC-LN-TGF- β 1, respectively.

2.2. Lentivirus production and CHO-S transduction

HEK 293T cells were cultured in T-150 tissue culture flasks until they reached 70% confluent when they were co-transfected by calcium phosphate precipitation for 24 hr with the following plasmids: pMD2G (10 μ g), psPAX2 (15 μ g) and lentiviral vector containing one of modified TGF- β 1 sequence (20 μ g). The next day, the medium was replaced with 30mL of DMEM+10%FBS containing sodium butyrate (10mM) that was previously shown to improve virus production [43]. Two days later, the supernatant was harvested, filtered, ultra-centrifuged at 50,000g for 2 hr and the lentiviral pellet was dissolved by 300 μ L fresh medium (DMEM+10%FBS) to yield 100x concentrated lentivirus stock.

One day prior to transduction, Chinese Hamster Ovary (CHO-S, Invitrogen, Eugene, OR) cells were seeded to a 24-well plate for adhesion. The following day, the lentivirus stock was diluted with DMEM+10%FBS and used to transduce CHO-S cells. The next the virus was removed and replaced with fresh culture medium.

2.3. Fusion TGF- β 1 Expression and Purification

The transduced CHO-S cells were cultured in T-150 flasks in DMEM+10%FBS until they reached confluence, when the medium was changed to serum free medium, pro-CHO-AT (Lonza) that was optimized for protein expression. After two rounds of 3-day production, all supernatants were pooled and filtered through 0.22 μ m bottle top filter to remove the cell

debris and other impurities and purified according to a previously published protocol [42, 44]. Briefly, the supernatant was first loaded to a HiTrap nickel column (GE Healthcare) and the bound histidine-tagged fusion TGF- β 1 protein was eluted into ten 0.5-ml fractions using imidazole buffer (50mM Tris-HCl, 150mM NaCl, 500mM imidazole, pH 8.0). The eluted fractions that contained TGF- β 1 fusion protein were determined by SDS-PAGE, pooled and applied to a HiTrap desalting column (GE Healthcare) for buffer exchange to 50mM glycine, 150mM NaCl at pH 3.0. Next, the fusion protein containing the mature TGF- β 1 (25.5~29kDa) was separated from LAP (~75kDa), by concentrating the low pH protein solution to half its original volume and subjecting it to gel filtration (Hiload 16/60 Superdex 75pg, GE Healthcare) with the mobile phase glycine buffer at pH 3.0. The TGF- β 1 containing fractions were pooled and dialyzed against TBS. After dialysis, BSA was added to a final concentration of 0.1% (w/v) and the TGF- β 1 concentration was determined by ELISA (Duoset, R&D systems).

2.4. Biological activity measurements

2.4.1. Luciferase assay—Mink lung epithelial cells stably transfected with a plasmid encoding for luciferase under the TGF-beta1 response promoter (PAI-L cells) were kindly provided by Dr. Rifkin (New York University) and the luciferase assay was done according to a previously published protocol [45]. Briefly, 50,000 PAI-L cells per well were seeded in 96-well tissue culture plate with opaque walls and cultured at 37°C, 10% CO₂ for 4 hr. Subsequently, the cells were washed with PBS and the medium was replaced with DMEM/0.1% BSA containing different concentrations of fusion TGF- β 1 protein as indicated. After incubation at 37°C, 10% CO₂ for 18 hr, the amount of luciferase was measured using a commercial kit (Promega) according to the manufacturer's instructions. Luminescence was detected by Synergy HT microplate reader (Biotek, Winooski, VT).

2.4.2. Proliferation inhibition assay—Mink lung epithelial cells CCL-64 (AATC) were suspended at 10,000 cells/mL in DMEM/10% FBS containing the indicated fusion TGF- β 1 concentration and cultured in 96-well tissue culture plates (100 μ l or 1,000 cells per well) for 4 days at 37°C, 10% CO₂. On day 4, MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, TCI America, Portland, OR) was added (10 μ L per well) for 4 hr and then removed and replaced with 10% SDS (150 μ L per well). After overnight incubation, the optical density (O.D.) of each cell lysate was measured at 570 nm using a plate reader (SpectraMax 340 Molecular Devices, Menlo Park, CA) and values were corrected by subtracting the O.D. at 650 nm. The corrected O.D. was converted to cell number using a previously established calibration curve.

2.5. Kinetics of growth factor release from fibrin hydrogels

Fibrin hydrogels were polymerized in 96-well plates that were pre-coated with 2% BSA (50 μ L per well) and covered by DMEM/0.1% BSA (100 μ L per well). Each hydrogel contained 1mg/mL fibrinogen, 2.5U/mL thrombin, 2.5mM Ca²⁺, 0.1PEU/mL FXIII and 100ng/mL peptide-TGF- β 1 or native TGF- β 1. The medium was collected at the indicated times and stored at -20°C until use. The TGF- β 1 concentration of each sample was determined by ELISA according to manufacturer's recommendations (Duoset, R&D systems).

2.6. Immunoprecipitation

Factor XIII (20 μ L, 50PEU/mL) was activated by incubation with thrombin (1 μ L, 500U/mL) in the presence of CaCl₂ (2.5 μ L, 200mM) for 1 hr at 37°C, followed by incubation with hirudin (10 μ L, 1000U/mL) for 30 min to inactivate thrombin and prevent polymerization of fibrinogen. Then the peptide/TGF- β 1 fusion protein was introduced (400ng in TBS) to a

final volume of 100 μ L and the mixture was added to an equal volume of fibrinogen (0.5 mg/mL in TBS) and incubated for 4 hr at 37°C. The solution was then incubated with anti-fibrinogen antibody (1/100 dilution; EMD Bioscience, San Diego, CA) under gentle rocking at 4°C overnight followed by incubation with 20 μ L of protein G agarose beads for 2 hr at 4°C under gentle rocking. After centrifugation (14,000g, 2 min) the supernatant was carefully removed and the pellet was washed three times sequentially each with TBS containing decreasing Triton X-100 concentrations (1, 0.5, 0.1%) and twice with pure TBS. At this time, some pellets were treated with plasmin (5 μ L, 25U/mL) for 30 min at 37°C. All immunoprecipitates were suspended in 20 μ L SDS loading buffer boiled at 95°C for 5 min, cooled on ice for 5 min, and centrifuged (14,000g, 3 min). The proteins were then subjected to western blotting (WB) using mouse-anti-TGF- β 1 (R&D Systems, Minneapolis, MN) and HRP-anti-mouse antibodies (Cell Signaling, Danvers, MA).

