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Transplantation of Engineered Cardiac Muscle Flaps in Syngeneic Rats

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Cardiac tissue engineering offers the prospect of a novel treatment for acquired or congenital heart defects. Previously, our studies have shown a significant mass of vascularized cardiac tissue can be generated using a vascularized tissue engineering chamber approach in nude rats. In this present study, syngeneic rats were investigated as an animal model for cardiac tissue engineering using the arteriovenous loop (AVL) chamber in the presence of a functional immune system. Neonatal cardiomyocytes implanted into the AVL chamber survived and assembled into a contractile flap confirming the basic features we previously showed in growing a cardiac construct. There was no significant loss of the assembled cardiac muscle from immune response. The engineered cardiac muscle flaps (ECMFs) formed were transplanted to the neck vessels of the same animal using a microsurgical technique, and all transplanted tissues remained contractile. The cardiac muscle volume of the control and transplant groups was estimated with histomorphometry using desmin and α-sarcomeric actin immunostaining, and there were no significant differences between the two groups. Finally, utilizing a novel model of transplantation, the ECMFs were transplanted to the heart of a recipient syngeneic rat as a vascularized tissue. The cardiac muscle within the transplanted ECMF was shown to survive and remain contractile for the 4-week post-transplantation period, and importantly, the cardiomyocytes retained the elongated, striated appearance of a mature phenotype. This study demonstrated the proof of concept for transplanting tissue-engineered cardiac muscle as a vascularized cardiac construct.

Introduction

In end-stage cardiac failure or congenital cardiac deformities, allogeneic transplantation remains the current treatment of choice.[1] The shortage of organ donors and the long-term effects of immunosuppression prompt an ongoing search for novel solutions. While cell therapy approaches obtained some evidence of clinical success, and many resources continue to be devoted to its research, the small benefits currently obtained with this method would probably restrain its implementation to the treatment of early heart failure postmyocardial infarction remodeling.[2] *De novo* engineering of contractile myocardial tissue for replacement is an interesting concept that may have clinical potential to provide a source of cardiac tissue for reconstruction.[3–6]

To date, many approaches to engineer myocardium were proposed,[7] and some of these have demonstrated clinical potential in small-animal models.[3,6] Generally, these approaches involved seeding cardiac cells in porous scaffolds[3] or forming a compact cell mass in the form of rings[8] or cell sheets[9] *in vitro*. In surgical terminology, the patches generated are cardiac grafts. In early transplantation studies performed in small animals,[3,9,10] these grafts were simply laid over normal or diseased myocardium, and survival relied on the ingrowth (angiogenesis) of host blood vessels derived from the surface of the myocardium. However, transplanting a nonvascularized myocardium that has a high metabolic rate cannot rely entirely on diffusion and *de novo* angiogenesis, for the loss of cardiac tissue is inevitable, thereby limiting its thickness.[11] Strategies have since been developed to prevascularize these cardiac grafts, allowing the process of inosculation to enhance the survival of the transplanted graft.[4,12–14]

Previously, our group has generated a significant mass of vascularized cardiac tissue using an arteriovenous (AV) loop tissue-engineering chamber approach in nude rats.[15] The cardiac tissue was demonstrated to contract spontaneously and responded to inotropes in a physiological fashion. More significantly, the cardiac flap formed has a defined vascular pedicle, which in theory allows immediate reperfusion after

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transplantation toe distant vessels with a standard microsurgical technique. However, to date, this engineered cardiac muscle flap (ECMF) has not been transplanted or examined morphologically some time after transplantation.

In the present study, we sought to demonstrate the transplantation potential of the ECMF in syngeneic rats and examined the morphological appearance of ECMF generated in inbred Sprague-Dawley (SD) rats using the AV loop (AVL) tissue-engineering approach. Here, we demonstrate the viability of transplanting a cardiac muscle flap to an ectopic site with direct attachment of the vascular pedicle to allow cardiac muscle volume to be retained. Finally, we transplanted the ECMF to the epicardium of a recipient syngeneic rat using a novel transplantation model to demonstrate the proof of concept for delivering a vascularized cardiac tissue flap to the surface of the heart while maintaining a significant mass of compact cardiac tissue post-transplantation.

Materials and Methods

All procedures were performed with the approval of the St Vincent's Hospital Animal Ethics Committee, and conformed to National Health and Medical Research Council (Australia) guidelines for animal welfare.

