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Implanted scaffold-free prevascularized constructs promote tissue repair

Caitlin A. Czajka, PhD¹, Bennet W. Calder, MD², Michael J. Yost, PhD^{2,*}, and Christopher J. Drake, PhD^{1,*}

¹Department of Regenerative Medicine and Cell Biology, Medical University of South Carolina, Charleston, South Carolina

²Department of Surgery, Medical University of South Carolina, Charleston, South Carolina

Abstract

To evaluate the anastomotic potential of prevascular tissue constructs generated from scaffold-free self-assembly of human endothelial and fibroblast cells, tissue constructs were implanted into athymic mice and immune-competent rats. Analysis of xenografts placed into hind limb muscle defects showed vascular anastomotic activity by 3 days following implantation and persisting for 2 weeks. Integration of the implanted prevascular tissue constructs with the host circulatory system was evident from presence of red blood cells in the implant as early as 3 days following implantation. Additionally, analysis of 3-day xenografts in the rat model showed activation of skeletal muscle satellite cells based on Pax-7 and MyoD expression. We conclude that prevascular tissue constructs generated from scaffold-free self-assembly of human endothelial and fibroblast cells are a promising tool to provide both vascular supply and satellite cell activation toward the resolution of skeletal muscle injury.

Keywords

Prevascular; scaffold-free; xenograft; anastomosis; vascularization; skeletal muscle; satellite cell; Pax-7; MyoD

Introduction

Tissue engineering approaches to skeletal muscle regeneration using both *in vitro* and *in vivo* means have been met with limited success, largely due to an inability to provide adequate vascularization^{1–4}. We have previously identified a method by which prevascular tissues are formed from scaffold-free self-assembly of human endothelial and fibroblast cells^{5,6}. During 3 days of *in vitro* culture, cells self-organized capillary-scale networks of endothelial cells and synthesized an extracellular matrix containing at least laminin, fibronectin, and collagen type I to form prevascular tissue constructs⁶. To evaluate the

Corresponding author: Michael J. Yost, PhD, Associate Professor, Department of Surgery, 173 Ashley Ave, CRI 605, MSC 508, Charleston, SC 29425-0508, Phone: 843-792-7789, Fax: 843-792-0429, yostm@musc.edu.

*Co-senior authorship

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anastomotic potential of these prevascular tissue constructs and their ability to facilitate skeletal muscle repair, tissue constructs were implanted into hind limb muscle defects in athymic mice and immune-competent rats, a model we have used previously to investigate muscle regeneration^{1,7,8}.

Skeletal muscle is the most prevalent tissue in vertebrates with the primary function of generating force in order to allow movement of the skeletal system⁹. Arranged into bundles of multinucleated myofibers, skeletal muscle has an inherent regenerative capacity due to the presence of resident mononuclear stem cells, called satellite cells. Satellite cells reside between the basal lamina – a region rich in laminin^{9,10} – and the sarcolemma (myofiber plasma membrane)¹¹. In addition to a role in regulating muscle turnover during homeostasis, activated satellite cells have been shown to be required for restoration of skeletal muscle tissue function following injury¹². Once activated, satellite cells upregulate fibronectin^{13,14}, proliferate and differentiate into myoblasts, and myoblasts then organize into myotubes that ultimately form hierarchically arranged muscle fiber bundles¹⁵. Muscle defects of a greater-than-superficial size are prone to infection, fibrous tissue formation, and scarring^{16,17} during which the hierarchical arrangement of muscle fibers is impaired, resulting in loss of tissue function.

We hypothesized that the use of our endothelial and fibroblast-derived prevascular tissue construct could aid in skeletal muscle repair by providing a temporary scaffolding permissive for vascular assembly and concomitant activation of resident satellite cells. This hypothesis is supported by the inherent regenerative capacity of skeletal muscle conferred by satellite cells^{9,11–13}; the observed inverse relationship between muscle wound size and vascularization/degree of wound healing; and the fact that extracellular matrix proteins synthesized by our constructs are present in the satellite cell microenvironment and implicated in their activation^{9,10,13,14}.

