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MEETING REPORTS

Pre-clinical Fracture Repair Investigations: Meeting Report from the 32nd Annual Meeting of the American Society for Bone and Mineral Research

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Michelle M. McDonald

Orthopaedic Research and Biotechnology, The Kids Research Institute, The Children's Hospital at Westmead, Westmead, Australia

The process of fracture repair involves a series of complex stages of cellular recruitment, differentiation and proliferation, followed by extensive matrix production and remodeling. When bone repair is compromised, often many of these stages are impaired. The prevalence of clinical cases where union at fracture sites is delayed or absent has prompted a large amount of pre-clinical research in the fracture repair field. A number of treatment options have recently arrived that show potential to help address the issue of impaired healing. However, in order to determine optimal intervention strategies for each fracture repair scenario, the mechanisms underlying both normal and delayed repair must be further understood.

At the 2010 ASBMR Annual Meeting, a number of presentations on pre-clinical fracture repair investigations expanded our knowledge in this area. The application of a number of new therapeutic agents targeted at osteoporosis to bone repair models was reported. Furthermore, new techniques were employed to examine the contributions of different cell populations and genetic pathways as well as to deliver cells or agents important to the repair process. By further developing our understanding of the fracture repair process, new tools and knowledge have been provided to investigators examining pathological bone repair. Furthermore, with both a plenary symposium dedicated to the contribution of the periosteum to bone regeneration and a focused *In Vivo* Working Group on fracture repair from bench to bedside, fracture repair gained more coverage as an important topic compared to previous ASBMR meetings.

The contribution of periosteal cells to bone repair has been widely examined and convincing data has been produced to confirm the importance of an intact periosteum, and its potential to provide BMP-2 to the injury site, in normal bone repair. The plenary symposium covered in detail recent work produced by a panel of experts in the field to examine closely the contribution of this cell layer to bone repair. A group directed by Dr. Celine Colnot has pioneered and established the validity of a periosteal transplant model in the mouse. Using this model with a number of reporter mice, Dr. Colnot's group has eloquently established a body of evidence to confirm a significant contribution from the periosteum to bone repair (1;2). The contribution of cells from this layer is clearly evident, although only locally restricted with little to no migration to other regions of the bone. The addition of muscle to this periosteal transplant model resulted in enhanced periosteal contribution. When considering unstable endochondral repair models and stable intramembranous repair, this group suggested that the inflammatory phase of repair is prolonged in a stable environment, and therefore proceeded to examine the role of matrix metalloproteinases (MMPs) in bone repair (3;4). When an MMP-9 knockout (KO) mouse was examined, stable fractures showed a cartilaginous callus. Interestingly, this feature was rescued when wild-type (WT) bone marrow was transplanted into these mice. When the reverse scenario was

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examined, however, where mutant bone marrow was transplanted into WT mice, stable fractures healed normally. Furthermore, periosteal transplants in the cortical defect model from MMP-9 KO mice into WT mice produced cartilage tissue. However, neither KO periosteum transplanted into WT animals, nor the reverse, produced cartilage tissue. Hence in the absence of MMP-9 in both the periosteum and bone marrow, cartilage tissue is favored after inflammation in bone repair models. Conversely, if only the bone marrow environment or the periosteum is MMP-9-null, cartilage tissue does not prevail in the callus. Taken together, these results confirm that both the periosteum and bone marrow contribute to control cell fate during bone repair.