2.7. Fibrin hydrogel preparation

Two solutions were prepared separately. The first one contained 12.5U/mL thrombin, 12.5mM Ca²⁺ and 0.5 PEU/mL Factor XIII with 100ng/mL peptide/TGF- β 1 fusion protein (LNG-TGF- β 1 or LN-TGF- β 1) or unmodified TGF- β 1. The second one contained 3.125mg/mL fibrinogen in TBS containing hHF-MSCs or bone marrow (BM) derived neonatal SMCs (1.25 $\times$ 10⁶ cells/mL). The two solutions were mixed at 1:4 ratio (100 μ L and 400 μ L, respectively) in a 48-well non-tissue culture plate. After 1hr, culture medium was added (DMEM containing 10% MSC-FBS and 2mg/mL ϵ -amino-*n*-caproic acid) to the cell-containing gels and was replenished daily.

2.8. Western blot

Human hair follicle mesenchymal stem cells (hHF-MSCs; 3 $\times$ 10⁵ per well) were seeded on 6-well plates in DMEM +10%MSC-FBS (Invitrogen, OR). The following day, culture medium (DMEM +10%MSC-FBS (Invitrogen, OR)) the cells were treated with 30ng/mL LNG-TGF- β 1 or LN-TGF- β 1 or native TGF- β 1. At indicated time, cells were lysed and lysates were subjected to WB using antibodies against pSmad2 (1:1000 dilution; Cell Signaling, Danvers, MA) and Smad2 (1:1000 dilution; Cell Signaling).

For fibrin hydrogels, the cells were embedded as described above and at the indicated times the gels were homogenized with lysis buffer. Lysates were subjected to WB using anti-pSmad2 or anti-Smad2 antibodies.

2.9. Fibrin gel compaction

Fibrin gels containing cells were prepared in 24-well non-tissue culture plates (1ml per well) that were pre-coated with 2% BSA. After 1hr, the gels were released from wells and culture medium was added (DMEM containing 2% FBS and 2mg/mL ϵ -amino-*n*-caproic acid) and replenished daily. The gels were photographed (UVP, Upland, CA) at the indicated times and images were analyzed by ImageJ. The area of each gel (A) was normalized to the initial area (A₀) and the ratio (A/A₀) was plotted as a function of time.

2.10. Tissue Engineered Vascular Constructs and their Contractility and Mechanical Properties

TGF- β 1 conjugated fibrin tissue equivalents were prepared as described previously [2, 8, 35, 46]. Briefly, BM-SMCs were suspended in fibrinogen and mixed with another solution containing thrombin, Ca²⁺, Factor XIII, and fusion TGF- β 1 at 4:1 ratio yielding fibrin gels with final concentration of 2.5mg/mL fibrinogen, 2.5U/mL thrombin, 0.1PEU/mL Factor XIII, 2.5mM Ca²⁺ and 50ng/mL TGF- β 1. Each construct contained 10⁶ cells per mL of fibrin that was polymerized around a 6.0 mm mandrel of poly(di-methyl siloxane) in the

middle of a well in a 24-well plate. The wells and the mandrels were pre-coated with 2% BSA for at least 20 min. After polymerization for 1 hr at 37°C, the gels were detached from the walls and incubated in 2 mL of DMEM containing 10% FBS supplemented with 2 mg/mL insulin, 300 mM ascorbic acid phosphate, and 2 mg/mL ϵ -amino-n-caproic acid. The media was replaced in the following day and every the other day thereafter.

After 2 weeks in culture, the length and wall thickness of each cylindrical tissue equivalent were recorded by digital camera and analyzed by ImageJ. Then the tissues were released from the mandrel, mounted in an isolated tissue bath and incubated in Krebs–Ringer solution. The tissues were continuously bubbled with 94% O₂, 6% CO₂ to obtain a pH of 7.4, a P_{CO2} of 38 mmHg, and a P_{O2} of 500 mmHg at 37°C. Each construct was mounted on stainless steel hooks through the lumen, one was fixed, and the other one was connected to a force transducer. Tissues were equilibrated at a basal tension of 1.0 g and constant length for 30–60 min. After equilibration, the thromboxane A2 mimetic U46619 (1 μ M), Endothelin-1 (20nM) or potassium chloride (KCl, 118mM) were added to the water bath in sequence after extensive washes to remove the previous vasoagonist and isometric contraction was recorded using a PowerLab data acquisition unit and analyzed with Chart5 (ADInstruments, Colorado Springs, CO).

2.11. Statistical Analysis

All experiments were performed three times with triplicate samples for each condition. Pairwise comparison was analyzed by two-tailed Student *t*-test and the data was considered statistically different when $p < 0.05$.

3. RESULTS

3.1. Genetic engineering and production of peptide/TGF- β 1 fusion protein

In this study we aimed at covalently conjugating transforming growth factor-beta1 (TGF- β 1) into fibrin hydrogels for applications in tissue engineering and regenerative medicine. To this end, we engineered a fusion protein between TGF- β 1 and the α_2 -plasminogen inhibitor I leader sequence (NQE₂VSP; denoted as N). The latter is recognized by factor XIII (FXIII) and is enzymatically conjugated into fibrin during polymerization [39, 47]. A plasmin cleavage site (GIYRSR denoted as G; [48]) was also introduced to promote cleavage of TGF- β 1 from the peptide upon degradation of fibrin by cells. The fusion protein was introduced into a lentiviral vector, which also encoded for enhanced green fluorescent protein (EGFP) and recombinant lentivirus was used to transduce Chinese hamster ovary-S (CHO-S) cells. Transduced CHO-S cells (>95% EGFP+) were expanded and used for TGF- β 1 production.

There were three possible sites for insertion of the peptide sequences: the N-terminus, the C-terminus or between the latency-associated-peptide (LAP) and mature-TGF- β 1 (Fig. 1a). Insertion at the N-terminus was ruled out because the peptide would be removed along with the LAP during the process of TGF- β 1 activation, thereby leaving mature TGF- β 1 unmodified. First, the peptide sequences were inserted at the C-terminus to generate three lentiviral vectors encoding for TGF- β 1-N, TGF- β 1-GN or TGF- β 1. Despite successful transduction of CHO-S cells (>95%) only the unmodified TGF- β 1 was produced at significantly higher levels compared to control non-transduced cells (TGF- β 1_CHO-S: 26.73 $\pm$ 0.32ng/mL-day; CHO-S: 1.62 $\pm$ 0.02ng/mL-day; $p < 0.05$). The media of control cells contained measureable amount of TGF- β 1 in the serum. However, media from TGF- β 1-GN or TGF- β 1-N transduced CHO-S cells did not contain TGF- β 1 above background levels (TGF- β 1-GN: 1.58 $\pm$ 0.03ng/mL-day; TGF- β 1-N: 1.59 $\pm$ 0.01ng/mL-day; $p > 0.05$ compared to control CHO-S cells). These results indicated that insertion of the peptide sequence at the C-

terminus hampered production of the fusion protein. In contrast, when the peptide sequences were introduced after the furin recognition sequence, RHRR between the LAP (L) and mature TGF- β 1 sequence, CHO-S cells produced significant amounts of LNG-TGF- β 1 or LN-TGF- β 1 protein (LNG-TGF- β 1: 25.7 ± 0.3 ng/mL-day; LN-TGF- β 1: 20.8 ± 0.12 ng/mL-day, $p < 0.05$ compared to control CHO-S cells), suggesting that the position between LAP and mature TGF- β 1 was the optimal insertion site.

