The Role of 14-3-3ε, an Intracellular Component of TNFR2 Receptor Complex, as a Key Player in Bone Healing

Lida Moradi, PhD; Chuan-Ju Liu, PhD

Department of Orthopaedics & Rehabilitation, Yale University School of Medicine, New Haven, CT, 06510, USA

Introduction: Our study elucidated the interaction between progranulin (PGRN), a multifaceted growth factor-like molecule, and TNF receptors (TNFRs), revealing its therapeutic efficacy against arthritis as previously reported (Tang et al., ***Science***, 2011; Zhao et al., ***ARD***, 2015; Fu et al., ***JCI***, 2021). Significantly, we engineered a protein termed "Atsttrin" by extracting three TNFR-binding domains from PGRN. Both PGRN and Atsttrin exhibited substantial capacity to induce chondrogenic and osteogenic differentiation *in vitro*. Notably, their therapeutic potential extended *in vivo*, demonstrating pronounced regenerative effects on articular cartilage and bone healing. This was achieved by activating the TNFR2 regenerative pathway while concurrently inhibiting the inflammatory TNFα/TNFR1 pathway. During our investigation, we identified 14-3-3ε as an indispensable intracellular component within TNFR2 complexes in chondrocytes, underpinning PGRN's regulatory control over chondrocyte metabolism. The pivotal role of 14-3-3ε was underscored by the significant attenuation of PGRN's therapeutic impact against osteoarthritis (OA) upon its global or chondrocyte-specific deletion. Mechanistically, the PGRN/TNFR2/14-3-3ε axis orchestrated ERK-dependent Elk-1 activation while concurrently repressing NF-κB signaling in chondrocytes. Consequently, the present study is dedicated to elucidating the requisite dependence on 14-3-3ε for mediating PGRN- and Atsttrin-facilitated fracture healing, thereby contributing to a deeper comprehension of the underlying molecular mechanisms. **Methods:** Thermo-responsive injectable chitosan-based hydrogels were prepared in our previous study (Moradi et al., ***Biomaterials***; 2023). CreERT2;14-3-3ε$^{flox/flox}$ mice were created by breeding RosaCreERT2 mice with 14-3-3ε $^{flox/flox}$ mice. To generate CreERT2;14-3-3ε knockout (KO) mice, tamoxifen was administered via intraperitoneal injection for five consecutive days into CreERT2;14-3-3ε $^{flox/flox}$ mice. Closed gravity femur fracture was induced in CreERT2;14-3-3ε KO mice followed by injecting PBS and Atsttrin-loaded hydrogel at the fracture site immediately post fracture. At day 21post-fracture, mice were euthanized, and femurs were dissected. New formed callus and regenerated femur tissues at the fracture site were observed and new bone formation assessed with histology, and MicroCT analysis. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Yale School of Medicine, Yale University, USA. The results were expressed as mean values ± SD. Statistical significance was assessed using ANOVA and Student's t test with GraphPad Prism software. P-value < 0.05 indicated statistically significant differences. **Results:** -Histology evaluation: WT and KO mice were subjected to a closed gravity fracture model followed by the injecting of 10 µL PBS, and hydrogel loaded with Atsttrin (8 µg of Atsttrin/10 µL of hydrogel) at the fracture site immediately after establishing the fracture. Histology evaluation using Safranin/O Fast green (**Figure 1**) demonstrated impaired fracture healing in 14-3-3ε KO mice treated with PBS and Atsttrin-loaded hydrogel compared to WT groups. On day 21, in PBS-treated WT mice, a slight amount of callus was observed, bone was healing, and the bone gap almost closed (**Figure 1a**). In the WT mice treated with Atsttrin-Loaded hydrogel (**Figure 1b**), bone gap at the fracture site was filled by bone minerals and completely healed, and the bone started remodeling and returned to normal geometries. In contrast in the KO-treated groups, we observed a little appreciable bone development, and the bone gap at the fracture site did not close and remained open with almost no sign of clear bony bridge formation (**Figure1c, d**). -MicroCT analysis: Femur fracture callus in 14-3-3ε KO mice remained less advanced with a transverse gap in the center of femur diaphysis, indicating the bony bridge of the fracture site was incomplete (**Figure 2**). At the same time, the WT mice displayed an almost completed bony bridge between neighboring femur fracture ends at the fracture site. Atsttrin-loaded hydrogel treatment exhibited a-complete-bony-bridge at fracture site that was entirely integrated with the adjacent femur ends. We also found significant differences for micro-CT parameters (**Figure 3**) including tissue mineral density (TMD), bone mineral density (BMD), and bone volume fraction (BV/TV) for WT and KO treated with PBS and Atsttrin-loaded hydrogel. The amount of new bone tissues formed in the WT mice treated with Atsttrin-loaded hydrogel group were significantly higher than those in the PBS treated groups as well as KO groups. As shown in **Figure 3** less amount of newly formed bone was observed in the KO groups, and TMD, BMD, and BV/TV showed even lower values than in the WT treated-PBS group. **Discussion:** In our previous study signaling molecule 14-3-3ε was identified as an intracellular component of TNFR2 complexes in chondrocytes in response to PGRN, and it is required for PGRN regulation of chondrocyte metabolism. We have also found that 14-3-3ε is downregulated in OA and its deficiency deteriorates OA. In addition, both global and chondrocyte-specific deletion of 14-3-3ε largely abolished PGRN's therapeutic effects against OA. Furthermore, PGRN/TNFR2/14-3-3ε signaled through activating ERK dependent Elk-1 while suppressing NF-κB in chondrocytes (Fu et al., ***ARD***, 2021). In this study we generated inducible 14-3-3ε KO mice to characterize the role of 14-3-3ε in bone healing in response to PGRN-derivative Atsttrin. PGRN and its derivative Atsttrin exert their therapeutic and protective effects in OA through dual mechanisms: primarily activating PGRN/TNFR2/14-3-3ε/Elk-1 anabolic pathway independent of TNFα and competing with TNFα to bind to TNFR1, thus simultaneously triggering PGRN/TNFR1/14-3-3ε/NF-κB anti-catabolic signaling. In line with our previous study, this study identifies 14-3-3ε as an inducible component of TNFR2 receptor complex in response to Atsttrin in bone fracture healing process where histology evaluation revealed decreased newly formed bone and incomplete bony bridge formation for 14-3-3ε KO mice relative to WT mice. Moreover, microCT analysis demonstrated amount of new bone tissues formed in the KO group were significantly lower than WT groups. **Conclusions:** This study identifies 14-3-3ε as an inducible component of TNFR2 receptor complex in response to PGRN derivative Atsttrin in bone healing and its deficiency play an important role in impairing of bone fracture regeneration. **Significance/Clinical Relevance:** This study is a great step forward in improving bone healing. We found out that Atsttrin can help promoting both cartilage and bone regeneration, and 14-3-3ε is a necessary part of this process. This discovery may lead to new target for better treatments of bones healing after injuries.

