Published in final edited form as:

Biomaterials. 2009 September ; 30(26): 4318–4324. doi:10.1016/j.biomaterials.2009.04.057.

Cell-Responsive Hydrogel for Encapsulation of Vascular Cells

Thomas P. Kraehenbuehl^{1,2}, Lino S. Ferreira^{3,4}, Prisca Zammaretti², Jeffrey A. Hubbell^{2,*}, and Robert Langer^{1,*}

¹Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA ²Institute of Bioengineering and Institute of Chemical Sciences and Engineering, Ecole Polytechnique Fédérale de Lausanne (EPFL), 1015 Lausanne, Switzerland ³Biocant, Centro de Inovação em Biotecnologia, 3060-197 Cantanhede, Portugal ⁴Center of Neurosciences and Cell Biology, University of Coimbra, 3004-517 Coimbra, Portugal

Abstract

The *in vitro* potential of a synthetic matrix metalloproteinase (MMP)-responsive polyethylene glycol (PEG)-based hydrogel as a bioactive co-encapsulation system for vascular cells and a small bioactive peptide, thymosin β 4 (Tp4), was examined. We show that the physical incorporation of Tp4 in this bioactive matrix creates a three-dimensional (3D) environment conducive for human umbilical vein endothelial cell (HUVEC) adhesion, survival, migration and organization. Gels with entrapped Tp4 increased the survival of HUVEC compared to gels without Tp4, and significantly up-regulated the endothelial genes vascular endothelial-cadherin and angiopoietin-2, whereas von Willebrand factor was significantly down-regulated. Incorporation of Tp4 significantly increased MMP-2 and MMP-9 secretion of encapsulated HUVEC. The gel acts as a controlled Tp4-release system, as MMP-2 and MMP-9 enzymes trigger the release. In addition, Tp4 facilitated HUVEC attachment and induced vascular-like network formation upon the PEG-hydrogels. These MMP-responsive PEG-hydrogels may thus serve as controlled co-encapsulation system of vascular cells and bioactive factors for *in situ* regeneration of ischemic tissues.

Keywords

Vascular tissue engineering; Biomimetic hydrogel; Matrix metalloproteinase (MMP); Human umbilical vein endothelial cells (HUVEC); Thymosin β 4

1. Introduction

A functional vascular system is essential for the formation and maintenance of most tissues in the body, and the lack of vascularization results in ischemic tissues with limited intrinsic

© 2009 Elsevier Ltd. All rights reserved.

*Correspondence to J. A. Hubbell (jeffrey.hubbell@epfl.ch) and R. Langer (rlanger@mit.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.

regeneration capacity. In recent attempts to stimulate the regeneration of functional blood vessels in ischemic tissues, endothelial cells and their precursors have been injected into the ischemic site [1,2,3,4]. Alternatively, angiogenic cytokines such as vascular endothelial growth factor (VEGF) have been administered by direct injection into the ischemic tissues or into the coronary artery [5,6,7]. Although improved regional blood flow was reported in preclinical ischemic heart and limb animal models, and also in human clinical trials, a prolonged effect is hampered by low efficiency of incorporation within the recipient's vasculature (less than 3% of injected cells may engraft, mainly due to cell death) [8,9], and rapid clearance of the cytokines from the ischemic site [10,11]. Subsequently, we and others have engineered biomaterials with the goal of preventing *anoikis* and improving functional engraftment, employing biodegradable materials as cell carriers and as cell ingrowth matrices [12,13,14,15,16], or alternatively as a protective environment for the controlled release of active cytokines [17,18,19,20,21]. Although elevated survival and engraftment have been reported, we sought to explore enhancement of cell survival and engraftment by co-encapsulating vascular cells and cytokines in a bioactive hydrogel environment common to both.

We have recently developed a 3D PEG-based synthetic hydrogel material as an extracellular matrix analog with key biochemical characteristics of natural collagenous matrices; MMP-sensitive peptides are used to crosslink telechelically-reactive branched PEG chains, producing a hydrogel matrix capable of cell-mediated proteolytic degradation and remodeling (Fig. 1A) [22]. These characteristics are also relevant in ischemic environments, where increased MMP-expression and activation has been observed [23,24,25]. Furthermore, the matrix-bound RGDSP adhesion peptide is co-incorporated into the matrix to promote cell adhesion via integrins that are known to be significant in vascular development and maintenance ($\alpha_5\beta_1$, $\alpha_v\beta_3$) [26]. Within these hydrogel matrices, we describe the physical incorporation of T β 4, a 43-amino-acid peptide previously shown to enhance survival of vascular cells and cardiomyocytes in ischemic environments [27,28,29], stimulate neovascularization after cardiac injury by inducing endogenous endothelial cell migration to the ischemic site [30,31], as well as play a key role in down-regulating expression of inflammatory molecules [32]. In this paper, we examined the *in vitro* potential of these synthetic MMP-responsive gels as a bioactive co-encapsulation system of HUVEC and T β 4.

