Video Article

Surgical Angiogenesis in Porcine Tibial Allotransplantation: A New Large Animal Bone Vascularized Composite Allotransplantation Model

Dimitra Kotsougiani¹, Caroline A. Hundepool¹, Joost I. Willems¹, Patricia Friedrich¹, Alexander Y. Shin¹, Allen T. Bishop¹

¹Microvascular Research Laboratory, Department of Orthopedic Surgery, Mayo Clinic

Correspondence to: Allen T. Bishop at Bishop.Allen@mayo.edu

URL: <https://www.jove.com/video/55238>

DOI: [doi:10.3791/55238](https://doi.org/10.3791/55238)

Keywords: Medicine, Issue 126, Pig, translational research, segmental bone defects, VCA, surgical angiogenesis, swine

Date Published: 8/13/2017

Citation: Kotsougiani, D., Hundepool, C.A., Willems, J.I., Friedrich, P., Shin, A.Y., Bishop, A.T. Surgical Angiogenesis in Porcine Tibial Allotransplantation: A New Large Animal Bone Vascularized Composite Allotransplantation Model. *J. Vis. Exp.* (126), e55238, doi:10.3791/55238 (2017).

Abstract

Segmental bone loss resulting from trauma, infection malignancy and congenital anomaly remains a major reconstructive challenge. Current therapeutic options have significant risk of failure and substantial morbidity.

Use of bone vascularized composite allotransplantation (VCA) would offer both a close match of resected bone size and shape and the healing and remodeling potential of living bone. At present, life-long drug immunosuppression (IS) is required. Organ toxicity, opportunistic infection and neoplasm risks are of concern to treat such non-lethal indications.

We have previously demonstrated that bone and joint VCA viability may be maintained in rats and rabbits without the need of long-term-immunosuppression by implantation of recipient derived vessels within the VCA. It generates an autogenous, neoangiogenic circulation with measurable flow and active bone remodeling, requiring only 2 weeks of IS. As small animals differ from man substantially in anatomy, bone physiology and immunology, we have developed a porcine bone VCA model to evaluate this technique before clinical application is undertaken. Miniature swine are currently widely used for allotransplantation research, given their immunologic, anatomic, physiologic and size similarities to man. Here, we describe a new porcine orthotopic tibial bone VCA model to test the role of autogenous surgical angiogenesis to maintain VCA viability.

The model reconstructs segmental tibial bone defects using size- and shape-matched allogeneic tibial bone segments, transplanted across a major swine leukocyte antigen (SLA) mismatch in Yucatan miniature swine. Nutrient vessel repair and implantation of recipient derived autogenous vessels into the medullary canal of allogeneic tibial bone segments is performed in combination with simultaneous short-term IS. This permits a neoangiogenic autogenous circulation to develop from the implanted tissue, maintaining flow through the allogeneic nutrient vessels for a short time. Once established, the new autogenous circulation maintains bone viability following cessation of drug therapy and subsequent nutrient vessel thrombosis.

Video Link

The video component of this article can be found at <https://www.jove.com/video/55238/>

Introduction

Large segmental osseous defects result from trauma, infection or limb-sparing surgery after malignancy. Current reconstructive options such as vascularized autogenous bone grafting, bone transport, prosthetic replacement, and cryopreserved necrotic allografts, used alone or in combination, are associated with significant morbidity and have high rates of complications^{1,2,3}.

The presence of a microvascular network is essential for formation and homeostasis of bone, supporting osteogenic, chondrogenic and mesenchymal stem cells required for bone repair⁴.

The transplantation of living allogeneic bone, a form of vascularized composite tissue allotransplantation (bone VCA), performed with microsurgical anastomosis of its nutrient pedicle, may represent a future reconstructive alternative. Like cryopreserved allogeneic bone, immediate stability is provided by closely matching bone defect morphology. Like autogenous vascularized graft, it provides the enhanced healing and remodeling of living bone tissue. The obstacle in any allotransplant procedure remains the need of long-term-immunosuppression (IS). The problem is more acute in musculoskeletal tissues, which require drug doses 2-3 times greater than organ transplants⁵. Concomitant risks including organ toxicity, malignancy, infection or development of graft-versus-host disease are difficult to justify in these nonlife-critical-applications⁶. However, episodes of acute and chronic rejection remain a major issue with current long-term IS⁷. Ongoing effort to closely match histocompatibility antigens, induce donor-specific tolerance and/or improve drug immunotherapy have not as yet routinely succeeded in permitting clinical drug-free tissue survival^{8,9}.

