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Modified heterotopic hindlimb osteomyocutaneous flap model in the rat for translational vascularized composite allotransplantation research

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Nov 29th 2018

Nandita Singh, Ph.D.
Senior Science Editor

Dear Dr. Singh:

Please accept this original research article entitled **Rat heterotopic osteomyocutaneous flap model of vascularized composite allograft rejection and vasculopathy** for review and consideration for publication in JoVE.

This paper details a modified VCA surgical method which allows for serial histological assessments and offers the opportunity to study various rejection paradigms. We believe this study would be of great interest to your readers.

This manuscript, in its current form or in part, has not been published nor is it currently under consideration for publication by any other journal. I declare that the co-authors have read the manuscript and approved its submission to JoVE. If you have any questions, please do not hesitate to contact me.

Sincerely,

Jason E. Beare

TITLE:

Modified Heterotopic Hindlimb Osteomyocutaneous Flap Model in the Rat for Translational Vascularized Composite Allotransplantation Research

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KEYWORDS:

Rat model, vascularized composite allotransplantation, transplantation, allorejection, surgical model, microvascular surgery

SUMMARY:

Vascularized composite allograft offers life-altering benefits to transplant recipients, but the biological causes of graft rejection and vasculopathy remain poorly understood. The rodent surgical model presented here offers a reproducible, clinically relevant model of transplantation, allowing researchers to evaluate rejection events and potential therapeutic strategies to prevent their occurrence.

ABSTRACT:

Vascularized composite allotransplantation (VCA) is a relatively new field in the reconstructive surgery. Clinical achievements in human VCA include hand and face transplants and, more recently, abdominal wall, uterus, and urogenital transplants. Functional outcomes have exceeded initial expectations, and most recipients enjoy an improved quality of life. However, as clinical experience accumulates, chronic rejection and complications from the immunosuppression must be addressed. In many cases where grafts have failed, the causative pathology has been ischemic vasculopathy. The biological mechanisms of the acute and chronic rejection associated with VCA and, especially, ischemic vasculopathy are important areas of research. However, due to the very

small number of VCA patients, the evaluation of proposed mechanisms is better addressed in an experimental model. Multiple groups have used animal models to address some of the relevant unsolved questions in VCA rejection and vasculopathy. Several model designs involving a variety of species are described in the literature. Here we present a reproducible model of VCA heterotopic hindlimb osteomyocutaneous flap in the rat that can be utilized for translational VCA research. This model allows for the serial evaluation of the graft, including biopsies and different imaging modalities, while maintaining a low level of morbidity.

INTRODUCTION:

Reconstructive surgery for the catastrophic tissue loss from amputation, blast injuries, malignancies, and congenital defects are limited by the availability of tissue from the patient and the additional morbidity caused at the donor site. In some cases, such as burn victims or quadrilateral amputees, viable tissue for reconstruction is not available from the patient. In 1964, the first modern hand transplant was performed in Ecuador. While this was a technical success, immunosuppression available at the time was insufficient to prevent rejection, and the graft was lost in less than 3 weeks[1]. In 1998 and 1999, the first hand transplants in the modern era of immunosuppression were performed in Lyon, France[2] and Louisville, Kentucky, USA[3]. For the first time, reconstructive surgeons could replace like with like. Face transplantation was first performed in 2005[4], and a number of other VCA grafts are now routinely being performed, such as abdominal wall[5], uterine, and urogenital transplants[6].

Unlike solid organ transplantation, most VCA techniques involve the presence of the highly antigenic donor skin. Clinical experience has determined that the acute rejection of the skin is relatively easy to control but may contribute to the chronic rejection of the underlying tissues and vessels, which do not respond well to treatment[7]. The vascular dysfunction associated with an alloimmune response is a more ominous obstacle for the field of VCA[7]. Macrovasculopathies lead to perfusion deficits, delayed healing, and proinflammatory conditions. Both confluent aggressive large-vessel vasculopathy and focal intimal hyperplasia occur in hand transplant recipients[7]. Additionally, microvasculopathies likely contribute to VCA complications as well and may even lead to rejection events. While both immune and nonimmune factors likely play a role in the vasculopathy of hand transplant recipients, the specific mechanisms promoting distal vessel dysfunction in VCA are not known, particularly in the context of low-grade, chronic rejection. These unanswered questions necessitate the development of an animal VCA model that will allow for the serial assessment of the graft during the clinical course of VCA rejection/maintenance and vasculopathy. Such a model will offer insights into the rejection and vasculopathy in the face of immunosuppression, infectious challenge, and/or other postoperative traumatic injury[8,9].

Presented here is an allogeneic rat VCA heterotopic hindlimb osteomyocutaneous flap model. Based on previously published VCA models, this procedure is technically easy to perform, reproducible in a large number, and exhibits minimal morbidity and discomfort to the recipient animal. This model was designed to allow clinical and histopathological assessments of VCA acceptance vs. rejection, as well as the opportunity to evaluate underlying immune and nonimmune mechanisms involved in rejection.

