Timing Matters: PR1P-Mediated VEGF Stabilization Differentially Regulates Endochondral Ossification and Fracture Healing Outcomes

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INTRODUCTION: Fracture healing requires precise coordination between angiogenesis and osteogenesis, processes critically regulated by Vascular Endothelial Growth Factor (VEGF). While VEGF is indispensable, its therapeutic application has been limited by instability, narrow dosing windows, and potential for aberrant vessel formation. Importantly, evidence suggests that not only the amount, but also the timing and duration of VEGF signalling are crucial determinants of successful endochondral ossification. PR1P, a novel VEGF-stabilizing peptide, prolongs VEGF activity within the fracture callus by protecting it from degradation. By amplifying the body's own VEGF rather than introducing supraphysiologic doses, PR1P avoids the pitfalls of direct VEGF therapy while sustaining angiogenic support. This property offers a powerful platform to investigate how short-term versus prolonged VEGF stabilization influences the trajectory of bone healing. The objective of this study was to determine how different durations of PR1P-mediated VEGF stabilization alter the biological and mechanical progression of fracture healing, with the goal of informing timing strategies for potential therapeutic use.

METHODS: All animal protocols were approved by the IACUC at Beth Israel Deaconess Medical Center. Nine-week-old female C57BL/6 mice underwent midshaft femur fractures that were stabilized by intramedullary nailing and assigned to control or PR1P-treated groups. Female mice were selected to avoid the confounding influence of testosterone, which has been reported to enhance fracture healing and could dilute or obscure the specific effects of PR1P. PR1P was administered intraperitoneally every other day at two dosing schedules: for 5 or 14 days, with control animals receiving saline. For analysis, femurs were harvested on days 5, 14, and 28 post-fracture. A total of 40 mice were used for RT-qPCR (n = 5 per group, 8 groups), and 17 mice were used for mechanical testing (n = 6 per group across 3 groups, with one sample loss).
RT-qPCR was performed on fracture callus tissue to evaluate angiogenic, osteogenic, and remodeling markers (Col1a1, Col2a1, Col10a1, Runx2, ALP, RANKL, OPG, MMP-13, OPN, OCN). Mechanical testing of fractured femurs was conducted by three-point bending to assess stiffness, failure load, and work to failure. Additional analyses, including µCT, histology, and CD31 immunohistochemistry, were performed to provide complementary context for bone morphology and vascularization.
For statistical analysis, ordinary one-way ANOVA with Sidak's multiple comparison test was used for qPCR datasets, while non-parametric ANOVA (Kruskal–Wallis test) with Dunn's post-hoc test was applied for mechanical testing. Statistical significance was set at $p < 0.05$.

RESULTS: RT-qPCR revealed distinct time-dependent effects of PR1P on fracture callus gene expression. Runx2 expression **(Figure 1a)** was significantly increased in the PR1P-14D group (9.36 ± 2.21, n=5) compared to the day-14 saline control (6.23 ± 1.16, n=5; p = 0.0009, one-way ANOVA with Sidak's post-hoc), consistent with enhanced osteogenic signalling following prolonged VEGF stabilization. In contrast, Col2a1 expression **(Figure 1b)** was significantly reduced in the PR1P-5D group (16.04 ± 17.01, n=5) relative to its day-14 saline control (52.36 ± 29.36, n=5; p = 0.0283), indicating accelerated cartilage resorption. The RANKL/OPG ratio **(Figure 1c)** was also significantly increased in PR1P-5D samples (1.90 ± 0.54, n=5) compared to controls (1.01 ± 0.65, n=5; p = 0.0172), reflecting a distinct remodeling response with short-term VEGF stabilization. Mechanical testing demonstrated a trend toward increased failure force in both PR1P-treated groups (PR1P-5D: [8.78 ± 3.93], n=6; PR1P-14D: [7.05 ± 3.56], n=6) compared to saline controls ([4.56 ± 0.78] n=5), although these differences did not reach statistical significance (p > 0.05, Kruskal–Wallis with Dunn's post-hoc).

DISCUSSION: These findings demonstrate that the timing of VEGF stabilization with PR1P distinctly alters the trajectory of fracture healing. The dosing schedules were selected based on the natural expression profile of VEGF, which peaks around day 5 post-fracture and declines toward baseline by day 14. Short-term PR1P treatment (5D) coincided with this peak, accelerating cartilage resorption and shifting the callus toward active remodeling, as evidenced by decreased Col2a1 expression and altered RANKL/OPG ratio. In contrast, prolonged PR1P administration (14D) sustained VEGF activity through the natural decline phase, enhancing osteogenic signalling via elevated Runx2 expression and aligning more closely with physiologic endochondral ossification. Together, these results suggest that short VEGF stabilization may "fast-track" the transition from cartilage to bone, whereas prolonged stabilization supports a more balanced healing process. Mechanical testing revealed trends toward improved failure force in both PR1P-treated groups, underscoring the functional relevance of these molecular signatures.

CLINICAL SIGNIFICANCE: These findings highlight that the duration of VEGF stabilization is a critical determinant of healing outcome. By accelerating remodeling or sustaining physiologic ossification depending on treatment duration, PR1P offers a tuneable strategy to enhance bone repair. This timing-dependent effect suggests that VEGF stabilization could be tailored to patient-specific needs, for example, using prolonged stabilization in elderly or diabetic patients with impaired healing, while shorter courses may be sufficient in otherwise healthy individuals. *As this study was conducted in a murine model, further validation in preclinical large-animal and clinical studies will be essential before translation to patient care.*

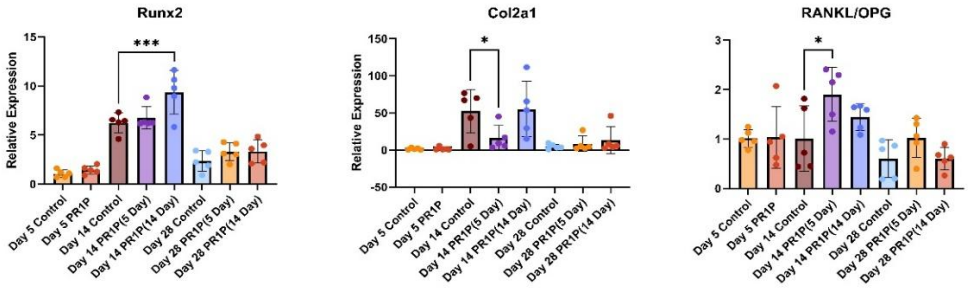

Figure 1. Relative expression of fracture callus genes at days 5, 14, and 28: (a) Runx2 (osteogenic marker), (b) Col2a1 (cartilage marker), (c) RANKL/OPG ratio (remodeling marker). Data shown as mean ± SD; $p < 0.05$, $^{**}p < 0.001$ vs. controls.