3.2. Fusion protein production and purification

Recombinant lentivirus encoding for LNG-TGF- β 1 or LN-TGF- β 1 (without EGFP) was used to transduce CHO-S cells and the transduced cells were expanded and used for protein production. The two proteins were purified by a two-step process of His-tag affinity chromatography followed by size exclusion chromatography and the TGF- β 1 containing fractions were dialyzed against TBS. As seen in Fig. 1b, SDS-PAGE revealed only one band at the molecular weight that corresponded to the fusion proteins (arrowhead). In addition, plasmin cleaved LNG-TGF- β 1 as evidenced by the molecular weight decrease but failed to cleave LN-TGF- β 1 which lacked the plasmin cleavage site. Furthermore, treatment with DTT reduced the disulfide bonds of both LNG-TGF- β 1 and LN-TGF- β 1 dimers as evidenced by the decrease in molecular weight by half, from about 28 kDa to 14 kDa. These results were verified by western blot (Fig. 1c) and the concentration of the protein stocks was measured by ELISA.

3.3. Biological activity of the peptide/TGF- β 1 fusion proteins

Next we employed two assays to test the biological activity of the fusion proteins. First, we examined the activity of the TGF- β 1-responsive plasminogen activator inhibitor (PAI) promoter in CCL-64 mink lung epithelial (CCL-64) cells, a stable cell line expressing luciferase under the PAI promoter [45]. CCL-64 cells were treated with different concentrations of LNG-TGF- β 1, LN-TGF- β 1 or commercial TGF- β 1, ranging from 5 pg/mL to 10 ng/mL and luciferase activity was measured the following day. As shown in Fig. 2a, all three proteins activated the PAI promoter and displayed similar dose response trends, reaching plateau at approximately 1 ng/mL.

Inhibition of CCL-64 proliferation has also been used widely as an indicator of TGF- β 1 activity [49]. Similar to PAI promoter activation, both LNG-TGF- β 1 and LN-TGF- β 1 inhibited CCL-64 proliferation to a similar extent as commercial TGF- β 1, for concentrations ranging from 5 pg/mL to 10 ng/mL (Fig. 2b). These two assays indicated that the activity of the two fusion proteins was comparable to that of native TGF- β 1.

3.4. Conjugation of fusion peptide/TGF- β 1 proteins to fibrinogen

Next we incorporated LNG-TGF- β 1, LN-TGF- β 1 or TGF- β 1 in fibrin hydrogels in the presence of FXIII and measured their release over time. As shown in Fig. 3a, unmodified TGF- β 1 was released from the gel fast with a half-time, $t_{50\%} \sim 12$ hr. In contrast, less than ten percent of LNG-TGF- β 1 or LN-TGF- β 1 was released from the gels even after six days, strongly indicating that the fusion proteins were conjugated in fibrin. Since every fibrinogen molecule has two α_2 -plasminogen inhibitor binding sites, high concentrations of fusion protein could be immobilized per milliliter of fibrin hydrogel. To test this hypothesis, fibrin gels were loaded with different concentrations of protein up to about 1,000 ng/mL in the presence of FXIII. After polymerization, the gels were overlaid with DMEM/0.1% BSA and the concentration of fusion proteins in the medium was measured by ELISA 12 hr later. As shown in Fig. 3b both fusion proteins remained in the hydrogels, demonstrating the high binding capacity of the fibrin matrix.

To provide direct evidence that the peptide/TGF- β 1 proteins bound to fibrinogen, we performed immunoprecipitation experiments as follows. Each peptide/TGF- β 1 protein was mixed with fibrinogen in the presence of various FXIII concentrations. For these experiments FXIII was activated by thrombin, which was subsequently inactivated by hirudin to prevent fibrin polymerization. After binding for 4 hours, the complexes were pulled down with anti-fibrinogen antibody and immunoblotted with a monoclonal anti-TGF- β 1 antibody. As shown in Fig. 4a, at 5 PEU/ml FXIII, we observed a high molecular weight complex (>220kDa), suggesting that the peptide/TGF- β 1 (MW~30kDa) bound to fibrinogen. Binding depended strongly on FXIII concentration, as the amount of TGF- β 1 in the high molecular weight complex decreased at lower FXIII concentrations. No band was detected when native TGF- β 1 was mixed with fibrinogen in the presence of 5 PEU/ml FXIII (data not shown).

Furthermore, treatment of the LNG-TGF- β 1 containing sample with plasmin yielded a band of molecular weight around 28kDa, which is about the molecular weight of TGF- β 1 (Fig. 4b), suggesting that plasmin treatment released TGF- β 1 from fibrinogen. In contrast, plasmin treatment of the LN-TGF- β 1 containing gels yielded a smear, possibly reflecting binding of LN-TGF- β 1 to different molecular weight fibrinogen fragments resulting from plasmin-mediated fibrinolysis.

3.5. Differential effects of immobilized vs. soluble TGF- β 1 on Smad2 phosphorylation

Furthermore, we investigated cell signaling in response to LN-TGF- β 1, LNG-TGF- β 1 or native TGF- β 1 by measuring the kinetics of Smad2 phosphorylation at different times after treatment. Human hair follicle-mesenchymal stem cells (hHF-MSCs, isolated in our group as described previously [1, 2]) were cultured in 6-well plate and treated with 30ng/ml of the indicated TGF- β 1 protein in solution. As shown in Figs. 5a, b the Smad2 phosphorylation kinetics induced by LN-TGF- β 1, LNG-TGF- β 1 or native TGF- β 1 was similar. Specifically, phosphorylation of Smad2 increased shortly after growth factor stimulation (30 min), remained high for 3hr and decreased thereafter reaching about 30%–40% of the initial level after 24hr (Fig. 5b). This result further supported the claim that engineered TGF- β 1 had similar biological activity to the native protein.