PROTOCOL:

All animal surgeries were performed in accordance with protocols approved by the University of Louisville's Institutional Animal Care and Use Committee (IACUC-approved protocol 18198) and the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals*[10]. Four-month-old male Brown-Norway (RT1.A^n) and 4-month-old male Lewis (RT1.A^l) rats were used as VCA donor and recipients, respectively.

1. Donor allograft harvest

1.1. Sedate the donor animal using vaporized isoflurane applied through a chamber.

1.2. Shave the graft donor area (hindlimb), as well as the groin and abdomen areas. Following that, treat with depilatory cream in order to reduce the amount of fuzz left by the clippers.

1.3. Deeply anesthetize donor animals using intraperitoneal (IP) ketamine (60 mg/kg)/xylazine (15 mg/kg)/acepromazine (2 mg/kg). Administer an initial dose of 0.2 mL/100 g of body weight and additional doses of 0.2 mL every hour.

1.4. Continuously monitor animals while under anesthesia for respiration, body temperature, and depth of anesthesia, using the toe pinch withdrawal reflex test.

1.5. Administer 30 U of heparin solution subcutaneously (SC) in the scruff area prior to the surgery to prevent clotting.

1.6. Wear a mask, a head cover, a disposable isolation gown, and sterile gloves.

1.7. Place the donor animal supine on a heating pad. Produce a sterile surgical field by prepping, scrubbing, and draping the surgical area.

1.8. Make a 3 cm skin incision in the groin concavity using scalpel blade #15 and check the inguinal fat pad laterally using iris scissors.

1.9. Expose the common femoral vessels and place a wire hook with an elastic band to retract the abdominal muscles.

1.10. Using a dissecting microscope (40x), dissect the pedicle proximally from the emergence of the common femoral vessels under the inguinal ligament and distally to the confluence of popliteal vessels into the graft.

1.11. Using microclips and bipolar jewelers' forceps, ligate and divide the large arterial and venous branches, such as lateral circumflex femoral vessels, superficial caudal epigastric vessels, the saphenous artery, and proximal caudal femoral vessels, to mobilize the main femoral vessels. Cauterize any small branches using fine bipolar forceps.

1.12. Make a skin incision from the center of the previous skin cut along the ventral side of the hindlimb, to the ankle area, using iris scissors.

1.13. Cut the gracilis muscle, as well as the other adductor muscles underneath it, in a vertical fashion to expose and ligate the medial proximal genicular vessels, deep-branching small vessels, and the sciatic nerve.

NOTE: At this point, on a separate surgical table, the other surgeon should intubate and anesthetize (2.5%–3% isoflurane) the recipient animal; this allows the surgeons to prepare the recipient surgical site in time for graft placement and minimize the graft's ischemic time.

1.14. On the donor animal, make circumferential skin incisions at the level of the knee and ankle. Disarticulate the knee and muscle, remove extraneous muscle and tissue, and make a vertical skin incision on the dorsal side of the hindlimb to free the graft. At this point, the graft (composed of fibula and tibia, covered with related muscles and skin island nourished by its perforators) is connected only by the pedicle.

1.15. Place small clamps as proximally as possible on the femoral artery and vein, and cut the pedicle as proximally as possible, near the inguinal ligament.

1.16. To flush the graft of blood, inject heparinized saline (30 U/mL) into the femoral artery using a 27 G flushing blunt cannula.

NOTE: Dilating the artery prior to the heparin flush allows easy access for insertion of the cannula. During the flush, closely monitor the outflow from the femoral vein. Once clear fluid exits the femoral vein, stop the flush.

1.17. Wrap the isolated graft in warm saline-soaked gauze and transport it immediately to the recipient animal's table. At this time, the recipient surgical site should already be prepared for vascular anastomosis.

1.18. After the graft harvest, immediately euthanize the donor rat via pneumothorax.

2. Recipient transplantation surgery

2.1. Following the sedation induction using vaporized isoflurane applied through a chamber, deeply anesthetize the recipient animal via a ventilator-controlled endotracheal tube and 2.5%–3% isoflurane.

NOTE: At this stage, the donor rat is still anesthetized.

2.2. Continuously monitor the heart rate, respiratory rate, body temperature, and depth of anesthesia of the recipient animal, using the toe pinch withdrawal reflex test.

2.3. In order to prevent dehydration and hypoglycemia, inject 2 mL of lactated Ringer's solution and 2.5% dextrose subcutaneously at the beginning and another 2 mL at the end of the surgery.

2.4. Shave the groin area and, then, treat it with depilatory cream in order to reduce the amount of fuzz left by the clippers.

2.5. Wear a mask, a head cover, a disposable isolation gown, and sterile gloves.

2.6. Place the animal supine on a heating pad. Apply ophthalmic ointment to prevent corneal abrasions during anesthesia. Produce a sterile surgical field by prepping, scrubbing, and draping the surgical area.