We have previously demonstrated the means to maintain bone VCA viability and enhance bone remodeling in small animal models by promotion of a new autogenous circulation within transplanted bone. This is done by the additional use of surgical angiogenesis from implanted autogenous tissue^{10,11,12}. Allogeneic bone segments are transplanted microsurgically with anastomosis of the nutrient bone segment pedicle. In addition host-derived vessels are implanted into the medullary canal of the allogeneic vascularized bone segment. During this 2-week process, patency of the allogeneic nutrient vessel is maintained with drug immunosuppression. After IS-withdrawal, the nutrient pedicle will eventually thrombose¹³. The new capillary bed, based on the host-derived vessels provides sufficient circulation to maintain tissue viability. Bone healing and remodeling are enhanced since osteogenesis and angiogenesis are coupled^{10,11,12}. No further immunotherapy is required and bone viability is maintained long-term despite an immunologically competent host and absence of donor-specific tolerance.

Translation of this novel method of bone allotransplantation into clinical practice should best be preceded by further study of healing, mechanical properties and immunology in a large animal model. The porcine model is ideal for such VCA research^{14,15,16}. Miniature swine are comparable in size and anatomy to man, allowing skeletal reconstruction using essentially identical surgical implants and techniques. Swine immunology is well defined, including swine leukocyte antigen (SLA) haplotypes and blood types, necessary for transplant surgery. Cell lineage studies are possible with sex-mismatched transplantation, as are detailed analyses of immune responses^{17,18,19,20,21}.

Here, we describe a bone VCA allotransplantation model in the Yucatan miniature swine, suitable for study of segmental bone loss and reconstruction. This model can be used to investigate the interplay of surgical angiogenesis and short-term IS on bone VCA survival and function, including osteocyte lineage, bone blood flow, healing and remodeling capacities, alloresponsiveness and biomechanics as well as to test other innovative immune modulatory strategies.

Protocol

The study was approved by the Institutional Animal Care and Use Committee (IACUC) at Mayo Clinic Rochester. Yucatan miniature swine were serving as both donors and recipients during this surgical VCA procedure. Pairing of donor and recipient was based upon DNA sequence swine leukocyte antigen (SLA) haplotyping to ensure a major mismatch in the SLAs^{22,23}. Animals were age- and weight-matched and of identical blood type. Two surgical teams simultaneously harvested a porcine tibial bone segment with its nutrient vessel from the donor and prepared the recipient to receive the orthopically-placed allogeneic tibial bone segment. Simultaneously with microvascular repair of the bone nutrient vessel, a recipient-derived arteriovenous bundle was placed within the tibial segment for autogenous angiogenesis.

1. Presurgical Preparations

1. Fast Yucatan miniature swine the day prior to the procedure and weigh them for controlled drug administration.
2. Sedate animals with Xylazine (2 mg/kg) and the combination of tiletamine HCL and zolazepam HCL (5 mg/kg), administered subcutaneously.
3. Place a peripheral catheter in an ear vein for intravenous drug and saline delivery and administer buprenorphine (0.18 mg/kg) and prophylactic antibiotics (1 g cefazoline intravenous and 5 mg/kg ceftiofur intramuscular).
4. Shave the right hindlimb and the left neck that will serve as harvest site for the vascularized tibial bone segment and site for the placement of the central venous catheter, respectively.
5. Check vital signs and the level of sedation by testing the relaxation of mouth muscles.
6. Intubate the animal with an appropriately sized endotracheal tube in sternal recumbency²⁴.
7. Transfer the miniature swine to the operation table and connect to a ventilator machine for maintenance of anesthesia by administering isoflurane (1-3%).
8. Confirm the anesthetic depth by testing palpebral, pupillary light and corneal reflexes.
9. Monitor oxygen saturation with a pulse oximeter transmission probe attached to the ear. Use a blood pressure cuff and temperature probe for intraoperative vital sign monitoring.
10. Place Yucatan miniature swine in a supine position on a warming pad. In addition, use a forced-air warming blanket during the operation to prevent hypothermia.
11. Use vet ointment on eyes to prevent dryness while under anesthesia.