2. Materials and Methods

2.1 Synthesis of PEG-vinylsulfone and peptides (RGDSP, MMP-substrate, T β 4)

PEG-vinylsulfone was synthesized adapting our previous protocol [33]. In brief, branched 8- or 4-arm PEG-OH ($M_w = 40,000$ g/mol for 8-arm PEG; $M_w = 20,000$ g/mol and $M_w = 15,000$ g/mol for 4-arm PEG) (Shearwater Polymers, Huntsville, AL) was dried by azeotropic distillation in toluene (VWR, Nyon, Switzerland) for 4 h. Toluene was distilled off and the residue dissolved in dichloromethane (Fisher Scientific, Wohlen, Switzerland). Sodium hydride (Sigma-Aldrich, Buchs, Switzerland) was added at 20-fold molar excess over OH-groups. Divinylsulfone (Fluka, Buchs, Switzerland) was added at a 50-fold molar excess over OH-groups. The reaction was carried out at room temperature under argon with

constant stirring for 24 h. After the addition of acetic acid (Fluka, Buchs, Switzerland), the mixture was filtered and concentrated by rotary evaporation. The polymer was then isolated by precipitation in ice-cold diethylether (Brunschwig, Basel, Switzerland) and filtered. Finally, the product was dried under vacuum, yielding 85%. The degree of PEG functionalization with vinylsulfone was determined by proton NMR spectroscopy (in CDCl_3) using a Bruker 400 spectrometer (Bruker BioSpin, Faellanden, Switzerland). Characteristic vinylsulfone peaks were observed at 6.1, 6.4, and 6.8 ppm. The degree of end group conversion was found to be $\approx 95\%$.

The integrin ligand peptide (Ac-GCGYGRGDSPG-NH₂), the substrates for MMP (Ac-GCRDGPQGIWGQDRCG-NH₂) and the T β 4 peptide (Ac-SDKPDMAEIEKFDDSKLKKTTETQEKNPLPSKETIEQEKQAGES-NH₂), were synthesized by solid phase peptide synthesis using NovaSyn TGR resin (Merck Biosciences, Laeufelfingen, Switzerland) with an automated peptide synthesizer (Chemspeed, Augst, Switzerland) with standard Fmoc chemistry. Purification was performed by mass-directed reverse phase-C₁₈ HPLC using a Waters Autopurification System. Separation and collection were performed by UV and mass directed software. Peptide sequences were confirmed by ion trap ESI mass spectrometry (all Waters, Baden-Daettwil, Switzerland).

2.2 Formation of PEG-hydrogels

Gel formation was done under physiological conditions as described elsewhere [33]. Briefly, the synthesis was carried out through Michael-type addition reaction of thiol-containing peptides onto vinylsulfone-functionalized PEG. PEG-vinylsulfone was dissolved in 0.3 M triethanolamine buffer pH 8.0 (Sigma Aldrich, St. Louis, MO, USA) to give a 10% (w/v) solution. A solution of the integrin ligand peptide (100 μM) in the same buffer was added to the PEG-vinylsulfone solution. After 10 min, the cell suspension, MMP-sensitive peptide in triethanolamine buffer was added (stoichiometric ratio between the crosslinking MMP-peptide and the PEG-arms: 1.2). The cross-linking reaction was continued for around 30 min, at 37° C. To enable cell survival analysis through flow cytometry, the cells were extracted from the matrix after controlled degradation with collagenase type IV. Gel degradation was carried out at standard incubator conditions (37° C, 5% CO₂) for 30 min with 2 ml collagenase type IV of 1.0 mg/mL in PBS (both from Invitrogen, Carlsbad, CA, USA).

2.3 Release of T β 4 from PEG- and Collagen-gels

T β 4 (40,000 ng/mL gel) was physically entrapped into 25 μL PEG- or collagen gels (Vitrogen 100, Cohesion, Palo Alto, CA, USA, at 2 mg/mL) by mixing it into the PEG-precursor solution before gelation, or collagen solution before initiating solidification by the addition of 0.1 M NaOH. T β 4 release from both materials was analyzed with a competitive ELISA kit (Bachem, Peninsula Laboratories, San Carlos, CA, USA). Samples were placed in 1 mL of PBS (pH 7.4) at standard incubator conditions (37° C, 5% CO₂) under orbital shaking. The buffer was collected every 12 h and refrigerated until further analysis. MMP-2 and MMP-9 at 100 ng/mL and 1,000 ng/mL were added at 72 h (both from R&D Systems, Minneapolis, MN, USA).