2.7. Make a 3 cm skin incision in the groin concavity using scalpel blade #15 and reflect the inguinal fat pad laterally using iris scissors.

2.8. Expose the common femoral vessels and place a wire hook with an elastic band to retract the abdominal muscles.

2.9. Ligate and divide Murphy branches.

2.10. Using 10-0 nylon interrupted sutures, anastomose donor vessels to recipient vessels via venous end-to-side technique and arterial end-to-end technique. Gradually release the clamps from the artery and then the vein. Monitor the anastomotic sites for bleeding and add additional sutures if needed.

2.11. Visually assess the vascular anastomosis in order to assure effective graft reperfusion.

2.12. Inset the graft into the inguinal pocket and orient it upside down, with the ankle joint superior and knee joint inferior.

2.13. Using tucking sutures, secure the graft to adjacent muscles. Close the skin via interrupted horizontal mattress skin absorbable 4-0 sutures.

2.14. Remove the recipient animal from anesthesia and wean it off the ventilator. Place the animal on a heating pad for thermal support.

NOTE: The overall operation time is between 3 to 4 h, depending on the surgeon's experience and acquaintance with the surgical procedure.

2.15. Administer meloxicam (1 mg/kg) for pain suppression and monitor until the animal is fully recovered and mobile.

3. VCA recipient monitoring

3.1. House the recipient rats singly and monitor them daily for clinical signs of pain, dehydration, weight loss, and decreased activity in addition to surgical failure (for the first 48–72 h) or rejection. Administer meloxicam (1 mg/kg) daily for the first 3 days for pain suppression.

3.2. Based on the research endpoint, choose an immunosuppressant drug to be administered.

4. Histology

4.1. Under inhaled isoflurane anesthesia (2.5%–3%), obtain the serial skin and underlying muscle biopsies from the donor graft at desired time points.

4.2. Close the wound with one to two stitches, using absorbable 4-0 sutures. Return the animal to its cage and allow it to recover from the anesthesia.

4.3. Fix the biopsied tissues in separate tubes in 10% formalin.

4.4. At the terminal time point and under inhaled isoflurane anesthesia (2.5%–3%), take a larger skin biopsy that spans the donor/recipient border. Carefully locate the vessel leash pair at the site of anastomoses; the proper site will be apparent due to the sutures. Take the desired vessel samples from the artery and/or vein. Fix all samples separately in 10% formalin.

4.5. Using a tissue processor (or other preferred embedding technique), paraffin-embed each biopsy into its own block. For skin samples, orient the tissue so that all epidermal and dermal layers may be seen in a single slice. For vessel samples, orient the vessels so that cross sections may be obtained.

4.6. Using a microtome, cut 6 µm-thick sections and apply them to slides for hematoxylin and eosin (H&E) staining.

4.7. Stain for H&E using a standard protocol.

4.8. Obtain representative images of all desired tissue samples using brightfield microscopy techniques.

REPRESENTATIVE RESULTS:

The rat VCA heterotopic hindlimb osteomyocutaneous flap model allows for long-term allograft survival under immunosuppression. The model is reliable, reproducible, and simple to perform. The flap is well hidden in the groin area and constitutes minimal morbidity and discomfort to the animal. The skin presentation is a clinical manifestation of the allograft's survival and rejection (**Figure 1**). The flap design allows for gross clinical monitoring and creates an opportunity for various imaging techniques, such as laser Doppler (**Figure 2**). Serial biopsies of the skin, muscle, and arteries make it possible to achieve histopathological follow-up and analysis at different rejection stages (**Figure 3**).

FIGURE AND TABLE LEGENDS:

Figure 1: Representative images from transplanted animals. (**A**) Syngeneic VCA long-term survival, without immunosuppression treatment, on postoperative day 45 (POD 45); note the difference in direction of fur growth due to the graft's inverted orientation. (**B**) Allogeneic VCA, treated daily with an immunosuppressant drug, on POD 5. (**C**) Allogeneic VCA long-term survival, treated daily with an immunosuppressant drug, on POD 40; note normal fur growth indicating proper perfusion of the graft, without signs of rejection. (D) Allogeneic VCA in rejection on POD 33. Immunosuppression treatment was stopped completely on POD 14; note the clinical signs of rejection (skin atrophy, desquamation, loss of fur).

Figure 2: Laser Doppler imaging system to monitor superficial skin revascularization of the allograft. The allograft presented was monitored on postoperative days 4, 14, and 64. The panels on the left show blood perfusion as measured by Doppler imaging, while the panels on the right show the area being imaged by the Doppler. Note the shift from minimal blood perfusion immediately post-VCA to full revascularization of the flap on day 64. This allograft was kept under proper immunosuppression without signs of rejection.