Isolation of neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes (rCMs) were isolated enzymatically from the ventricles as previously described (Neonatal rCM isolation system, Worthington Biochemical).[16] In brief, ventricles from inbred SD rat neonates (1–3-days old; Experimental Medicine and Surgery Unit, St. Vincent's Hospital, Melbourne, Australia) were minced into 1–3-mm^3 fragments and incubated in 50 μg/mL trypsin solution overnight. The next day, samples were transferred into a trypsin inhibitor and warmed in a 37°C water bath for 2 min, and digested with collagenase at 37°C for 45–60 min. After incubation, the digested tissue was triturated 10 times to release the cells, filtered through a 70-μm filter, and incubated at room temperature for 20 min to allow complete digestion of the partially degraded collagen. The cell suspension was centrifuged at 1200 rpm for 5 min, and cardiomyocytes in the pellet were resuspended in the culture medium (DMEM/F12 with HEPES, 5% horse serum, 1% antibiotic/antimycotic solution supplemented by 3 mM pyruvic acid, 2 g/L bovine serum albumin, 100 mg/mL ampicillin, 4 mg/mL transferrin, 0.7 ng/mL sodium selenite, 5 mg/mL linoleic acid, and 100 mM ascorbic acid) and plated on flasks coated with 10 mg/mL fibronectin (Sigma-Aldrich). Viable cells were counted in trypan blue by a hemocytometer, and 6×10^6 rCMs in 50 μL of cardiomyocyte medium were suspended in 200 μL hydrogel (Matrigel™; BD Biosciences Pharmingen) and kept on ice until implantation.

Preparation of vascularized tissue-engineering chamber and implantation of cardiomyocytes

An AVL was constructed in the groin of adult male inbred SD rats (270–510 g) using a technique previously described for nude rats.[15] In brief, the loop was fabricated by interposing a femoral vein graft between the femoral artery and vein. The loop was laid inside the base of a polycarbonate chamber (0.5-mL internal volume, 1.3-cm internal diameter, and 0.5-cm height; Department of Chemical Engineering, The University of Melbourne, Melbourne, Australia). The base of the chamber was anchored to the inguinal ligament and surrounding tissues with sutures. This was followed by the implantation into the chamber of 250 μL of the Matrigel cell suspension of rCMs from donor neonatal rats born in the same inbred colony. Finally, the lid of the chamber was applied, and the wound was closed.

Autologous transplantation of ECMF to ectopic site

Six weeks after implantation of rCMs, the ECMF formed in the groin chambers was explored (see Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/tea). Vascular patency and contractility of the muscle flaps were assessed. Appearances of the flaps were recorded as photographs and videos. This was followed by the dissection of the left carotid artery and jugular veins in the same animal. The cranial ends of the vessels were ligated with small titanium vessel-ligating clips (Horizon™; WECK®), as close to the distal branching point as possible. This was to match the luminal size of the pedicle of the flaps. The caudal end of the vessels was clamped, and the lumen flushed with heparinized saline. Exposed tissues on the neck were covered with damp gauze, and attention was shifted back to the groin. The contracting ECMF was raised based on the femoral vessels, ligating at the inguinal ligament (preserving as much length as possible). The flap was then flushed with 4°C heparinized saline through the arterial end, until perfusate ran clear from the venous end. The flaps were then anastomosed to the neck vessels using a microsurgical technique (end-to-end anastomoses with 10-0 nylon suture). Cold ischemia times were ~30–40 min. After the flap had resumed contraction, ~1 min after reperfusion, the neck wound was closed in layers after careful hemostasis. All tissues were then harvested 2 weeks post-transplantation. In the control group, tissue was explored, but left *in situ* (after removing the polycarbonate chamber at 6 weeks) while leaving the flap pedicle intact. The wound was then closed.

Harvest of cardiac muscle flaps

At 2 weeks postsurgical intervention (control or transplant group), the rats were anesthetized, and the flaps were explored and examined for vascular patency and contractility.[15] Spontaneous contractions were recorded with videomicroscopy to record the contractile activity of all constructs. The contractility of the flaps was easily distinguished from the femoral pulse of the animal (control) or by occluding the strongly pulsatile carotid artery. The flaps were then ligated and removed from the animal for histology.

Histological analysis

The volume and weight of the flaps were measured as described previously,[15,17] and then, the harvested flap tissues were fixed in 4% paraformaldehyde for 24 h. Tissues were divided into serial 2-mm-thick transverse slices that were embedded in paraffin, and 5-μm-thick histological sections were made and routinely stained with hematoxylin and eosin (H&E) and Masson's trichrome for evaluation of general morphology. Muscle cells with antibodies to α-sarcomeric

actin (1:200; Dako), troponin I (1:100; Santa Cruz Biotechnology), and desmin (1:100; Dako) were detected by immunohistochemistry. Gap junctions were identified using connexin-43 (1:2000; Sigma-Aldrich). Macrophages were identified with ED1 (1:800, Serotec). Negative controls used mouse IgG at the concentration of the primary antibody. Peroxidase activity was visualized with diaminobenzidine (Dako), and hematoxylin was used as a nuclear counterstain.

