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RESEARCH ARTICLE

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Bone vascularized composite allotransplantation model in swine tibial defect: Evaluation of surgical angiogenesis and transplant viability

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Introduction: In prior small animal studies, we maintained vascularized bone allotransplant viability without long-term immunotherapy. Instead, an autogenous neoangiogenic circulation is created from implanted vessels, sufficient to maintain bone viability with only 2 weeks immunosupression. Blood flow is maintained despite rejection of the allogeneic vascular pedicle thereafter. We have previously described a large animal (swine) pre-clinical model, reconstructing tibial defects with vascularized tibial allotransplants. In this manuscript, autologous angiogenesis is evaluated in this model and correlated with bone viability.

Materials and methods: Allogeneic tibial segments were transplanted across a major swine leukocyte antigen mismatch. Microvascular repair of the bone VCA pedicle was combined with intraosseous implantation of an autogenous arteriovenous (AV) bundle. The bundle was ligated in group 1 ($n = 4$), and allowed to perfuse in group 2 ($n = 4$). Three-drug immunotherapy was given for 2 weeks. At 16 weeks micro-CT angiography quantified neoangiogenic vessel volume. Bone viability, rejection grade, and bone healing were analyzed.

Results: A substantial neoangiogenic circulation developed from the implanted AV-bundle in group 2, with vessel density superior to ligated AV-bundle controls (0.11 ± 0.05 vs. 0.01 ± 0.01, $P = .029$). Bone allotransplant viability was also significantly enhanced by neoangiogenesis ($78.7 \pm 4.4\%$ vs. $27.7 \pm 5.8\%$, $P = .028$) with higher bone healing scores (21.4 ± 2.9 vs. 12.5 ± 3.7, $P = .029$). Ligated control tibias demonstrated disorganized bone morphology and higher local inflammation ($P = .143$).

Conclusion: Implantation of autogenous AV bundles into vascularized bone allotransplants resulted in the rapid formation of a neoangiogenic autogenous blood supply in a swine tibia model that maintained bone viability, improved bone healing, and minimized rejection.

1 | INTRODUCTION

Current reconstructive options for large skeletal defects are problematic. Transplantation of living allogeneic bone potentially offers both excellent stability and preserved viability with no donor site morbidity, as seen in commonly used vascularized autogenous bone flaps (Belt, Dickinson, & Theile, 2005; Gao, Ketch, Eladoumikdachi, & Netscher, 2001). As a form of vascularized composite tissue allotransplantation, or VCA, long-term immune modulation is required to prevent rejection (Innis et al., 1991; Shores, Imbriglia, & Lee, 2011; Siemionow & Ozturk, 2011). The toxicity and complications of standard immunosuppressive drug therapy is of major concern for such non-life-critical indications. Strategies to maintain VCA viability by other means require further study (Ruiz et al., 2013). A promising alternative in small animal segmental defect models is the use of autologous surgical angiogenesis (Larsen, Pelzer, Friedrich, Wood, & Bishop, 2011). Implantation of autogenous vessels within the bone VCA at the time of transplantation results in autogenous vessel angiogenesis, replacing the allogeneic bone circulation after only 2 weeks' immunosuppression. After cessation of drug therapy the

nutrient vessels thrombose, but bone perfusion is thus maintained indefinitely by the newly created vasculature (Giessler, Zobitz, Friedrich, & Bishop, 2009; Kremer et al., 2013; Larsen, Friedrich, & Bishop, 2010).

In our previous investigation, the porcine tibia blood supply anatomy was demonstrated, and a model of segmental bone loss/reconstruction developed for study of vascularized bone allotransplantation methods in a large animal model (Kotsougiani et al., 2017). A preliminary study demonstrated successful healing, ongoing bone remodeling and excellent limb function (Kotsougiani et al., 2017).

In this second part of this report, we have evaluated the extent of intraosseous angiogenesis in tibial VCAs in groups with implanted arteriovenous (AV) bundles, and in a control VCA transplant group with a ligated AV bundle.