2. Harvest of a Vascularized Tibial Bone Segment

1. Wash the right leg of each miniature swine with povidone-iodine solution. Dry the skin with a sterile towel and drape the extremity in a sterile fashion. Envelop and isolate the limb with an iodine impregnated adhesive incision drape to minimize the risk of contamination.
2. Perform an incision with a scalpel anterolaterally in the hindlimb, beginning at the knee joint, extending distally along the anterior ridge of the tibia to the tibiotalar joint.
3. Dissect the skin and the subcutaneous tissue with scissors and retract the anterior compartment muscles from the tibia laterally.
NOTE: Release of the tibialis anterior muscle origin facilitates exposure. The interosseous membrane is now exposed.
4. Identify the cranial tibial artery and vein (to be later used as the arteriovenous bundle for the surgical angiogenesis).
NOTE: The cranial tibial artery and vein lie on the anterior surface of the interosseous membrane.
5. To improve the operative field of view, release a part from the tibial anterior muscle from its insertion and remove a part of the tibial ridge by using an oscillating saw.
6. Protecting the cranial tibial vessels, incise the interosseous membrane beginning at the level of the tibial tubercle with a scissor.
7. Visualize the caudal tibial vessels, running distally beneath the membrane.
NOTE: They branch from the cranial tibial vessels and give rise to the nutrient pedicle of the tibial diaphysis just distal to the tubercle. It is now possible to visualize the nutrient foramen and vessels entering the tibia on its posterior lateral surface just distal to the tibial tubercle.
8. Tag the nutrient pedicle with a microclamp. Do not detach the vascular pedicle.
9. Identify a muscle branch in the tibial anterior compartment near the nutrient foramen; this may be used for the anastomosis to the vascularized bone allotransplant nutrient vessel. Mark the muscle branch with a microclamp.
10. **Harvest of a 3.5 cm tibial bone segment including the vascular pedicle.**

1. Use a cutting jig to ensure a precise and reproducible bone resection. Position and fix the cutting jig on the medial surface of the tibia to include the nutrient foramen and vessels.
 1. Guided by the jig, perform parallel bone cuts with an oscillating saw to remove a 3.5 cm tibial segment. Use the same positioning and jig for both donor and recipient animals to maximize size- and shape-match.
11. Once both cuts have been made with the oscillating saw, rotate the tibial bone segment to visualize the nutrient pedicle on the posterior surface. Divide the nutrient pedicle at its origin from the cranial tibial artery with scissors. Dissect and free the tibial segment with scissors, leaving a thin cuff of periosteum and muscle on its surface.
12. Retract the tibial bone segment and elevate the tibial bone segment with its vascular pedicle with a sharp clamp, leaving the cranial tibial artery in place.

NOTE: The vascularized bone segment is now ready for microvascular transfer and a 3.5 cm tibial bone defect has been created in each Yucatan miniature swine.
13. Ligate the cranial tibial vessels at the ankle with absorbable polyglactin 3-0 sutures, freeing them with a cuff of perivascular tissue to create an arteriovenous (AV) bundle. Leave the sutures at least 5 cm long to ease the implantation into the tibial bone segment.

3. Orthotopic Tibial Bone VCA Reconstruction in Combination with Surgical Angiogenesis

1. **Exchange the harvested tibial bone segments with its nutrient pedicles between the two animals to use them as bone VCAs.**
 1. To allow passage of the cranial tibial arteriovenous (AV) bundle into the tibial bone segment, remove the V shaped segment from the proximal junction site using the oscillating saw.
 2. Drill a hole of 0.5 cm diameter in the distal part of the tibial bone defect site and into the medullary canal of the tibial bone segment and introduce the recipient AV bundle that has been ligated distally, into the intramedullary canal to promote subsequent autogenous new blood supply.
2. **Place the vascularized tibial bone segment orthotopically into the recipient defect.**
 1. Anastomose the nutrient pedicle of the tibial bone segment to the prepared muscle branch of the tibial anterior compartment in an end-to-end manner using the simple interrupted suture technique and 9-0 sutures²⁵.
3. Confirm patency of the microvascular anastomosis using the milking test²⁶.
4. **Achieve osteosynthesis by using a 9-hole 3.5 mm locking plate.**
 1. Place the 9-hole plate on the tibia anteromedially. Fix the plate with three bicortical screws above and below the tibial bone segment. Additionally, place unicortical screws in the tibial bone segment for internal fixation. To confirm correct positioning of the bone VCA and plate, use anteroposterior and lateral radiographs.
5. Perform fascial and layered skin closure using interrupted 3-0 and 2-0 absorbable sutures. Finally seal the wound with an occlusive transparent dressing.