Dr. Regis O'Keefe followed this discussion with an expansion of a presentation given at the *In Vivo* Working Group seminar, covering both his own work and that of colleagues that has explored the periosteum and inflammation during bone repair. Initial experiments performed by Dr. O'Keefe's group confirmed that without a live stem/progenitor cell population, bone grafts are unable to produce normal periosteal healing (5;6). Through a complex series of experiments using reporter mice and genetic manipulation, this group has gone further to suggest links, in a complex model, between BMP-2 from the periosteum or injury with Cox-2, Ihh, and the Wnt β-catenin pathway to control chondrogenesis and osteogenesis in bone repair (7-11). This work provides some explanation for the impaired healing in the aged population, with Cox-2-null mice exhibiting a repair phenotype similar to that of aged mice (12). The investigators' specific studies on the role of BMP-2 (11) correspond well with research presented at the meeting by Vicki Rosen and colleagues, who examined the effect of complete BMP-2 ablation on bone repair (13). In the complete absence of BMP-2, fracture calluses fail to form. However, when the control of BMP-2 deletion is driven by markers of early and late osteoblast differentiation, impressive differences in callus formation were noted. When Col2.3, a late osteoblast differentiation marker, drove BMP-2 deletion, fracture healing proceeded normally. However, when deletion of BMP-2 was driven by expression of prx1, an early skeletal progenitor cell marker, fracture callus formation was completely absent. This finding confirmed that the role BMP-2 plays during bone repair is to stimulate progenitor cell commitment to osteoblasts, rather than to act during later osteoblast differentiation. Furthermore, a number of BMP and Wnt antagonists were increased in the absence of callus formation, implicating roles for these agents in regulating cellular differentiation during repair.

An extension of the work exploring the role of the periosteum in repair was presented in a paper confirming the contribution of progenitor cells from a periosteal origin (14). The implantation of 5 different bone scaffolds seeded with periosteal-derived bone progenitor cells in subcutaneous pockets in nude mice resulted in bone formation after 8 weeks that had followed an endochondral ossification process. A complete lack of cell incorporation in the implant led to no bone formation at all implants. When cells were included, NuOss, a de-proteinized bovine bone mineral, was the superior scaffold when compared to the others. However, at 8 weeks post-implantation only 13% of the implant region was new bone, which suggests that additional pro-osteogenic signals may be required to enhance bone formation in this model. As shown by Dr. Colnot and colleagues, an active bone marrow environment combined with periosteal cells may be required for optimal bone healing. However, the periosteum clearly has strong anabolic potential during bone repair, and while harnessing this potential for clinical application in both bone repair and pathology has yet to be translated, it certainly is an exciting prospect.

The number of therapeutic applications to enhance bone repair is increasing in pre-clinical research, with many showing great potential. In one study, PHTPP, an estrogen receptor β antagonist, was administered during cortical bone repair in ovariectomized mice (15). PHTPP treatment altered the pattern of volumetric bone accrual over the

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course of the repair model, appearing to advance the rate of repair. These changes were more evident in the intramedullary region of bone, with more than 2-fold increases in BV/TV in this region at early time points. However, by 21 days the treatment group showed no difference in BV/TV at this site. Mechanical testing of the samples at this late stage confirmed improved repair with increased recovery of normal load and energy to failure in PHTPP-treated mice compared to controls. It would be of interest to assess the mechanical properties at an earlier time point, when the differences in BV/TV are more evident.

The treatment of healing fractures with recombinant myostatin (GDF-8) was also explored in a study by Mark Hamrick and colleagues (16). *In vitro* analyses confirmed a reduction in chondrogenesis from bone marrow-derived mesenchymal stem cells (BMSCs) in the presence of myostatin. *In vivo* treatment of fibula fractures in mice with local myostatin led to union in all samples but reduced callus bone volume in treated mice at 15 days post-fracture. Analysis at 10 days post-fracture revealed a reduction in cartilaginous callus area with GDF-8 treatment, suggesting that the reduced callus size at 15 days was a result of the impaired chondrogenesis after the initial injury.

Combination therapy with LIPUS and alendronate was examined in a rat cancellous osteotomy model (17). This approach did not lead to any additional effects of this combined therapy. Interestingly, the authors failed to demonstrate any effect in this model with alendronate alone. In contrast, LIPUS alone enhanced bone formation at the repair site. The lack of effect with alendronate suggests that the 2-week time point examined was too early to detect any effects of an anti-resorptive agent.