Methods

Prevascular tissue fabrication

Human cell tissue constructs were generated as previously described⁶. Briefly: cell suspension containing human adipose microvascular endothelial cells (ScienCellTM, Carlsbad, CA) and normal dermal fibroblasts (Lonza, Walkersville, MD) at a 1:4 ratio, respectively, was seeded at high density into non-adherent agarose troughs approximately 0.9cm × 0.1cm × 0.5cm and cultured in 1:2 M200 (Gibco[®], Grand Island, NY) + LSGS (Gibco[®]) + 1% PS (Corning Cellgro[®], Manassas, VA): Hi-glucose DMEM (Gibco[®]) +10% FBS (Corning) + 1% PS for 3 days. Prior to seeding, mixed cell suspension was labeled with CellTrackerTM Red CMTPX (Molecular Probes[®], Carlsbad, CA) for rat implantation or QTrackerTM 655 (Molecular Probes[®]) for mouse implantation according to manufacturer's directions. To verify prevascular network organization, following day 3 of culture some constructs were fixed with paraformaldehyde and indirect immunofluorescence labeling performed using anti-human CD31 (BD Pharmingen, 550389, 1:40).

Animal surgery

All animal procedures were conducted under the approval of the Institutional Care and Animal Use Committee (IACUC) of the Medical University of South Carolina (AAALAC accredited as of 1987). A skeletal muscle injury was created in the left hind limb of isoflurane-anesthetized adult male Sprague Dawley[®] (Charles River Labs, Wilmington, MA) or Nu/Nu Athymic mice (Charles River Labs). After adequate anesthesia was established, the left hind limb was clipped if appropriate, prepped, and draped in sterile fashion. A ~0.5–1cm-long longitudinal incision in the skin of the dorsal aspect of the leg was made and with a combination of blunt and sharp dissection a small area of the underlying biceps femoris muscle was exposed. A 1–2mm incision was made in the muscle with microscissors and a pocket created in the avascular plane between the biceps femoris and vastus muscle group with careful blunt dissection. The pocket was then irrigated to remove any blood or debris. A prevascular tissue construct was placed into the pocket, the biceps femoris reapproximated with simple interrupted 5.0 (for rat) or 7.0 (for mouse) Prolene[®] sutures (Ethicon, Bridgewater, NJ), and the skin closed using Gluture[®] topical tissue adhesion (Abbott, Abbott Park, IL). Following buprenorphine administration, animals were monitored until ambulatory and then daily for duration of experiments. Rats (n=4) were observed for 3 days and mice for 3 (n=3), 7 (n=3) or 14 (n=6) days prior to sacrifice. Xenografts and surrounding host tissue were excised *en bloc* (~1cm³ sample size) prior to animal euthanasia. For controls, tissue sections were excised from the right hind limb (no surgery) and/or from the left hind limb of animals having undergone sham surgery (no prevascular tissue construct introduced into incision site).

Paraffin tissue sections

Paraformaldehyde-fixed rat explant tissue samples were embedded in paraffin blocks and 7µm sections cut onto adherent slides, capturing a cross-sectional view of the xenograft-host interface. Slides were cleared using Histo-Clear[™] II (National Diagnostics, Charlotte, NC) and ethanol gradients prior to standard Hematoxylin and Eosin staining or indirect immunofluorescence labeling with anti-rat Pax-7 (sc81648, 1:50) and anti-rat MyoD (sc-760, 1:50) antibodies. Nuclei were labeled with Hoechst 33342 (Molecular Probes[®], 1:1000).

Frozen tissue sections

Athymic mouse explant tissues were embedded in Tissue-Tek[®] O.C.T. Compound (Electron Microscopy Sciences, Hatfield, PA) and flash frozen on dry ice prior to cryosectioning. 10µm sections were cut onto adherent slides, capturing a cross-sectional view of the xenograft-host interface. Slides were fixed briefly in 4% paraformaldehyde prior to indirect immunofluorescence labeling with anti-mouse CD31 antibody (BD Pharmingen 553370, 1:50). Nuclei were labeled with Hoechst 33342 (Molecular Probes[®], 1:1000).

