Vascular pedicle and microchannels: simple methods toward effective *in vivo* vascularization of 3D scaffolds

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ABSTRACT

Poor vascularization remains a key limiting factor in translating advances in tissue engineering to clinical applications. Vascular pedicles (large arteries and veins) isolated in plastic chambers are known to sprout an extensive capillary network. This study examined the effect vascular pedicles and scaffold architecture have on vascularization and tissue integration of implanted silk scaffolds.

Porous silk scaffolds with or without microchannels were manufactured to support implantation of a central vascular pedicle, without a chamber, implanted in the groin of Sprague Dawley rats and assessed morphologically and morphometrically at 2 and 6 weeks. At both time points, blood vessels, connective tissue and an inflammatory response infiltrated all scaffold pores externally, and centrally when a vascular pedicle was implanted. At week 2, vascular pedicles significantly increased the degree of scaffold tissue infiltration, and both the pedicle and the scaffold microchannels significantly increased vascular volume and vascular density. Interestingly, microchannels contributed to increased scaffold vascularity without affecting overall tissue infiltration, suggesting a direct effect of biomaterial architecture on vascularization. The inclusion of pedicles and microchannels are simple and effective pro-angiogenic techniques for engineering thick tissue constructs as both increase the speed of construct vascularization in the early weeks post *in vivo* implantation.

INTRODUCTION

Effective cell survival within thick 3D tissue engineered scaffolds requires their engagement with an adequate vascular network. This remains a significant hurdle to overcome in most forms of tissue/organ regeneration. Numerous approaches have been trialed to increase the degree and reduce the time frame of adequate angiogenesis in 3D engineered constructs, including: **i)** angiogenic growth factors to enhance vascularization from the host tissues^[1-4] (termed extrinsic vascularization), **ii)** implantation of an **isolated** arteriovenous pedicle or loop (AVL) within a chamber. This provides an angiogenic stimulus, promoting extensive capillary sprouting from the pedicle^[5-7] (termed intrinsic vascularization), and promotes the survival of stem/progenitor or differentiated cells **implanted within the chamber**,^[8] and **iii)** pre-vascularization techniques where tissues (**including capillary networks**) are assembled *in vitro* using co-culture of endothelial cells and tissue specific cells **generally within a scaffold**.^[9, 10] Capillary-like structures assembled *in vitro*, and then transplanted *in vivo* **can** form functional connections with host capillaries, a process termed inosculation **that depends on a number of processes including angiogenic sprouting from the host capillary bed towards the preformed capillaries**.^[11, 12] **All three techniques have had some success in promoting vascularization of thicker engineered tissues. However, complete vascularization in a short time frame, that eliminates the occurrence of ischaemic necrosis is still beyond our reach, but remains essential for effective clinical application of engineered tissues.** No single vascularization technique has proven effective across multiple applications, prompting research efforts into combinatorial approaches involving multiple vascularization cues.^[13, 14]

It is now well recognized that the physical and chemical features of biomaterials directly affect cell behavior, including vascularization.^[15, 16] For example, in addition to allowing

space for the growth of interconnected blood vessels within the scaffold, pore size and pore morphology have been shown to influence the degree of vascularization through mechanisms involving at least in part activation and polarization of macrophages.^[16] Similarly, microchannels engineered into 3D scaffolds have been extensively used to enhance mass transport of solutes to cells and vascularization of 3D bioengineered tissues.^[13]

Scaffolds derived from *Bombyx mori* silk fibroin (referred to as 'silk' throughout) have been used to engineer a number of tissues including heart,^[18] bone,^[19] cartilage,^[20] arteriovenous grafts,^[21] esophagus,^[22,] nerve,^[23] urethra,^[24] and liver.^[25] Silk-based biomaterials show great promise in regenerative medicine applications, but rapid vascularization of large, 3D silk constructs remains challenging. When microchannels (500 μm $\varnothing$, 1 mm spacing) were introduced into 2 mm thick 3D porous silk scaffolds implanted subcutaneously in mice, they significantly improved cell, connective tissue and vascular ingrowth into the scaffolds compared to scaffolds without the microchannels.^[26] Microchannels also improved vascular ingrowth compared to pre-vascularization with endothelial cells, but the combination of microchannels and endothelial cells had the greatest positive effect.^[27]

Given the success of isolated vascular pedicles in rapidly generating a microvascular arterio-capillary-venous network,^[6] the present study sought to implement this method of vascularization within 7 mm thick porous silk scaffolds to assess the speed and degree of vascular penetration within the scaffold. In addition to the interconnected porous silk scaffold architecture, microchannels were introduced into the scaffolds to determine if they additionally aided in rapid vascularization. Thus both intrinsic and extrinsic methods of scaffold vascularization,^[28] were employed in the study to stimulate vascular growth into the construct.

MATERIALS and METHODS

Manufacture of the Silk Scaffolds

Silk fibroin solution was prepared from *Bombyx mori* silk cocoons as previously described.^[29] Briefly, silk cocoons were boiled in 0.02M sodium carbonate solution for 30 min to remove sericin. Silk fibroin fibers were washed in milliQ water, air dried, and solubilized in 9.3 M lithium bromide (4 ml/g silk fibers) for 4 h at 60°C. The solution was dialyzed against milliQ water for 3 days using SnakeSkin™ dialysis tubing (3500 MWCO). The concentration of the silk solution was determined by drying a known volume and massing the remaining solids. This protocol resulted in a 6-8 % wt/v silk solution. Silk was stored at 4°C until required.

3D, porous silk scaffolds were manufactured by freezing and lyophilizing the silk solution in a custom-made chamber (**Figure 1**). Square chambers (25 mm³, wall thickness 2.5 mm) were manufactured using polytetrafluoroethylene (PTFE) with a 4.5 mm Ø hole drilled in two chamber walls at 15 mm from the bottom of the chamber, to allow for the insertion of the vascular pedicle (**Figures 1A, B**). The chamber was assembled by threading a 4 mm Ø Nylon tube through the holes in the chamber walls in the presence or absence of a linear wire array (**Figure 1 C**).