Figure 3: H&E histopathology of allograft in syngeneic vs. allogeneic transplants. (**A**) Skin biopsy of a syngeneic allograft on POD 45 (10x magnification); note the normal morphology of the skin components (epidermis, adnexa, and no sign of mononuclear cell infiltration). (**B**) Skin biopsy of an allogeneic allograft in rejection on POD 75, treated daily with a lower dose of an immunosuppressant (10x magnification); note epidermal atrophy, adnexa atrophy, mononuclear cell infiltration, perivascular infiltration, and capillary thrombosis. (**C**) Muscle biopsy of a syngeneic allograft on POD 45 (10x magnification); note the normal morphology of the striated muscle. (**D**) Muscle biopsy of an allogeneic allograft in rejection on POD 98, treated daily with a lower dose of an immunosuppressant (10x magnification); note the muscle atrophy and mononuclear cell infiltration. (**E**) Femoral artery biopsy of a syngeneic allograft on POD 45 (20x magnification); note the normal morphology of the artery. (**F**) Femoral artery biopsy of an allogeneic allograft in rejection on POD 98, treated daily with a lower dose of an immunosuppressant (20x magnification); note the intimal hyperplasia, narrow lumen, and perivascular infiltration. *For panels **A**–**D**, the scale bar = 200 μm. For panels **E** and **F**, the scale bar = 100 μm.

DISCUSSION:

In developing this model of VCA, several key issues were considered. First, it was important to include intact bone (tibia and fibula), bone marrow, and skin in the graft. While clinical hand transplants from adult donors do not transfer significant amounts of actively hematopoietic marrow, studies of the role of the bone marrow niche are better mirrored using an intact, vascularized bone rather than a cut long bone, which results in fibrosis of the exposed marrow. Moreover, the closed bone osteomyocutaneous flap design reduces the risk of infection and bleeding. Both bone marrow and skin are highly immunogenic tissues, which may be used to trigger an immune response if desired. Second, it was not necessary for the graft to be functional,

eliminating the need for an orthotopic model that requires complex osteosynthesis and re-enervation of the graft. This also prevents some of the well-known troublesome consequences of orthotopic models, such as a prolonged surgical procedure and animal discomfort[11,12]. However, it is important to note that the heterotopic design does not allow functional outcome measures of bone and cartilage, as well as muscle function, all of which are of significant interest in VCA research. Third, the graft needed to be accessible to imaging systems, clinical follow-up, and serial biopsies. Finally, for throughput purposes, the grafting surgeries needed to be readily performed without complications. With these considerations in mind, a modified rat osteomyocutaneous model of VCA was developed in which the distal hindlimb, between the knee and ankle of the donor (Brown-Norway), including the overlying skin and associated vasculature, was transplanted into the inguinal region on the recipient (Lewis). In this case, the vascular supply to the graft occurred via the femoral artery and vein anastomoses.

Because the skin is an important key factor to monitor VCA rejection, specific care was taken in preparing the graft in order to preserve the small artery perforators supporting skin perfusion. When establishing this model, we performed preliminary experiments using indocyanine green (ICG) angiography (results not shown) to confirm the model's skin perfusion design.

Since the graft is oriented upside down, such that the distal part of the graft is superior and the proximal part of the graft is inferior, a long pedicle is required in order to avoid kinking. Therefore, it should be emphasized that the donor femoral vessels should be divided as proximally as possible and that the recipient femoral artery should be divided as distal as possible.

The simultaneous participation of two surgeons is recommended during the final preparation/isolation of the donor graft; one surgeon should finish the graft isolation, while the other surgeon anesthetizes and intubates the recipient animal and begins preparing the vessels for anastomoses. If the space and equipment are available, a third surgeon could prepare a second recipient animal and both donor legs may be used for VCA grafts. Surgeons must coordinate with one another to ensure minimal graft ischemic time prior to anastomoses. In our experience, most of the postoperative mortalities are attributed to anesthesia technique. If possible, we recommend that a different member of the team should be in charge of anesthesia monitoring during the surgery. It goes without saying that, in order to perform this model successfully, a trained surgeon with basic microvascular techniques is required. Depending on the surgeon's experience, the model can be achieved successfully following two to six surgery attempts.

Syngeneic rats may be used as a control group to account for healing dynamics unrelated to rejection. The contralateral leg of the recipient rat may also be used as a control, especially when performing imaging and biopsies.

Fur regrowth over the transplanted skin is one of the best indications of successful allograft perfusion. On the other hand, fur loss, skin erythema, and de-epithelization may indicate a rejection event and decreased blood supply to some parts of the flap. In a very advanced rejection stage, the skin may show necrosis and exfoliation. A decrease in the allograft muscle

mass is shown in an advanced stage because of denervation atrophy. The animals usually lose body weight (up to 10%) in the first 7–10 days, but then recover and thrive. We recommend adding nutritionally fortified water gel (e.g., DietGel Recovery) in the first few postoperative days to support the recipient rat nutrition. In a very small number of animals (two out of over 50 experimental animals), we witnessed skin infection and autophagy.

