

Periosteal stem cells are essential for bone revitalization and repair

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Bone is unique in that its repair involves tissue regeneration instead of scar formation. The generation of new bone requires mesenchymal stem cells that proliferate and then differentiate and drive the reparative process. The complex process of bone repair involves formation through endochondral and intramembranous ossification, as well as remodeling, which is dependent upon osteoclasts. A necessary component of these formation and remodeling activities is re-vascularization of the injured bone segment and newly forming bone. We initiated a series of investigations defining the role of the mesenchymal stem cell population in a bone repair.

Structural allograft bone represents one condition where inadequate repair occurs. Segmental allografts are used to replace areas of bone loss due to tumors or other conditions and have a high complication rate including infection, non-union, and fracture. Non-unions occur in approximately 20% of the cases and frequently require additional bone grafting procedures. Even more problematic is the development of an allograft fracture. Allograft fractures occur in approximately 30% of allografts and typically appear between 2 and 5 years after successful placement of the allograft. Since allografts only re-vascularize and remodel in the 1-2 millimeter segment attached to the host bone, the remainder of the allograft is subject to stress injury and eventually fails due to lack of re-vascularization and inability to remodel microdamage.

We developed a murine model to study the role of stem cells in allograft repair. A 4 mm mid-diaphyseal segment is removed from the femur by osteotomizing the bone using a

saw. A 4 mm cortical bone graft is then inserted into the segmental defect and stabilized by a 22-gauge metal pin placed through an intramedullary marrow cavity. Grafting procedures are performed between inbred C57BL/6 mice with identical genetic background (autograft), or mice with genetically different backgrounds (allograft). Autografts involve live bone graft transplantation and mimic human autograft surgery. The graft is carefully dissected out of muscles, briefly washed with warm phosphate saline buffer and immediately transplanted into the mice (live autograft transplantation). For allograft transplantation, a devitalized bone graft that undergoes processing is transplanted, also similar to the human clinical situation. Allograft bone is obtained from a genetically different mouse strain. The bone is scraped free of all tissues, extensively washed, sterilized with 70% ethanol, rinsed in saline to remove residual ethanol and then fresh frozen at -70°C for at least 1 week prior to transplantation. A similar processing procedure is performed on autograft bone and this is referred to as isograft.

Our findings show that autograft bone undergoes complete remodeling and incorporation while both allograft and isograft bone form bone and heal only at the host-graft junction. Thus in autografts, there is bone formation all along the graft surface. Bone forms along the surface with the presence of cartilage and bone cells. The graft segment eventually undergoes complete osteoclast remodeling with formation of new marrow. Histology and Micro-CT demonstrate re-vascularization of the graft. In contrast, allograft and isograft transplants have bone and cartilage formation only at the host-allograft junction and along the remainder of the graft there is no bone formation, and a marked reduction in vascularization is observed. Since both isograft and allograft bone function in a similar manner, the findings show that the decrease in bone formation is not due to an immune response to the allograft tissue. The similar findings in the two grafts and differences from the live autograft suggest that stem cells along the graft surface drive the reparative response.

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Additional experiments were performed to examine this hypothesis. The first set of experiments, autograft segments from Rosa 26 mice were used and implanted into wild type mice so that the fate of the periosteal stem cells could be followed. All cell progeny from Rosa 26 mice express beta-galactosidase and can be stained blue using histochemical methods. Using this model, we observed proliferation of mesenchymal stem cells all along the implanted autograft. By day 3, the cell layer was 3-5 cells thick and by day 5 it was 7-10 cells thick. By day 7 chondrocytes and osteoblast differentiation was present. Staining showed that the stem cells attached to the autograft surface gave rise to chondrocytes, osteoblasts, and surprisingly, to endothelial cells as well.

In a second set of experiments, mouse mesenchymal cells derived from C3H 10T 1/2 (C9 cells), were seeded onto bone allografts at a density of 1.5×10^5 cells per graft and cultured for one additional hour at 37°C in DMEM before use. C9 cells were genetically engineered to produce human recombinant BMP2. C9 cell coated allografts induced bone bridging bone callus around the entire bone allograft segment. Bone formation and remodeling was similar to that observed in autografts. Micro-CT demonstrated that C9 coated allografts produced 3-fold more new bone around the graft at 4 weeks and 9 weeks compared to allograft alone. Stiffness and ultimate torque of control allografts were only 10% of the corresponding autograft ($n=9$, $p<0.05$) at 9 weeks, while C9 coated allograft displayed the same stiffness and ultimate torque as those of autograft. C9 treated allograft also exhibited significantly higher torsional rigidity than autograft controls. These experiments show that adult stem cell-based and gene enhanced tissue engineering may offer novel and exciting therapeutic approaches to augment bone allograft healing and repair.