To manufacture scaffolds with microchannels, a linear wire array was placed in the chamber prior to the Nylon tube. Linear wire arrays were assembled using 508 µm Ø Teflon-coated stainless steel wires spaced 1 mm apart in a polydimethylsiloxane (PDMS) base (5 mm thick) as previously described.^[30] The wires were grouped into 4x6 and 3x6 arrays with 5.4 mm spacing between the two arrays (**Figure 1B**). To make porous scaffolds without the microchannels, a 5 mm thick PDMS base without the wires was placed at the bottom of the chamber.

The 3% wt/v silk solution was poured into the chambers, frozen at -20°C and lyophilized as previously described.^[30] The chambers were wrapped in aluminum foil and autoclaved to induce beta-sheet formation in silk (thus stabilizing the scaffolds in aqueous environments) and to sterilize the scaffolds. Silk scaffolds were hydrated in PBS under sterile conditions and removed from the chambers, following the removal of the Nylon tubing. Linear wire arrays were removed from the silk scaffolds as previously described^[30] leaving porous silk scaffolds with a large 4 mm Ø channel (for the placement of the pedicle) in the middle and in half the conditions an array of microchannels running the length of the scaffold perpendicular to the large channel (**Figure 1D**). The scaffolds were trimmed to 12 mm Ø and 7 mm thickness using a biopsy punch and razor blades and a cut was made across the scaffold bisecting the 4 mm Ø channel, but leaving a small attached hinge region on one side (**Figure 1E**). This allowed the scaffolds to be opened for easy positioning at the implant site and placement of the pedicle in the large channel, followed by closing of the scaffold around the pedicle.

Characterization of the silk scaffold morphology

Scaffolds were cut with a razor blade to reveal different region and lyophilized to dry. Scaffolds were sputter coated with gold and imaged using a Hitachi 3400 scanning electron microscope.

Animals

The animals used, their care and maintenance and all surgical procedures were approved by St Vincent's Hospital, Melbourne Animal Ethics Committee (Protocol 031/12), according to National Health and Medical Research Council of Australia guidelines. Adult male Sprague

Dawley rats (8-10 weeks old) were used in these experiments and were purchased from ARC, Perth, Western Australia.

Surgical Protocol

The experimental groups (**Figure 2A**) were as follows:

Group A: *Side one:* Silk scaffold without microchannels, and no vascular pedicle; *Contra-lateral side:* Silk scaffold with microchannels, and no vascular pedicle; **Group B:** *Side one:* Silk scaffold without microchannels, plus a vascular pedicle (no anastomosis); *Contra-lateral side:* Silk scaffold with microchannels, plus a vascular pedicle (no anastomosis); **Group C:** *Side one:* Silk scaffold without microchannels, plus a vascular pedicle with anastomosis; *Contra-lateral side:* Silk scaffold with microchannels, plus a vascular pedicle with anastomosis. Each subgroup had N=5-8 scaffold implantations, and the study overall used 30 rats.

Rats explored at 2 weeks had pedicle/scaffold constructs implanted for all 6 groups. Rats explored at 6 weeks had only 4 construct groups, Groups A and B (as above).

Operation 1 [Described for Group C, see below for details of Operation 1 for Groups A and B]. All rats had the anti-biotic Baytril (7ml/L water) added to their drinking water the day before surgery and for two days post-operatively to counter any possible wound infections. All rats were placed under general anaesthesia: a mixture of 0.75 ml of ketamine stock solution (100 mg/ml), 0.5 ml of xylazine stock solution (20 mg/ml) and 0.75 ml water for injection. This anaesthetic was injected intra-peritoneally (i.p.) at 20 ml per 100 g body weight, achieving a final dose of 75 mg/kg of ketamine and 10 mg/kg of xylazine.

Under sterile conditions in an animal surgical theatre, the groins and upper legs of the rats were rendered hair-free using a depilatory cream and the skin then decontaminated using chlorhexidine solution. Adult male Sprague Dawley rats (250 g approximately) were treated pre-operatively with the analgesic Carprofen (5 mg/kg, s.c.). With the animal supine an incision was made from the inguinal ligament down the inside of the leg to approximately midway to the knee. The femoral vessels were exposed and the femoral artery and vein cleared of connective tissue over a distance of 2-3cm. In the mid-section of this cleared area the artery and vein were sectioned and clamps applied to each cut end. The cut ends of the artery were then re-anastomosed back together using 10/0 nylon micro-sutures, approximately 6-8 stitches per anastomosis, and similarly the cut ends of the femoral vein were anastomosed together (**Figure 2B**). The purpose of creating the anastomosis was to **promote fibrin leakage outside the large vessels in a confined space (the scaffold), which is known to promote angiogenic sprouting from large vessels.** ^[6]

A pre-made silk scaffold in a circular shape, (approximately 12 mm Ø and 7 mm thick) was applied around the cleared artery and vein. This was achieved by the scaffold being sectioned in two halves in its horizontal mid-line parallel to the upper and lower surfaces, but remaining 'hinged together' by a small area of intact scaffold on one side. Centrally a large channel (approximately 4 mm Ø) running the length of the scaffold diameter had been formed during scaffold manufacture and this channel was used to house the femoral artery and vein. The two halves of the scaffold were closed over the pedicle and the scaffold sutured together with stitches on either side (**Figure 2C**). The scaffold was also sutured to neighboring muscle tissue. This operative procedure was repeated in the opposite groin. Silk scaffolds in one leg had a scaffold without microchannels implanted, whilst the scaffold on the opposite side was

with microchannels, and had an identical operative procedure. The overlying skin wounds were closed with metal clips and the rats resuscitated.

Operation 1 for Group B: This was identical to Group C, except that no anastomoses were created in the femoral artery and vein, but these vessels (in both groins) were cleared of connective tissue and placed inside the scaffold as in Group C.