In conclusion, the modified model of heterotopic, allogeneic hindlimb osteomyocutaneous VCA graft presented here offers a reproducible, versatile transplantation paradigm. Serial biopsies and imaging offer information on the time course of rejection events. The variety of clinical symptoms that may be studied with this method make it a highly adaptable translational model with the potential for numerous insightful discoveries in the years to come.

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DISCLOSURES:

The authors have nothing to disclose.

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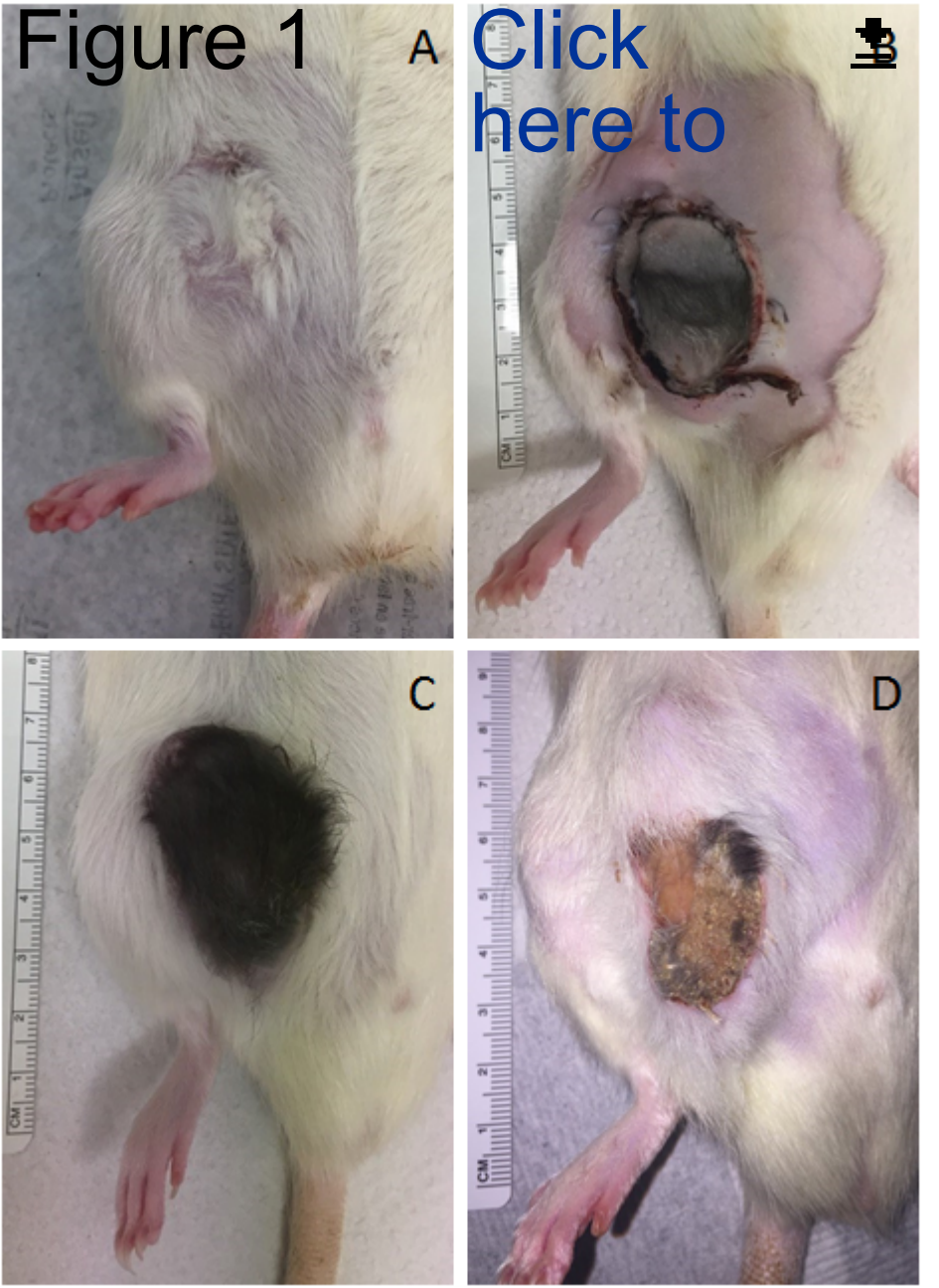
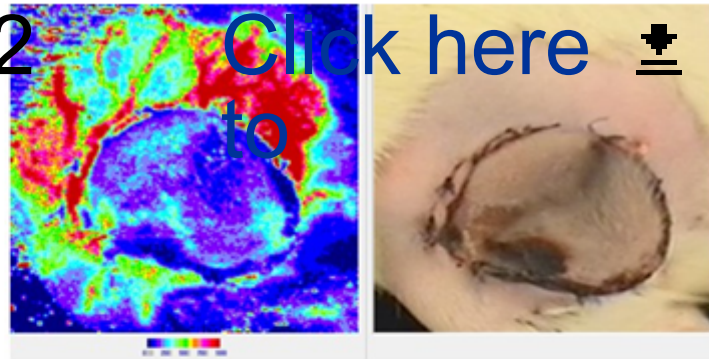
Figure 1