Next we attempted to examine whether conjugation of TGF- β 1 in fibrin hydrogels affected cell signaling dynamics in a 3D context. To this end, LN-TGF- β 1, LNG-TGF- β 1 or native TGF- β 1 were incorporated in fibrin hydrogels along with hHF-MSCs, while maintaining the same protein to cell number ratio as in 2D culture (0.1 pg/cell). As in 2D cultures, native TGF- β 1 induced only transient Smad2 phosphorylation on day 1, which decreased significantly on day 2 and diminished at later times (Fig. 5c, d). On the other hand, LNG-TGF- β 1 and LN-TGF- β 1 induced sustained phosphorylation of Smad2 up to 4 days, suggesting that immobilization of the growth factor had significant effect on the activity of the signaling pathway.

3.6. The effect of immobilized TGF- β 1 on fibrin hydrogel compaction

It is well known that TGF- β 1 improves MSC and SMC contractility, but the effects of surface immobilized TGF- β 1 have not been investigated in a 3D context. To this end, we compared the compaction of fibrin hydrogels containing immobilized LNG-TGF- β 1, LN-TGF- β 1 or unmodified TGF- β 1 (100ng/mL). These experiments were conducted with hHF-MSCs and ovine bone marrow derived smooth muscle progenitor cells (BM-SMCs) that were isolated in our laboratory as described previously [1, 2, 7, 8].

Hydrogels containing immobilized TGF- β 1 compacted to a similar extent as those treated with unmodified TGF- β 1 reaching to approximately 20% of their original area after 96 hr

(Fig. 6a, c). In contrast, fibrin hydrogels in the hHF-MSCs and BM-SMCs control groups (no TGF- β 1 treatment) compacted to much lesser extent reaching 60% and 70% of their original area, respectively. Addition of the TGF- β 1 receptor I kinase inhibitor, SB431542, decreased compaction to the level of control for hHF-MSC (Fig. 6b) and diminished compaction for ovine BM-SMC, (Fig. 6d).

3.7. The effect of conjugated TGF- β 1 on vascular contractility

Next we investigated the effects of immobilized TGF- β 1 on vascular reactivity of fibrin based vascular constructs. To this end, BM-SMC containing fibrin was polymerized around mandrels in the presence of 50ng/mL of LNG-TGF- β 1, LN-TGF- β 1 or soluble TGF- β 1. As we reported previously [2, 7, 8, 46], after two weeks in culture the cells compacted the gels to less than 5% of their original volume forming vascular ring-shaped constructs. These tissue rings were connected to a force transducer in an isolated tissue bath and vascular contractility was measured in response to receptor (U46619 and Endothelin-1) and non-receptor (KCl) mediated agonists. As shown in Fig. 7, the tissue engineered vascular constructs (TEVs) conjugated with LNG-TGF- β 1 or LN-TGF- β 1 exhibited about two times higher vascular reactivity to all vasoagonists compared to those containing native TGF- β 1. This result demonstrated that the mode of TGF- β 1 presentation had significant effect on the force generation ability of SMCs in the context of 3D tissue constructs.

4. DISCUSSION

Fibrin has been used widely for tissue regeneration such as tissue engineered blood vessels [8, 33–35], wound healing [28, 29, 50], or protein and gene delivery [32, 40, 41, 51, 52]. The goal of this study was to fabricate fibrin hydrogels with covalently bound TGF- β 1 to promote cellular function in a 3D context. Our results showed that the peptide/TGF- β 1 fusion remained biological active and was successfully incorporated into fibrin even at high concentrations. TGF- β 1 that was conjugated into fibrin hydrogels induced sustained phosphorylation of Smad2 and increased the vascular contractility in response to receptor and non-receptor mediated agonists as compared to soluble native growth factor. These results suggest that fibrin-immobilized TGF- β 1 may be used to provide localized signaling that may enhance the function of engineered tissues in vitro and potentially also in vivo.

Introduction of the peptide between the LAP and mature TGF- β 1 yielded a functional protein that could still be processed inside the cell and secreted as mature TGF- β 1 dimer as suggested by the 2-fold reduction of its molecular weight by DTT. In addition, the peptide/TGF- β 1 retained the full biological activity of TGF- β 1 as shown by PAI promoter activation and by reduced proliferation of lung epithelial cells. On the other hand, production of the fusion protein failed when the peptide was introduced at the C-terminus of TGF- β 1. Although the reason is unclear, the crystal structure of mature TGF β 1 might provide a possible explanation. Specifically, the C-termini of the two monomers that make up the TGF- β 1 dimer are restricted in space within the inner core of the protein facing each other [53]. Introduction of extra amino acids on each end might have prevented correct folding, thereby causing protein degradation.

Many tissue regeneration approaches require that growth factor release is tuned in concert with cellular demand. The current study demonstrates a biomimetic strategy to covalently conjugate TGF- β 1 within fibrin gels using an enzymatic reaction and allow for cell-mediated release upon fibrin degradation. Indeed, modified TGF- β 1 was conjugated into fibrin gels in a FXIII concentration dependent manner and was retained within the hydrogels even at high concentrations. Immunoprecipitation experiments showed that LNG-TGF- β 1 and LN-TGF- β 1 bound to fibrinogen but only LNG-TGF- β 1 that contained the plasmin cutting site was released by treatment with plasmin. This is important as cells degrade fibrin

by activating plasminogen to plasmin locally on the cell surface and the rate of fibrin degradation can be delayed by fibrinolytic inhibitors e.g. aprotinin or ϵ -amino-caproic acid. Therefore, attenuating fibrin degradation may allow time for TGF- β 1 stimulated deposition of new extracellular matrix e.g. collagen to replace fibrin and enhance the mechanical properties of the constructs.

In vivo TGF- β 1 is secreted as a complex with latency-associated propeptide (LAP) and targeted to specific locations in the extracellular matrix through binding to latent TGF- β binding protein (LTBP) [54]. However, most studies are conducted with soluble pre-activated TGF- β 1 and therefore, they are missing the effects of targeting and immobilization on cell signaling. Interestingly, we found that conjugation of peptide/TGF- β 1 had significant impact on cell signaling in a 3D context compared to native growth factor, despite similarities in their biological activity when provided in solution. Specifically, both LNG-TGF- β 1 and LN-TGF- β 1 stimulated continuous phosphorylation of Smad2 for at least four days when conjugated within fibrin hydrogels. In contrast, native TGF- β 1 induced only transient Smad2 phosphorylation for only one day, suggesting that pathway activation is critically dependent on TGF- β 1 presentation. Since TGF- β 1 signaling was shown to be tightly regulated by internalization of TGF- β 1-receptor complex [55], immobilized TGF- β 1 may induced sustained pathway activation by preventing or delaying receptor internalization.