Operation 1 for Group A: The femoral artery and vein in both groins were left intact and were not dissected out. The scaffolds were placed in both groins in approximately the same position as in Groups B and C and sutured to neighboring tissues.

Operation 2 (all groups): Two or 6 weeks after implantation of the silk scaffolds the rats were anaesthetized (as above) and the skin wounds reopened.

Femoral artery patency was determined using the ‘milking’ patency test on an area of femoral artery immediately distal to the silk scaffold. This involves the use of two forceps to ‘milk’ the blood out of the artery and observe the rebounding of the artery when it refills with blood. Refilling of the artery with blood will only occur if the artery is patent.

Scaffold harvest: The femoral pedicles and attached scaffolds (Groups B and C, Figure 2D and E) or scaffolds alone (Group A) were removed from the rat and immediately immersed in fixative (4% paraformaldehyde) for subsequent processing in paraffin. The rat was euthanized with an overdose of lethabarb ip. (200 mg/kg using a 24 gauge needle) as soon as the pedicle/scaffold was removed from the animal. Note that all two week groups were completed first. As the two week results indicated that there was no advantage to tissue infiltration or vascularization in the group where the pedicle included an anastomosis, this Group (Group C) was not completed for the six week assessments.

All harvested tissue was processed for paraffin embedding. Histological/immunohistochemical assessment and morphometric analysis of the tissues for connective tissue and blood vessel ingrowth into the scaffold was completed.

Histology

Intact whole silk scaffolds with or without a vascular pedicle were processed to paraffin embedding and sectioned vertically into 5µm thick sections. All scaffolds had several central sections stained routinely for haematoxylin and eosin, and Masson's trichrome stain. The general morphology of the scaffold and tissue ingrowth was determined from examination of these sections on an Olympus Microscope Olympus BX61 microscope.

Immunohistochemistry

von Willebrand factor (vWF) immuno-labelling

The wash buffer used throughout this procedure was phosphate-buffered saline plus 0.05% Tween20 (v/v). Dewaxed and rehydrated paraffin sections went through antigen retrieval in 10 mM citric acid pH 6.0 at 95 °C for 20 min. Slides were cooled at room temperature for 20 min then treated with 3% (v/v) hydrogen peroxide for 10 min at room temperature. This was followed with Dako serum-free protein block for 30 min at room temperature. For detection of vessels *in vivo*, sections were incubated with rabbit anti-human von Willebrand factor (Millipore AB7356 1:200 in 20% Dako normal goat serum) for 60 min at room temperature. Sections were then incubated with biotinylated secondary antibodies (Vector Laboratories goat anti-rabbit, 1: 300 in Dako antibody diluent) for 30 min at room temperature followed by

Vectastain Elite ABC HRP Kit (Vector Laboratories) for 30 min at room temperature. Finally, Dako Liquid DAB+Substrate Chromogen System was applied for 5 min at room temperature followed by haematoxylin counterstain and coverslips were mounted with Entellan mounting medium (Merck).

ED1 immunolabelling

ED1 immunolabelling was used for macrophage identification, with immune labelling performed on a Dako Autostainer. After dewaxing and hydration, peroxidase activity was quenched with 3% hydrogen peroxide in 50% methanol for 5 min, followed by antigen retrieval with proteinase K for 4 min. TBS or PBS (with 0.05% Tween-20) washes were performed between steps. Protein blocking was performed with Dako protein block and primary ED1 antibody (mouse anti-rat ED1, Serotec, Raleigh) was added without wash for 60 min (room temperature) at 1:800 in Dako antibody diluent. Primary antibody was detected with rabbit anti mouse-biotin at 1:400 for 30 min. ED1 labelling was detected using Vector ABC Elite at 1:400 for 30 minutes followed by a TBS wash and subsequent color development with Dako liquid DAB for 5 min. All sections were then counterstained with haematoxylin for 1-2 min in ammonia water, dehydrated, cleared and mounted in DPX. For negative controls mouse IgG at the same concentration was substituted for the primary antibody.

Imaging

Haematoxylin and eosin, Masson's trichrome and vWF and ED1 immune-labelled section images were photographed on an Olympus BX61 upright microscope using analySIS software with 4x, 10x, 20x or 40x objectives.

Morphometry

A number of tissue ingrowth parameters were measured in N=5 scaffolds/group morphometrically, including: (1) The percent volume of the scaffold pores filled with invading host tissue at 2 and 6 weeks, (2) The percent volume of the unfilled scaffold pores at 2 and 6 weeks, (3) The percent volume of silk material remaining in the scaffold at 2 and 6 weeks, (4) The percent vascular volume of the entire scaffold at 2 and 6 weeks, (5) The percent vascular volume of the infiltrating tissue into the scaffold at 2 and 6 weeks, and (6) The number of blood vessels/mm² of the entire scaffold at 2 and 6 weeks.

Complete central scaffold sections immunostained with von Willebrand Factor were counted in all specimens in the 2 and 6 week groups. Using Stereo Investigator V11 software (MBF Bioscience), a blinded observer (Y-WG) traced around the external circumference of the scaffold sections. The area of the tissue section traced was recorded. Tissue outside the scaffold was not included in the trace. Ten percent of the tissue area traced was randomly selected for analysis at a magnification of X20, with a grid of 16 points applied to each field.

The first 4 parameters listed above were determined by counting the grid points that fell on invading host connective tissue, blood vessels within that tissue (excluding the implanted pedicle), and empty space within the silk scaffold pores. The percent volume of the infiltrating tissue, empty pore space, and vascular volume of the entire scaffold was determined by dividing the number of grid points falling on each morphological feature, by the total number of points counted for that scaffold section and multiplying by 100. The percent vascular volume of the infiltrating tissue only, (point 5 above) was determined by dividing the number of points falling on vWF positive blood vessels (points falling on the vessel lumen and the vessel wall) divided by the total points falling on the infiltrating tissue multiplied by 100.

