IN VIVO RETROVIRAL-BASED GENE THERAPY WITH LIM MINERALIZATION PROTEIN (LMP)-1 ENHANCES FRACTURE HEALING

*Strohbach, CA; **Rundle, CH; **Chen, S-T; **Wergedal, JE; **Linkhart, TA; *Baylink, DJ; **Lau, K-HW; **Strong, DD
*Loma Linda University and **The Musculoskeletal Disease Center, Jerry L Pettis Veterans Administration Medical Center, Loma Linda, CA
Cassandra.Strohbach@med.va.gov

Introduction
Successful gene transfer with viral vectors and subsequent gene therapy in an orthopedic setting has led to the possibility for development of new treatments for bone defects and fractures. LIM mineralization protein-1 (LMP-1) is a novel intracellular osteogenic factor associated with bone development in vivo that has been implicated in the BMP signaling pathway. Our laboratory has developed a direct in vivo retroviral gene delivery model to test the effect of overexpression of BMP-4 on fracture repair in a rat model (1). However, these studies, as well as others, suggested that the use of BMPs as a gene therapy in a clinical setting may be limited 1) because BMP-4 stimulates supra-periosteal bone formation but does not accelerate bony union after application to the rat femur fracture model and 2) because of a requirement for high amounts of BMPs for osteoinduction in primates (2). In this study we evaluated the effectiveness of LMP-1 gene therapy to promote bone formation and bony union in the rat femur fracture model. LMP-1 was chosen based on its osteoinductive effect in spinal fusion models, and because circumstantial evidence suggests that LMP-1 mediates its effects through BMP-dependent and BMP-independent pathways (2-7). We hypothesized that LMP-1 gene therapy would enhance fracture healing but unlike BMPs, would accelerate bony union.

Materials and Methods
A murine leukemia virus (MLV)-based retroviral vector was used to target the expression of the human LMP-1 or control transgenes into cultured murine osteoblasts. Additionally, LMP-1 was 5'-tagged with influenza hemaglutinin (HA-LMP-1) to facilitate its identification. Effects of increased HA-LMP-1 and control transgene expression on osteoblast mineralization in vitro were evaluated at day 21 by alizarin red and von Kossa staining.

Femur fractures were produced in 12-week-old male Fischer 344 rats by the three-point bending technique (8). The retroviral vector was applied directly into the periosteum at the fracture site in a percutaneous injection at one day post-fracture; this approach targeted our retroviral vector to periosteal cells stimulated to proliferate in response to injury. Healing in HA-LMP-1 treated and β-galactosidase control rats was evaluated by X-ray analysis at 7, 10, 14 and 21 days, and by pQCT and histology at 21 days after fracture (n=3 each). Statistical analysis was performed by t-test. Immunohistochemistry with anti-HA and anti-BMP-4 antibodies was used to identify the cells that expressed HA-LMP-1 and BMP-4 protein, respectively. All animal procedures were conducted with the approval of the institutional IACUC in accordance with US law.

Results
Marrow stromal cells and calvarial osteoblasts transduced with the MLV-HA-LMP-1 produced the expected 52 kDa HA-LMP-1 protein recognized by anti-HA tag antibodies in western immunoblots. Osteoblasts transduced with HA-tagged LMP-1 and cultured for 21 days, under conditions that promote mineralization, showed increased alizarin red staining and von Kossa staining indicating increased mineral deposition in response to HA-LMP-1. By contrast, cells transduced with control vector did not show more mineralization than untransduced cells. These results suggested that the HA-LMP-1 was expressed in cells normally expected to mediate fracture repair, and that HA-LMP-1 was functional in increasing osteoblast differentiation.

Radiographic evidence suggested that mineralized tissue was augmented at 21 days in fractures of each animal injected with MLV-HA-LMP-1, as compared to β-galactosidase control animals (Figure 1). No heterotopic bone formation was observed. Examination of the LMP-1-treated fracture histology at 21 days by Safranin Orange staining also suggested that the increased mineralized tissue was increased bone formation that coincided with reduced cartilage (c), fibrous tissue (f), and improved union at the fracture gap (F) at 21 days (Figure 1, insets). This effect occurred well before the 4-5 weeks normally required for bony union (i.e., healing) in this fracture model. Previous studies also established that the HA tag did not affect fracture repair. None of the control fractures displayed augmented bone formation at 21 days.

pQCT analysis at the fracture site at 21 days post-fracture revealed that HA-LMP-1 therapy significantly increased the bone mineral content of the mineralized callus to 4.57 ± 0.76 from 2.39 ± 0.87 in the controls (p=0.04), and the area of the mineralized callus to 12.11 ± 2.02 from 7.64 ± 1.55 in the controls (p=0.05). The area of the soft callus tissues was not significantly different at 21 days of healing. None of these callus parameters were significantly different in response to HA-LMP-1 therapy earlier than 21 days post-fracture.

Discussion
We chose to compare fracture healing with a transgene, LMP-1, that not only mediated its bone forming effects by increasing local BMP expression (4, 5), but also by increasing responsiveness to BMPs through Smad signaling (6) and by other novel but poorly defined mechanisms (7). Our previous gene therapy studies on fracture healing with MLV-BMP-4 (1) indicated that while the therapy strongly increased callus formation (mostly cartilage) that remodelled normally, the therapy did not produce bony union of the fracture gap more rapidly than in the controls.

To our knowledge, this is the first study demonstrating that local expression of LMP-1 from a retroviral vector increases bone formation and enhances bony union in a normally healing fracture model. When applied to an established in vivo model of fracture repair by the same injection technique, LMP-1 gene therapy 1) enhanced the union of bony callus tissues over the fracture, and 2) promoted this healing without the production of heterotopic bone. Furthermore, while immunohistochemistry demonstrated that LMP-1 expression was consistent with enhanced differentiation of the osteoblast lineage, the lack of colocalized BMP-4 expression and heterotopic bone production strongly suggested that LMP-1 therapy was not mediated directly through BMP-4 production. Our studies demonstrate that MLV-LMP-1 gene therapy could be effective for therapeutic fracture applications.

Acknowledgements
This study was supported by a special appropriation to the J.L. Pettis Memorial VAMC Musculoskeletal Disease Center, and was performed at facilities provided by the Department of Veterans Affairs.

References