Click here to

Figure 2 Click here to

POD4



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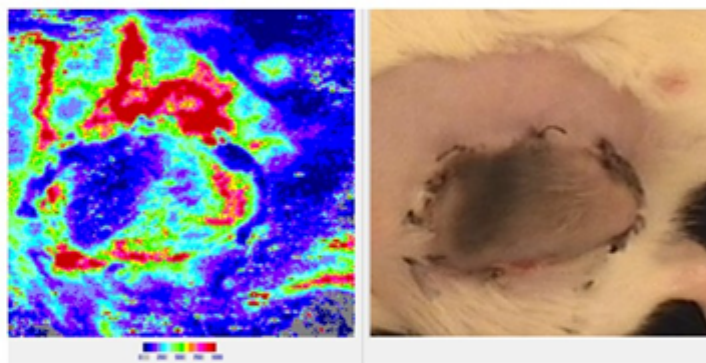

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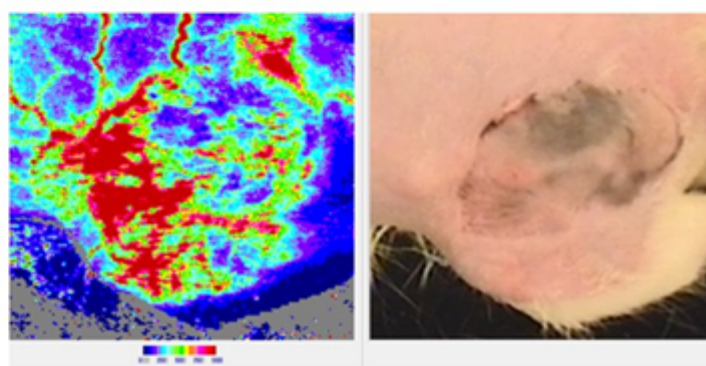