The final parameter (point 6 above) - the blood vessel density was determined by counting in each of the fields used for point counting (as above) the number of vWF⁺ blood vessel profiles (vWF⁺ blood vessels in cross or longitudinal section). Following the 'forbidden line rule' two of the grid field perimeter lines (in each field) were selected as forbidden lines and blood vessel profiles that fell over these lines were excluded from the vessel counts. The total number of vessels for the entire section was determined by adding all the individual field counts, which were multiplied by 10 (as only 10% of the tissue section was counted) and then divided by the total area so that the vessel density of the scaffold section was expressed as number of blood vessels/mm² of scaffold area.

Statistical analyses

Morphometric counts are expressed as mean +/- standard error of the mean (SEM) for each group. Statistical significance of morphometric counts was determined using two-way ANOVA on Graph Pad Prism version 7, with a subsequent Bonferroni post hoc test used to determine statistical significance between individual groups. A *p* value, of less than 0.05 was considered statistically significant. **All morphometric assessments were made on N=5 scaffolds/experimental group.**

[Note: no transformations, or normalization of data occurred, and all outliers were included in statistical testing].

RESULTS

Scaffold Manufacture

3D porous silk scaffolds with or without microchannels were manufactured to interface with the vascular pedicle (**Figure 1A**). The manufacturing process involved assembling a custom-

made freezing chamber (**Figure 1B-C**) in which the silk solution was frozen and lyophilized. Following silk scaffold stabilization, the chamber was disassembled and the scaffolds removed (**Figure 1D**). The scaffolds were trimmed for implantation and a cut made across the scaffold bisecting the central channel, but leaving a small attached hinge region on one side (**Figure 1E**). This allowed the scaffolds to be opened for easy positioning at the implant site and placement of the pedicle in the central channel, followed by closing of the scaffold around the pedicle. The scaffolds were robust and easy to handle and manipulate during the manufacturing process and the surgical procedure, with no scaffold deformation or damage observed.

SEM analysis of the scaffold morphology demonstrated an open, interconnected porosity throughout the scaffold, including at the central channel and microchannel walls (**Figure 1F-I**). The pores were interconnected and irregular in shape with an approximate pore diameter of 200 μm .

Figure 1: Fabrication and morphology of silk scaffolds for vascular pedicle implants.

(A) Schematic representation of the silk scaffold implanted with the vascular pedicle.

(B) Components required to assemble the chamber for silk scaffold manufacture, including a custom made polytetrafluoroethylene (PTFE) chamber, a linear wire array made

by immobilizing 500 μm $\varnothing$ Teflon coated wires in a polydimethylsiloxane (PDMS) base as previously described^[32] and 4 mm $\varnothing$ nylon tubing. **(C) Assembled fabrication chamber** into which silk solution is poured to generate silk scaffolds. The assembled chamber with the silk solution is frozen at -20°C , lyophilized and autoclaved to induce β -sheet formation in the silk protein and sterilize the scaffolds. Following scaffold hydration in PBS, the chamber is disassembled and the silk scaffold removed. **(D) Silk scaffold following chamber disassembly**, showing the 4 mm $\varnothing$ vascular pedicle channel and an array of 500 μm $\varnothing$ microchannels spanning the scaffold. **(E) Silk scaffold trimmed to dimensions for implantation** (12 mm $\varnothing$ and 7 mm thick). The scaffold was cut horizontally bisecting the pedicle channel and leaving a small attached ‘hinge’ region, allowing the scaffold to be opened for the pedicle placement. **(F-I) Scanning electron micrographs of the silk scaffolds, showing the morphology** of the pedicle channel **(F)**, scaffold bulk **(G)**, and microchannels, which show porous morphology similar to that of the scaffold bulk **(H-I)**.

Pedicle patency and macroscopic appearance of the scaffold at harvest

Scaffold implantation surgery was successfully achieved in all groups, without any difficulties. This was aided by the fact that there was room in the rat groin for the relatively large silk scaffold used, and that the femoral vascular pedicle runs through this area and is easily identified.

At harvest **in the two week vascular pedicle without anastomosis (with and without microchannels) 5/6 pedicles were patent (83% patency) and all other vascular pedicles in the remaining four groups demonstrated 100% patency** when assessed by the ‘milking patency test’ **(Supplementary Data, Table 1)**. The scaffolds were readily identified in the groin and had not moved from their original position. The scaffolds were gently separated

from surrounding tissues, and on external examination they appeared pink with healthy tissue surrounding the scaffold (**Figures 2D and E**). There were no signs of infection, pus or fluid accumulation around any scaffold.

Figure 2: Implantation of silk scaffolds with a vascular pedicle. Schematic representation of the three surgical protocols combining a vascular pedicle with a porous silk scaffold. Scaffolds with and without microchannels were placed bilaterally in the groins of rats for 2 or 6 weeks. Group A received scaffolds without the pedicle, Group B scaffolds included the pedicle, but no anastomosis and Group C received scaffolds with the pedicle and anastomosis as described below. **(B)** Representative Group C implantation, showing the pedicle with anastomoses (arrow) in the artery and vein was placed centrally in the scaffold halves. **(C)** Bi-lateral implantation of the scaffolds (+/- microchannels) sutured into position in the groin. **(D)** Scaffold harvest at week 2, showing the pedicle (Group C, pedicle with anastomosis) and scaffold without microchannels. **(E)** Representative harvested scaffold appears pink indicating good tissue infiltration at 2 weeks. This was a Group C scaffold with microchannels. Black suture material surrounds the patent pedicle proximal and distal to the scaffold.