Morphometric analysis

Both α-sarcomeric actin immunostaining and desmin immunostaining were analyzed by morphometry with a computer-generated 12-point square grid (CAST System; Olympus Denmark).[15] Fields were sampled systematically, such that 4%–8% of the specimen was assessed with a minimum of 757 points/specimen. The tissue was categorized into desmin- or α-sarcomeric-actin-immunostained cardiac muscle and other tissues (including new nonmuscle tissue and vessels). The total volume of cardiac muscle or of nonmuscle tissue was calculated by multiplying the percentage of each tissue (determined above) by the total tissue construct volume at harvest. The amount of collagen content was determined by capturing digital images with a slide scanner (Aperio ScanScope®) on Masson's trichrome-stained sections. The Positive Pixel Count algorithm from the proprietary image analysis program (ImageScope®) was used to measure the collagen content. Patches of assembled cardiac muscle tissue (stained red, with a morphological appearance of cardiomyocytes) were outlined with a freehand pen tool to define multiple regions of interest in each section. The threshold was adjusted to segment only pixels with blue (i.e., collagen) staining (hue = 0.66, width = 0.2, color saturation threshold = 0.04), and this was then used to measure the total area of collagen within the outlined area. The percentage of the collagen area (%) in cardiac muscle tissue was expressed as total collagen (in area mm^2)/total cardiac muscle (in area mm^2) tissue per slide.

Transplantation of vascularized ECMFs to the heart in syngeneic rats

For transplantation studies, ECMFs were generated in donor animals for 6 weeks. At week 6, the ECMFs were explored and raised along the femoral-iliac-aortic vascular axis until just caudal to the renal vessels. A recipient rat matched for body weight was intubated and ventilated. The left carotid artery and left jugular vein were dissected as described above. This was followed by a free transfer of the ECMFs with a long pedicle to the recipient vessels. This was achieved by the anastomosis of the abdominal aorta to carotid artery and inferior vena cava to jugular in an end-to-end fashion. A left thoracotomy was then performed in the recipient animal, exposing the anterior surface of the heart. On the surface of the flap that would be in contact with the epicardium, a small square area of capsule was manually dissected and removed, to allow contact between the cardiac muscle content of the flap and the epicardium. The flap was then secured to the epicardium (after removal of the pericardium) with 6-0 prolene sutures. A small window was created by excising a segment of rib at the entry point of the pedicle to prevent occlusion of the vessel. The wound was closed, and animals were recovered. The transplanted tissues were harvested 4 weeks post-transplantation with the whole heart. Tissues were sectioned into four equal portions along the axis of the heart. The segments were fixed with 4% paraformaldehyde for 48 h, and embedded in paraffin for routine histology. Five-micrometer-thick histological sections were made and stained for H&E and Masson's trichrome for general morphology. The survival and microscopic appearance of cardiomyocytes were assessed by desmin immunostaining.

Statistical analysis

Data are expressed as mean ± standard error of the mean. An unpaired Student's *t*-test (two-tailed) was used to compare two groups. $p < 0.05$ was considered significant.

Results

Establishing in vivo *cardiac tissue engineering using vascularized tissue-engineering chamber in syngeneic rats*

Spontaneous contraction. We implanted 6×10^6 rCMs into the AVL tissue-engineering chamber using inbred SD rats ($n = 6$, Fig. 1A, B). Eight weeks postimplantation, the chambers were explored as previously described.[15] New contractile tissues were formed in the tissue-engineered flaps (Fig. 1C). The ECMFs were contracting independently from the host's own pulse at a rate of 106 ± 7 bpm.

Histology and immunohistochemistry. Immunohistochemistry showed the assembly of implanted cardiomyocytes into patches of cardiac muscle-like tissue distributed predominantly around the AVL (Fig. 1D). Sarcomeric striations representing mature contractile apparatus of functional cardiomyocytes were demonstrated in serial sections with desmin, α-sarcomeric actin, and troponin I immunostaining (Fig. 2A–C). Connexin-43 immunostaining demonstrated the presence of gap-junction plaques (Fig. 2D).

H&E staining showed variable inflammation with multiple inflammatory foci particularly evident at sites of the residual Matrigel. Inflammatory infiltrates were found in variable degrees in different constructs. While some were intense and infiltrated the assembled cardiac muscle patches, the most inflammatory infiltrate tended to be present at the periphery of the flap (Fig. 2E). The flap anatomy was consistently composed of an outer fibrous capsule, inner loose connective tissue, and centrally the vascular pedicle surrounded by muscle cells (Fig. 2F). ED-1 staining showed that macrophages were mainly distributed within the capsular and loose connective tissue regions, with some scattered around granulation tissues emanating from the AVL and occasional Matrigel remnants (Fig. 2G, H).

Tissue mass and composition. Collagen content of the cardiac patches was 29.0% ± 7.5%. The weight and volume of these 8-week tissues were 142 ± 23 mg and 109 ± 23 μL, respectively.