The aim of this study was to evaluate the efficacy of autogenous angiogenesis and short-term immunosuppression to maintain bone VCA viability and reconstruct segmental bone defects.

2 | MATERIALS AND METHODS

Eight Yucatan mini-pigs were used (Sinclair Bio Resources, LLC, Missouri), paired such that each had a major SLA mismatch. Each pair was operated upon simultaneously serving as donor and a recipient of a bone VCA. All experiments were performed under the direction of the Institutional Animal Care and Use Committee and performed according to established National Institutes of Health guidelines.

2.1 | Operative procedure

In each of 4 surgical procedures, a pair of swine was anesthetized simultaneously on adjacent operating tables. Xylazine and Telazol were used for induction, maintaining anesthesia with Isoflurane. The right hind limb was used. Two surgical teams harvested 3.5 cm proximal vascularized tibial diaphyseal segments as described previously, to include the constant nutrient artery from the caudal interosseous vessels (Kotsougiani et al., 2017). The harvested bones were exchanged to reconstruct each tibial defect with a bone VCA. Bone perfusion was restored by anastomosis of the nutrient pedicle to an adjacent muscular branch supplying the anterior compartment muscles of the hindlimb. In addition autogenous vessels from the recipient lower limb (cranial tibial vessels) were mobilized and next implanted within the medullary canal of the bone VCA proximal of the anastomosis (Figure 1) using our published method to induce surgical angiogenesis (Kotsougiani et al., 2017). Stabilization was achieved by plate osteosynthesis (DePuy Synthes Vet, West Chester, Pennsylvania). Each pig was assigned randomly to either a control or an experimental group. The AV bundle was ligated in control group 1, and remained patent in experimental group 2.

To inhibit rejection and ensure adequate initial perfusion of the allogeneic tibial segment, immunosuppression was administered postoperatively for 2 weeks. The immunosuppressive protocol used was based on the experience in current human hand and face transplantation and a porcine intestinal transplantation model (Gruessner et al., 2004; Hautz et al., 2010). A three-drug therapy was used, consisting of tacrolimus (FK 506) (Sandoz Inc., Princeton, New Jersey), mycophenolate mofetil (MMF) (Sandoz Inc., Princeton, New Jersey) and methylprednisolone (Pfizer Inc., New York, New York). The initial drug dose given for FK 506 was 0.8–1.5 mg/kg and for MMF 50–70 mg/kg daily. Blood trough levels, checked every 2–3 days for a total of 2 weeks, were used to adjust the immunosuppressive therapy aiming for 5–30 ng/mL for FK 506 and 1–3.5 μg/mL for MMF (Gruessner et al., 2004). Methylprednisolone was reduced gradually starting with 500 mg/day (Table 1).