General scaffold morphology

Two weeks

On histological examination a vascularized connective tissue infiltration into individual scaffold pores occurred around the entire peripheral circumference of all scaffold groups at 2 weeks (**Figure 3A**). The infiltrating tissue consisted of highly cellular collagenous extracellular matrix, its collagen component identified as blue in Masson's trichrome sections (**Figure 3E-F**). Connective tissue cells included fibroblasts, macrophages and other white blood cells, and cells of the blood vessels (endothelial cells, pericytes and smooth muscle cells). Macrophages (ED⁺) were numerous and covered the silk material almost continuously where tissue infiltration had occurred, and giant cells were common at the silk surface confirming a foreign body reaction (**Figures 3D**). Occasionally, tissue infiltration extended from the periphery towards the middle of the scaffold in advance of elsewhere at the periphery, this advanced tissue infiltration appeared to be associated with the presence of channels (**Figure 3B**). There were still large scaffold areas where empty scaffold pores were common – these were positioned deep into the scaffold (**Fig 3A, E-F**). In scaffolds that included a vascular pedicle (Groups B and C) tissue filling of the pores also occurred centrally around the vascular pedicle (**Figure 3A, C, and F**). In some areas the tissue infiltration from the periphery had met the tissue infiltration from the central vascular pedicle (**Figure 3A**).

Blood vessel ingrowth into the scaffold at two weeks: At two weeks blood vessels were clearly seen entering external pores (**Figure 3G**) of all scaffolds and infiltrating throughout the scaffold. In scaffolds containing a vascular pedicle (Groups B and C) capillary branching off the pedicle into surrounding areas including into the pores of the scaffold was observed (**Figure 3H**).

Six weeks

The tissue infiltration into the scaffold pores was far more extensive at 6 weeks than at 2 weeks and most scaffolds regardless of their group demonstrated approximately three quarters or more of pores filled with vascularized connective tissue (**Figure 4A**). The cellular components of the vascularized connective tissue were similar to the 2 week groups, with an extensive collagen component within the cellular infiltration evident from Masson's trichrome staining in all Groups (**Figure 4B-E**). Blood vessels were evident in all areas of tissue infiltration (**Figure 4 F-H**). The macrophage response (ED1⁺ cells) to the silk scaffold material was very strong in some scaffold areas at 6 weeks, but in other areas of the same scaffolds ED1⁺ staining was considerably reduced (**Figure 4I**). This variable ED⁺ response was not apparent in 2 week scaffolds.

Blood vessel ingrowth into the scaffold at six weeks: At six weeks the pattern of vascularization from both external surface host vessels, and from the centrally placed vascular pedicle had not changed, but was more extensive than at 2 weeks and it exactly mirrored the extent of connective tissue infiltration (**Figure 4B, E and F-H**).

Silk scaffold material at 2 and 6 weeks: The silk scaffold material that formed the walls of the pores throughout the construct appeared as thin blue, pink or red lines depending on the stain used (indicated in **Figures 3D, G** and **Figures 4, B, F**) in an irregular pattern between the invading connective tissue at 2 and 6 weeks. There were no significant differences in the amount of silk observed between different groups at each time point, but there was an overall

decrease in the silk content from week 2 to week 6 ($p=0.0004$), indicating likely silk degradation (**Supplementary Data, Figure 1**).

Figure 3. Histological and immunohistochemical analyses of the silk scaffolds harvested at two weeks post-implantation. (A-C) Haematoxylin and eosin staining showing (A) Low magnification images of representative central, vertical scaffold sections for each experimental group at 2 weeks. A complete rim of pink tissue infiltration is seen in the peripheral scaffold pores (*). Unfilled scaffold pores appear white. Arrows indicate the centrally positioned vascular pedicle. In each scaffold including a pedicle an area of pink tissue infiltration is also seen around the pedicle. Scale bars are 2 mm. **(B)** The peripheral tissue infiltration in a scaffold with microchannels **(and including a pedicle with anastomosis)** indicates advanced tissue infiltration from the periphery into the center of the scaffold via the microchannels (arrows). Scale bar is 500 μ m. **(C)** The central vascular pedicle including the patent femoral artery (black arrow) and patent femoral vein (blue arrow) surrounded by vascular connective tissue infiltration (pink material indicated with *). **Scaffold without microchannels with a pedicle with an anastomosis.** Scale bar is 500 μ m. **(D)** Immunostaining for macrophages (ED1⁺, brown area), showing the periphery of a 2

week scaffold (left) where a numbers of macrophages are evident (arrow). Higher magnification of ED⁺ area (right) shows individual ED1⁺ macrophages (black arrow) and giant cells (blue arrow) which occurred frequently around the silk scaffold material (empty arrows). Scale bars are 200 μ m (left) and 50 μ m (right). **Scaffold with microchannels with a pedicle without an anastomosis.** (E-F) Masson's trichrome staining showing the cellular tissue infiltration in red/purple and collagen in blue. (E) Vertical central scaffold (without microchannels) section without a vascular pedicle. Peripheral purple material is tissue infiltration. Higher magnification image illustrates the composition of the tissue infiltration, which is identical to a scaffold with a pedicle (F). Yellow arrow indicates collagen (stained blue) deposited in the scaffold pores. (F) Vertical central scaffold (with microchannels) section with a central vascular pedicle without an anastomosis (black arrow). Higher magnification image showing collagen deposition (blue color indicated by yellow arrows) in the scaffold pores. Black arrow indicates vascular pedicle. Scale bars are 2 mm (top) and 500 μ m (bottom). (G-H) Von Willebrand factor immunostaining (vWF⁺, brown) for blood vessel endothelium. (G) A vWF⁺ capillary (black arrow) extends from the tissue external (*) to the scaffold, and into the scaffold cellular connective tissue. Empty arrow indicates silk scaffold material. **Scaffold without microchannels and no pedicle.** Scale bar is 100 μ m. (H) Area around the central femoral vein. Black arrows indicate vWF⁺ capillaries sprouting from the vein wall into the surrounding connective tissue. Blue arrows indicate vWF⁺ capillaries extending away from the vein towards the scaffold. **Scaffold with microchannels and a pedicle without an anastomosis.** Scale bar is 200 μ m.