Ectopic transplantation of ECMF in syngeneic rats

Surgical outcomes. To test the robustness of the ECMF, we transplanted the flap to an ectopic site in an autologous fashion (Fig. 3A, B). At harvest, 2 weeks postintervention

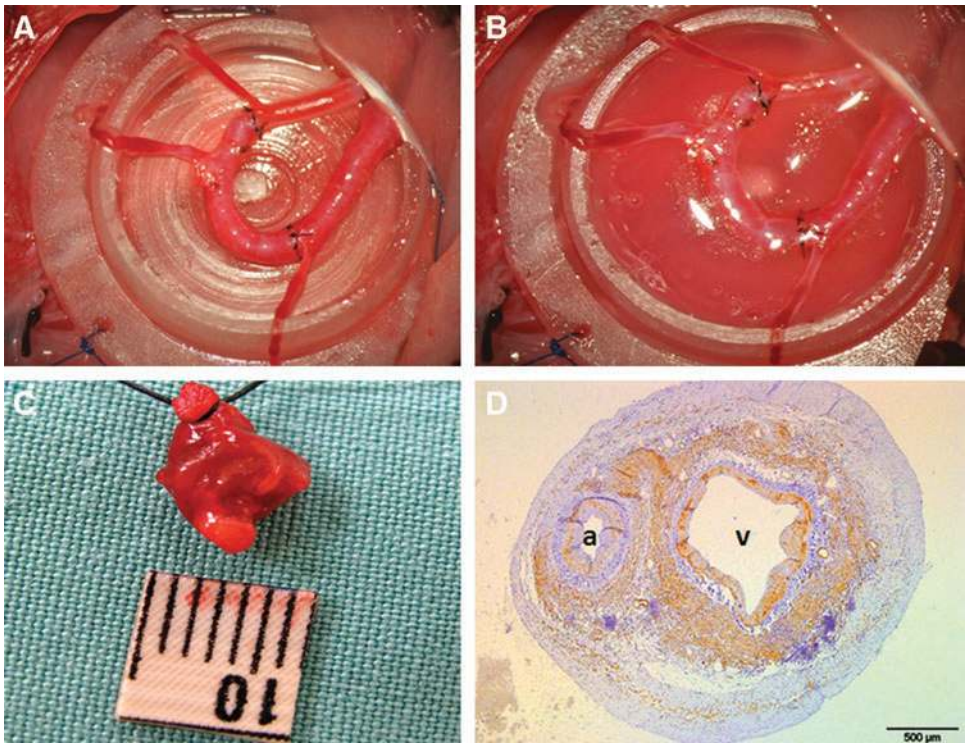

FIG. 1. *In vivo* arteriovenous loop (AVL) tissue-engineering approach in syngeneic rats. **(A)** An AVL fashioned in the groin of inbred Sprague-Dawley rats was placed into the base of a polycarbonate chamber. **(B)** Neonatal cardiomyocytes suspended in Matrigel were implanted into the chamber. **(C)** Gross appearance of the engineered myocardial flap at 8 weeks. **(D)** Cross section of the 8-week myocardial flap showing the patent femoral artery (a) and vein (v) with cardiac muscle-like tissue stained with desmin immunohistochemistry (scale bar = 500 µm). Color images available online at www.liebertpub.com/tea