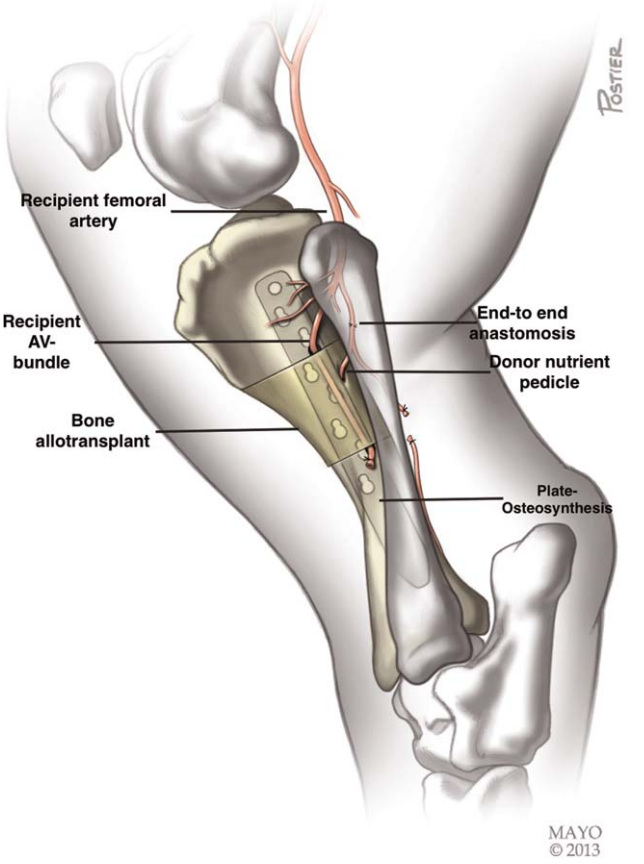

FIGURE 1 Drawing of the surgical technique. A tibial allogeneic bone transplant with its nutrient pedicle was used to reconstruct a porcine tibial defect in a recipient animal. Vascular anastomosis was performed to a branch of the recipient femoral artery in an end-to-end fashion. The cranial tibial artery and venae comitantes were ligated distally and raised as an AV bundle, implanted within the allogeneic bone transplant. Fixation was performed with plate-osteosynthesis

TABLE 1 Group distribution

AV-bundle	Group 1 Ligated	Group 2 Patent
Immunosuppression (weeks)	2	2
Survival time (weeks)	16	16
n	4	4

AV, arteriovenous.

2.2 | Postoperative treatment

All Yucatan mini-pigs were maintained in single-animal pens and allowed immediate weight bearing as tolerated with food and water ad libitum. Postoperative infection prophylaxis consisted of an aminoglycoside in combination with a third generation cephalosporin. The experiment was terminated after 16 weeks.

2.3 | X-ray evaluation of segmental bone defect healing

Radiographs were taken at 2, 4, 6, 10, and 16 weeks (Min-R 2000 Film, Eastman Kodak, Rochester, New York) at 80 kV and 5 mAs in two different oblique views. Osseous healing as well as allotransplant appearance was graded at each time point by two independent and blinded observers (Giessler, Zobitz, Friedrich, & Bishop, 2008; Taira, Moreno, Ripalda, & Forriol, 2004).

2.4 | Microangiography

After a 16-week survival period, animals were euthanized with pentobarbital 100 mg/kg IV, (Fatal-Plus, Vortech Pharmaceuticals, Dearborn, Michigan). The femoral vessels were cannulated and irrigated with 500 mL heparinized saline, followed by 125 mL of a yellow radiopaque angiographic polymer (MICROFIL MV-122, Flow Tech, Carver, Massachusetts). Sufficient perfusion was confirmed visually by a yellow color change in the hoof and femoral vein.

2.5 | Micro-CT angiography

Two cm long samples from the central portion of the VCA were fixed in 10% buffered formalin for 48 h and decalcified using Decalcifying Solution (Thermo Fisher Scientific, Chesire, WA, UK) over a period of 5–6 weeks until complete, based upon X-ray imaging. Intraosseous vessels were imaged by micro-CT scanning (Inveon PET CT, Siemens Medical Solutions USA, Inc., Marvern, Pennsylvania). Using image analysis software (PMOD Technologies, Zurich, Switzerland) vessel volume within the allogeneic tibial bone segment was measured. Vessel volume was assessed by an independent observer, blinded for the AV bundle patency. Grey-scale thresholding was used to differentiate between Microfil perfused blood vessels and bone, on a series of adjacent slices, each with a slice thickness of 0.027 cm, to build a 3D image (Figure 2).

2.6 | Histological grading

Another segment of the tibial VCA was dehydrated in graded ethanol and embedded in glycol methylmethacrylate. Five-μm thick horizontal sections were cut with a microtome (Leica 20651, Leica Microsystems Inc., Buffalo Grove, Illinois) and stained with hematoxylin/eosin. True color digital images were obtained by using the ImageScope Aperio software (Leica Biosystems Inc., Buffalo Grove, Illinois).

Bone viability was analyzed in six randomly selected fields with 20× magnification on horizontal sections by using the osteocyte count (Kremer et al., 2013). All bone lacunae were counted, expressing viability as the ratio of osteocyte-containing lacunae to the total number of lacunae using ImageJ software (National Institutes of Health, Bethesda, Maryland). All analyses were performed by two independent and blinded investigators.

