PERFUSABLE BRANCHING MICROVESSEL BED FOR VASCULARIZATION OF ENGINEERED TISSUES

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INTRODUCTION

Tissue engineering may offer alternative treatment options for tissue and organ replacement but challenges related to vascularization *in vitro* and *in vivo* remain. Functional vasculature is required to grow tissues beyond 0.02cm thick, due to the diffusional limitations in oxygen supply.[1] Generation of complex multi-cell type tissues starting from autologous progenitors is limited by the application of soluble growth factors. Here, we devised a method to engineer a prototype vascular network consisting of two branching vessels and a microvascular bed suitable for rapid vascularization of engineered tissues *in vitro*. The novelty of our approach lies in the integrated use of topographical cues and sustained release of growth factors to guide the outgrowths from a human artery and a vein resulting in a connected microvascular bed.

We demonstrate the utility of this vascular network in engineering a vascularized myocardium. Functional properties of myocardium are afforded by cardiomyocytes, a cell type sensitive to oxygen limitations due to their high metabolic activity. Direct perfusion of the engineered cardiac tissues has been shown to improve cell viability.[1, 2] However, it also causes non-physiologic shear stress on cardiomyocytes, thus affecting their phenotype.[3] Presence of microvascular bed in engineered cardiac tissues *in vitro* may provide a biomimetic milieu for functional cell assembly.

Recent breakthrough studies enable fabrication of microvascular structures with barrier function[4]; however, in cases where geometry of tubules was controlled, there was no facile way of seeding cells in the parenchymal space as it was occupied by a synthetic or natural polymer.[4, 5] Additionally, these approaches do not enable facile removal of the engineered microvascular bed from the microfabricated mold or microfluidic device used to house the vasculature. Importantly, we used a biomimetic approach here where cells undergo proliferation and migration in response to topography and angiogenic factors, rather than forcing endothelial cells to attach to predetermined regions in the polymer or hydrogel substrate.

We report here findings recently recently published in PNAS.[6] We previously developed a collagen-chitosan hydrogel that was capable of releasing thymosin β4 (Tβ4) over 28 days. [7] Tβ4 is an angiogenic and cardioprotective peptide that enhances cardiomyocyte survival by induction of coronary vascularization and upregulation of Akt activity.[8, 9] Here we applied the developed hydrogel onto micropatterned PDMS substrates with 25μm, 50μm or 100μm wide grooves (Figure 1A) (Figure 1 courtesy of Chiu et al.[6]) Subsequently, mouse, rat or human arterial and venous explants were placed at the two ends of the substrate to promote outgrowth of capillaries and form an organized vascular structure that is anchored by two parent explants.

METHODS AND RESULTS

Tβ4 also stimulated synthesis and autocrine secretion of VEGF previously.[10] Here, we observed enhanced VEGF secretion from explants cultivated on Tβ4-hydrogels compared to Tβ4-free gels. The branch density and branch length of the capillary outgrowths from mouse arterial and venous explants were both increased with the addition of encapsulated Tβ4 in the hydrogel coating. More importantly, the outgrowths were oriented in the direction of the microgrooves, and formed a connection between the two parent explants by Day 21

(Figure 1B). This process could be accelerated to 14 days by addition of soluble hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF). The engineered capillary network structure could be removed from the PDMS substrate while maintaining structural integrity (Figure 1C, D). Moreover, the capillary outgrowths had characteristics of developed vascular structures with open lumens (Figure 1E-J) and expressed endothelial cell markers, CD31 and von Willebrand factor. The network was perfusable as demonstrated using fluorescently labelled dextran. When cardiomyocytes were seeded onto the vascular structures, they formed synchronously beating tissues with improved excitation threshold compared to cardiomyocytes seeded onto plain hydrogel coated substrates (Figure 1K). The maximum capture rate remained the same (Figure 1L). The cardiomyocytes also exhibited improved cell morphology and cell-cell junctions as evaluated by the Troponin T (Figure 1M, N) and Connexin-43 immunostaining respectively (Figure 1O). Importantly, this approach is not species specific. It can be used to engineer a rat (Figure 1P) and human (Figure 1Q) microvascular bed *in vitro*. Capillary outgrowths, obtained from human umbilical arteries and veins, aligned along the topographical cues provided by the micropatterned surfaces. The human capillaries were composed of CD31-positive endothelial cells. By Day 21, the outgrowths connected between the parent artery and vein and exhibited open lumens (Figure 1Q).