Figure 4. Histological and immunohistochemical analyses of the silk scaffolds harvested at six weeks post-implantation. (A) Heamatoxylin and eosin staining of central scaffold sections at 6 weeks of scaffolds with or without microchannels implanted without the pedicle, or with the pedicle but without anastomosis. Scale bars are 2 mm. (B-E) Masson's trichrome staining of central scaffold sections at 6 weeks, showing representative scaffold sections (insets, scale bars are 2 mm) and higher magnifications of different scaffold areas of interest. (Scale bars are 100 μ m). (B) The tissue filling the pores consists of collagen (blue) and cellular infiltrate (red). Note a blood vessel (full of blood) in the middle of the pore (black arrow). Scaffold material (yellow arrow) without microchannels can be seen at the edges of the image. **No pedicle was present in this group. (C) Boundary between tissue filled pores on the right hand side of the image and unfilled pores on the left. * indicates unfilled pores. Red strands are scaffold material. **(Scaffold without microchannels and no pedicle).** (D) Connective tissue in the scaffold pores. The blue staining areas are collagen; the red areas are cells infiltrating the pores, many of which are macrophages. Black arrows indicate giant cells. **Scaffold without microchannels and with a pedicle.** (E) Area**

around the femoral vein surrounded by collagen (blue) in which numerous blood vessels, some containing blood (black arrows) are seen – branches off the femoral vein (**black arrow**), and an arteriole (yellow arrow) which has sprouted from the femoral artery. **Scaffold with microchannels and a pedicle.** (F-H) **Von Willebrand factor immunostaining (vWF⁺, brown) for blood vessel endothelium.** (F) The external surface of the scaffold at 6 weeks showing numerous vWF⁺ blood vessels outside the scaffold (blue arrow) and one vWF⁺ vessel clearly seen entering (black arrow) the scaffold from the external surface. Empty arrow indicates scaffold material. **Scaffold without microchannels and no pedicle.** Scale bar is 100 μ m. (G) Long vWF⁺ blood vessels (black arrows) seen extending through a scaffold with **no microchannels and without a vascular pedicle** at 6 weeks. Scale bar is 200 μ m. (H) vWF⁺ vessels (black arrow) sprouting off the femoral vein wall at 6 weeks and large numbers of these vessels (blue arrow) seen extending away from the vein towards the scaffold with microchannels. Scale bar is 100 μ m. (I) **Immunostaining for macrophages (ED1⁺, brown area),** showing ED1⁺ stained scaffold section, demonstrating a very strong macrophage (black arrow, macrophage) presence in some scaffolds (left, **with microchannels and a pedicle**), but not in others (right) where the macrophage response is greatly reduced at 6 weeks. (**Scaffold without microchannels and with a pedicle**). * indicates pedicle. Scale bars = 200 μ m.

Morphometric analysis

Two-way ANOVA was used to analyze the effect of the vascular pedicle and the effect of microchannels in the scaffolds on five parameters related to the degree of tissue infiltration and the level of vascularization in the scaffold pores.

Two weeks

Tissue infiltration in the scaffold (Figure 5A): The presence of a vascular pedicle overall was found to have a significant positive effect on tissue infiltration ($p=0.0005$). Scaffolds including a pedicle had an increased percent volume of tissue infiltration compared to scaffolds without a pedicle. Bonferroni's post hoc test demonstrated significantly increased tissue infiltration in the *pedicle (no anastomosis) plus microchannels group* compared to the *no pedicle plus microchannels* ($p<0.05$) and the *no pedicle no microchannels group* ($p<0.005$). While there was a trend toward higher tissue infiltration in the presence of microchannels, the differences were not significant.

Percent volume of unfilled (empty) scaffold pores (Figure 5B): Overall the presence of the pedicle had a significant effect on the percent volume of unfilled pores ($p=0.0011$). Bonferroni's post-hoc test indicated that the *pedicle (no anastomosis) plus microchannels group* had significantly less percent volume of unfilled pores compared to the *no pedicle, no microchannels group* ($p<0.05$). The overall effect of microchannels within the scaffold was not significant.

Percent vascular volume of scaffold (Figure 5C): Overall the presence of the pedicle had a significant effect on percent vascular volume of the scaffold ($p=0.0006$), and also the presence of microchannels had a significant effect ($p=0.0127$). Bonferroni's post-hoc test demonstrated that the *pedicle with anastomosis plus microchannels* had a significantly greater percent vascular volume than the *no pedicle, no microchannels group* ($p<0.05$), whilst the *pedicle (no anastomosis) plus microchannels group* had significantly greater percent vascular volume than the *no pedicle plus microchannels group* ($p<0.05$) and the *no pedicle, no channels group* ($p<0.005$).

Percent vascular volume of infiltrating tissue (Figure 5D): Overall the presence of the pedicle, or microchannels had no effect on the infiltrating tissue percent vascular volume (PVV). This result is not surprising as the degree of vascularity within the infiltrating tissue would be expected to be the same in all scaffolds groups.

Vascular density - number of blood vessels/mm² (Figure 5E): Overall the presence of the pedicle ($p=0.030$) and the microchannels ($p=0.0063$) had a significant effect on the vascular density of the scaffolds at 2 weeks. The *pedicle (no anastomosis) plus microchannels group* had a significantly greater vascular density than the *no pedicle, no microchannels group* ($p<0.05$).

Figure 5

Figure 5: Morphometric analysis of silk scaffolds at 2 weeks.

(A) The percent tissue infiltration of the entire scaffold in all 6 groups at 2 weeks. Two-way ANOVA indicated an overall significant effect on increased tissue infiltration with the presence of the vascular pedicle ($p=0.0005$). Bonferroni's post-hoc test indicated a significant difference between the *pedicle (no anastomosis) plus microchannels* group compared to the *no pedicle plus microchannels* ($p<0.05^*$) and the *no pedicle no microchannels* group ($p<0.005^{***}$). The presence of the microchannels had no effect on tissue infiltration.

(B) The percent volume of unfilled scaffold pores at 2 weeks. Two-way ANOVA indicated an overall significant effect of the presence of the pedicle which decreases the percent volume of unfilled scaffold pores ($p=0.0011$). Bonferroni's post-hoc test indicated a significant difference between the pedicle (no anastomosis) with microchannels and the no pedicle, no microchannels group ($p<0.05$). The presence of the microchannels had no effect on the percent volume of unfilled pores.