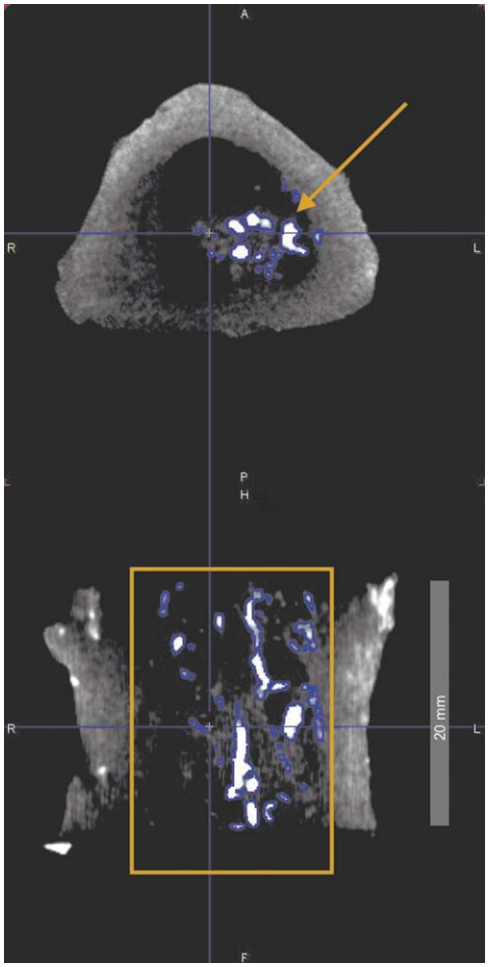

FIGURE 2 Representative illustration of the microangiographic-CT based measurement of the vessel volume in allogeneic tibial allotransplants. First contours were drawn around the bone segment and fluorescent blood vessels were detected by gray/scale thresholding semiautomatically (blue lines) marking the first region of interest (ROI) on a horizontal section (arrow). Next the ROI was transferred semiautomatically to neighboring slices including the complete allogeneic bone specimen to calculate the volume of interest (VOI), representing the vessel volume within the allogeneic bone segment (rectangle)

Bone rejection was graded using a 4 point scale previously described, ranging from no immune response (grade 0) to mild (grade 1), moderate (grade 2), and severe (grade 3) rejection (Buttemeyer, Jones, Min, & Rao, 1996; Larsen et al., 2010).

The patency of the implanted AV bundle was analyzed by presence/absence of Microfil® contrast on histologic sections.

2.7 | Statistical methods

A nonparametric test (Mann–Whitney *U* test) was used to compare groups for bone viability, vessel volume and bone healing scores (BHS), as the data were not normally distributed. The paired Wilcoxon signed-

TABLE 2 Results

Animal	AV-bundle patency	Vessel volume (mm^3) A: Allotransplant C: Contralateral	Bone viability allotransplant (%)	Bone healing score at 16 weeks (out of 25 possible points)
Group 1	Thrombosed	A: 0.01 ± 0.01 C: 0.03 ± 0.02	27.7 ± 5.8	12.5 ± 3.7
Group 2	Patent	A: 0.11 ± 0.05 C: 0.01 ± 0.01	78.7 ± 4.4	21.4 ± 2.9

Values are means and standard deviations.
AV, arteriovenous.

rank test was used for microangiographic-CT analysis of treated and contralateral tibias of each animal. Histological rejection was compared with the Fisher exact test for ordered contingency tables. All statistical tests were two-sided with P values of $<.05$ considered significant. Results are reported as percentage of the total or the mean and standard deviation (SD). Data in this manuscript were analyzed using GraphPadPrism™ Version 5.0 (Graphpad software, La Jolla, California).

3 | RESULTS

All Yucatan mini-pigs recovered from surgery and remained healthy during the experiment. Ambulation on all four limbs was demonstrable from the first postoperative day in all animals. No infections occurred. No adverse effects occurred with the short-term use of immunosuppressive medication. Results are shown in Table 2.