4. Central Venous Catheter Placement in Jugular External Vein

1. **For postoperative drug administration and immunosuppressive (IS) drug level monitoring, place a venous catheter into the external jugular vein using an open technique. Perform the placement at the conclusion of the allotransplantation procedure under anesthesia (see section 1).**
 1. Perform an anterolateral incision in the neck with a scalpel. Dissect the subcutaneous tissue with scissors and expose the left jugular vein.
 2. Place a Hickman catheter into the jugular vein through a small hole in the external jugular vein and secure it with non-absorbable sutures. Exteriorize the catheter in the back by tunneling subcutaneously.
 3. Secure the catheter in place to the skin and close the neck in layers using interrupted 3-0 and 2-0 absorbable sutures.
 4. Place occlusive bandages over the incision. Use a fishnet bandage to hold the bandages and the catheter in place.

5. Postoperative Treatment and Follow Up

1. Immediately after the operation, treat the Yucatan miniature swine with an intramuscular injection of carprofen (4 mg/kg) for postoperative analgesia. Administer buprenorphine (0.18 mg/kg) to treat pain of high intensity as needed.
2. Allow the pig to recover for 60 min and then return the pig to a special intensive care unit pan and monitor closely until complete recovery.
3. Move the Yucatan miniature swine to a normal cage and provide ad libitum access to water and food.
4. Administer tacrolimus (0.8-1.5 mg/kg/day) and mycophenolate mofetil (MMF) (50-70 mg/kg/day) orally and methylprednisolone sodium succinate intravenously (starting with 500 mg/day) for two weeks.
5. Adjust daily doses of immunosuppressive drugs according to trough blood levels, aiming for 5.0-30.0 ng/mL for tacrolimus and 1.0-3.5 µg/mL for MMF, respectively. Reduce the dose of methylprednisolone gradually until the maintenance dose of 50 mg per day is reached.
6. Administer prophylactic antibiotics gentamicin (3 mg/kg intravenously) and ceftiofur (5 mg/kg intramuscularly) for two weeks.

Representative Results

The described technique was successfully performed in four SLA major mismatched Yucatan miniature swine and segmental tibial defects reconstructed using size-matched tibial VCA. Simultaneous nutrient vessel repair of the bone allotransplant and implantation of an AV bundle from the recipient animal within the allotransplant medullary canal permitted both immediate bone circulation and development of a new autogenous blood supply over time (**Figure 1**). At 16 weeks a neoangiogenic circulation had been established within all tibial VCAs, visualized by Micro-computed tomographic (micro-CT) angiography after injection of a radiopaque angiographic polymer (125 ml) into the femoral vessels and decalcification of the tibial VCA (**Figure 2**).

Figure 1: Orthotopic tibial bone VCA procedure. Diagram showing the surgical procedure. **(A)** Donor procedure: Harvest of a tibial bone segment with its nutrient pedicle. **(B)** Exchange of the tibial bone segments between major SLA mismatched pigs. **(C)** Recipient procedure: Arteriovenous bundle implantation: cranial tibial vessels are carefully inserted into the medullary canal. **(D)** Microvascular anastomosis of the nutrient pedicle to the muscle branch of the anterior tibial compartment and plate osteosynthesis of the tibial diaphysis. Used with permission of Mayo Foundation for Medical Education and Research. All rights reserved. [Please click here to view a larger version of this figure.](#)

Figure 2: Representative 3D micro-CT angiographic image of a decalcified tibial VCA segment. The neovascularized circulation (yellow arrow) is depicted after perfusion with a radiopaque silicon solution. [Please click here to view a larger version of this figure.](#)

Miniature swine showed no signs of distress or self-mutilation. All wounds healed without infection and animals ambulated normally, ultimately able to bear full weight on the operated right limb from the first postoperative day on. At the study endpoint at 16th weeks all Yucatan miniature swine had gained over 150% of their original body weight (pretransplant: 56.0 ± 6.1 versus 16 weeks posttransplant: 84.5 ± 6.0).

