In **Chapter 2**, we describe a novel method of living bone allotransplantation combining microvascular repair of the nutrient circulation, implantation of host-derived arteriovenous (AV) bundles, and short-term immunosuppression. We hypothesized that neoangiogenesis from the implanted vessels would maintain graft viability and circulation after withdrawal of FK506 (Tacrolimus) immunosuppression. In this study, vascularized femoral allotransplantation was performed between DA and PVG rats. In addition to microsurgical pedicle anastomoses, a saphenous AV bundle from the recipient animal was implanted in the medullary space. Ninety-seven rats were randomly allocated to groups differing in immunosuppression and AV bundle patency. Implanted vessels significantly improved capillary density and bone blood flow in non-immunosuppressed, and immunosuppressed groups respectively. A lower incidence of spontaneous AV bundle thrombosis was found with FK506 treatment. More viable osteocytes were seen at 4 weeks when the AV bundle was patent.

In **Chapter 3**, the same model is used to measure bone blood flow, neoangiogenic capillary density, along with histologic analysis of osteocyte viability and degree of rejection after an 18 week survival period in thirty-eight transplant recipients. Independent variables were patency of the implanted AV bundle, and the use of 2 weeks’ FK-506 immunosuppression. Bone blood flow and capillary density were significantly greater in transiently immunosuppressed recipients with a patent AV bundle. This experimental study demonstrated that neoangiogenesis from implanted host-derived AV bundles combined with short term immunosuppression can maintain blood flow in bone allotransplants in the long term.

**Chapter 4** is a validation of this method of composite tissue allotransplantation (CTA) by comparison to isotransplants and conventional allografts, and investigates whether donor-specific tolerance has occurred. Ninety-three rats underwent femoral allotransplantation, isotransplantation or allografting. Paired comparison allowed evaluation of AV bundle effect (ligated versus patent), bone allogenicity (isogeneic rather than allogeneic transplants), and initial tissue circulation/viability (allotransplant versus allograft). Again, two weeks of immunosuppression maintained blood flow. At 18 weeks, skin grafts from donor, recipient and third-
party rats tested for immune competence and donor-specific tolerance. At 21 weeks bone circulation was quantified and new bone formation measured by quantitative histomorphometry. Final circulatory status depended upon both initial graft viability and successful neoangiogenic circulation development. Cortical blood flow was highest in allotransplants, intermediate in isografts and absent in others. Capillary proliferation and new bone formation was highest and equivalent in allo- and isografts with patent AV bundles, and less in other animals. Donor and third-party-type skin grafts were rejected, indicating immunocompetence without donor-specific tolerance.

In Chapter 5, we investigate the role of angiogenic cytokine delivery with biodegradable microspheres to enhance the process of neoangiogenesis from the implanted AV bundle. Forty-three microsurgical femoral allotransplantations were performed from DA to PVG rats. Poly(D,L-lactide-co-glycolide) microspheres loaded with buffer, basic fibroblast growth factor (FGF2), vascular endothelial growth factor (VEGF), or both were inserted intramedullarily along with a recipient-derived AV bundle. FK-506 was administered daily for 14 days, then discontinued. At 28 days, bone blood flow was measured using hydrogen washout. Microangiography, histologic and histomorphometric analysis were performed. Capillary density was greater in the FGF2+VEGF group (35.1%) than control (13.9%) (p<0.05), and a linear trend was found from control, FGF2, VEGF, to FGF2+VEGF (p<0.005). Bone formation rates were greater with VEGF (p<0.01) and FGF2+VEGF (p<0.05). VEGF or FGF2 alone increased blood flow more than when combined. Histology rejection grading was low in all grafts. Local administration of vascular and fibroblast growth factors were found to augment angiogenesis, bone formation and bone blood flow from implanted blood vessels of donor origin in vascularized bone allografts after removal of immunosuppression.