(C) The percent vascular volume of the entire scaffold at 2 weeks. Two-way ANOVA indicated an overall significant effect of the pedicle increasing scaffold vascularization ($p=0.0006$) and the effect of microchannels which overall significantly increased vascularization ($p=0.0127$). Bonferroni's post-hoc test demonstrated that the *pedicle with anastomosis plus microchannels group* had a significantly greater percent vascular volume than the *no pedicle, no microchannels group* ($p<0.05^*$), whilst the *pedicle (no anastomosis) plus microchannels group* had significantly greater percent vascular volume than the *no pedicle plus microchannels group* ($p<0.05^*$) and the *no pedicle, no microchannels group* ($p<0.005^{***}$).

(D) The percent vascular volume of the infiltrating vascularized connective tissue at 2 weeks was similar for all groups. Neither the presence of the pedicle or the microchannels had any effect.

(E) The vascular density (blood vessel profiles/mm²) at 2 weeks. Two-way ANOVA indicated an overall significant effect of the pedicle which significantly increased vascular density ($p=0.030$), and the effect of the microchannels also significantly increased vascular density ($p=0.0063$). Bonferroni's post-hoc test indicated the *pedicle (no anastomosis) plus microchannels group* had a significantly greater vascular density than the *no pedicle, no microchannels group* ($p<0.05^*$).

For all data in Figure 5 morphometric counts were taken from N=5 scaffolds/group.

Six weeks

Overall there was no effect of the pedicle or the microchannels on tissue infiltration or vascularization in scaffolds at 6 weeks (Figure 6) other than in the percent volume of empty scaffold pores for which the presence of the pedicle had a significant effect ($p=0.042$) (Figure 6B). Group B (with pedicle) had less than 5 % unfilled scaffold whilst the no pedicle group (Group A) had 7-13% of the scaffold empty. Therefore, even at this relatively late time point of 6 weeks the presence of the pedicle was having a positive influence in the scaffold. These tissue parameters indicate generally that tissue ingrowth continued between 2 and 6 weeks. The significantly decreased tissue infiltration, percent vascular volume, and number of blood vessels/mm² that scaffolds without pedicles had at 2 weeks was counteracted over time gradually as tissue ingrowth (from the scaffold exterior) continued to fill the empty scaffold pores.

Comparisons of the percent tissue infiltration between 2 and 6 weeks show that at 2 weeks all percent tissue infiltration is in the range of 25% to 50%, whilst at 6 weeks it is close to 80% for all 4 groups. Similarly, the percent vascular volume of the entire scaffold at 2 weeks ranged between 1.0-2.5% for all groups, but was close to 4-5% for all four groups examined at 6 weeks, and the vascular density was between 12-25 blood vessels/mm² at 2 weeks for all groups, and between 40-60 blood vessels/mm² at 6 weeks for all groups.

In conclusion, the vascular pedicle and the silk scaffold microchannels both significantly increased vascularization at 2 weeks *in vivo* in porous silk scaffolds. From 2 to 6 weeks, tissue infiltration and blood vessel ingrowth from the scaffold exterior continued, and this was more marked in scaffolds without pedicles and microchannels, which appear to have

greater tissue ingrowth/vascularization during this period so that they effectively ‘catch up’ to silk scaffolds with pedicles and channels by 6 weeks.

Figure 6

Figure 6: Morphometric analysis of the silk scaffolds at 6 weeks. (A) The percent tissue infiltration of the entire scaffold in all 4 groups at 6 weeks. Two-way ANOVA indicated no overall effect caused by the presence of the pedicle, or the presence of the microchannels. There were no significant differences between individual groups. (B) The percent volume of unfilled scaffold pores at 6 weeks. Two-way ANOVA indicated an overall significant effect of the presence of the pedicle ($p=0.042$), but no effect of the microchannels. Bonferroni's

post-hoc test did not demonstrate any significant differences between individual groups. **(C)** The percent vascular volume of the entire scaffold at 6 weeks. Two way ANOVA indicated no effect of the pedicle or the microchannels at this time point. There were no significant differences between individual groups. **(D)** The percent vascular volume of the infiltrating tissue at 6 weeks. Two way ANOVA indicated no effect of the pedicle or the microchannels at this time point. There were no significant differences between individual groups. **(E)** The number of blood vessels/mm² of scaffold at 6 weeks. Two way ANOVA indicated no effect of the pedicle or the microchannels at this time point. There were no significant differences between individual groups.

For all data in Figure 6 morphometric counts were taken from N=5 scaffolds/group.

DISCUSSION

The presence of a vascular pedicle within silk scaffold constructs had a significant positive impact on the percent volume of tissue infiltration and most importantly significantly increased blood vessel percent volume and blood vessel density throughout the scaffold at two weeks. The effect of microchannels within the silk scaffold also significantly increased vascularization within the scaffold at 2 weeks having a positive effect on percent vascular volume and vascular density. Neither factor, the pedicle or the microchannels had an effect on vascularization at 6 weeks, indicating that it is the first few weeks after *in vivo* implantation that both are significant in increasing silk scaffold vascularization. The capillary sprouting of the vascular pedicle within the silk scaffold parallels previous studies where vascular pedicles have been used to vascularize tissues in pre-fabrication techniques.^[31] Additionally, vascular pedicles isolated within a plastic chamber sprout extensive

arteriolar/capillary/venous networks,^[2, 6] and have been shown to support the growth of engineered tissues, including cardiac muscle,^[8] adipose tissue,^[2] skeletal muscle,^[32, 33] bone,^[34] and other soft tissues.^[7] This current study suggests that the chamber is not essential to the process, as the thick silk scaffold in the current study effectively replaced the chamber creating a protected space for pedicle capillaries to sprout and vascularize the scaffold.