Two weeks of immunosuppression, consisting of tacrolimus, mycophenolate mofetil (MMF) and methylprednisolone succinate were used to maintain blood flow through the nutrient pedicle until a new autogenous blood supply had been established within the allogeneic bone allotransplant. During the 2 week immunosuppression periodic blood samples were taken from the jugular catheter to assess drug blood levels. Doses were adjusted to maintain trough blood levels of 5-30 ng/ml for tacrolimus and 1-3.5 $\mu\text{g/ml}$ for MMF (**Table 1**). No drug related complications occurred and aimed trough levels for tacrolimus and mycophenolate mofetil could be achieved (**Figure 3** and **Figure 4**).

Immunosuppressant	Initial dose	Trough levels	Maintenance dose
Tacrolimus	0.8-1.5 mg/kg/day	5-30 ng/ml	
Mycophenolate Mofetil	50-70 mg/kg/day	1-3 $\mu\text{g/ml}$	
Methylprednisolone sodium succinate	500 mg		50 mg

Table 1: Short term immunosuppression protocol. Depicted is the immunosuppressive protocol for the first 2 weeks post transplantation with the starting dose for Tacrolimus, Mycophenolate mofetil and Prednisolone. Additionally aimed trough levels for Tacrolimus and Mycophenolate mofetil and the maintenance dose of Prednisolone are shown.

Figure 3: Trough blood levels for Tacrolimus. The median and interquartile range of the achieved trough levels for Tacrolimus over the first 2 weeks post transplantation are depicted. Error bars denote the interquartile range. [Please click here to view a larger version of this figure.](#)

Figure 4: Trough blood levels for Mycophenolate mofetil. The median and interquartile range of trough blood levels of Mycophenolate mofetil over the short-term immunosuppression period of 2 weeks are shown. Error bars denote the interquartile range. [Please click here to view a larger version of this figure.](#)

Despite cessation of immunosuppression after two weeks, periodic radiologic evaluation at different time points (2, 4, 6, 10 and 16 weeks) of the operated right hind limb with x-rays revealed progressive bone healing over the study period of 16 weeks when graded by two independent and blinded observers (**Figure 5**)^{27,28}. Micro-CT analysis at 16th weeks was used to quantify both volume and density of callus, as well as bridging bone formation at the host/bone VCA junctions and bone VCA allotransplant appearance²⁷. Maintenance of internal fixation without loss of reduction or losing, promoted by the new autogenous blood supply, could be demonstrated²⁸. Osseous union was achieved in all tibias (**Figure 6**).

Figure 5: Bone healing progression over 16 weeks. To define the bone healing progression a nonlinear regression model was used. The value R^2 was used to define the fit of the model to the data. Using a scoring system, based on anteroposterior and lateral radiographs the osseointegration of the bone VCA into the segmental bone defect was scored with a maximum value of 25 points at different time points over the study period (2, 4, 6, 10 and 16 weeks) by two independent and blinded observers^{30,31}. The nonlinear regression model depicts the median and interquartile range for the bone healing values over the study period ($R^2 = 0.931$) showing a continuous bone healing progression approximating the value of 25 at 16 weeks. Error bars denote the interquartile range. [Please click here to view a larger version of this figure.](#)

Figure 6: Three-dimensional reconstruction of the porcine tibial diaphysis after micro-computed tomographic evaluation. Representative three-dimensional computed image of the reconstructed tibia with internal plate fixation at 2x actual size. At 16 weeks complete union after tibial bone VCAs with surgical angiogenesis is shown. [Please click here to view a larger version of this figure.](#)

Histological analysis on hematoxylin-eosin undecalcified stained sections, using a previously described scale grading rejection (none, mild, moderate and severe) revealed no signs of severe rejection, whereby mild and moderate signs of rejection could be demonstrated in three pigs (Figure 7)²⁹.