In Chapter 6, we study the long-term effects of angiogenic cytokine delivery with biodegradable microspheres on our CTA model. Again, forty-three microsurgical femoral allotransplantations were performed from DA to PVG rats and microspheres loaded with buffer, FGF2, VEGF, or both were inserted intramedullarily along with
a recipient-derived AV bundle. FK-506 was administered daily for 14 days, then discontinued. The survival time was increased to 18 weeks, when bone blood flow was measured and microangiography, histologic histomorphometric and alkaline phosphatase analysis were performed. Bone blood flow was greater in the FGF2+VEGF group than control and VEGF groups (p=0.04). Capillary density was greater in the FGF2 group than in the VEGF and FGF2+VEGF groups (p<0.05). Bone viability, histomorphometric analysis and measurement of alkaline phosphatase did not vary significantly between groups. We again found local administration of vascular and fibroblast growth factors to augment angiogenesis and bone blood flow but that bone growth was not enhanced in the long term.

In Chapter 7 we further apply the findings from our CTA studies and the studies using biodegradable microspheres to conventional allografts. This is an intermediate step to using our findings in a clinical setting. Rat femoral diaphyseal allografts were frozen at -80 °C, and heterotopically transplanted over a major histocompatibility mismatch. The saphenous AV bundle was inserted into the intramedullary canal. Growth factor was encapsulated into microspheres and inserted into the graft, allowing for a localized and sustained drug release. Forty rats were randomly allocated to four groups: I) Phosphate buffered saline, II) FGF2, III) VEGF and IV) FGF2+VEGF. At 4 weeks, angiogenesis was measured by the hydrogen washout method and by microangiography. Bone remodeling was evaluated by quantitative histomorphometry and histology. Bone blood flow was significantly higher in groups III and IV compared to control (p<0.05). Similarly, bone remodeling was higher in VEGF groups. FGF2 alone had little effect on allograft revascularization. No synergistic effect was observed with use of both cytokines. Delivered in microspheres, VEGF proved to be a potent angiogenic cytokine, increasing cortical bone blood flow and subsequent bone formation in frozen allografts.

In order to elucidate the mechanisms underlying successful composite tissue allotransplantation by modulation of immunosuppression, a new method enabling determination of lineage of selected cells in our model of vascularized bone allotransplantation is described in Chapter 8. Real-time PCR was performed on DNA
from laser capture microdissected cortical bone regions to determine the extent of chimerism in the bone allotransplants. To do so, we analyzed the relative expression ratio of the sex-determining region Y (Sry) gene, specific only for male recipient rat DNA, to the cyclophilin housekeeper gene. We found substantial transplant chimerism in cortical bone (range 77-97%). Rats without immunosuppression and with a patent AV bundle revealed significantly higher chimerism than those with immunosuppression and a ligated AV bundle. We include a description of the laser capture microdissection of selected bone regions, and the calculation of the relative expression ratio.

Chapter 9 outlines the application of our model to joint allotransplantation. Microvascular knee CTA was performed in 9 rats across a major histocompatibility barrier with both pedicle repair and implantation of host-derived AV bundles. In the control group (N=3), the pedicle was ligated. Immunosuppression was given daily. Joint mobility, weight-bearing, pedicle patency, bone blood flow and sprouting from AV bundles were assessed at three weeks. All but the non-revascularized control knees had full passive motion and full weight bearing. One nutrient pedicle thrombosed prematurely. Blood flow was measurable in transplants with patent nutrient pedicles. Implanted AV bundles produced new vascular networks on angiography. This new rat microsurgical model permits further study of joint allotransplantation. Patency of both pedicles and implanted AV bundles was maintained, laying a foundation for future studies.