Microangiography verified patency of all group 2 AV bundles, as well as the development of a neoangiogenic circulation from the implanted vessels penetrating endosteal bone (Figure 3). Mean intraosseous vessel volumes of group 2 allotransplants were statistically higher in comparison to group 1 (0.11 ± 0.05 vs. 0.01 ± 0.01, $P = .029$). Comparison with the opposite side (undisturbed tibia) was not significant in either group (0.01 ± 0.01 vs. 0.03 ± 0.02, $P = .25$). However, mean vessel volumes of group 2 allotransplants showed a trend toward higher values as compared to undisturbed tibia from the contralateral side (0.11 ± 0.05 vs. 0.01 ± 0.01, $P = .125$).

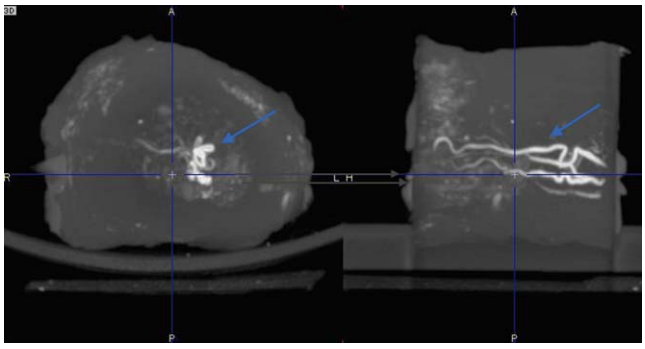

FIGURE 3 Representative, reconstructed 3D microangiographic-CT image of an allogeneic tibial bone segment from group 2. The bone vasculature (blue arrow) was depicted after perfusion with a fluorescent silicone solution (Microfil)

Allotransplants in group 2 had higher osteocyte counts than controls on their endosteal surfaces ($P = .028$). The mean osteocyte viability ratio was 78.7 ± 4.4% in group 2 as compared to 27.7 ± 5.8% in group 1. Group 1 bone morphology was markedly disorganized with local inflammation, rated mild in 1, moderate in 1, and severe in 2 animals. Rejection grading in group 2 was rated as none in 1, mild in 2, and moderate in 1. These differences were not significant ($P = .143$) (Figure 4A,B).

The combination of locked plate osteosynthesis and matched bone VCA interposition maintained limb alignment and function in all animals. One fracture occurred at 16 weeks in a group 2 tibia. Progression of bone healing was noted in both groups. However, mean BHS were significantly higher in group 2 at 16 weeks (21.4 ± 2.9 vs. 12.5 ± 3.7 of 25 total possible points, $P = .029$) (Figure 5).

4 | DISCUSSION

Transplantation of living allogeneic bone has seldom been performed clinically. Hofmann and colleagues transplanted three femoral segments and six whole knee joints for tumor, infection-related or traumatic loss of bone (Hofmann et al., 1998; Hofmann, Kirschner, Gonschorek, & Buhren, 1998; Hofmann, Kirschner, Gonschorek, & Buhren, 1999). All femoral nutrient vessels occluded, associated with infection in one requiring transplant removal (Kirschner et al., 1998). Long-term immunosuppression was insufficient to maintain VCA viability, although the two remaining femora provided a functional reconstruction. One later required a total knee arthroplasty complicated by postoperative cytomegalovirus infection. Doi et al. treated a child with congenital tibial pseudarthrosis with the mothers' fibula as a bone VCA. Early pedicle thrombosis occurred despite continuous drug immunosuppression (Doi, Kawai, & Shigetomi, 1996). The non-viable fibular segment healed 12 months later but required a corrective osteotomy for angular deformity 6 years later, presumably the result of stress fracture. New and innovative therapeutic concepts are required if bone VCA is to be used in the future.