DISCUSSION

Introduction of topographical cues was essential for formation of branches with open lumens, as Tβ4-gel alone on smooth PDMS substrates led to cell outgrowth, but no formation of luminal structures. While controls with uncoated grooved substrates led to tube formation by migrated cells, these vascular structures were not well organized. This points to the importance of localization of the Tβ4 molecules to the groove/ridge space, since Tβ4 was present in the culture medium and not localized to the grooves in this control case. Topographical cues themselves, could act in enabling tube formation by one of the following factors: contact guidance, change in the local mechanical properties of the environment or by locally increasing the concentration of autocrine growth factors. Atomic force microscopy nano-indentation measurements indicated that all gel coated PDMS substrates exhibited a similar Young's modulus (~1kPa), thus differences in local mechanical properties were not affecting the tube formation. A simplified mathematical model was derived to show that the presence of grooves and the groove width affected the local concentration of $VEGF_{165}$, assumed to be secreted as a point source from one single cell located at the bottom of the groove. The model values are not absolute as several simplifying assumptions were used. However, the relative local VEGF concentrations, as normalized to the value for flat substrate at point (0, 0), show that groove dimensions can locally increase the concentration of the autocrine growth factors (by 26, 13 and 7 times for the 25, 50 and 100 μm grooves respectively) when compared to the flat control. This process can likely result in the optimal groove width for the tube formation: although the narrower grooves will concentrate the autocrine growth factors more, grooves that are too narrow will impose physical constraints on the migrating cells, thus resulting in the optimal groove width in the intermediate range (e.g. 50μm) as observed here.

CONCLUSIONS

We have shown that controlled release of Tβ4 in conjunction with the application of topographical cues guided endothelial outgrowths from an artery and a vein, and aided the organization of capillaries into functional microvasulature. The resulting capillary structure was used as a vascular bed for growing vascularized engineered cardiac tissues. This approach enables the engineering of a prototype vascular network consisting of two branching vessels suitable for cultivation of different parenchymal cell types. This is the only approach that allows facile removal of the vasculature from the microfabricated substrate and easy seeding of the tissue specific cell types in the parenchymal space.