2.4 Cell culture in PEG-hydrogels

HUVEC were grown in monolayer cultures in EGM-2 medium (both cells and medium from Lonza, Walkersville, MD, USA). For seeding into PEG-hydrogels, single cells (500,000 cells per gel) were mixed into the gel precursor solution. After the crosslinking reaction at 37°C, the cell-seeded gels were cultured in low attachment 24-well plates with 1.0 mL EGM-2 medium for 7 d under serum starved conditions at a concentration of 0.2% (10 times lower than the concentration typically recommended by Lonza for endothelial cell culture). The medium was exchanged every third day.

2.5 Cell attachment on top of PEG-hydrogels

A total of 30,000 cells were seeded on top of swollen 3D PEG-hydrogels in low attachment 24 well plates. The cells were allowed to attach for 4 h, after which the samples were washed three times with 1 mL PBS. Phase contrast images (Axiovert 200, Carl Zeiss Microimaging, Thornwood, NY, USA) were taken at 10 randomly selected locations (area of $1 \times 2 \text{ mm}^2$) on the matrix. The number of attached cells was determined by manually counting between 20 and 120 cells per location.

2.6 FACS analysis

For quantitative cell survival analysis by flow cytometry, gels were digested as described above for cell extraction at 7 d after seeding, and single cells were extracted. The cells were washed with ice-cold PBS prior to incubation with propidium iodide (Sigma Aldrich, St. Louis, MO, USA) for 3 min. The samples were analyzed by flow cytometry using a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA). Between 80,000 and 100,000 cell events were counted per sample. Processing of the data was performed using FlowJo software (TreeStar, Ashland, OR, USA).

2.7 Quantitative real time-polymerase chain reaction (qRT-PCR) analysis

For isolating RNA from the cells at 7 d after seeding, the gels containing Tp4 or without Tp4 (control) were homogenized and total RNA was isolated with Trizol Reagent (Invitrogen, Carlsbad, CA, USA) following the supplier's protocol. Total RNA was quantified by a Nanodrop Spectrometer (Nanodrop 1000, Thermo Scientific, Wilmington, DE, USA) and 1 µg was used for the Reverse Transcription. Reverse Transcription was performed on 1 µg total RNA by using TaqMan Reverse Transcription Reagents (Applied Biosystems/Roche Molecular Systems, Branchburg, NJ, USA) following the supplier's protocol. qRT-PCR was performed using Power SYBR Green PCR Master Mix and the detection using a StepOnePlus Real-Time PCR System (both Applied Biosystems, Foster City, CA, USA). Primer sequences and reaction conditions are given in Supplementary Information, Table 2. Quantification of target genes was performed relatively to the reference GADPH gene: $\text{relative expression} = 2^{[-(\text{CtSample} - \text{CtGAPDH})]}$. The mean minimal cycle threshold values (Ct) were calculated from quintuplicate reactions.

2.8 Statistical analysis

The results of the cell experiments are shown as mean values ($\pm$ SD) of samples performed typically in sextuplicates in two independent experiments. Tp4 release study was performed

in sextuplicates in two independent experiments as well, and in triplicates after addition of MMP-2 and MMP-9. qPCR was performed in quintuplicates. Characterization of T β 4 by HPLC was performed in a single experiment. Comparative analysis was performed with two-tailed student T-test using S-PLUS software. Differences between two data sets were considered statistically different when $P < 0.05$.

3. Results

3.1 Release of physically entrapped T β 4

To examine the ability of synthetic PEG-gels to serve as carriers of physically entrapped Tp4 (Fig. 1A), we systematically altered the network crosslink density. We showed that PEG-gels formed from reactive 8-arm PEG (stoichiometric ratio between crosslinker peptide and PEG-molecules: 1.2) were able to retain T β 4 over 72 h (Fig. 1B). Decreasing the number of arms from 8 to 4 or lowering the molecular weight from 20 kDa to 15 kDa at constant stoichiometric ratio resulted in an initial burst of around 70% of the incorporated Tp4 within the first 12 h, followed by a slower phase of release without significant difference between 12 h and 72 h. As a comparator to the synthetic PEG-gels, which mimic key characteristics of natural collagenous matrices [22], we used collagen type I gels; these collagen gels released the incorporated Tp4 in an initial burst of around 80%, followed by a significantly faster release (from 24 h on) up to 72 h as compared to the 4-arm PEG-gels.