Surgical angiogenesis, while not a new concept has never been used clinically for allotransplantation purposes. Hori et al. implanted veins, arteries and AV bundles into intact bone and isolated bone segments in a canine model. In all cases, active proliferation of new blood vessels and formation of new bone occurred (Hori et al., 1979). AV bundles have been used clinically to revascularize necrotic bone and

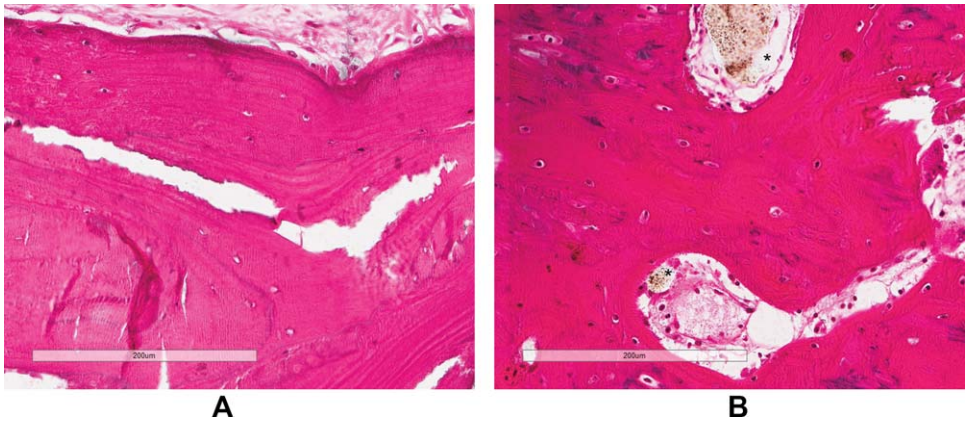

FIGURE 4 (A, B) Horizontal, hematoxylin–eosin stained sections of tibial allotransplants (20× magnification, scale bar = 200 μm). Microfil-filled vessels were displayed brown (asterisk). (A) Representative sample from group 1 allotransplants with altered bone morphology, irregular cortical bone, locally nonviable woven bone and less than one-third osteocyte containing lacunae. (B) Representative sample from group 2 allotransplants in which more than two-thirds of the lacunae were filled with normal osteocytes

engineer vascularized bone grafts (Fan et al., 2014; Kneser et al., 2006; Shin, Bishop, & Berger, 1998).

We have previously reported successful maintenance of bone VCA viability in rat and rabbit femora by development of a neoangiogenic autogenous blood supply, requiring only 2 weeks of immunosuppression (Giessler et al., 2009; Kremer et al., 2013; Larsen et al., 2010, 2011). Allotransplant viability and osseointegration was enabled by this neoangiogenic circulation, although thrombosis of the nutrient bone VCA pedicle occurred after cessation of the short-term IS. Survival was not the result of donor-specific tolerance (Larsen et al., 2011). With long-term immunosuppression, bone viability results in seeding (microchimerism) of allogeneic cells, detected in spleen, thymus, peripheral blood and bone marrow (Muramatsu, Kurokawa, Kuriyama, Taguchi, & Bishop, 2005; Muramatsu, Valenzuela, & Bishop, 2003). This may well be less with only short-term IS, reducing the risk of graft-versus-host disease. Repopulation of the bone VCA with autogenous cells occurs in either instance (Pelzer, Larsen, Friedrich, Aleff, & Bishop, 2009). Using laser-capture microdissection, we have demonstrated these autogenous cells particularly in areas of new bone formation (Pelzer et al., 2009). These small animal findings are encouraging, but might not be applicable in the clinical setting. The need to further test efficacy in a larger animal model was the motivation for this experiment. We have been able to reconstruct segmental defects of the porcine tibia with bone VCAs without the need of long-term IS by the use of surgical angiogenesis. Bone formation and remodeling increased without adverse mechanical effects (Kotsougiani et al., 2017).