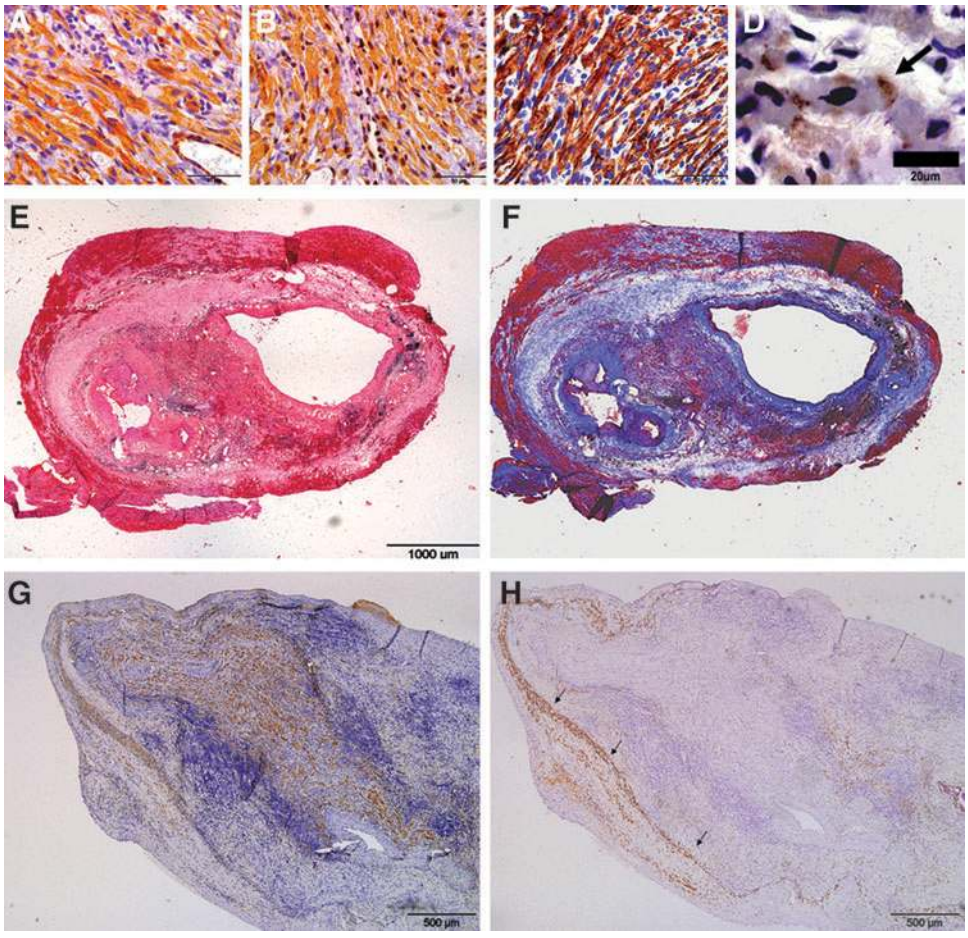

FIG. 2. Representative micrographs that show the characterization of cells in the flap at 8 weeks. **(A)** Desmin-stained cardiomyocytes (brown), **(B)** α-sarcomeric-actin-stained cardiomyocytes (brown), **(C)** Troponin-I-labeled cardiomyocytes (brown) **(A–C,** scale bar = 50 µm**)**, **(D)** connexin-43 immunostained intercalated disc-like junctions (arrow) (scale bar = 20 µm), **(E)** hematoxylin and eosin- and **(F)** Masson's trichrome-stained flap cross sections showing distribution of inflammatory infiltrate (scale bar = 1000 µm). Consecutive sections of the same flap with **(G)** desmin staining to identify cardiac muscle and **(H)** ED-1 staining for macrophages (arrows) (scale bar = 500 µm). Color images available online at www.liebertpub.com/tea

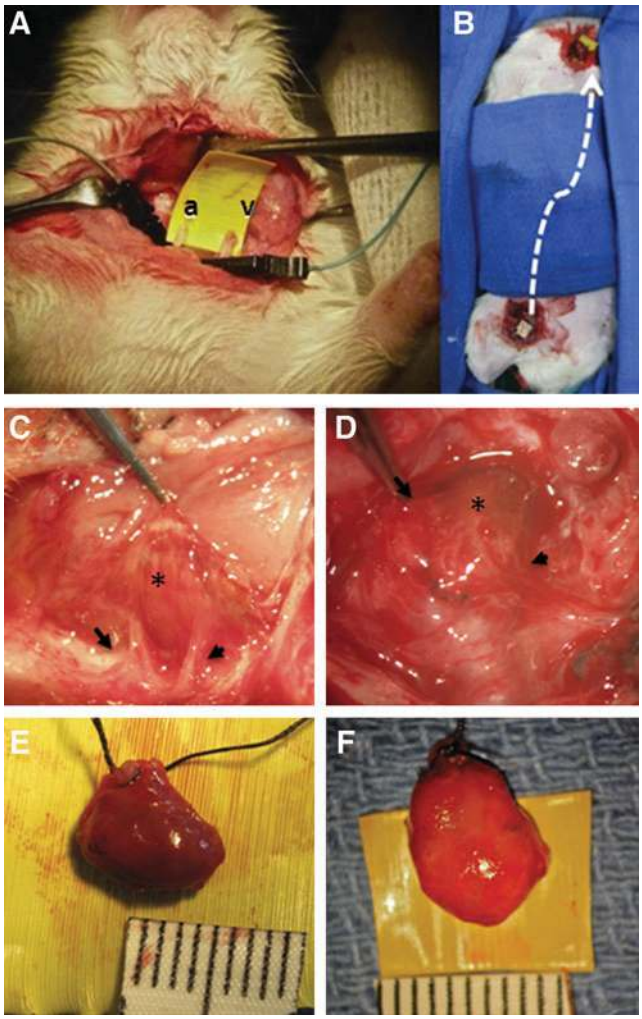

FIG. 3. Ectopic transplantation of engineered cardiac muscle flap (ECMF) in syngeneic rats. **(A)** Autologous neck transplant model, a = carotid artery, v = vein with arrow showing transplantation from groin to neck. **(B)** Control flap tissue and **(C)** transplanted tissue **(D)** with arrow showing integration with surrounding soft tissues at exploration (flap labeled *). **(E)** Control & **(F)** transplanted flap tissue after harvest. Color images available online at www.liebertpub.com/tea

(Supplementary Table S1), the control and transplanted flaps were integrated with the surrounding soft tissues without further obvious encapsulation with fibrous capsule (Fig. 3C–F). All control flaps had patent pedicle, and the transplanted flaps had patent anastomoses.