Figure 7: Representative image of a horizontal hematoxylin-eosin stained section from a tibial VCA. Radiopaque silicon solution-filled vessels are displayed brown (asterisk). Mild endosteal infiltration and reaction (thick arrow) is seen with over two thirds of the lacunas filled with osteocytes (small arrow) in accordance with viable bone. 10X magnification. Scale bar = 300 μ m. [Please click here to view a larger version of this figure.](#)

Discussion

The transplantation of vascularized allogeneic bone (bone VCA) may represent a future reconstructive option for large segmental osseous defects. However, the need of long-term-immunosuppression (IS) and its significant side effects required for bone VCA survival are difficult to justify in these nonlife-critical-applications⁶.

Although inbred strains of the laboratory rat have been used extensively in allotransplantation research to test various approaches for avoidance of long-term-immunosuppression, porcine models may provide significant advantages^{8,9}. The Yucatan mini pig is ideal for study of the complex process of bone VCA rejection. Physiologically, the new bone formation rate is comparable to man (pigs 1.2-1.5 μ m per day; humans 1.0-1.5 μ m per day respectively)³². Anatomic similarities enable the use of orthotopic bone reconstruction using essentially identical surgical implants and techniques. Perhaps most importantly, the well-defined porcine alloresponse -made possible by progress in porcine cytokine detection and development of anti-porcine cluster of differentiation antibodies- makes this and other VCA studies more rigorous³³.

As in any similar clinical application, the method of porcine tibial bone defect reconstruction using bone VCA is technically demanding, requiring a two team approach with sufficient surgical expertise in microvascular surgery and bone reconstruction in order to achieve reproducible results. Strict maintenance of sterile intraoperative conditions and perioperative antibiotic prophylaxis are obligatory to decrease the risk of infectious complications.

In previous studies using rats and rabbits short-term IS maintained viability of vascularized bone allotransplants in the first 2 weeks through perfusion of the bone VCA through its allogeneic nutrient vessel. After immunosuppression withdrawal recipient derived vessels within the medullary canal provided neovascularization allowing long-term bone VCA healing and viability^{10,11,12}. At the study endpoint, substantial allotransplant chimerism could be detected^{34,35,36}. We have moved forward and applied our rat and rabbit well-established methodology on the porcine model. This model is feasible to test a new means to maintain tissue viability without long-term IS in bone VCA research, using surgical angiogenesis from implanted autogenous vessels combined with short-term IS, effectively switching the bone circulation from allogeneic to autogenous vessels.

A major advantage of this model over other existing porcine bone containing VCA models is its orthotopic design enabling functional evaluation of weight bearing and assessment of mechanical properties, data which are especially sparse^{14,37}. The complex mechanism of local and systemic bone VCA rejection can easily be monitored through radiologic and histologic evaluation of the allotransplanted tibial bone segment as well as molecular biologic analysis of the peripheral blood. Ultimately the low morbidity of the surgical bone VCA procedure enables long-term bone VCA survival and analysis.

Stable internal fixation, proper allogeneic tibial bone segment apposition and limb alignment are crucial to allow ambulation of the pigs on the first postoperative day and require careful presurgical planning. The method we have selected using a special designed cutting jig for precise and reproducible bone resection combined with plate osteosynthesis is sufficiently stable for permitting rigid fixation in allotransplants, even in those with minimal size mismatches.

One limitation of the presented technique is that it does not allow assessment of different tissue components as skin and muscle besides the vascularized bone component. While a composite flap including different tissue components is possible, this model has been designed to study exclusive bone allotransplantation as the immunogenicity of various VCA tissue components varies³⁸.

In conclusion, this article provides information for establishing a reproducible large animal model with defined genetics for bone VCA research. This model may serve as basis for future studies investigating the influence of surgical angiogenesis on bone blood flow and bone remodeling and might eliminate the need of long-term immunosuppression. Furthermore it can be used to delineate the complex process of bone VCA rejection and test other innovative immune modulatory strategies. Defined SLA-haplotypes and quantification of SRY-genes in sex-mismatched pigs may allow determination of the extent of chimerism of the allotransplant and peripheral blood.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

The authors thank the Division of Media Support Services, Mayo Clinic Rochester, MN for video production as well as Georgios Kotsougianis for editing of the video. The excellent artwork was conducted by Jim Postier, Rochester, MN. Additionally, the authors wish to thank the German research foundation (Deutsche Forschungsgemeinschaft) for providing salary support for Dr. Dimitra Kotsougiani (DFG grant: KO 4903/1-1). This work was supported by a generous gift from Tarek E. Obaid. This work was performed in the Microvascular Research Laboratory, Department of Orthopedic Surgery Mayo Clinic Rochester, MN.