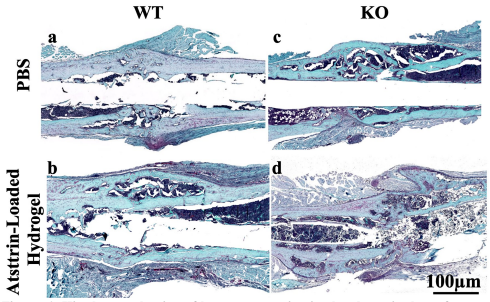

Figure. 1. Histology evaluation of bone regeneration in closed gravity bone fracture mouse model. Longitudinal sections of the fracture callus area at postoperative day 21 are shown by Safranin O/Fast green staining images for WT (control) (a, b), and 14-3-3ε KO mice (c, d) treated with PBS and Atsttrin-loaded hydrogels. Cartilaginous matrix is specifically marked in a reddish color while mineralized tissue is observed as blue. Endochondral ossification was delayed in the KO mice group treated with PBS and Atsttrin-loaded hydrogel compared with the WT group. The scale bar represents 100 µm.

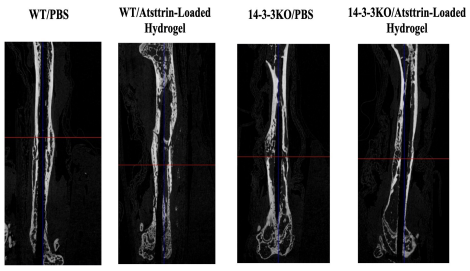

Figure. 2. Micro-computed tomography (MicroCT) analysis. MicroCT analysis indicates that callus formation and the subsequent bone remodeling process impaired in KO mice compared to WT mice during fracture healing. Representative microCT longitudinal section images of femurs reveal the state of healing of the bone fractures after 3 weeks in PBS, and Atsttrin-loaded hydrogel groups. The images were used to qualitatively assess callus size and healing state.

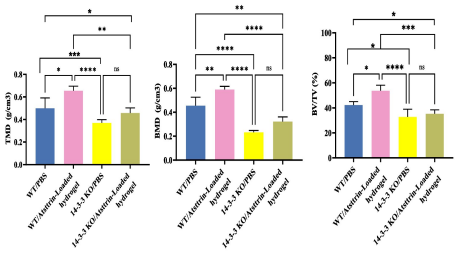

Figure. 3. Quantitative evaluation of fracture callus in WT and KO mice by the analyzing parameter of microCT including tissue mineral density (TMD; g/cm3), bone mineral density (BMD; g/cm3), and bone volume fraction (BV/TV, %), at day 21 after inducing closed gravity femur fracture followed by treatment with PBS (n = 3), and Atsttrin-loaded hydrogel (n = 3). Values are presented as mean ± SD. Significance was determined via One-Way ANOVA followed by Dunnet's test for multiple comparisons. *p < 0.05, **p < 0.01, *** p <0.001, **** p <0.0001.