The kinetics of signaling pathway activation by conjugated or native TGF- β 1 correlated with the function of 3D vascular constructs. Fibrin gel compaction was equally effective with fusion peptide/TGF- β 1 or native TGF- β 1 proteins, as all of them stimulated Smad2 phosphorylation during the first 24–48hr, when the greater part of gel compaction occurred. However, for longer time scales e.g. 2 weeks as required for generation of cylindrical vascular constructs, tissues with conjugated growth factors generated twice the amount of force in response to receptor (ET-1, U46619) and non-receptor (KCl) acting vasoagonists, as compared to those containing native TGF- β 1 supplied at the same concentration. These results indicated that immobilizing peptide/TGF- β 1 in a 3D context enhanced tissue function possibly by sustaining the intensity of the signaling response for much longer times compared to the native factor.

This conjugation strategy may be potentially applied to promote stem cell differentiation in 3D fibrin hydrogels. Supplementation of TGF- β 1 has been employed to induce differentiation of MSC into chondrocytes or osteocytes [56, 57]. In particular, chondrogenic differentiation was enhanced by cell aggregation in the presence of high TGF- β 1 concentration [11–13, 58, 59]. This inspired some investigators to develop controlled release TGF- β 1 biomaterials to promote chondrogenesis [60–64]. We propose that LNG-TGF- β 1 or LN-TGF- β 1 decorated fibrin hydrogels might be an effective platform to induce MSC differentiation to chondrogenic lineage. This hypothesis is currently under investigation.

5. Conclusions

We demonstrated a method to covalently incorporate TGF- β 1 onto fibrinogen molecules in a FXIII-dependent manner. We engineered fusion proteins of TGF- β 1 with mono- or bi-functional peptides containing the FXIII substrate and a plasmin cutting site to facilitate cell-mediated cleavage of TGF- β 1 from the peptide. The fusion proteins LN-TGF- β 1 and LNG-TGF- β 1 displayed similar biological activity compared to commercially available TGF- β 1 in solution and were covalently conjugated to fibrinogen in a FXIII dose-dependent manner. Accordingly, when immobilized into fibrin-based constructs, TGF- β 1 sustained the activity of the downstream signaling pathway leading to increased vascular contractility. This

biomimetic strategy may be applied to other myogenic tissues including heart, intestine or bladder extending its applications in the field of regenerative medicine.

Acknowledgments

This work was supported by grants from the National Institutes of Health (R01 HL086582) and the New York State Stem Cell Science (NYSTEM Contract #C024316) to S.T. Andreadis.

References

1. Liu JY, Peng HF, Gopinath S, Tian J, Andreadis ST. Derivation of functional smooth muscle cells from multipotent human hair follicle mesenchymal stem cells. *Tissue Eng Part A*. 2010; 16:2553–64. [PubMed: 20236033]
2. Liu JY, Peng HF, Andreadis ST. Contractile smooth muscle cells derived from hair-follicle stem cells. *Cardiovasc Res*. 2008; 79:24–33. [PubMed: 18316325]
3. Narita Y, Yamawaki A, Kagami H, Ueda M, Ueda Y. Effects of transforming growth factor-beta 1 and ascorbic acid on differentiation of human bone-marrow-derived mesenchymal stem cells into smooth muscle cell lineage. *Cell Tissue Res*. 2008; 333:449–59. [PubMed: 18607632]
4. Gong Z, Niklason LE. Small-diameter human vessel wall engineered from bone marrow-derived mesenchymal stem cells (hMSCs). *The FASEB J*. 2008; 22:1635–48.
5. Wang D, Park JS, Chu JS, Krakowski A, Luo K, Chen DJ, et al. Proteomic profiling of bone marrow mesenchymal stem cells upon transforming growth factor beta1 stimulation. *J Biol Chem*. 2004; 279:43725–34. [PubMed: 15302865]
6. Yao L, Liu J, Andreadis ST. Composite fibrin scaffolds increase mechanical strength and preserve contractility of tissue engineered blood vessels. *Pharm Res*. 2008; 25:1212–21. [PubMed: 18092140]
7. Han J, Liu JY, Swartz DD, Andreadis ST. Molecular and functional effects of organismal ageing on smooth muscle cells derived from bone marrow mesenchymal stem cells. *Cardiovasc Res*. 2010; 87:147–55. [PubMed: 20097675]
8. Liu JY, Swartz DD, Peng HF, Gugino SF, Russell JA, Andreadis ST. Functional tissue-engineered blood vessels from bone marrow progenitor cells. *Cardiovasc Res*. 2007; 75:618–28. [PubMed: 17512920]
9. Long JL, Tranquillo RT. Elastic fiber production in cardiovascular tissue-equivalents. *Matrix Biol*. 2003; 22:339–50. [PubMed: 12935818]
10. Ross JJ, Tranquillo RT. ECM gene expression correlates with in vitro tissue growth and development in fibrin gel remodeled by neonatal smooth muscle cells. *Matrix Biol*. 2003; 22:477–90. [PubMed: 14667840]
11. Kawaguchi J, Mee PJ, Smith AG. Osteogenic and chondrogenic differentiation of embryonic stem cells in response to specific growth factors. *Bone*. 2005; 36:758–69. [PubMed: 15794925]
12. Kim JH, Do HJ, Yang HM, Oh JH, Choi SJ, Kim DK, et al. Overexpression of SOX9 in mouse embryonic stem cells directs the immediate chondrogenic commitment. *Exp Mol Med*. 2005; 37:261–8. [PubMed: 16155402]
13. Chiou M, Xu Y, Longaker MT. Mitogenic and chondrogenic effects of fibroblast growth factor-2 in adipose-derived mesenchymal cells. *Biochem Biophys Res Commun*. 2006; 343:644–52. [PubMed: 16554022]
14. Wahl SM, Chen W. TGF-beta: how tolerant can it be? *Immunol Res*. 2003; 28:167–79. [PubMed: 14713712]
15. Strober W, Fuss I, Boirivant M, Kitani A. Insights into the mechanism of oral tolerance derived from the study of models of mucosal inflammation. *Ann N Y Acad Sci*. 2004; 1029:115–31. [PubMed: 15681751]
16. Powrie F. Immune regulation in the intestine: a balancing act between effector and regulatory T cell responses. *Ann N Y Acad Sci*. 2004; 1029:132–41. [PubMed: 15681752]