References

1. Ham, S. J. *et al.* Limb salvage surgery for primary bone sarcoma of the lower extremities: long-term consequences of endoprosthetic reconstructions. *Ann Surg Oncol.* **5**, 423-436 (1998).
2. Niimi, R. *et al.* Usefulness of limb salvage surgery for bone and soft tissue sarcomas of the distal lower leg. *J Cancer Res Clin Oncol.* **134**, 1087-1095 (2008).
3. Tukiainen, E., Asko-Seljavaara, S. Use of the Ilizarov technique after a free microvascular muscle flap transplantation in massive trauma of the lower leg. *Clin Orthop Relat Res.* 129-134 (1993).
4. Schipani, E., Maes, C., Carmeliet, G., Semenza, G. L. Regulation of osteogenesis-angiogenesis coupling by HIFs and VEGF. *J Bone Miner Res.* **24**, 1347-1353 (2009).
5. Murray, J. E. Organ transplantation (skin, kidney, heart) and the plastic surgeon. *Plast Reconstr Surg.* **47**, 425-431 (1971).
6. Ravindra, K. V., Wu, S., McKinney, M., Xu, H., Ildstad, S. T. Composite tissue allotransplantation: current challenges. *Transplant Proc.* **41**, 3519-3528 (2009).
7. Lantieri, L. *et al.* Face transplant: long-term follow-up and results of a prospective open study. *Lancet.* **388**, 1398-1407 (2016).
8. Brent, L. B. Tolerance and its clinical significance. *World J Surg.* **24**, 787-792 (2000).
9. Utsugi, R. *et al.* Induction of transplantation tolerance with a short course of tacrolimus (FK506): I. Rapid and stable tolerance to two-haplotype fully mhc-mismatched kidney allografts in miniature swine. *Transplantation.* **71**, 1368-1379 (2001).
10. Giessler, G. A., Zobitz, M., Friedrich, P. F., Bishop, A. T. Host-derived neoangiogenesis with short-term immunosuppression allows incorporation and remodeling of vascularized diaphyseal allogeneic rabbit femur transplants. *J Orthopaedic Res.* **27**, 763-770 (2009).
11. Kremer, T. *et al.* Surgical angiogenesis with short-term immunosuppression maintains bone viability in rabbit allogeneic knee joint transplantation. *Plast Reconstr Surg.* **131**, 148e-157e (2013).
12. Larsen, M., Friedrich, P. F., Bishop, A. T. A modified vascularized whole knee joint allotransplantation model in the rat. *Microsurgery.* **30**, 557-564 (2010).
13. Ohno, T., Pelzer, M., Larsen, M., Friedrich, P. F., Bishop, A. T. Host-derived angiogenesis maintains bone blood flow after withdrawal of immunosuppression. *Microsurgery.* **27**, 657-663 (2007).
14. Ibrahim, Z. *et al.* A modified heterotopic swine hind limb transplant model for translational vascularized composite allotransplantation (VCA) research. *J Vis Exp.* (2013).