Imaging

Stereo microscopic images were obtained using an Olympus DP80 2CCD color/monochrome camera system with Olympus cellSens Dimension 1.9 software (Olympus Imaging America Inc., Center Valley, PA) and a Dage-MTI CCD300-RC camera system

(Dage-MTI, Michigan City, IN) with Scion ImagePC software (NIH, Gaithersburg, MD). Confocal image arrays were acquired as Z-stacks (1.5µm step size) using a Leica TCS SP5 AOBS Confocal Microscope System (Leica Microsystems Inc., Buffalo Grove, IL). Paraffin and frozen slides were imaged using a SPOT RT3 Camera and Software v4.7 (Diagnostic Instruments Inc., Sterling Heights, MI) on a Leica DMR epifluorescence microscope (Leica Microsystems Inc). Images were compiled, analyzed, and auto-enhanced using ImageJ (NIH, Bethesda, MD) and Adobe Photoshop software (Adobe Systems Inc, San Jose, CA).

Statistics

Vascularity was quantified from fluorescence microscopic images of mouse samples (n=3 control and 3, 7, 14 days). Single-channel binary images were created from CD31 and Hoechst co-labeled samples and the black-to-white ratios, indicative of stained-to-unstained areas, quantified for each channel using an ImageJ plugin. The values obtained for CD31-labeled images were normalized by dividing by the corresponding Hoechst-labeled images in order to account for possible gross differences in cellularity among images. Mean normalized ratios were analyzed by ANOVA (VassarStats, Poughkeepsie, NY) at a significance level of $p < 0.05$. Satellite cell activation was assessed from fluorescence microscopic images of rat samples (n=2 control, n=4 3 days). Satellite cells were defined as Pax-7+ cells co-labeled or closely adjacent to Hoechst-labeled cells. Mean satellite cell number was assessed by Student's t-test at a significance level of $p < 0.05$.

Results

Human endothelial and fibroblast cells at a 1:4 ratio seeded at high density in non-adherent agarose molds formed rod-shaped tissues over the course of three days in culture as previously described^{5,6}. Confocal immunofluorescence analysis of whole mount tissues immunolabeled with anti-human CD31 confirmed the presence of organized networks of human endothelial cells (Figure 1).

In order to assess the *in vivo* anastomotic potential of prevascular tissue constructs, constructs were implanted into hind limb muscle defects in an athymic mouse model. To identify the implanted tissue from the host tissue, prevascular constructs were labeled with Qtracker™ 655 during *in vitro* culture. When tissue from the wound site was analyzed 3, 7, and 14 days post-implantation, the prevascular tissue constructs could be clearly delineated by Qtracker™ 655 expression (Figure 2). A histological boundary between the implant and the host tissue was also observed at 3 and 7 days post-implantation (Figure 2A–B). Immunolabeling of these tissue sections with anti-mouse CD31 revealed the presence of vascular networks in the Qtracker™ 655-positive implanted tissues (Figure 2B).

Having established that implanted tissue constructs could be delineated from the host tissue and that the prevascular tissues became vascularized by the host, we next investigated whether prevascular tissue constructs could facilitate skeletal muscle repair in a hind limb muscle defect model in immune-competent rats. Stereo microscopic analysis of xenografts excised 3 days following implantation in the rat model revealed both discernable vascular network patterns and the presence of red blood cells throughout the xenograft (Figure 3). The identity of the xenograft was confirmed via CellTracker™ Red expression (data not

shown). Further, histochemical analysis of sections from tissue excised 3 days following implantation in the rat hind limb muscle defect model revealed that the observed red blood cells were within the lumens of blood vessels within the xenograft (Figure 4). Diversity in blood vessel diameter was also noted throughout the xenograft.

In order to investigate the effects of prevascular tissue construct implantation on skeletal muscle repair mechanisms, sections from tissue excised 3 day post-implantation in the rat model were labeled for Pax-7 and MyoD, markers of activated satellite cells and myoblasts, respectively. As Figure 5A shows, Pax-7-positive cells were associated with muscle fibers adjacent to the site of implantation. Cells expressing MyoD were found in cord-like arrangements within the xenografts (Figure 5B).