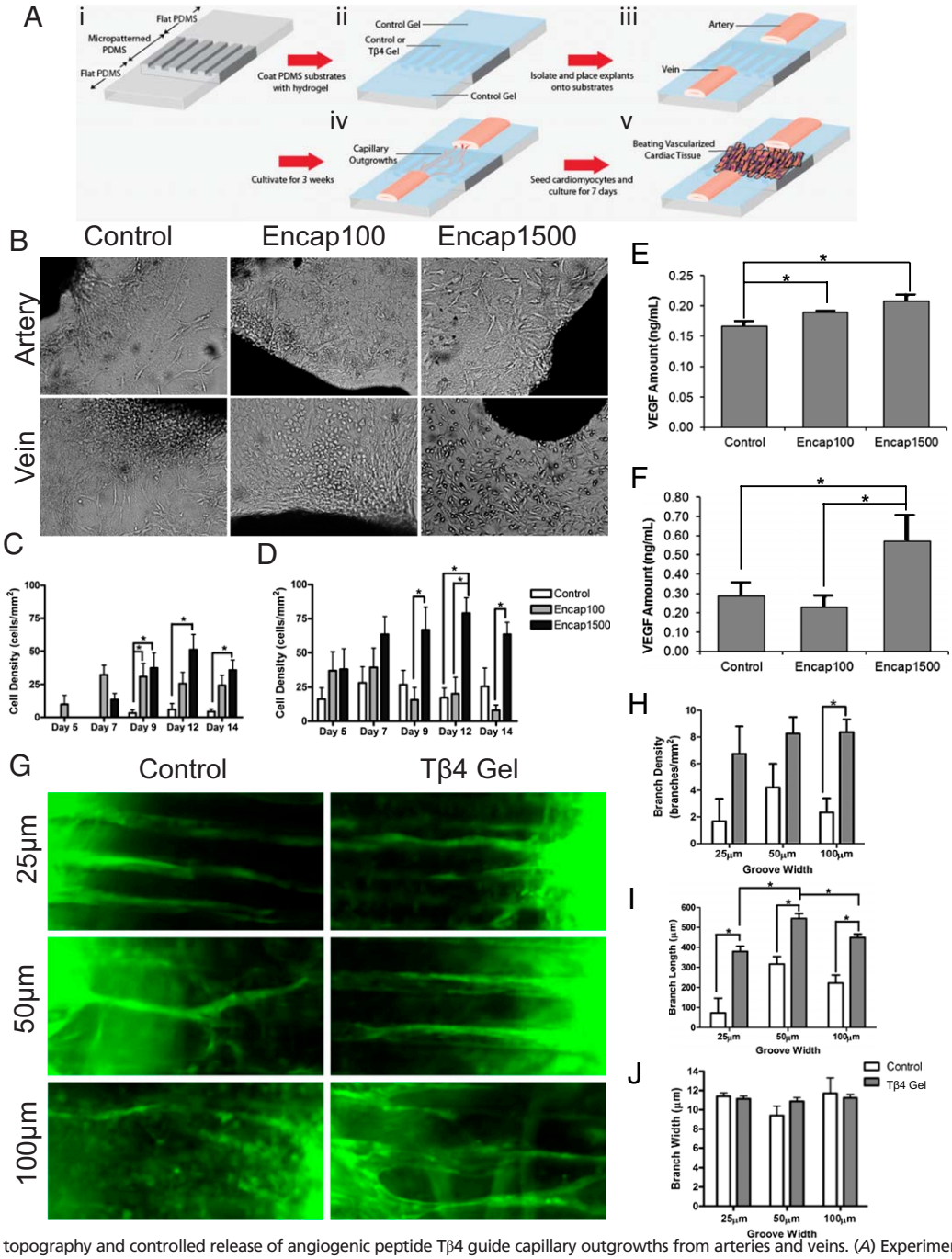

Fig. 1. Substrate topography and controlled release of angiogenic peptide Tβ4 guide capillary outgrowths from arteries and veins. (*A*) Experimental setup. (*i*) PDMS substrates were fabricated using standard soft lithography methods and (*ii*) were coated with collagen-chitosan hydrogel with or without Tβ4. (*iii*) Arteries and veins were isolated and placed on the two ends of the substrate and (*iv*) were cultivated for 2 wk with HGF or VEGF supplementation or for 3 wk without any growth factors. (*v*) Cardiomyocytes were seeded onto the engineered vascular bed and cultured for additional 7 d to grow a beating, vascularized cardiac tissue. (*B*) Representative images of cell outgrowths from mouse artery and vein explants on nonpatterned substrates at day 14 of cultivation ($n = 3$ per group). The asterisks indicate the location of explants; arrows indicate locations of outgrowths. (Scale bars, 200 μm.) (*C*) Density of migrated cells from artery explants at various time points. (*D*) Density of migrated cells from vein explants at various time points. (*E* and *F*) Autocrine VEGF secretion was dependent on the Tβ4 dose and correlated with cell outgrowth density. (*E*) Total amount of VEGF measured with artery explants between days 12 and 14 ($n = 3$ per group; $P = 0.0034$, one-way ANOVA; $P < 0.05$ for control vs. both Encap100 and Encap1500). (*F*) Total amount of VEGF measured with vein explants between days 12 and 14 ($n = 3$ per group, $P = 0.0036$ for control vs. Encap1500; $P = 0.0055$ for Encap100 vs. Encap1500). (*G–J*) Tβ4 increased the branch length of capillary outgrowths from mouse artery and vein explants at day 14, with the most pronounced effect on substrates with grooves 50-μm wide. (*G*) Representative images of outgrowths ($n = 10–12$ per group). (Scale bars, 100 μm.) (*H*) Branch density on grooves with different widths (a branch is defined by the distance between two nodes or between a node and the end of a tube; $P = 0.0002$, two-way ANOVA; $P < 0.01$, Bonferroni posttest for control vs. Tβ4 gel groups on 100-μm grooves; $P = 0.7078$ within Tβ4 gel groups). (*I*) Average branch length on grooves with different widths ($P < 0.0001$, two-way ANOVA; $P < 0.05$ for control vs. Tβ4 gel on 25-μm grooves; $P < 0.001$ for control vs. Tβ4 gel on 50-μm and 100-μm grooves; $P < 0.001$ for 50-μm grooves vs. both 25-μm and 100-μm grooves in Tβ4 gel groups). (*J*) Average branch width on grooves with different widths ($P = 0.3782$). Micropatterned PDMS substrates had grooves 25, 50, and 100 μm in width and 65 μm in height. Experimental groups include hydrogel without Tβ4 (control) and hydrogel with 1,500 ng encapsulated Tβ4 (Tβ4 gel). The asterisks in graphs indicate statistically significant differences between groups ($P < 0.05$, two-way ANOVA with Bonferroni posttests for cell density, branch density, branch length, and branch width; one-way ANOVA with post hoc Tukey tests for VEGF expression).

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