Spontaneous contraction. Before the exploration at 6 weeks, the control and the transplant group animals had contraction rates of 90 ± 6 bpm and 97 ± 12 bpm, respectively (data not shown). At 8-week harvest, the ECMFs in the control animals were contracting spontaneously at 107 ± 28 bpm. The transplanted flaps were also found to be contracting spontaneously at a rate of 91 ± 7 bpm (Table 1). Statistical analysis did not show significant differences between the control and transplanted group contraction rate.

Histology and immunohistochemistry. Cardiomyocytes were present in both the control and transplant flaps at 8-week harvest by desmin and α-sarcomeric actin immunostaining (Supplementary Fig. S1A, B). In the transplanted flaps, inflammation was similar to that of controls, with intense inflammatory patches present among the loose connective tissue, but minimal inflammatory infiltrate within the assembled muscle component of the patch (Supplementary Fig. S1C). Connexin-43 was expressed around the cardiomyocyte periphery. These gap-junction-like structures were predominantly distributed along the longitudinal axis of the border of adjacent cardiomyocytes (Supplementary Fig. S1D). Some adjacent cells had concentrated gap-junction plaques at the intercalated disc, similar to mature adult rat heart muscle (Supplementary Fig. S1E). ED-1 immunostaining demonstrated a consistent distribution of macrophages concentrating at the loose connective tissue and fibrous capsule interface (Supplementary Fig. S1F), and none were observed within the substance of the cardiac muscle patches.

Table 1. Comparison of Weight, Volume, Contraction Rate, Collagen Content, and Cardiac Muscle Volume Between Control and Transplanted Flaps

	Control (n = *4–6*)	*Transplant* (n = *7*)
Weight of flap (μg)	94 ± 12	117 ± 10
Volume of flap (μL)	79 ± 10	84 ± 11
Contraction rate (bpm)	107 ± 28	91 ± 7
Collagen content (%)	31.24 ± 3.01	28.16 ± 3.11
Cardiac muscle volume (μL)		
α-Sarcomeric actin	6.25 ± 0.73	6.49 ± 1.18
Desmin	4.92 ± 0.65	6.02 ± 1.02

There were no significant differences between the two groups. All values are expressed as mean ± standard error of the mean.

Tissue mass and composition. The weights of the harvested tissues of the control group and transplanted groups were 94 ± 12 μg and 117 ± 10 μg, respectively (Table 1). The volumes of the harvested tissues from the control group were 79 ± 10 μL and 84 ± 11 μL, respectively (Table 1). There were no significant differences in the weights and volumes of the tissues harvested between the control and transplant group.

There were no significant differences in collagen content between the control (31.24% ± 3.01%) and transplant groups (28.16% ± 3.11%) (Table 1). Morphometric analysis of the muscle tissue volume (Table 1) using immunostaining did not show any significant differences between the cardiac muscle volume of the control (desmin 4.93 ± 0.65 μL; α-sarcomeric actin 6.26 ± 0.73 μL, $n = 6$) and transplant groups (desmin 6.02 ± 1.00 μL; α-sarcomeric actin 6.49 ± 1.18 μL, $n = 7$). Counting between desmin and α-sarcomeric actin immunostaining slides did not show significant differences.

Survival of ECMF in the transplantation to epicardium

To demonstrate the proof of concept for transplantation of the ECMF to the heart, a novel model using major abdominal and neck vessels was developed. All vascular anastomoses were patent at 4 weeks post-transplantation of the ECMF to the heart surface of recipient animals ($n = 6$). At harvest, the flap remained apposed to the anterior surface of the heart, and connective tissue of the ECMFs was integrated with the