3.2 MMP-mediated release of T β 4

To characterize the potential for release mediated by cellular-activated MMPs, we added MMP-2 and MMP-9, both at the concentrations of 100 ng/mL and 1,000 ng/mL. We found that gels degrade and release T β 4 upon addition of exogenous MMP-2 and MMP-9 (Fig. 1C-F). The release was not significantly different between the two concentrations (100 ng/mL and 1,000 ng/mL) for both, MMP-2 and MMP-9, with one exception for MMP-9 at 120 h. However, we show a significantly faster release for MMP-2 at both concentrations (100 ng/mL and 1,000 ng/mL) as compared to MMP-9-mediated release within the first 24 h after addition (Fig. 1C,D). Significant gel weight loss was observed for high concentrations of MMPs, specifically at 1,000 ng/mL (Fig. 1E,F). T β 4 was demonstrated itself to be cleaved by MMP-2 and MMP-9, but only at concentrations higher than 1,000 ng/mL (Supplementary Information, Table 1)

3.3 T β 4 effects on survival of HUVEC

To assess the effect of T β 4 on the survival of HUVEC in tissue culture plates, the cells were grown in EGM-2 medium containing low serum concentration (0.2%, 10 times lower than the concentration typically recommended by Lonza for endothelial cell culture) and variable concentration of T β 4. We found a significant increase in survival of HUVEC over 7 d after addition of 1,000 ng/mL T β 4, as compared to control samples without T β 4 (Fig. 2A). Addition of T β 4 at 10 ng/mL and 100 ng/mL resulted in significantly higher cell survival as compared to the control after 48 h.

To examine the potential pro-survival effect of Tp4 on HUVEC encapsulated in 3D PEG-gels, as it was reported with cardiomyocytes grown on laminin-coated slides [27], we

physically entrapped T β 4 and cultured the gel-encapsulated cells under serum-starved conditions. Survival of HUVEC was significantly increased at 7 d for all T β 4 concentrations, at 400 ng/mL, 4,000 ng/mL and 40,000 ng/mL gels (all 25 μ L gels), as compared to PEG-gels without entrapped T β 4 (Fig. 2B). The highest survival was at 40,000 ng/mL, with a 25% higher survival rate as compared to the control samples without entrapped T β 4.

3.4 T β 4 effects on release of MMP-2/9 from HUVEC

MMPs are crucial in angiogenesis, as they degrade the basement membrane, which allows sprouting from existing blood vessels [23]. Here, we show that the secreted MMP-2 and MMP-9 levels were significantly elevated at 7 d when co-encapsulating T β 4 at the highest concentration of 40,000 ng/mL gel (25 μ L gels) with the HUVEC (Fig. 2C,D). Furthermore, the production of MMP-2 (Fig. 2C) was around 2,000 times higher than that of MMP-9 (Fig. 2D).

3.5 T β 4 effects on cell attachment

T β 4 facilitated cell attachment upon the PEG-hydrogels (Fig. 3A). The incorporation of 40,000 ng/mL gel (25 μ L gels) T β 4 increased the number of attached HUVEC 2-fold at 4 h after seeding. In addition, we show that T β 4 facilitates vascular-like network formation within the first 12 h after seeding (Fig. 3B). The cells appeared more elongated and showed capillary-like structures. At 24 h, we did not detect a difference between the T β 4-treated and the non-treated samples.

3.6 T β 4 effects on vascular gene expression by HUVEC

To examine the effect of the 3D bioactive gel and the entrapped T β 4 on the co-encapsulated HUVEC, we analyzed vascular gene expression under serum-starved conditions, 7 d after seeding (Fig. 4). While the expression of some genes (PECAM1, CD34, Tie2, Flk-1) was not significantly different in cells cultured in gels containing T β 4 (40,000 ng/mL gel, 25 μ L gels) as compared to gels without T β 4, cells cultured in 3D gels with T β 4 showed an up-regulation of vascular endothelial (VE)-cadherin (7-fold), which is involved in normal vascular development and stabilization, and endothelial survival [34], as well as Angiopoietin-2 (Ang-2) (2-fold), relevant for angiogenic outgrowth, vessel remodeling and maturation [35]. Furthermore, we found significant down-regulation of von Willebrand factor (vWF) (4-fold), which is recognized to be predictive at elevated levels for adverse cardiovascular events associated with endothelial dysfunction [36].

4. Discussion

Here, we explored the *in vitro* potential of synthetic, MMP-responsive hydrogels displaying vasculo-typic adhesion morphogens, for efficient encapsulation of vascular cells while acting as a controlled drug release system of T β 4 (Fig. 1A). Our data indicates that the physical incorporation T β 4 in the PEG-based hydrogel can create a supportive 3D environment for HUVEC adhesion, survival, migration and vascular-like network organization.