17. Staines NA, Derry CJ, Marinova-Mutafchieva L, Ali N, Davies DH, Murphy JJ. Constraints on the efficacy of mucosal tolerance in treatment of human and animal arthritic diseases. *Ann N Y Acad Sci.* 2004; 1029:250–9. [PubMed: 15681763]
18. Okamoto A, Kawamura T, Kanbe K, Kanamaru Y, Ogawa H, Okumura K, et al. Suppression of serum IgE response and systemic anaphylaxis in a food allergy model by orally administered high-dose TGF-beta. *Int Immunol.* 2005; 17:705–12. [PubMed: 15837712]
19. Wahl SM, Swisher J, McCartney-Francis N, Chen W. TGF-beta: the perpetrator of immune suppression by regulatory T cells and suicidal T cells. *J Leukoc Biol.* 2004; 76:15–24. [PubMed: 14966194]
20. Goncalves LM, Epstein SE, Piek JJ. Controlling collateral development: the difficult task of mimicking mother nature. *Cardiovasc Res.* 2001; 49:495–6. [PubMed: 11166262]
21. Lu L, Stamatas GN, Mikos AG. Controlled release of transforming growth factor beta1 from biodegradable polymer microparticles. *J Biomed Mater Res.* 2000; 50:440–51. [PubMed: 10737887]
22. Lu L, Yaszemski MJ, Mikos AG. TGF-beta1 release from biodegradable polymer microparticles: its effects on marrow stromal osteoblast function. *J Bone Joint Surg Am.* 2001; 83-A(Suppl 1):S82–91. [PubMed: 11314800]
23. Peter SJ, Lu L, Kim DJ, Stamatas GN, Miller MJ, Yaszemski MJ, et al. Effects of transforming growth factor beta1 released from biodegradable polymer microparticles on marrow stromal osteoblasts cultured on poly(propylene fumarate) substrates. *J Biomed Mater Res.* 2000; 50:452–62. [PubMed: 10737888]
24. DeFail AJ, Chu CR, Izzo N, Marra KG. Controlled release of bioactive TGF-beta 1 from microspheres embedded within biodegradable hydrogels. *Biomaterials.* 2006; 27:1579–85. [PubMed: 16140372]
25. Drinnan CT, Zhang G, Alexander MA, Pulido AS, Suggs LJ. Multimodal release of transforming growth factor-beta1 and the BB isoform of platelet derived growth factor from PEGylated fibrin gels. *J Control Release.* 2010; 147:180–6. [PubMed: 20381553]
26. Jung Y, Chung YI, Kim SH, Tae G, Kim YH, Rhie JW. In situ chondrogenic differentiation of human adipose tissue-derived stem cells in a TGF-beta1 loaded fibrin-poly(lactide-caprolactone) nanoparticulate complex. *Biomaterials.* 2009; 30:4657–64. [PubMed: 19520426]
27. Ueda H, Hong L, Yamamoto M, Shigeno K, Inoue M, Toba T, et al. Use of collagen sponge incorporating transforming growth factor-beta1 to promote bone repair in skull defects in rabbits. *Biomaterials.* 2002; 23:1003–10. [PubMed: 11791902]
28. Bannasch H, Fohn M, Unterberg T, Bach AD, Weyand B, Stark GB. Skin tissue engineering. *Clin Plast Surg.* 2003; 30:573–9. [PubMed: 14621305]
29. Geer DJ, Andreadis ST. A novel role of fibrin in epidermal healing: plasminogen-mediated migration and selective detachment of differentiated keratinocytes. *J Invest Dermatol.* 2003; 121:1210–6. [PubMed: 14708628]
30. Geer DJ, Swartz DD, Andreadis ST. Fibrin promotes migration in a three-dimensional in vitro model of wound regeneration. *Tissue Eng.* 2002; 8:787–98. [PubMed: 12459057]
31. Peretti GM, Xu JW, Bonassar LJ, Kirchhoff CH, Yaremchuk MJ, Randolph MA. Review of injectable cartilage engineering using fibrin gel in mice and swine models. *Tissue Eng.* 2006; 12:1151–68. [PubMed: 16771631]
32. Zisch AH, Schenk U, Schense JC, Sakiyama-Elbert SE, Hubbell JA. Covalently conjugated VEGF--fibrin matrices for endothelialization. *J Control Release.* 2001; 72:101–13. [PubMed: 11389989]
33. Grassl ED, Oegema TR, Tranquillo RT. A fibrin-based arterial media equivalent. *J Biomed Mater Res A.* 2003; 66:550–61. [PubMed: 12918038]
34. Isenberg BC, Williams C, Tranquillo RT. Small-diameter artificial arteries engineered in vitro. *Circ Res.* 2006; 98:25–35. [PubMed: 16397155]
35. Swartz DD, Russell JA, Andreadis ST. Engineering of fibrin-based functional and implantable small-diameter blood vessels. *Am J Physiol Heart Circ Physiol.* 2005; 288:H1451–60. [PubMed: 15486037]