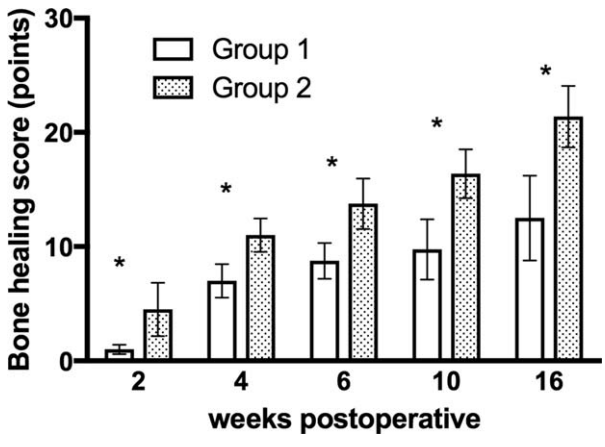

FIGURE 5 Bar chart of the bone healing scores at 2, 4, 6, 10, and 16 weeks postoperative. The mean values and standard deviations were depicted. The asterisks represent significant results between group 1 with a ligated AV bundle and group 2 with a patent AV-bundle

New bone formation during fracture healing depends upon angiogenesis, allowing subsequent migration of osteoprogenitor cells. A similar process occurs adjacent to the neoangiogenic circulation within bone allotransplants (Glowacki, 1998; Maes et al., 2010; Schipani, Maes, Carmeliet, & Semenza, 2009).

Vascularization in long bones is provided by periosteal and intramedullary vessels, connected through Volkmann's canals with centrifugal flow directed toward the periosteal vessels (Marenzana & Arnett, 2013). Insufficient flow leads to hypoxia, inhibiting osteogenesis and favoring osteoclasis and bone resorption (Arnett, 2010). In the current study, we demonstrated that implantation of an AV bundle and subsequent angiogenesis in the medullary canal of a vascularized allogeneic bone segment will rapidly replace the former intramedullary vascular network with autogenous vasculature. This ultimately exceeded undisturbed (contralateral) bone vessel volume. That endosteal bone viability was improved by this process is not surprising, as these vessels supply the oxygen, soluble factors, and numerous cell types needed to maintain bone VCA-viability (Coultas, Chawengsaksophak, & Rossant, 2005; Kanczler & Oreffo, 2008). We have shown the lineage of these cells to be from the recipient animal, the result of gradual replacement of allogeneic cells (Pelzer et al., 2009). This is associated with a decreased local inflammatory response in bone VCAs after AV bundle implantation. We found similar enhancement of bone healing in our previous porcine tibial bone VCA study at all time points analyzed (Kotsougiani et al., 2017). This resulted in an almost completed osseointegration of the bone VCA into the skeletal tibial defect at 16 weeks, which allowed complete weight-bearing in this orthotopic model. No adverse

biomechanical effects of angiogenesis were seen in rabbit femoral or porcine tibial VCAs, however (Giessler et al., 2008; Kotsougiani et al., 2017).

This process is certainly not complete at the 16-week time interval chosen in this study. It is likely that the autogenous vessels will continue to flow, osteosynthesis will improve, and further remodeling of the allogeneic bone will progress over time. Further study is required to analyze the influence of the implanted AV-bundle on the systemic as well as local immune response in this porcine model with a larger group size. An analysis of the transplant and systemic chimerism is required in order to understand the extent of cell trafficking. Documentation if failure to develop donor-specific tolerance has occurred at the study endpoint should be performed prior to implementation into clinics.

5 | CONCLUSION

Bone allotransplantation has proven problematic in the limited clinical experience due to acute and chronic rejection, bone necrosis and infection. Opportunistic infection and complications of sustained immunosuppressive drug therapy make current VCA transplant methods problematic in bone-only tissue. We have proposed a novel alternative transplantation method that does not require life-long drug immunosuppresion or tolerance induction. The promising results of autologous vessel implantation within long bone vascularized allotransplants reported in small animal models are affirmed in this initial large animal study.

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FINANCIAL DISCLOSURES

The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

ETHICAL REVIEW COMMITTEE STATEMENT

All animal procedures were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) at Mayo Clinic Rochester (A171213). The study was performed in accordance with the ethical standards in the 1964 Declaration of Helsinki and with relevant regulations of the US Health Insurance Portability and Accountability Act.

CONFLICT OF INTEREST

All authors declare no conflict of interest.

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