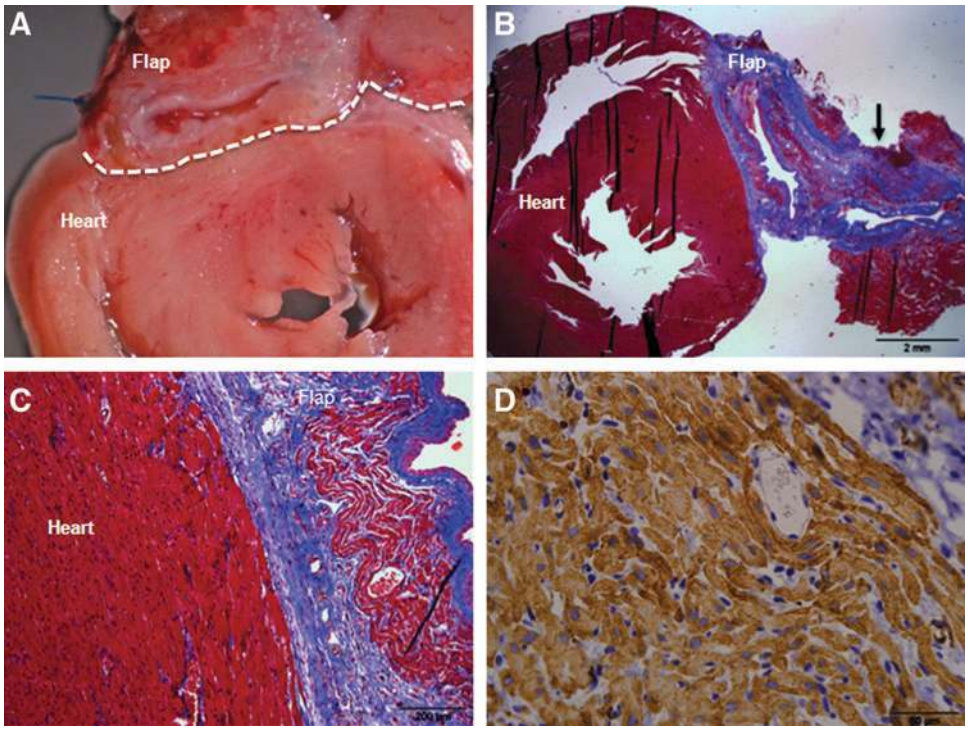

FIG. 4. Transplantation of ECMF to the epicardium of the syngeneic rat heart. **(A)** Macroscopic image of the integrated vascularized ECMF and rat's heart 4 weeks after transplantation. The dotted line represents the interface between the epicardium and the transplanted flap. **(B)** Masson's trichrome staining showing cross section of ECMF patching on the heart with its pedicle (arrow) intact (scale bar = 2 mm). **(C)** Masson's trichrome staining showing cardiac muscle in red separated by fibrous connective tissue (scale bar = 200 μm). **(D)** Desmin immunostaining showing sarcomeric striation of ECMF muscle cells (scale bar = 50 μm). Color images available online at www.liebertpub.com/tea

epicardial surface of the host heart (Fig. 4A, B). Masson's trichrome staining showed continued presence of a compact muscle tissue mass in the ECMF, indicating substantial muscle cell survival through transplantation and confirmed connective tissue integration of the ECMF and epicardium (Fig. 4C). Desmin immunostaining confirmed that mature striated CMs survived the transplantation (Fig. 4D). There was no direct contact observed between cardiac muscles of the ECMF and the myocardium, largely due to the substantive connective tissue capsule of the ECMF and the epicardial connective tissue layer.

Discussion

In this study, we have shown that a pedicled cardiac muscle flap generated using an *in vivo* tissue-engineering approach can be transplanted, and that it maintains its contractility and cardiac muscle volume for at least 4 weeks. We further progressed our AVL cardiac tissue-engineering model to demonstrate survival of implanted cardiomyocytes in syngeneic rats without the need for immunosuppression. We then demonstrated the transplantability of the flap in an autologous transplantation model to an ectopic site. Our data showed that contractility and cardiac muscle volume were maintained after removal of the flap from the chamber space, and after transplantation and revascularization. The morphology of the transplanted flap was comparable to the control flap and our previous publication of flaps generated in nude rats.[15,16] Finally, we devised a technique for transplanting these heterotopic cardiac flaps to the epicardium of rats and examined their morphology in detail. These studies demonstrate the potential for cardiac muscle constructs generated using the AVL *in vivo* tissue-engineering approach as suitable for transplantation, in an autologous fashion, using an immunocompetent small-animal model.

In our previous study, we demonstrated the survival and assembly of neonatal cardiomyocytes implanted into an AVL tissue-engineering chamber in nude rats.[15] In the present study, we established a syngeneic rat model and managed to generate a contractile myocardial flap with minimal impact of inflammation on muscle formation within the flap and contractility. Several tissue-engineering approaches have demonstrated implantation potential of *in vitro* engineered cardiac tissue in small animals,[10,13,18,19] using nude rats or syngeneic rats with immunosuppression of the recipients of the tissue-engineered cardiac grafts. There is some evidence for immunogenicity of the reagents used to generate engineered cardiac constructs, and as upon transplantation of the construct, the cardiomyocytes were subjected to an immune response from the host in the study conducted by Zimmermann *et al.*[10] In their study, without immunosuppressants, syngeneic rCMs were completely degraded after implantation. In our studies, the cells we implanted successfully survived the implantation up to 8 weeks with continuing contractility. While foci of inflammation were present in the flap, we showed long-term survival of implanted cardiac muscle cells, assembly of cells from a suspension at implantation into a compact cardiac muscle-like tissue, and maintenance of functional contraction. Upon ED-1 staining, foreign-body giant cells and macrophages containing residual hemosiderin were identified and predominantly localized to a layer just deep to the fibrous capsule (Fig. 2G, H), which probably represents sites of early thrombus overlain by ongoing remodeling in this AVL chamber model.[20] This clearly indicates that reagents used in the AVL *in vivo* approach did not contain substances that can elicit a sufficient immune response from the host to destroy the cardiac muscle cells. This remained true (Fig. 4A, B) after the cardiac muscle flap was transplanted to the epicardium of a syngeneic recipient.

