

Bone-targeted Nanoparticle Drug Delivery System Immunomodulation for Enhanced Fracture Healing

Baixue Xiao¹, Emmanuela Adjei-Sowah¹, Jared Mereness¹, Ming Yan¹, and Danielle S. W. Benoit^{1,2}

¹University of Rochester, Rochester, NY, ²University of Oregon, Eugene, OR

dbenoit@uoregon.edu

Disclosures: Baixue Xiao (N), Emmanuela Adjei-Sowah (N), Jared Mereness (N), Ming Yan (N), and Danielle S. W. Benoit (N)

INTRODUCTION: Despite major advances in surgical procedures, a significant percentage (~10%) of fractures still do not heal, leading to delayed unions or even non-unions. Therefore, novel, and minimally invasive approaches are urgently needed to prevent and/or treat these debilitating and costly non-union fractures. To this end, we pioneered the development of a novel nanoparticle (NP) system composed of poly(styrene-maleic anhydride)-b-poly(styrene) (PSMA-b-PS) functionalized with bone-targeting tartrate-resistant acid phosphatase (TRAP) binding peptide (TBP) ¹⁻³. We have previously shown the accumulation of these bone-targeting NPs (TBP-NP) at fractures is 2-3-fold higher versus untargeted or scrambled peptide-targeted NP¹. Wnt/ β -catenin agonists (AR28) have been investigated to not only enhance osteoblastic activity for regenerative cells, but also promote alternative (M2) macrophage (M Φ) polarization. Furthermore, our recent work shows that the anionic nature of the PSMA-b-PS NPs promote M2 M Φ polarization. ⁴ This knowledge combined with the growing understanding of the role of M Φ in fracture healing, motivated us to investigate the underlying healing mechanism of TBP-NP delivery of AR28.

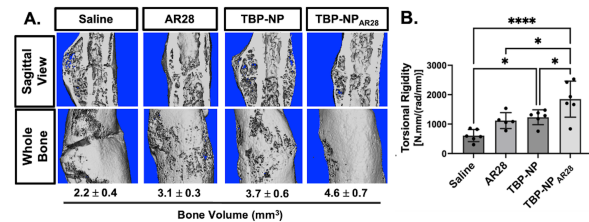


Figure 1. TBP-NP-AR28 treatments expedite fracture healing. Scale bar = 1 mm. Data represented as mean \pm standard deviation, n = 6. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

fragmentation chain transfer (RAFT) polymerization^{1,2}. Mid-diaphyseal femur fractures were created in mice and were followed by saline, free AR28, unloaded TBP-NP, and TBP-NP-AR28 injections (2 mg/kg AR28 basis, 30 mg/kg NP basis) 3 days post-fracture (dpf). Fracture healing was analyzed by microcomputed tomography (μ CT) and biomechanical testing (Fig 1). Cells from fracture callus were collected and stained with cell surface markers to identify the TBP-NP-targeted cells (Fig 2A). To further investigate the osteoimmunomodulatory role of M Φ in fracture healing, histological analysis was performed to characterize the M Φ spatial localization at fractures on 10 dpf (Fig 2B). To quantitatively evaluate M2/M1 ratios, flow cytometry was performed over time (Fig 2C). To explore the underlying mechanism of enhanced fracture healing via delivery of TBP-NP-AR28 at a gene expression level, bulk RNA sequencing analysis was performed (Fig 2D, E).