15. Solla, F. *et al.* Composite tissue allotransplantation in newborns: a swine model. *J Surg Res.* **179**, e235-243 (2013).
16. Ustuner, E. T. *et al.* Swine composite tissue allotransplant model for preclinical hand transplant studies. *Microsurgery.* **20**, 400-406 (2000).
17. Ho, C. S. *et al.* Molecular characterization of swine leucocyte antigen class II genes in outbred pig populations. *Anim Genet.* **41**, 428-432 (2010).
18. Ho, C. S. *et al.* Molecular characterization of swine leucocyte antigen class I genes in outbred pig populations. *Anim Genet.* **40**, 468-478 (2009).
19. Morin, N., Metrakos, P., Berman, K., Shen, Y., Lipman, M. L. Quantification of donor microchimerism in sex-mismatched porcine allotransplantation by competitive PCR. *BioTechniques.* **37**, 74-76, 78, 80-71 (2004).
20. van Dekken, H., Hagenbeek, A., & Bauman, J. G. Detection of host cells following sex-mismatched bone marrow transplantation by fluorescent in situ hybridization with a Y-chromosome specific probe. *Leukemia.* **3**, 724-728 (1989).
21. Leonard, D. A. *et al.* Vascularized composite allograft tolerance across MHC barriers in a large animal model. *Am J Transplant.* **14**, 343-355 (2014).
22. Smith, D. M., Martens, G. W., Ho, C. S., Asbury, J. M. DNA sequence based typing of swine leukocyte antigens in Yucatan miniature pigs. *Xenotransplantation.* **12**, 481-488 (2005).
23. Ho, C. S. *et al.* Nomenclature for factors of the SLA system, update 2008. *Tissue Antigens.* **73**, 307-315 (2009).
24. Kaiser, G. M., Heuer, M. M., Fruhauf, N. R., Kuhne, C. A., Broelsch, C. E. General handling and anesthesia for experimental surgery in pigs. *J Surg Res.* **130**, 73-79 (2006).
25. Alghoul, M. S. *et al.* From simple interrupted to complex spiral: a systematic review of various suture techniques for microvascular anastomoses. *Microsurgery.* **31**, 72-80 (2011).
26. Acland, R. Signs of patency in small vessel anastomosis. *Surgery.* **72**, 744-748 (1972).
27. Kotsougiani, D. *et al.* Recipient-derived angiogenesis with short term immunosuppression increases bone remodeling in bone vascularized composite allotransplantation: A pilot study in a swine tibial defect model. *J Orthopaedic Res.* (2016).
28. Riegger, C. *et al.* Quantitative assessment of bone defect healing by multidetector CT in a pig model. *Skeletal Radiol.* **41**, 531-537 (2012).
29. Buttemeyer, R., Jones, N. F., Min, Z., Rao, U. Rejection of the component tissues of limb allografts in rats immunosuppressed with FK-506 and cyclosporine. *Plast Reconstr Surg.* **97**, 139-148; discussion 149-151 (1996).
30. Taira, H., Moreno, J., Ripalda, P., Forriol, F. Radiological and histological analysis of cortical allografts: an experimental study in sheep femora. *Arch Orthop Trauma Surg.* **124**, 320-325 (2004).
31. Giessler, G. A., Zobitz, M., Friedrich, P. F., & Bishop, A. T. Transplantation of a vascularized rabbit femoral diaphyseal segment: mechanical and histologic properties of a new living bone transplantation model. *Microsurgery.* **28**, 291-299 (2008).
32. Laiblin, C., & Jaeschke, G. [Clinical chemistry examinations of bone and muscle metabolism under stress in the Gottingen miniature pig--an experimental study]. *Berliner und Munchener tierarztliche Wochenschrift.* **92**, 124-128 (1979).
33. Saalmuller, A. Characterization of swine leukocyte differentiation antigens. *Immunol Today.* **17**, 352-354 (1996).
34. Pelzer, M., Larsen, M., Friedrich, P. F., Aleff, R. A., Bishop, A. T. Repopulation of vascularized bone allotransplants with recipient-derived cells: detection by laser capture microdissection and real-time PCR. *J Orthopaedic Res.* **27**, 1514-1520 (2009).
35. Muramatsu, K., Kurokawa, Y., Kuriyama, R., Taguchi, T., Bishop, A. T. Gradual graft-cell repopulation with recipient cells following vascularized bone and limb allotransplantation. *Microsurgery.* **25**, 599-605 (2005).
36. Muramatsu, K., Bishop, A. T., Sunagawa, T., Valenzuela, R. G. Fate of donor cells in vascularized bone grafts: identification of systemic chimerism by the polymerase chain reaction. *Plastic and reconstructive surgery.* **111**, 763-772; discussion 773-764 (2003).
37. Vossen, M. *et al.* Bone quality and healing in a swine vascularized bone allotransplantation model using cyclosporine-based immunosuppression therapy. *Plast Reconstr Surg.* **115**, 529-538 (2005).
38. Lee, W. P. *et al.* Relative antigenicity of components of a vascularized limb allograft. *Plast Reconstr Surg.* **87**, 401-411 (1991).