The positive influence of the vascular pedicle particularly in the first two weeks suggests a future role for the pedicle in thick construct vascularization. The surgical technique is straight forward, and if a vascular pedicle of appropriate size is in the vicinity of scaffold implantation it could be implanted through a scaffold of any type. The significant advantage of the central pedicle is that it generates a capillary network and a blood flow in the central core of a 3D scaffold within 3-5 days.^[6] The approach for generating the central channel in this study was chosen for its simplicity and access without specialized equipment, but advances in additive manufacturing, and in particular bioprinting technologies are making fabrication of sophisticated scaffold designs increasingly accessible.^[13] The use of the anastomosis within the pedicle was of no advantage to vascularization or tissue infiltration, possibly because the pedicle wall was damaged in the anastomosis region limiting capillary sprouting. Therefore, a vascular anastomosis should not be included in the protocol. This makes the surgical approach simpler and less time consuming. The vascularization provided by the pedicle and by incorporated scaffold microchannels would be particularly important if cells were seeded within the scaffold pores prior to implantation, and would diminish the time period for oxygen and nutrients to reach implanted cells and diminish the likelihood of ischaemic necrosis of these cells.

It was interesting to note that even though the microchannels engineered into the porous silk scaffolds did not enhance overall tissue infiltration in this model, they had a significant positive effect on the vascular volume, suggesting that microchannels provide vascularization cues beyond empty space for tissue ingrowth. The exact effects of microchannels are difficult to isolate as introduction of microchannels into 3D scaffolds affects a number of properties known to influence cellular interactions, including mechanical properties, degradation rates and surface area available for protein interactions.^[13] Microchannels have been shown to affect the expression of signaling molecules, likely through changes in the local cellular microenvironments. For example, in one study ceramic scaffolds with 250 μm microchannels were found to increase the expression of angiogenic factors hypoxia-inducible factor 1-alpha, placental growth factor and C-X-C chemokine receptor type 4 and promote the formation of small blood vessels, while 500 μm microchannels increased vascular endothelial growth factor A expression and formation of larger blood vessels.^[35] Finally, cells are known to respond to local topographical cues and may respond to microchannel shape and curvature.^{[36,}
^{37]} Just as pore size and shape have been shown to affect vascularization via macrophage polarization responses,^[16] it is possible similar effects are induced by microchannels and warrant further investigation.

The use of the vascular pedicle and the microchannels although significantly increasing the silk scaffold vascularization in the first two weeks, including early vascularization in the scaffold core, did not achieve complete scaffold vascularization. The Group with the best tissue infiltration and vascularization at 2 weeks was Group B *scaffold with channels plus a pedicle (no anastomosis)* having slightly less than 50% of the scaffold infiltrated with tissue. Thus there was half of the scaffold without tissue infiltration and vascularization at 2 weeks. In tissue infiltrated areas the degree of tissue vascularization was good [percent vascular

volume (PVV) of infiltrating tissue approximately 4-5.5% at both time points]. This is however lower than the percent vascular volume of the rat arteriovenous loop pedicle isolated in a plastic chamber, where at 2 weeks the PVV was 15%, and 20% at 6 weeks.^[6] However, the level of vascularization achieved in our study is similar to that of isolated mouse flow-through pedicles embedded in Matrigel in a chamber (without growth factors).^[2]

The factors that determine the degree of vascular pedicle capillary sprouting are likely to be multifactorial based on the shear stress/flow within the pedicle vessels,^[38] the volume of pro-angiogenic fibrin exudate around the pedicle,^[39, 40] the degree of scaffold/tissue hypoxia, and the scaffold/tissue elements directing biomechanical signaling that will initiate cell proliferation and migration.^[41]

Techniques such as the use of angiogenic growth factors around the pedicle can increase the degree of early angiogenesis,^[2] although there are a number of issues related to their use including method and site of delivery, controlled spatio-temporal release, rapid growth factor degradation, and possible complications related to vasodilation, abnormal blood vessel growth, excess tissue proliferation and promotion of cancer.^[28, 42] Another technique would be to implant within the scaffold cell types known to be pro-angiogenic, such as mesenchymal stem cells, or their paracrine secretions,^[43] which could further increase the degree of scaffold vascularization by 2 weeks. Another possibility is to pre-vascularize the scaffold with patient-derived endothelial cells prior to implantation. If patient cells were seeded uniformly into the silk scaffold pores with human fibrin *in vitro*, and formed an extensive interconnecting network throughout the scaffold,^[27, 44, 45] *in vivo* implantation would enable the donor network to inosculate with capillaries sprouting from the central pedicle and external surface in a few days to rapidly completely vascularize a silk scaffold of

any dimensions. Finally, the effect of scaffold features, including pore and channel morphology, and mechanical properties, on the extent of tissue vascularization should be explored in a systematic manner.

The thickness of the scaffold should also be taken into account. The scaffold used here was thick (7 mm) and therefore rapid vascularization through-out may be impossible. Thinner scaffolds would reduce the time for complete tissue infiltration and scaffold vascularization, but scaffold thickness will be dictated by the size requirements of the specific tissue/organ being bio-engineered.

Conclusions

Overall, this study demonstrates a simple, effective method of vascularizing thick 3D constructs *in vivo* by implanting a vascular pedicle in the construct. A pedicle was directly implanted into a porous silk scaffold in the absence of a plastic chamber, which highlights that this is a simple and accessible surgical technique for most tissue locations that have an accessible artery and vein, making the technique clinically relevant and translatable. The study further demonstrates that an array of microchannels engineered into a 3D scaffold has a positive effect on the extent of vascularization that is independent of tissue ingrowth and is therefore a simple, accessible scaffold design feature that can be employed across a range of biomaterial types and tissue engineering applications.

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In an *in vivo* study, the presence of vertical microchannels within a thick porous silk scaffold, and the placement of the femoral artery and vein through the scaffold centrally, both resulted in significant increases in scaffold blood vessel ingrowth by two weeks as measured by the percent vascular volume, and density/mm² of blood vessels within the scaffold.

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