We demonstrate that our synthetic hydrogel scaffold material, mimicking key biochemical degradative characteristics of collagen matrices, is able to retain the physically entrapped T β 4 over time (Fig. 1B), and to release it “on-demand”, as MMP-2 and MMP-9 enzymes trigger gel degradation and release (Fig. 1C-F). The mechanism of retaining T β 4 in the small-meshed PEG-matrix (mesh size: 5-10 nm, mesh size collagen at least 2 orders of magnitude higher [37]) may mainly be physical hindrance. This is supported by the fact that only smallest-meshed 8-arm gels were able to retain a major fraction of T β 4 over time, whereas the 4-arm gels released the incorporated T β 4 mainly after 12 h similar to the collagen matrices (Fig. 1B).

Small cleavage products from the T β 4-peptide can provide pro-angiogenic stimuli *in vitro* and *in vivo* [30]. We thus analyzed the cleavage of T β 4 at different concentrations of MMP-2 and MMP-9. We demonstrated that cleavage occurs mainly at concentrations higher than 1,000 ng/mL (Supplementary Information, Table 1). Since HUVEC in this study (500,000 cells/gel) release less than 100 ng/mL MMP-2 within 7 d, and MMP-9 may even be less (Fig 2C,D), we suggest little to no cleavage products from T β 4 mediated by cell-associated MMP-2 and MMP-9 proteases. However, we cannot eliminate that HUVEC release other proteases that might degrade T β 4. It is likely in the situation of use that most of the T β 4 is released intact.

Our synthetic hydrogels with physically entrapped T β 4 increased the survival of HUVEC significantly (Fig. 2A,B). Even small concentrations induced significantly elevated cell survival in the two-dimensional environment (10 ng/mL) as well as in the 3D bioactive hydrogels (400 ng/mL gel, 25 μ L gels). Similar pro-survival effects of T β 4 were recently described *in vitro* by using corneal epithelial cells [38] or C2C12 myoblasts, and *in vivo* after coronary artery ligation in mice [27]. T β 4 was found to form a functional complex with the particularly interesting new cysteine-histidine-rich protein (PINCH) and integrin-linked kinase (ILK), resulting in early activation of the survival kinase Akt [27].

An additional pathway may be involved in the increased survival. We demonstrate significantly up-regulated VE-cadherin gene expression (7-fold) of HUVEC in the 3D bioactive hydrogels induced by the T β 4 co-encapsulated in the gel (Fig. 4). Others have shown that the lack of VE-cadherin in mice induced endothelial apoptosis due to impaired transmission of pro-survival signaling through VEGF-A to Akt-kinase [34]. We thus speculate that two pathways could have contributed to the observed increased cell survival, one through the up-regulated ILK, and another through elevated VE-cadherin expression.

We found that Ang-2 was also significantly up-regulated (2-fold) as a consequence of the co-entrapped T β 4 (Fig. 4). In adult mice and humans, Ang-2 expression has been reported to be restricted to sites of vascular remodeling [35,39]. This suggests that T β 4 may play a role in stimulating neovascularization of ischemic tissues through the activation of Ang-2 of co-delivered vascular cells in the 3D bioactive hydrogel, as well as potentially exert an effect on the host vascular cells. This potential correlation between T β 4 and VE-cadherin, and Ang-2 has not been previously shown, to our knowledge.

The biomaterial scaffold that we are employing responds by local degradation to cell-derived MMP-2 and MMP-9, as well as potentially other MMPs. We demonstrate that co-entrapment of T β 4 induces the release of MMP-2 and MMP-9 from HUVEC encapsulated in our hydrogels (Fig. 2C,D), which could accelerate remodeling. This observed effect of T β 4 incorporation on MMP activation is in line with studies conducted with HUVEC in a two-dimensional environment and *in vivo* experiments on wound healing [40,41]. In these studies, the central actin-binding domains were identified to be responsible for the increased protease levels. MMPs secreted by HUVEC enhance matrix remodeling and thus enable cell migration of the incorporated cells, and may also facilitate cell ingrowth of the recipient's cells *in vivo* [22]. The incorporated T β 4 may possibly induce a bi-directional communication between the incorporated cells and the matrix as the increased MMP-expression is “answered” by the matrix through controlled release of the incorporated T β 4 [22].

Whether vascular cells are co-delivered in a hydrogel scaffold or invade from the host tissues, rapid stimulation of vascular organization is critical for survival of cells within the implant. To explore this, we investigated the vascular organizing and angiogenic potential of the bioactive gels as a controlled drug release system of entrapped T β 4. We show that gels containing T β 4 increase the attachment of HUVEC 2-fold within 4 h after seeding (Fig. 3A), and are able to induce more rapid vascular-like network formation on top of the scaffold (Fig. 3B). A similar accelerated vascular-like organization has been previously observed in T β 4-transfected HUVEC seeded upon Matrigel matrices [42]. It is possible that the released T β 4 from the 3D MMP-sensitive PEG-gels may act as a chemoattractant to the surrounding vascular cells, and the bioactive gel itself may provide both biophysical and biochemical stimuli to facilitate attachment and organization.