A number of pathways were examined in fracture models through either genetic manipulation or pharmaceutical intervention. Protease-activated receptor-2 (PAR-2) is present on mature osteoblasts where it is activated by proteases present during inflammation, and activation of this receptor leads to reduced osteoclast differentiation (18). Mice deficient in PAR-2 show increased resistance to cartilage degradation and increased subchondral bone in a model of arthritis (19). In this context, fracture repair in PAR-2-deficient mice was examined in a study presented at this year's meeting (20). PAR-2-null mice, unlike WT mice, showed delayed healing as confirmed by reduced BV/TV measured by μCT and reduced torsional strength compared to the intact controls 7 weeks after fracture. Histological examination at earlier time points would be of interest to determine the role inflammation played in this outcome.

Fracture repair in mice with deletion of the gap junction protein connexin 43 (Cx43) specifically in osteoblasts was examined at numerous stages post-fracture (21). Endochondral repair was delayed in KO mice with persistence of cartilaginous callus at 21 days. Further hard callus remodeling once union had been achieved was also delayed, with woven bone callus persisting 35 days post-fracture. Evidently, this gap junction protein plays an important role in the contribution that mature osteoblasts play during endochondral bone repair and hard callus remodeling. Further assessment of bone formation and resorption parameters in the fracture calluses may confirm if one or both of these processes are altered in the absence of Cx43.

On a therapeutic note, a number of investigators explored how modulation of the Wnt/β-catenin pathway through either genetic deletion of the endogenous Wnt inhibitor sclerostin (*Sost*), or by neutralizing this protein with an antibody, enhanced bone repair. *Sost*-null mice exhibited enhanced union and hard callus remodeling in an externally-fixed closed tibial fracture model (22). Endochondral repair appeared advanced, with a smaller, more dense hard callus formed in *Sost* KO mice compared to WT littermates. The authors have planned further time points to exclude differences in mechanical stability in early repair that may have modified the repair response. In agreement with this result, a neutralizing

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antibody to sclerostin administered to rats after closed fracture production produced fracture calluses with higher bone volume and higher peak load compared to controls (23). With the combination of these promising results and the emerging outcomes of clinical trials of sclerostin antibody in the treatment of osteoporosis, clinical application of sclerostin antibody to impaired fracture repair scenarios is inevitable.

Pathological bone repair is common and thus is a key area of pre-clinical fracture research; a number of studies in this area were presented at this year's meeting. Patients with type 1 neurofibromatosis (NF1) have an underlying osteopenic phenotype (24). More striking, however, is the pseudarthrosis and extremely poor bone repair characteristic of these patients. David Little and colleagues explored this clinical pitfall using a genetic mouse model. In order to recapitulate the pseudarthosis seen clinically, this team used a floxed heterozygous *Nf1* mouse and knocked out the second *Nf1* allele locally using a cre-adenovirus injection at the site of fracture (25). By doing so, a fibro-cartilaginous callus formed more closely resembling the clinical scenario. This group now plans to manipulate this fracture environment with therapeutics such as those presented at this year's conference.

Consumption of alcohol has been associated with delays in or complete absence of healing after fracture. In a pre-clinical model, alcohol treatment impaired repair such that callus volume was reduced and matrix production altered, impairing the mechanical integrity of the callus at 14 days (26). Examination of the Wnt/β-catenin pathway revealed in part the mechanism behind this phenomenon. Both reduced β-catenin protein expression and reduced Wnt-mediated transcription were found in alcohol-compromised calluses. Although the authors agree that dysregulation of this pathway may be only part of the mechanism, the potential of emerging new therapeutics that enhance this pathway, such as sclerostin antibody, in their model may now be explored.

As clinical management of patients increasingly requires the combination of orthopedics and endocrinology approaches, demands on pre-clinical investigations of fracture repair are escalating. As elegantly outlined by Dr. Louis Gerstenfeld during the *In Vivo* Working Group, many new analytical techniques have allowed for advancement in the fracture repair field. By combining these new tools that examine biochemical, structural and histological properties, our three-dimensional understanding of the complex processes of bone repair has become more comprehensive, aiding the design of pre-clinical studies aimed at manipulating the many pathways involved in fracture repair. Armed with these new tools and the knowledge they facilitate, the fracture repair field will be better able to meet the needs of clinicians.

Conflict of Interest: Dr. McDonald reports that she is the first author on one of the abstracts referred to in this article and that the work in that abstract was funded by Novartis.

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