36. Flanagan TC, Sachweh JS, Frese J, Schnoring H, Gronloh N, Koch S, et al. In vivo remodeling and structural characterization of fibrin-based tissue-engineered heart valves in the adult sheep model. *Tissue Eng Part A*. 2009; 15:2965–76. [PubMed: 19320544]
37. Syedain ZH, Tranquillo RT. Controlled cyclic stretch bioreactor for tissue-engineered heart valves. *Biomaterials*. 2009; 30:4078–84. [PubMed: 19473698]
38. Black LD 3rd, Meyers JD, Weinbaum JS, Shvelidze YA, Tranquillo RT. Cell-induced alignment augments twitch force in fibrin gel-based engineered myocardium via gap junction modification. *Tissue Eng Part A*. 2009; 15:3099–108. [PubMed: 19338433]
39. Schense JC, Hubbell JA. Cross-linking exogenous bifunctional peptides into fibrin gels with factor XIIIa. *Bioconjug Chem*. 1999; 10:75–81. [PubMed: 9893967]
40. Sakiyama-Elbert SE, Panitch A, Hubbell JA. Development of growth factor fusion proteins for cell-triggered drug delivery. *FASEB J*. 2001; 15:1300–2. [PubMed: 11344120]
41. Geer DJ, Swartz DD, Andreadis ST. Biomimetic delivery of keratinocyte growth factor upon cellular demand for accelerated wound healing in vitro and in vivo. *Am J Pathol*. 2005; 167:1575–86. [PubMed: 16314471]
42. Zou Z, Sun PD. Overexpression of human transforming growth factor-beta1 using a recombinant CHO cell expression system. *Protein Expr Purif*. 2004; 37:265–72. [PubMed: 15358346]
43. Lei P, Andreadis ST. Stoichiometric limitations in assembly of active recombinant retrovirus. *Biotechnol Bioeng*. 2005; 90:781–92. [PubMed: 15812799]
44. Zou Z, Sun PD. An improved recombinant mammalian cell expression system for human transforming growth factor-beta2 and -beta3 preparations. *Protein Expr Purif*. 2006; 50:9–17. [PubMed: 16901717]
45. Abe M, Harpel JG, Metz CN, Nunes I, Loskutoff DJ, Rifkin DB. An assay for transforming growth factor-beta using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. *Anal Biochem*. 1994; 216:276–84. [PubMed: 8179182]
46. Yao L, Swartz DD, Gugino SF, Russell JA, Andreadis ST. Fibrin-based tissue-engineered blood vessels: differential effects of biomaterial and culture parameters on mechanical strength and vascular reactivity. *Tissue Eng*. 2005; 11:991–1003. [PubMed: 16144435]
47. Sakiyama SE, Schense JC, Hubbell JA. Incorporation of heparin-binding peptides into fibrin gels enhances neurite extension: an example of designer matrices in tissue engineering. *FASEB J*. 1999; 13:2214–24. [PubMed: 10593869]
48. Hervio LS, Coombs GS, Bergstrom RC, Trivedi K, Corey DR, Madison EL. Negative selectivity and the evolution of protease cascades: the specificity of plasmin for peptide and protein substrates. *Chem Biol*. 2000; 7:443–53. [PubMed: 10873836]
49. Ikeda T, Lioubin MN, Marquardt H. Human transforming growth factor type beta 2: production by a prostatic adenocarcinoma cell line, purification, and initial characterization. *Biochemistry*. 1987; 26:2406–10. [PubMed: 3475130]
50. Bannasch H, Unterberg T, Fohn M, Weyand B, Horch RE, Stark GB. Cultured keratinocytes in fibrin with decellularised dermis close porcine full-thickness wounds in a single step. *Burns*. 2008; 34:1015–21. [PubMed: 18395990]
51. Raut SD, Lei P, Padmashali RM, Andreadis ST. Fibrin-mediated lentivirus gene transfer: implications for lentivirus microarrays. *J Control Release*. 2010; 144:213–20. [PubMed: 20153386]
52. Lei P, Padmashali RM, Andreadis ST. Cell-controlled and spatially arrayed gene delivery from fibrin hydrogels. *Biomaterials*. 2009; 30:3790–9. [PubMed: 19395019]
53. Hinck AP, Archer SJ, Qian SW, Roberts AB, Sporn MB, Weatherbee JA, et al. Transforming growth factor beta 1: three-dimensional structure in solution and comparison with the X-ray structure of transforming growth factor beta 2. *Biochemistry*. 1996; 35:8517–34. [PubMed: 8679613]
54. Hyttiainen M, Penttinen C, Keski-Oja J. Latent TGF-beta binding proteins: extracellular matrix association and roles in TGF-beta activation. *Crit Rev Clin Lab Sci*. 2004; 41:233–64. [PubMed: 15307633]

55. Clarke DC, Brown ML, Erickson RA, Shi Y, Liu X. Transforming growth factor beta depletion is the primary determinant of Smad signaling kinetics. *Mol Cell Biol.* 2009; 29:2443–55. [PubMed: 19223462]
56. Centrella M, Horowitz MC, Wozney JM, McCarthy TL. Transforming growth factor-beta gene family members and bone. *Endocr Rev.* 1994; 15:27–39. [PubMed: 8156937]
57. Centrella M, McCarthy TL, Canalis E. Transforming growth factor-beta and remodeling of bone. *J Bone Joint Surg Am.* 1991; 73:1418–28. [PubMed: 1918129]
58. Hwang NS, Kim MS, Sampattavanich S, Baek JH, Zhang Z, Elisseeff J. Effects of three-dimensional culture and growth factors on the chondrogenic differentiation of murine embryonic stem cells. *Stem Cells.* 2006; 24:284–91. [PubMed: 16109760]
59. Pelttari K, Steck E, Richter W. The use of mesenchymal stem cells for chondrogenesis. *Injury.* 2008; 39 (Suppl 1):S58–65. [PubMed: 18313473]
60. Degat MC, Dahri-Correia L, Lavigne F, Meunier A, Sedel L, Correia J, et al. Benzylaminated dextran-modified hydrogels: a long-term bioactive TGF-beta1 carrier. *J Biomed Mater Res A.* 2009; 91:1178–88. [PubMed: 19148927]
61. Han Y, Wei Y, Wang S, Song Y. Enhanced chondrogenesis of adipose-derived stem cells by the controlled release of transforming growth factor-beta1 from hybrid microspheres. *Gerontology.* 2009; 55:592–9. [PubMed: 19672054]
62. Hu X, Ma L, Wang C, Gao C. Gelatin hydrogel prepared by photo-initiated polymerization and loaded with TGF-beta1 for cartilage tissue engineering. *Macromol Biosci.* 2009; 9:1194–201. [PubMed: 19890886]
63. Park JS, Yang HJ, Woo DG, Yang HN, Na K, Park KH. Chondrogenic differentiation of mesenchymal stem cells embedded in a scaffold by long-term release of TGF-beta 3 complexed with chondroitin sulfate. *J Biomed Mater Res A.* 2010; 92:806–16. [PubMed: 19280636]
64. Solorio LD, Fu AS, Hernandez-Irizarry R, Alsberg E. Chondrogenic differentiation of human mesenchymal stem cell aggregates via controlled release of TGF-beta1 from incorporated polymer microspheres. *J Biomed Mater Res A.* 2010; 92:1139–44. [PubMed: 19322820]

Schematic of the fusion peptide/TGF- β 1 expression plasmid. **(a)** The optimal position for peptide insertion was between the LAP and mature TGF- β 1 domains, right after the furin site (RHRR; point I). The fusion proteins pro-LNG-TGF- β 1 or pro-LN-TGF- β 1 are secreted as pro-peptides and then cleaved at RHRR to yield LNG-TGF- β 1 or LN-TGF- β 1, respectively. The mutation C33 to S33 prevented binding to the latent TGF- β 1 binding protein; 8xHis was used for protein purification and rat serum albumin (RSA) leader sequence was introduced to increase protein expression. **(b)** SDS-PAGE of purified LNG-TGF- β 1 (lanes 1–3) and LN-TGF- β 1 (lanes 4–6). Samples were untreated (lanes 1, 4, solid arrowhead), or treated with plasmin (lanes 2, 5, open arrowhead) or DTT (lanes 3, 6, pound sign). **(c)** Purified LNG-TGF- β 1 (lanes 1, 2) and LN-TGF- β 1 (lanes 3, 4) were immunoblotted with anti-TGF- β 1 antibody. Samples were untreated (lanes 1, 3) or treated with plasmin (lanes 2, 4).