Conclusions

Here, we explored the *in vitro* potential of synthetic, cell-responsive hydrogels displaying vascular adhesion epitopes for encapsulation of vascular cells while acting at the same time as a controlled drug release system. Our data indicates that the physical incorporation of the small bioactive peptide, T β 4, in the PEG-based hydrogel can create a supportive 3D environment for HUVEC adhesion, survival, migration and vascular-like network organization. These MMP-responsive PEG-hydrogels may thus potentially serve as a controlled model system to better understand biophysical and biochemical factors governing vascular development, or as a controlled co-encapsulation system of vascular cells and cytokines for *in situ* regeneration of ischemic tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the Biopolymer facility at the Massachusetts Institute of Technology for help with peptide synthesis and characterization with HPLC. This work was supported in part by NIH (grant HL060435) and the European Union's 6th Framework Program Expertises. TPK was supported by the Swiss National Science Foundation (grant number 120938), and a Rotary Ambassadorial Scholarship. LSF was supported by a Marie Curie-Reintegration Grant and the MIT-Portugal program.

References

1. Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat Med.* 1999; 5:434–8. [PubMed: 10202935]
2. Urbich C, Heeschen C, Aicher A, Dernbach E, Zeiher AM, Dimmeler S. Relevance of monocytic features for neovascularization capacity of circulating endothelial progenitor cells. *Circulation.* 2003; 108:2511–6. [PubMed: 14581410]
3. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell.* 2003; 114:763–76. [PubMed: 14505575]
4. Miranville A, Heeschen C, Sengenès C, Curat CA, Busse R, Bouloumié A. Improvement of postnatal neovascularization by human adipose tissue-derived stem cells. *Circulation.* 2004; 110:349–55. [PubMed: 15238461]
5. Ferrara N, Alitalo K. Clinical applications of angiogenic growth factors and their inhibitors. *Nat Med.* 1999; 5:1359–64. [PubMed: 10581076]
6. Schumacher B, Pecher P, von Specht BU, Stegmann T. Induction of neoangiogenesis in ischemic myocardium by human growth factors: first clinical results of a new treatment of coronary heart disease. *Circulation.* 1998; 97:645–50. [PubMed: 9495299]
7. Baumgartner I, Pieczek A, Manor O, Blair R, Kearney M, Walsh K, et al. Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. *Circulation.* 1998; 97:1114–23. [PubMed: 9537336]
8. Jackson KA, Majka SM, Wang H, Pocius J, Hartley CJ, Majesky MW, et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J Clin Invest.* 2001; 107:1395–402. [PubMed: 11390421]
9. Mooney DJ, Vandenburgh H. Cell delivery mechanisms for tissue repair. *Cell Stem Cell.* 2008; 2:205–13. [PubMed: 18371446]
10. Lazarous DF, Shou M, Stiber JA, Dadhania DM, Thirumurti V, Hodge E, et al. Pharmacodynamics of basic fibroblast growth factor: route of administration determines myocardial and systemic distribution. *Cardiovasc Res.* 1997; 36:78–85. [PubMed: 9415275]
11. Kim TK, Burgess DJ. Pharmacokinetic characterization of 14C-vascular endothelial growth factor controlled release microspheres using a rat model. *J Pharm Pharmacol.* 2002; 54:897–905. [PubMed: 12162707]
12. Kipshidze N, Chekanov V, Chawla P, Shankar LR, Gosset JB, Kumar K, et al. Angiogenesis in a patient with ischemic limb induced by intramuscular injection of vascular endothelial growth factor and fibrin platform. *Tex Heart Inst J.* 2000; 27:196–200. [PubMed: 10928508]
13. Niklason LE, Gao J, Abbott WM, Hirschi KK, Houser S, Marini R, et al. Functional arteries grown in vitro. *Science.* 1999; 284:489–93. [PubMed: 10205057]
14. Zisch AH, Lutolf MP, Ehrbar M, Raeber GP, Rizzi SC, Davies N, et al. Cell-demanded release of VEGF from synthetic, biointeractive cell ingrowth matrices for vascularized tissue growth. *FASEB J.* 2003; 17:2260–2. [PubMed: 14563693]
15. Ehrbar M, Metters A, Zammaretti P, Hubbell JA, Zisch AH. Endothelial cell proliferation and progenitor maturation by fibrin-bound VEGF variants with differential susceptibilities to local cellular activity. *J Control Release.* 2005; 101:93–109. [PubMed: 15588897]
16. Jain RK, Au P, Tam J, Duda DG, Fukumura D. Engineering vascularized tissue. *Nat Biotechnol.* 2005; 23:821–3. [PubMed: 16003365]
17. Langer R. New methods of drug delivery. *Science.* 1990; 249:1527–33. [PubMed: 2218494]
18. Ferreira LS, Gerecht S, Fuller J, Shieh HF, Vunjak-Novakovic G, Langer R. Bioactive hydrogel scaffolds for controllable vascular differentiation of human embryonic stem cells. *Biomaterials.* 2007; 28:2706–17. [PubMed: 17346788]
19. Lee KY, Peters MC, Anderson KW, Mooney DJ. Controlled growth factor release from synthetic extracellular matrices. *Nature.* 2000; 408:998–1000. [PubMed: 11140690]
20. Richardson TP, Peters MC, Ennett AB, Mooney DJ. Polymeric system for dual growth factor delivery. *Nat Biotechnol.* 2001; 19:1029–34. [PubMed: 11689847]