The hydrogen washout method of bone blood flow measurement used in our studies is unique in that it is a modification of existing techniques that are greatly more cumbersome. In Chapter 10 the background for measurement of blood flow in both clinical and experimental settings is described. We develop on the hydrogen washout method with modifications including modern hydrogen sensors, a micromanipulator for probe placement and custom software that greatly simplifies the original technique. The hydrogen washout method requires breathing a gas mixture containing hydrogen until the level of hydrogen in the tissue reaches equilibrium. The hydrogen flow is then stopped. The rate of tissue hydrogen washout that follows is proportional to tissue blood flow. Because of technical difficulties and the risk of
explosion with hydrogen tanks, hydrogen washout has not been frequently used in the past. We describe a straightforward and safe way of using this method to measure bone blood flow in the laboratory.

Although we were using the modified hydrogen washout technique, its use in bone tissue had not yet been validated. To this end, in Chapter 11, we compare cortical bone blood flow measurements obtained by radioactive-labeled microsphere entrapment with those from hydrogen washout. Blood flow was measured in tibial cortical bone of 12 New Zealand White rabbits by radioactive microsphere entrapment and by hydrogen washout. Besides a control group (n=6), four animals were treated with systemic epinephrine (0.8 μg/kg/min) (group 2) and two with nitroprusside (100 μg/kg/min) (group 3). Furthermore, 9 femora from 7 rats were isolated on their vascular pedicles and repeated bone blood flow measurements were made with the hydrogen washout method to confirm reproducibility. An average flow of 2.3±2.0 mL/min/100 g was obtained with the microsphere method and 2.0±0.5 mL/min/100 g with the hydrogen washout method. There was a significant correlation and agreement: R²=0.97 (p < 0.01). No consistent flow variations were found with systemic vasoactive drug administration. Hydrogen washout provided reproducible results and showed high sensitivity to flow changes.

Any treatise on a new method of composite tissue allotransplantation is incomplete without an analysis of the potential risks. Chapter 12 provides a clinical example on which to base this discussion. Studies show nerve allotransplants to require immunosuppression only until end-organ connections are made, without the subsequent cessation of these drugs having an effect on functional outcome. Although the tissue is different from that of our previous studies, the mechanism is similar to the replacement of bone cells that occurs in our CTA model. Currently, nerve allotransplantation is the only comparable method being used in a clinical setting. In the pediatric population, with increased disease burden and nerve regeneration, and the frequent availability of a living-related donor, this forms an all the more attractive solution to nerve reconstructive problems. However, the risks of immunosuppression cannot be underemphasized, and deserve more attention in the current CTA literature. We provide a case report of an infant who received a living-related nerve
allograft from his father who was positive for Epstein-Barr virus. He quickly developed a symptomatic EBV viral infection concurrent with immunosuppressant drugs. While the patient suffered only a short illness when the immunosuppression was stopped prematurely, the EBV infection could have developed into a life-threatening post-transplant lymphoproliferative disorder (PTLD). This is a mononucleosis-type lymphoma induced by the EBV, and the potential risks of PTLD, that he is predisposed to developing, will have to be monitored for over his life. This case highlights the importance of considering the potentially fatal risks associated with the clinical application of a CTA procedure, even when short-term immunosuppression is used.

In conclusion, we have described a novel method of composite tissue allotransplantation that permits the long-term survival of bone and joint allotransplants after cessation of immunosuppression. We have further explored the boundaries of this experimental model by assessing the short-term and long-term effects of growth factors and further applied the growth factor administration method to conventional allografts. The mechanisms behind bone tissue survival have been analyzed, in particular with reference to the role of tolerance to donor tissue and transplant chimerism. New methods in experimental CTA research have been developed, notably the use of calibrated microwave rapid decalcification and modified hydrogen washout blood flow measurement. One example of clinical application of CTA is discussed with emphasis given to caution because of the risks incurred even with short-term immunosuppression.

In the future, we expect CTA to be more widely used and accepted. Although outside the realm of segmental defects, hand and face transplantation have been at the forefront of CTA advances in the post-organ transplant era. Possibly the lessons learned from those cases may be combined with our research in structural CTA with short-term immunosuppression to provide a new option in reconstructive surgery. It is essential that in the reconstruction of non-life critical defects, the risk to the patient be minimized so as to do no further harm.