Figure 3

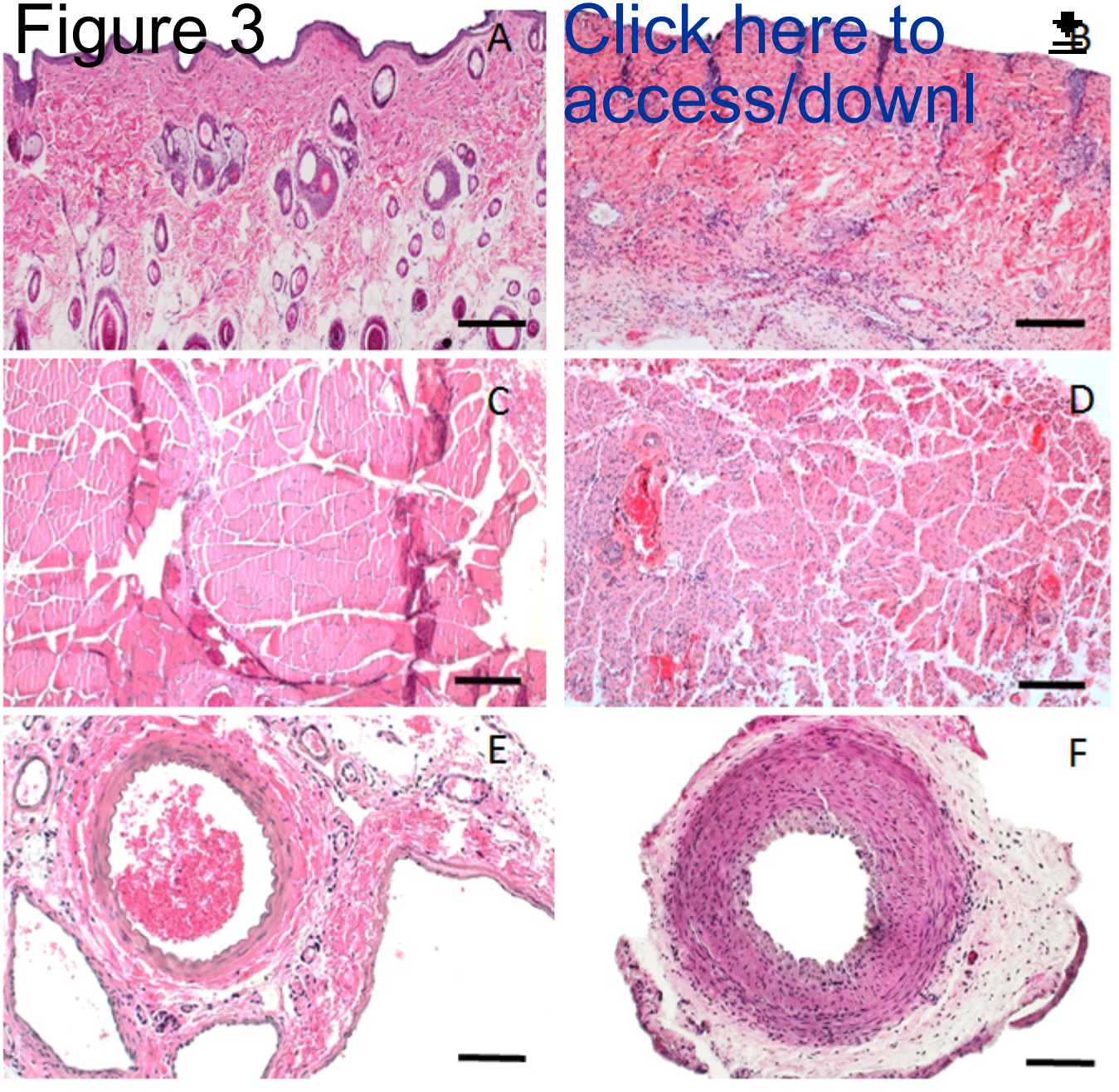

Table of Materials

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Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Acepromazine	Henry Schein	5700850	
Adventitia Scissors	ASSI	SAS15R8	
Approximator Clamp (Double)	ASSI	ABB2V, ABB22V	
Approximator Clamp (single)	FST	00398-02	
Clamp Applying Forceps	ASSI	CAF4	
Dissecting Scissors	ASSI	SDS18R8	
Flushing blunt needle 27G	SAI		
Heparin Sodium	Sagent	25021-400-30	
Isoflurane	Patterson Veterinary	14043-704-06	
Jewelers Bipolar	ASSI	103000BPS03	
Jewelers forceps #3	FST	11231-30	
Ketamine HCl 100 mg/ml	Zoetis	043-304	DEA License required
Lactated Ringer Solution	Hospira	0409-7953-03	
Lactated Ringer Solution + 5% Dextrose	Hospira	0409-7953-09	
Meloxicam	Henry Schein	11695-6925-2	
Micro forceps	ASSI	JFAL3	
Micro needle holder	ASSI	B138	
Prograf (Tacrolimus) 5 mg/ml	Astellas	0469-3016-01	
Suture, 10-0 Prolene	Ethicon	W2790	or 10-0 Ethilon (2830)
Suture, 4-0 Coated Vicryl	Ethicon	J714D	
Vessel Dilator Forceps	ASSI	D5AZ	
Xylazine	VetOne	13985-612-50	

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Dear editors and reviewers,
Thank you for the insightful comments. We have revised this paper based on the suggestions; the revisions to the manuscript may be found in red text. Please find our responses to each reviewer comment below.

Editor comments:

Q1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thank you for the suggestion – proofreading was done.

Q2. Please revise lines 220-224 to avoid previously published text.

We are unaware of any previously published text that matches our wording. This is the most detailed yet succinct description of our model that we can provide.

Q3. Please provide an email address for each author.

Thanks for the comment. The authors emails are listed below:
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Q4. Please abbreviate liters to L to avoid confusion.

We have made the requested changes.

Q5. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

Thank you for the comment. The numbering of the Protocol was adjusted as indicated in JoVE Instructions for Authors.

Q6. Lines 91 and 143: Please specify the age and gender of the donor and recipient animals and describe how the sedation is done.

Please find the specified gender and age of donor and recipient animals in line 81. In line 87 and in line 135-136 there is a description of the sedation technique ("…using vaporized isoflurane applied through a chamber.")

Q7. Line 105: How large is the incision?

Length of incision was added. Please see line 100 and line 149.

Q8. Please also briefly describe how to collect the images and H&E histopathology data presented in the Representative Results section.

We have added a section to the protocol for Histology, lines 175-193.

Q9. Representative Results: Please describe the figures in more details.

We have added more details to the figure descriptions. Please see lines 206-234.

Q10. Figure 2: Please describe what the left and right panels are in the figure legend.

We apologize for the confusion. Text has been added to the figure legend; see lines 216-218.

Q11. References: Please do not abbreviate journal titles.

Journal titles were corrected to full name.

Q12. Table of Equipment and Materials: Please sort the items in alphabetical order according to the name of material/equipment.

Changes have been made as requested.

Reviewer #1:

Q1. In the last paragraph of the Introduction you explain the intention of the submitted manuscript, which is to present an allogeneic rat VCA heterotopic osteomyocutaneous flap model (line 77). This VCA model could be from everywhere from the rat (abdomen, hindlimb, etc.). You should consider explaining what kind of VCA you are presenting in the manuscript.

Thank you for the suggestion, we have added in line 2, 42, 73, 88, 196, 253, and 289 that this is a hindlimb VCA.

Q2. Line 93, Are you shaving or using depilatory cream? This is confusing.

We agree that this is not clear enough and apologize for the confusion. We do both, shaving followed by depilatory cream, in order to reduce the amount of fuzz left by the clippers. Please see corrected text in lines 88-89 and 143-144.