21. Arras M, Mollnau H, Strasser R, Wenz R, Ito WD, Schaper J, et al. The delivery of angiogenic factors to the heart by microsphere therapy. *Nat Biotechnol.* 1998; 16:159–62. [PubMed: 9487522]
22. Lutolf MP, Weber FE, Schmoekel HG, Schense JC, Kohler T, Müller R, et al. Repair of bone defects using synthetic mimetics of collagenous extracellular matrices. *Nat Biotechnol.* 2003; 21:513–8. [PubMed: 12704396]
23. Hiraoka N, Allen E, Apel IJ, Gyetko MR, Weiss SJ. Matrix metalloproteinases regulate neovascularization by acting as pericellular fibrinolysins. *Cell.* 1998; 95:365–77. [PubMed: 9814707]
24. Heymans S, Luttun A, Nuyens D, Theilmeier G, Creemers E, Moons L, et al. Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure. *Nat Med.* 1999; 5:1135–42. [PubMed: 10502816]
25. Webb CS, Bonnema DD, Ahmed SH, Leonardi AH, McClure CD, Clark LL, et al. Specific temporal profile of matrix metalloproteinase release occurs in patients after myocardial infarction: relation to left ventricular remodeling. *Circulation.* 2006; 114:1020–7. [PubMed: 16923753]
26. Francis SE, Goh KL, Hodivala-Dilke K, Bader BL, Stark M, Davidson D, et al. Central roles of alpha5beta1 integrin and fibronectin in vascular development in mouse embryos and embryoid bodies. *Arterioscler Thromb Vasc Biol.* 2002; 22:927–33. [PubMed: 12067900]
27. Bock-Marquette I, Saxena A, White MD, Dimaio JM, Srivastava D. Thymosin beta4 activates integrin-linked kinase and promotes cardiac cell migration, survival and cardiac repair. *Nature.* 2004; 432:466–72. [PubMed: 15565145]
28. Smart N, Rossdeutsch A, Riley PR. Thymosin beta4 and angiogenesis: modes of action and therapeutic potential. *Angiogenesis.* 2007; 10:229–41. [PubMed: 17632766]
29. Marx J. Biomedicine. Thymosins: clinical promise after a decades-long search. *Science.* 2007; 316:682–3. [PubMed: 17478695]
30. Smart N, Risebro CA, Melville AA, Moses K, Schwartz RJ, Chien KR, et al. Thymosin beta4 induces adult epicardial progenitor mobilization and neovascularization. *Nature.* 2007; 445:177–82. [PubMed: 17108969]
31. Hinkel R, El-Aouni C, Olson T, Horstkotte J, Mayer S, Müller S, et al. Thymosin beta4 is an essential paracrine factor of embryonic endothelial progenitor cell-mediated cardioprotection. *Circulation.* 2008; 117:2232–40. [PubMed: 18427126]
32. Sosne G, Chan CC, Thai K, Kennedy M, Szliter EA, Hazlett LD, et al. Thymosin beta 4 promotes corneal wound healing and modulates inflammatory mediators in vivo. *Exp Eye Res.* 2001; 72:605–8. [PubMed: 11311052]
33. Kraehenbuehl TP, Zammaretti P, Van der Vlies AJ, Schoenmakers RG, Lutolf MP, Jaconi ME, et al. Three-dimensional extracellular matrix-directed cardioprogenitor differentiation: systematic modulation of a synthetic cell-responsive PEG-hydrogel. *Biomaterials.* 2008; 29:2757–66. [PubMed: 18396331]
34. Carmeliet P, Lampugnani MG, Moons L, Breviario F, Compernelle V, Bono F, et al. Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell.* 1999; 98:147–57. [PubMed: 10428027]
35. Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, et al. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science.* 1997; 277:55–60. [PubMed: 9204896]
36. Spiel AO, Gilbert JC, Jilma B. von Willebrand factor in cardiovascular disease: focus on acute coronary syndromes. *Circulation.* 2008; 117:1449–59. [PubMed: 18347221]
37. Raeber GP, Lutolf MP, Hubbell JA. Molecularly engineered PEG hydrogels: a novel model system for proteolytically mediated cell migration. *Biophys J.* 2005; 89:1374–88. [PubMed: 15923238]
38. Sosne G, Siddiqi A, Kurpakus-Wheater M. Thymosin-beta4 inhibits corneal epithelial cell apoptosis after ethanol exposure in vitro. *Invest Ophthalmol Vis Sci.* 2004; 45:1095–100. [PubMed: 15037574]
39. Ramsauer M, D'Amore PA. Getting Tie(2)d up in angiogenesis. *J Clin Invest.* 2002; 110:1615–7. [PubMed: 12464666]