Subsequently, we showed that these cardiac muscle flaps maintained their cardiac muscle volume and contractility despite removal of the protective chamber for 2 weeks. After removing the chamber, the cardiac muscle volume did not show signs of regression (Supplementary Fig. S2). In previous characterization of our AVL tissue-engineering chamber, a rigid noncollapsible chamber was thought to induce tissue growth, with its mean tissue volume peaking between day 7 and 10, followed by a plateau after day 14 with an ongoing remodeling process occurring thereafter.[20] It seems that after cardiomyocytes have fully assembled into patches, a similar plateau of mean volume is reached,[15] after which the presence of the chamber space no longer contributes to the maintenance of the tissue. This is consistent with the observation of Shimizu *et al.*,[18] who implanted preformed cardiomyocyte sheets over femoral vessels in stages, generating a thick myocardial sheet without any protective mechanism.

Finally, we transplanted the autologous ECMF to an ectopic site, and consistently, for the first time using the AVL tissue-engineering approach, show no change in the cardiac muscle volume after 2 weeks post-transplant. Other transplant studies performed in small animals have demonstrated thin grafts with survival of cardiomyocytes on the surface of host epicardium. While this is encouraging, upscaling of these grafts to repair a significant mass of myocardium will be limited by the blood supply. This is, to date, the first study to quantitatively demonstrate maintenance of the cardiac muscle volume after transplantation, instead of merely demonstrating cardiomyocyte survival. In our transplant to the epicardium, a significant mass of cardiac muscle was transplanted and demonstrated to survive up to 4 weeks post-transplantation. Leor *et al.*[21] developed a cardiac patch composed of a porous alginate scaffold seeded with fetal rCMs. In one of their follow-up studies,[22] the patch was prevascularized *in vivo* by implanting it into the peritoneum, followed by using it to replace a 5-mm ventricular wall defect in a small-animal model. Immunostaining for cardiac markers revealed no survival of cardiomyocytes at 4-week harvest. This demonstrated the extreme oxygen dependency of adult cardiac tissues, requiring almost immediate perfusion of its intrinsic vasculature to survive. Our strategy allows immediate perfusion of the cardiac muscle tissue upon transplantation. Our model also showed that the ECMF withstands ischemia time not dissimilar to that required for the heterotopic, transplanted rat heart in cardiac transplant studies.[23] Our approximate ischemia time from clamping of the vessels to reperfusion of the flap was 40 min.

Despite manually removing the fibrous capsular layer of the cardiac muscle flap before its application to the epicardium, to improve potential for muscle cell contact on the surfaces of flap and epicardium, no obvious cellular integration of cardiac muscle tissues of the flap into the recipient's myocardium was seen. Instead, connective tissues with high collagen content were present at the contact surface. There have been a few studies that demonstrated possible gap-junction formation[10] and electrophysiological integration of cardiac grafts and recipient's heart,[6,9] despite similar fibrous tissues being seen in those studies. Further electrophysiology studies will be required to investigate electrical integration between the cardiac muscle flap and the recipient's heart. Even so, the vascularized cardiac muscle flap can act as a wrap-around flap and potentially be paced similar to the dynamic latissimus dorsi cardiomyoplasty.[24] The weakness of that procedure was a fatigable skeletal muscle that could not maintain an effective cardiac contraction rate; however, the use of true cardiac muscle as a wrap-around flap with pacing may resolve this weakness.

In summary, we have demonstrated the clinical potential of a vascularized cardiac muscle flap. The flap can be transplanted with no significant loss of muscle volume and maintained contraction in syngeneic rats. This novel model demonstrates its potential clinical application for repair of the heart. Further study to assess the functional performance of the ECMF in rats with injured hearts is underway.

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Disclosure Statement

Professor Morrison is an inventor on the Vascularized Tissue Graft patent and entitled to proceeds derived from commercialization of the patent, and is a board member and employee of the O'Brien Institute, which has an interest in the company charged with the commercialization of the Vascularized Tissue Graft patent. The remaining authors report no competing financial interests.

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