Finally, a murine fracture-healing model was used to understand the decreased potential for fracture healing during aging. Closed, stabilized femur fractures were created in 6-week and 1-year-old C57/B6 mice. Calcified callus and evidence of bone union were observed in young fractures at days 10 and 14, respectively, but were only present in aged fractures after 14 and 18 days. Compared to aged mice, increased calcification and vascularization were observed by micro-CT in young mice at days 10 and 14. By day 18, calcification and vascularization were similar in young and old animals, consistent with the radiographic findings, and the overall volume of cartilage callus was similar. Histological evaluation was consistent with these observations. Sections from 14-day fractures demonstrated union and presence of a bone callus in young mice, while old mice continued to have a cartilaginous callus.

In young mice *col2* appeared earlier and peak levels were observed by 7 days compared to maximal expression at 10 days in aged mice. While peak expression of the chondrocyte maturation marker, *colX*, occurs at day 10 in both young and aged mice, the level of expression is much higher in young mice. Moreover, while both *col2* and *colX* expressions are essentially gone in young mice by day 14 consistent with com-

pletion of the endochondral phase of repair, expression of these cartilage matrix genes persists until day 21 in aged mice, consistent with the delay in vascularization of the callus.

Two peaks of *osteocalcin* expression were observed. An early peak occurred at day 3 consistent with initial intramembranous ossification along the periosteal bone surface. Interestingly, similar *osteocalcin* levels were observed in young and old mice at this time. A second peak occurred during the endochondral phase of repair with bone formation occurring on the cartilaginous template. In the endochondral phase, *osteocalcin* expression occurred earlier, was of greater magnitude, and returned more quickly to basal levels in young mice consistent with more rapid completion of endochondral ossification.

Finally, aged fractures have dysregulation of genes anabolic for fracture repair. *COX-2* expression immediately preceded cartilage formation with peak levels observed at day 5 and with return to basal levels upon completion of endochondral bone formation. Young mice had substantially elevated *COX-2* expression compared to aged mice. While young mice also had peak *BMP-2* expression by day 5, the increase in *BMP-2* expression was delayed in aged fractures with maximal levels observed between 10 and 14 days. Interestingly, both noggin and chordin levels peak immediately following maximal *BMP-2* expression (day 7 in young and day 14 in aged fractures) suggesting that a subsequent decrease in BMP signaling may have importance in fracture repair.

Discussion. Several models were used to examine the role of stem cells and the expression of genes involved in bone repair. Structural allografts can restore the size and shape of a resected bone segment, but have limitations due to lack of revitalization of the dead segment of bone. Our work shows that the primary impairment limiting this response is the presence of mesenchymal stem cells capable of driving the response. Periosteum and bone surface lining cells are capable of initiating this response. However, this response is absent in processed and devitalized allograft and autograft bone, demonstrating that stem cells located in the local host environment alone are not capable of initiating the repair response. Using Rosa 26 mice, we were able to show that the transplanted stem cell population provides precursors for chondrocytes, osteoblasts, and endothelial cells. Finally, mesenchymal cells expressing recombinant human BMP2 (rhBMP-2) seeded onto the bone allograft surface generate a response that resembles periosteal bone formation and restore the biomechanical strength of the grafted femur. The study further indicates that adult stem cell-based and gene enhanced tissue engineering may offer novel and exciting therapeutic approaches to augment bone allograft healing and repair.

Our examination of fracture repair demonstrates that the delayed fracture healing that occurs in aged mice is related to alterations in multiple interdependent stages of fracture repair, including chondrogenesis, chondrocyte maturation and terminal differentiation, vascularization, primary endochondral bone formation, and remodeling. Interestingly, two

genes known to stimulate repair, *BMP-2* and *COX-2* have decreased and/or delayed expression in aging fractures. *BMP-2* and *COX-2* had maximal expression in young fractures at 5-days, a period of mesenchymal stem cell (MSC) proliferation and maximal chondrogenesis. Altogether the findings indicate that the impairment of fracture healing that occurs with aging involves essentially all stages of the process and suggest that the dysregulation of genes involved in the

early events of fracture repair affect the entire healing cascade. The findings suggest intrinsic differences in the ability of stem cells at different ages to express critical genes involved in the repair process. The aging fracture model provides an excellent mechanism to test the hypothesis that interventions affecting early events will accelerate all stages of fracture repair and have the greatest potential to improve healing in the elderly.