40. Philp D, Scheremeta B, Sibbliss K, Zhou M, Fine EL, Nguyen M, et al. Thymosin beta4 promotes matrix metalloproteinase expression during wound repair. *J Cell Physiol.* 2006; 208:195–200. [PubMed: 16607611]
41. Malinda KM, Goldstein AL, Kleinman HK. Thymosin beta 4 stimulates directional migration of human umbilical vein endothelial cells. *FASEB J.* 1997; 11:474–81. [PubMed: 9194528]
42. Grant DS, Kinsella JL, Kibbey MC, LaFlamme S, Burbelo PD, Goldstein AL, et al. Matrigel induces thymosin beta 4 gene in differentiating endothelial cells. *J Cell Sci.* 1995; 108:3685–94. [PubMed: 8719875]

Figure 1.

(A) Scheme of co-encapsulation of HUVECs with T $\beta 4$ in 3D MMP-responsive PEG-hydrogels. Reactive branched PEGs are crosslinked with bifunctional peptides, which are designed to be MMP substrates. The crosslinked gels that result are also functionalized with integrin-binding peptides. (B) Passive release (i.e., in the absence of MMPs) of T $\beta 4$ from PEG-hydrogels and collagen type I gels. 8-arm PEG-gels ($M_w = 40$ kDa) were able to retain the physically incorporated T $\beta 4$ peptide over time, whereas collagen type I gels released around 80% in an initial burst within the first 12 h. Crosslinking of branched PEGs with fewer arms was also less effective for entrapment. (C,D) Both MMP-2 (C) and MMP-9 (D) addition resulted in gel degradation. (E,F) MMP exposure resulted in T $\beta 4$ release, for both MMP-2 (E) and MMP-9 (F).

Figure 2.

(A,B) Survival of HUVECs under serum-starved conditions. (A) Survival in tissue culture plates up to 7 d significantly increased at all time points for the T β 4 concentration of 1000 ng/mL. (B) Significantly higher cell survival was observed at 7 d when co-encapsulated in the 3D PEG-hydrogels (8-arm, $M_w = 40$ kDa) with T β 4 at concentration of 400 ng/mL, 4,000 ng/mL and 40,000 ng/mL gel (all 25 μ L gels). (C,D) Release of MMP-2 and MMP-9 from HUVECs co-encapsulated in 3D PEG-hydrogel with T β 4 at 7 d. (C) Significantly elevated MMP-2 production was observed at 7 d with T β 4 incorporation at 400 ng/mL, 4,000 ng/mL and 40,000 ng/mL gel. (D) MMP-9 levels were around 2,000 times smaller than observed for MMP-2 at 7 d. Only the highest concentration of T β 4 of 40,000 ng/mL gel increased the release of MMP-9 significantly. * denotes significance as compared to the control sample, with $P < 0.05$.

Figure 3.

Cell attachment and vascular-like network formation upon PEG-hydrogels under serum-starved conditions. (A) Incorporation of T β 4 (40,000 ng/mL gel, 25 μ L gels) increased the number of attached HUVECs 2-fold (upper image in inset) as compared to gels without T β 4 (lower image) 4 h after seeding (scale bar: 50 μ m). (B) T β 4 facilitates vascular-like network formation of HUVECs upon PEG-hydrogels within the first 12 h after seeding. The cells appeared more elongated and formed capillary-like structures with incorporation of Tp4. No differences in vascular-like network formation were observed after 24 h. Scale bar: 50 μ m. * denotes significance as compared to the control sample, with $P < 0.05$.

Figure 4.

Endothelial gene expression as assessed by qRT-PCR at 7 d after co-encapsulating HUVECs and Tβ4 (40,000 ng/mL gel, 25 μL gels) in the MMP-sensitive PEG-hydrogels. Tβ4 up-regulated the expression of VE-cadherin (7-fold) and Ang-2 (2-fold) in HUVECs as compared to the control gels without Tβ4, whereas it significantly down-regulated the expression of vWF (4-fold). * denotes significance as compared to the control sample, with $P < 0.05$.