Q3. Line 100, Where are you injecting Heparin?

We inject Heparin subcutaneously in the scruff area. Please see corrected text in line 95.

Q4. Line 140, Please include the information, that the rat is still anesthetized

Thank you for the suggestion. We added this information in lines 136-137.

Line 162, How do you test patency of the anastomosis? Are you using the milking test?

Thank you for the comment. Basically, we prefer to test anastomosis patency by observation without handling the vessel at all. We look for distal expansile pulsation or wriggling of the vessel. We sometimes use the "uplift test" when we are in doubt or when we suspect longitudinal pulsation or false wriggling. We seldom use "empty and refill test" because it is aggressive and traumatic, however it is being used when indicated.

Q5. Please insert the overall operation time, which would be of great interest to the reader.

The overall operating time was added in lines 164-166.

Q6. Lines 214-218, The heterotopic design of the model is a definite disadvantage regarding functional outcome measures of bone and cartilage, as well as muscle function, which is of significant interest in VCA-research. However, by choosing a heterotopic model, you avoid lengthy operation times due to complex osteosynthesis and nerve-coaptation. I recommend addressing these criticisms.

This is a valid concern. We added these criticisms in lines 247-250.

Reviewer #2:

Major Concerns:
Q1. The authors mention that they are describing a VCA model of chronic rejection and vasculopathy. However, all methods descried in the protocol is the surgical part of the transplantation. Under the monitoring section, the authors need to clearly define how they induce chronic rejection following transplantation. The dosage, the duration and frequency of Tacrolimus injections must be given, so that the reader will be able to

reproduce the experiments. At the current version, nobody will be able to induce a chronic rejection.

Thank you for your concerns. Our original intent in this method paper was to describe our modification of the heterotopic hindlimb osteomyocutaneous flap model in the rat. As we suggest, this model could assist in research of VCA chronic rejection and other translational research. After further consideration, we have decided to delete all mention of chronic rejection, with the exception of a brief mention that further study of this phenomenon is required in the Introduction. We agree with the reviewer that the title and some part of the text are not accurate. We have changed the paper title to be more precise, and also the text that relates to our method of immunosuppression. Please be advised that we are looking to publish our rejection protocols using this model in a different paper in the near future. Similar papers on animal VCA model that were published in JoVE did not describe the immunosuppression protocols utilized as well.

Q2. The authors need to describe the macroscopic skin changes also histologic changes related to chronic rejection and show these changes in the model described here.

Thank you for the suggestion. As mentioned above, we have removed the chronic rejection model from the text. We added more detailed descriptions of the macroscopic and histologic changes to the figures legends. Please note that these are only representative images that support the description of the surgical model.

Minor Concerns:
Q1. Figure 2 is redundant. The authors showed images of acute rejection. The need to provide figures of chronic rejection.

Thank you for the comment. The authors agree with the reviewer that Figure 2 does not represent rejection. However, Figure 2 does provide an example of the imaging possible in this model design, as well as the superficial revascularization over time of an allograft maintained under proper immunosuppression without rejection.

Q2. The statements in the discussion such as: ''By applying different immunosuppression protocols, features of various types of rejection can be observed, such as acute rejection, chronic rejection and even vasculopathy.''and ''Lower doses of Tacrolimus were used for chronic rejection models, and complete removal of Tacrolimus induced acute rejection.'' has no justification. There is neither literature support nor result in the study for these statements.

As mentioned previously, we have removed text relating to chronic rejection from the manuscript. These sentences have also been removed.

Q3. In the conclusion, the statements "Using this model, rejection may be studied from either an acute or a chronic perspective." and "Additionally, differences in biological/immunological vasculopathy vs mechanical vasculopathy may be examined as underlying complications leading to graft failure" are not the conclusion of the current manuscript. The chronic rejection part is not presented in the protocol.

These sentences have been removed from the conclusion.

Q4. The authors divide the femoral vessels as proximal as possible in the donor rat, which makes the pedicle vascular pedicle quite long. Is there a specific reason for this long pedicle, how they avoid kinking of the pedicle? This issue must be discussed in the discussion section.

Thank you for the comment. As we described in lines 159-160, the graft is oriented upside down, in a way that the distal part of the graft is superior and the proximal part of the graft is inferior. Therefore, a long pedicle is required so we could avoid kinking. In our experience, we did not encounter a kinking problem. We added this issue into the discussion in lines 261-265.

Q5. During the transplantation, the authors anastomosed donor vessels to recipient vessels via venous end-to-side technique and arterial end-to-end technique. Is there a reason for performing end to side venous anastomosis?

This is an important question. We believe that venous end to end anastomosis is necessary in this model. This technique prevents any venous drainage complications of the ipsilateral recipient leg distal to the anastomosis. Also, we find the venous end to side anastomosis technically easier to perform compared to end to end.

Thank you again for the thoughtful and insightful suggestions. It is our hope that the updated manuscript will be acceptable for publication in JoVE.