Discussion

This study investigated the anastomotic ability of prevascular tissue constructs generated from scaffold-free self-assembly of fibroblast and endothelial cells with interest in skeletal muscle repair in athymic mice and immune-competent rats. We have previously reported the fabrication and biomaterial properties of our prevascular tissues^{5,6}. The results reported herein suggest that – in addition to synthesizing a matrix permissive for rapid vascularization⁶ – the prevascular tissue constructs also serve to activate skeletal muscle resident stem cells in a rat hind limb muscle defect model.

Immunofluorescence analysis illustrating vascularization throughout the prevascular construct implanted into the athymic mouse model for 3, 7, and 14 days utilized an anti-mouse antibody in order to highlight host vasculature. By 7 days following implantation into the athymic mouse model, CD31-labeled vessels can be clearly identified amongst QtrackerTM-expressing cells within the xenograft, indicating that the host's own vasculature perfuses the construct at this time point (Figure 2B). This finding is important because it suggests that the xenograft is acting as a temporary scaffolding rather than a permanent implant. It could be argued that as a temporary scaffold the gradual degradation of the xenograft is advantageous, providing a niche that fosters the persistence of nascent angiogenic host vessels along with the migration of activated satellite cells and putative myotube assembly.

The stereo microscopic analysis of 3-day xenografts from the rat model (Figure 3) showed striking resemblance to the prevascular network patterns indicated by CD31 labeling prior to implantation (Figure 1). These results suggest that the prevascular networks were functional during early anastomosis *in vivo*; i.e., host blood was able to utilize the human-derived prevascular networks following implantation. Diversity in vessel caliber throughout the xenograft was a surprising finding, considering that the prevascular networks were of uniform capillary-size prior to implantation. This result further confirmed that the construct rapidly anastomosed, by which prevascular networks underwent remodeling likely due to hemodynamics associated with blood flow.

Whether the human prevascular networks retained functionality longer than 3 days would require more eloquent analysis to determine. The presence of QtrackerTM in the 3, 7, and 14

day (Figure 2) athymic mouse samples indicates that the xenograft construct persisted at these time points. However, as QtrackerTM was used to label both endothelial and fibroblast cell types simultaneously during *in vitro* construct fabrication, it is difficult to ascertain whether both cell types remain at these time points and similarly difficult to determine whether the size and morphology of the construct resembles that of its pre-implantation state.

The limitation of xenograft studies using immune-competent model systems, such as the rat hind limb muscle defect model used for this study, is that of the adaptive immune response: namely foreign body reactions and fibrous capsule formation¹⁸. However, use of the immune-competent rat model was also advantageous as the 3-day time point allows us to investigate the initial inflammatory reaction. While a clear histological separation between the xenograft and host tissue is evident at 3 days post-implantation in both the mouse (Figure 2A) and rat (Figure 4A) models, the amount of necrotic tissue at this border zone was surprisingly minimal (Figure 4A–B). Additionally, host myofibers retained intact sarcolemma in this border zone (Figure 4B, asterisk), indicating presence of the prevascular construct did not exacerbate the surgically-induced muscle injury even in the immune-competent models.

Evidence of Pax-7 and MyoD expression within the xenograft is perhaps the most promising result (Figure 5). Considering that the xenograft was composed of solely endothelial and fibroblast cells, Pax-7 and MyoD expression indicates that not only were host satellite cells activated by 3 days following implantation but also that activated cells were able to migrate into the injury site by this time point. That satellite cells have been shown to upregulate fibronectin in response to injury¹⁴ suggests that the presence of fibronectin as a matrix protein synthesized in our initial prevascular construct could have enhanced the activation of resident stem cells, resulting in the Pax-7 expression noted in Figure 5A. Furthermore, the organization of MyoD+ cells into cord-like arrangements within the injury site suggests that in addition to promoting satellite cell activation the xenograft construct also supports the differentiation of these progenitor cells into myoblasts and concordant formation of nascent myofibrils. Together, these results support further research into the use of prevascular tissues – particularly with respect to the unique vascular-permissive matrix synthesized – for skeletal muscle regeneration.