RESULTS: μ CT of fractures treated with TBP-NP-AR28 showed a more mature anatomical shape of cortical bone with better union and more organized woven bone at the bridging callus than other groups (Figure 1A). Additionally, TBP-NP-AR28 treated mice showed ~4-fold greater torsional rigidity than saline controls (Fig 1B), suggesting expedited fracture healing. Interestingly, analysis of cells from the callus revealed that ~60% of TBP-NPs at fractures were taken up by M Φ (Fig. 2A) rather than conventional regenerative cell types. Histological analysis of the fracture sites at 10 dpf revealed that all the groups had F4/80⁺CD206⁺ cells at the medullary area. Interestingly, unlike other groups, the TBP-NP-AR28 treated group showed F4/80⁺CD206⁺ cells also present at the periosteum (Fig 2B, yellow arrows) and on the cortical bone surface (Fig 2B, red framed inserts), further indicating more rapid resolution of inflammation and transition to bone repair. Flow cytometry data suggested that TBP-NP-AR28 increased M2/M1 ratio, which is associated with enhanced fracture healing in both bone formation volume and mechanical properties (Fig 2C). Bulk RNAseq suggested that the enhanced fracture healing observed with TBP-NP-AR28 is associated with the upregulation of M2 M Φ genes and downregulation of M1 M Φ genes, resulting in elevated M2/M1 ratios (Fig 2D, E).

DISCUSSION: This study shows that macrophages are crucial in fracture healing. The M2/M1 ratio correlated with healing outcomes, and TBP-NP-AR28 upregulated this ratio by promoting M2 and inhibiting M1 macrophage polarization. Furthermore, quantification of fracture-localized AR28 and TBP-NP-AR28 to further understand the pharmacokinetics and pharmacodynamics of this bone-targeted NP system is warranted. In addition, the impact of NP-mediated healing in more clinically relevant models, including advanced age, rheumatoid arthritis, and diabetes, which are highly associated with nonunion fractures, is worthwhile. Altogether, enhanced fracture healing via delivery of TBP-NP-AR28 increased M2/M1 ratio.

SIGNIFICANCE/CLINICAL RELEVANCE: The importance of macrophages in various diseases, preferential accumulation at fractures, and regulation of macrophage polarization, highlight a potent therapeutic benefit of bone-targeted NP DDS.

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METHODS: PSMA-b-PS-based NPs were synthesized via reversible addition-

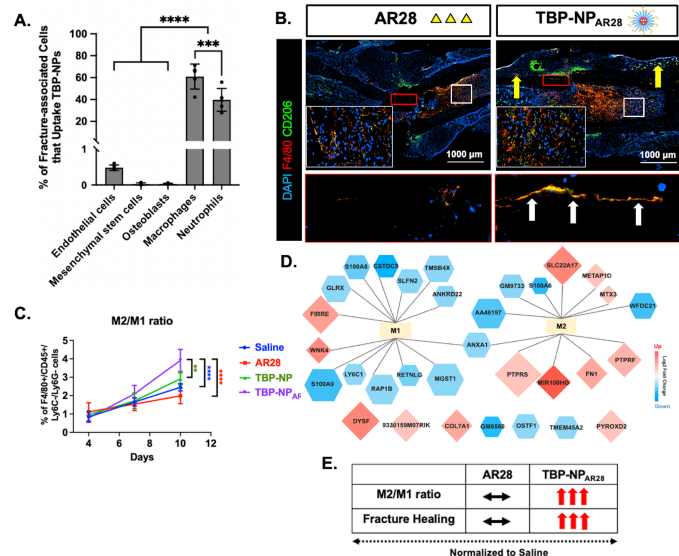


Figure 2. Bone-Targeted NP delivering AR28 expedites fracture healing through M Φ modulation. A: Most TBP-NPs at fractures are taken up by M Φ . B: TBP-NP-AR28 exhibited distinct spatial distribution of M Φ at fractures. Immunofluorescence staining of F4/80, iNOS, and CD206, where M2 M Φ were identified as F4/80⁺CD206⁺. Scale bars = 1000 μ m. C: Flow cytometry analysis of M2/M1 ratio, gated as double positive for MHCII/CD38 (M1 M Φ) and CD206/CD163 (M2 M Φ), at day 4, 7, and 10 post-fracture; D, E: Bulk RNAseq analysis indicated that TBP-NP-AR28 enhanced fracture healing via increasing M2:M1 ratios. D) The network of the top 30 differential genes and their associated inflammatory response, plotted by Cytoscape; E) Summary of the relationship between M2:M1 ratios, and fracture healing. Data represented as mean \pm standard deviation (SD), n=4. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, as determined by two-way ANOVA, Tukey's multiple comparison test.