Figure 2.

LNG-TGF-β1 and LN-TGF-β1 maintained the TGF-β1 bioactivity. Modified proteins increased the PAI promoter activity (a); and decreased proliferation (b) of mink lung epithelial (CCL-64) cells in a dose dependent manner similar to native TGF-β1. The growth inhibition index was defined as $1 - \frac{C_{treated}}{C_{control}}$, where $C_{treated}$ and $C_{control}$ denote the final cell number of treated and control (no TGF-β1 treatment) samples after 4 days in culture. All values are mean±SD of triplicate samples in a representative experiment (n=3).

Figure 3.

Kinetics of release from fibrin hydrogels. **(a)** LNG-TGF-β1, LN-TGF-β1 or native TGF-β1 were loaded in fibrin hydrogels at 100ng/ml and the gels were overlaid with DMEM/0.1% BSA. At the indicated times points the medium was removed and replenished with fresh medium. The amount of protein in the medium was measured by ELISA for TGF-β1. **(b)** LNG-TGF-β1 or LN-TGF-β1 were loaded in fibrin gels at different concentrations as indicated. The gels were overlaid by DMEM/0.1%BSA and 12 hr later the TGF-β1 concentration in the medium was measured by ELISA. All values are the mean±SD of triplicate samples in a representative experiment (n=3). The symbol (*) denotes p<0.01 between modified and native TGF-β1 at the indicated time point.

Figure 4. Modified TGF- β 1 proteins bind to fibrinogen in a FXIII dependent manner. **(a)** LNG-TGF- β 1 or LN-TGF- β 1 (400 ng/ml) were mixed with fibrinogen (0.5mg/ml) containing thrombin-activated FXIII at the indicated concentration. After 4hr, the complexes were immunoprecipitated by anti-fibrinogen antibody and immunoblotted with anti-TGF- β 1. **(b)** Immunoprecipitated complexes were resuspended in plasmin (0.1 U) and incubated for 30 min at 37°C before they were subjected to western blotting.

Figure 5.

Effects of TGF-β1 immobilization on Smad2 phosphorylation. **(a)** Kinetics of Smad2 phosphorylation in 2-D culture. hHF-MSCs were seeded in a 6-well plate (3×10^5 cells/well) and the following day LNG-TGF-β1, LN-TGF-β1 or unmodified TGF-β1 was added to each well. At the indicated times, cells were lysed and lysates were subjected to WB. **(b)** At each time point the intensity of phosphor (p)-Smad2 was normalized to that of total Smad2 and reported as fold-increase over the initial ratio at 30min. **(c)** Kinetics of hHFMSCs SMAD2 phosphorylation in 3-D culture. Fusion or native TGF-β1 (100ng/mL) was copolymerized with hHF-MSCs (10^6 cells/mL) in fibrin hydrogels. At the indicated times, cells were lysed and lysates were subjected to WB. **(d)** At each time point the intensity of p-Smad2 was normalized to total Smad2 and reported as fold-change over the initial ratio on day 1. All values are the mean $\pm$ SD of quadruplicate samples in a representative experiment ($n=4$). The

symbols (*) or (#) denote $p < 0.01$ or $p < 0.05$, respectively, between modified and native TGF- β 1 at the indicated time point.

Figure 6.

Modified TGF-β1 proteins enhanced fibrin gel compaction by embedded mesenchymal stem cells and smooth muscle cells. **(a)** hHF-MSCs or **(c)** BM-SMCs were embedded in fibrin hydrogels (10^6 cells/ml) that contained either conjugated fusion TGF-β1 (LNG-TGF-β1 or LN-TGF-β1) or native TGF-β1. Control gels were not treated with TGF-β1. At the indicated times, the projected area of each gel (A) was measured and plotted as a fraction of the initial area (A_0). **(b)** and **(d)** All conditions are the same as in **(a)** and **(c)**. The area of each gel was measured after 48hr in culture in the presence (white bars) or absence (black bars) of the TGF-β1 receptor inhibitor, SB431542. All values are the mean $\pm$ SD of triplicate samples in a representative experiment ($n=3$). The symbol (*) in **(a)** and **(c)** denotes $p < 0.01$ between the

indicated sample and the corresponding control at the same time point. The symbol (*) in (b) and (d) denotes $p < 0.01$ between SB431542-treated and untreated samples within each group.

Figure 7.

Conjugated TGF-β1 increased BM-SMC contractility. Fibrin-based cylindrical constructs were prepared with 50 ng/ml of LNG-TGF-β1, LN-TGF-β1 or native TGF-β1 and cultured for two weeks around 6 mm cylindrical mandrels. Vascular reactivity was measured in response to U46619 (1μM), Endothelin-1 (ET-1; 20nM) or KCl (118mM). All values are the mean±SD of triplicate samples in a representative experiment (n=3). The symbol (*) denotes p<0.01 between the indicated samples and those treated with native TGF-β1.

Table 1

Primers List

For_1	AAAGGGGCTAGCGCCACCATGAAGTGGGTAACCTTTCTCCTCCTC
Rev_1	AACACCGGTTCAAGTGCAGTGCAGGAGCGCACGATCA
Rev_2	AACACCGGTTCAATTTGTTCTTGGACACTGGGACCCCCACCGCTGCCGATGTATCTGCTTCGACCACCTCCGCTGCTGCACTTGCAGGAGCGCAC
Rev_3	AACACCGGTTCAATTTGTTCTTGGACACTGGGACCCCCACCGCTGCTGCACTTGCAGGAGCGCACGATCA
Rev_4	AACCTCGAGTCAGTGCAGTGCAGGAGCGCACGATCA
For_LNG	AATCAAGAACAAGTCAGTCCCGGTGGGGTAGCGGCATCTACAGAAGCCGAGTGGTGAAGCGCCCTGGATACCAACTACTGCTTCA
Rev_LNG	GCTTCCACCACCTCGGCTTCTGTAGATGCCGCTACCCCCACCGGACTGACTTGTCTTGATTTCGGCGGTGCCGGGAGCTGT
For_LN	AATCAAGAACAAGTCAGTCCCGGTGGGGTAGCGCCCTGGATACCAACTACTGCTTCA
Rev_LN	GCTACCCCCACCGGACTGACTTGTCTTGATTTCGGCGGTGCCGGGAGCTGT