Many attempts to utilize tissue engineering for skeletal muscle wound repair have focused on incorporation of isolated muscle-derived cells, such as myoblasts or satellite cells, into tissue constructs for implantation^{1–3,10,19,20}. Our results from this study indicate it is possible to promote innate satellite cell activation as a mechanism of skeletal muscle wound repair without implantation of skeletal muscle-derived cells. Additionally, our results highlight the importance of the vasculature during skeletal muscle wound repair. Given that fibrous tissue formation occurs concomitant with a lack of adequate vascularization following muscle injury, one could argue that a patent vascular network is key to regenerating functional tissue. It is possible large-volume muscle defects result in fibrous tissue formation rather than functional muscle regeneration because the means through which the mechanism(s) of skeletal muscle regeneration is conveyed becomes disrupted; in other words, an interruption in a continuous vascular network throughout the musculature

such as that occurring due to injury might result in scar formation rather than functional muscle regeneration due to a loss of the ability for skeletal muscle to mount a coordinated wound healing response. Considering the multitude of steps known to be required for successful skeletal muscle regeneration, the necessity of a coherent vasculature to serve as a coordinating pathway during large volume muscle injury repair seems plausible.

Conclusions

Prevascular tissue constructs generated by scaffold-free self-assembly of human endothelial and fibroblast cells rapidly anastomose with host vasculature and are retained for at least 2 weeks in hind limb skeletal muscle defects in athymic mice. Evidence of remodeling of prevascular vessel size following implantation and concomitant activation and migration of innate satellite cells 3 days following implantation in hind limb muscle defects in immune-competent rats suggests that adequate functional vascularization and/or a vascular-permissive matrix enhances skeletal muscle regeneration. In sum, the results of these studies indicate that prevascular tissue constructs offer a promising solution for skeletal muscle regeneration both by activating innate satellite cells and by providing a solution to the long-standing issue of inadequate vascularization associated with volumetric muscle injury.

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Figure 1.
Confocal image projection of one end of a rod-shaped 1:4 human endothelial cell to fibroblast-derived prevascular tissue construct after 3 days of culture. Human endothelial cells are labeled with anti-CD31 (red) and show organization of a primary network pattern *in vitro*. 20×

Figure 2.

Immunofluorescence images of 10µm frozen section of prevascular tissue xenografts and surrounding host tissue after 3 (A), 7 (B), and 14 (C) days in hind limb muscle defects in athymic mice. The prevascular constructs were pre-labeled with Qtracker™ 655 Quantum Dots (yellow) during *in vitro* culture. CD31-labeled blood vessels (red) indicate xenograft vascularization by the host vasculature. Nuclei are labeled in blue. 20×. Quantification of vasculature of day 7 constructs showed a significant difference as compared to controls, and day 7 vasculature was likewise significantly different than day 14 ($p < 0.05$, ANOVA).

Figure 3.

Stereo microscopic images of a prevascular tissue xenograft 3 days after implantation in an immune-competent rat. A: *en face* view of the xenograft (yellow dashed line) attached to host muscle. B: Red blood cells are visible in vascular network patterns throughout the xenograft (C: higher magnification).

Figure 4.

Light microscopic images of Hematoxylin and Eosin stained tissue sections of a prevascular tissue xenograft 3 days after implantation in an immune-competent rat. Xenograft is outlined with dashed yellow line (A); host muscle is oriented to the bottom region of the images (yellow asterisks). Presence of red blood cells in the lumens of vessels (yellow arrows) indicates the xenograft anastomosed to the host vasculature. Image in C shows a higher magnification region from the middle of the xenograft, distal from the implant-host border zone. A: 10×; B: 20×; C: 40×.

Figure 5.

Immunofluorescence images of tissue sections of a prevascular tissue xenograft 3 days after implantation in a hind limb muscle defect in an immune-competent rat showing activated satellite cells migrating into the xenograft. A: Pax-7 (red), Hoechst (blue); 20×. B: MyoD (red), Hoechst (blue); 20× confocal image projection. Arrows indicate activated satellite cells (A) and nascent myotubes (B). Quantification of satellite cells was not significantly different at day 3 compared to sham controls ($p < 0.05$, Student's t-test).