

14-3-3ε: a novel negative regulator of osteogenesis crucial for progranulin-induced fracture healing

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Disclosure: None. **Introduction:** In the previous investigation, we reported the interaction between progranulin (PGRN), a growth factor-like glycoprotein with multiple functions, and TNF receptors (TNFRs), and its anti-inflammatory and chondroprotective roles in both inflammatory arthritis and osteoarthritis (Tang et al., Science, 2011; Zhao et al., ARD, 2015; Fu et al., JCI, 2021). This achievement was possible largely through the activation of the TNFR2 regenerative pathway. Additionally, signaling molecule 14-3-3ε was isolated as an inducible factor recruited to TNFR2 receptor complex in response to PGRN stimulation. Further, the critical significance of 14-3-3ε was demonstrated by observing a substantial reduction in PGRN's therapeutic effectiveness against arthritis upon deletion of 14-3-3ε. The objective of the current study is to determine the role of 14-3-3ε in osteogenesis and bone regeneration as well as its potential involvement in PGRN-stimulated fracture healing. **Methods:** C57BL/6J mice and CreERT2;14-3-3ε^{fllox/fllox} mice were used in the study. CreERT2;14-3-3ε^{fllox/fllox} mice were generated by crossbreeding RosaCreERT2 mice with 14-3-3ε^{fllox/fllox} mice. To establish CreERT2;14-3-3ε knockout (KO) mice, tamoxifen was intraperitoneally injected for five consecutive days into CreERT2;14-3-3ε^{fllox/fllox} mice. C57BL/6J mice served as the control group for this investigation. Bone marrow stem cells were isolated from the femurs and tibias of the mice for *in vitro* study. The gravity induced Bonnarens and Einhorn bone fracture model was used for *in vivo* study. Both the bone marrow mesenchymal stem cells (BMSCs) and the mice with fractures were subjected to treatment with either PBS or PGRN. Injections of PBS and PGRN were administered at the fracture site at intervals of 0, 5, 10, 15, and 20 days following the surgical procedure. Histological staining and MicroCT were performed to determine the importance of 14-3-3ε regulating bone metabolism. All procedures involving animals were ethically reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Yale School of Medicine, Yale University, USA. The outcomes were presented as the mean values ± SD. Statistical significance was determined using Student's t-test and ANOVA with GraphPad Prism software. A p-value below 0.05 denoted statistically significant differences. **Results: 14-3-3ε is a negative regulator of osteogenesis and required for PGRN-enhanced osteogenesis:** BMSCs, obtained from WT and 14-3-3ε KO mice, were exposed to either a growth medium or an osteogenic culture medium, in the absence or presence of 200ug PGRN. Within the WT group, Alizarin red staining demonstrated that the presence of PGRN increased mineral deposition, leading to the development of clustered nodules over a 21-day incubation period. Conversely, among KO group, the absence of 14-3-3ε notably intensified mineral deposition, resulting in the formation of nodular aggregates. Depletion of 14-3-3ε appeared to mitigate PGRN-induced mineral deposition in the KO group (Figure 1). **14-3-3ε ablation stimulates bond formation and 14-3-3ε signaling is crucial for PGRN-induced fracture healing:** Callus samples from WT and KO mice were harvested at day 10, 16 and 22 post surgery. After fixation and decalcification, Safranin O/Fast green staining was conducted for the sample section. The findings revealed that PGRN-treated mice in the WT group exhibited an expedited chondrogenic process. In contrast, the absence of 14-3-3ε did not significantly affect chondrogenesis on its own. However, in the KO group, the lack of 14-3-3ε appeared to attenuate the chondrogenic effects induced by PGRN (Figure 2). MicroCT analysis revealed that femur fracture callus in WT-PBS mice exhibited less advanced with a transverse gap in the center of femur diaphysis, indicating the bony bridge of the fracture site was incomplete. At the same time, the KO mice displayed an almost completed bony bridge between neighboring femur fracture ends at the fracture site. PGRN treatment exhibited a complete bony bridge at fracture site that was entirely integrated with the adjacent femur ends in the WT group; however, PGRN failed to further stimulate fracture healing in KO mice (Figure 3). **Discussion:** Diverging from our prior study, this study unexpectedly identified 14-3-3ε as a previously-unrecognized negative regulator of osteogenesis and bone regeneration. PGRN, known to stimulate both chondrogenesis and osteogenesis, lost its chondrogenic and osteogenic activities in 14-3-3ε deficient BMSCs. Collectively, these findings suggest that upon PGRN treatment, 14-3-3ε is recruited to the TNFR2 receptor complex, transitioning from its role as an inhibitor of osteogenesis to a critical signaling facilitator, essential for PGRN/TNFR2-driven enhancement of fracture healing. Notably, in contradiction, the deficiency of 14-3-3ε did not exert a substantial impact on chondrogenesis by itself. Nevertheless, within the KO group, the absence of 14-3-3ε seemed to lessen the chondrogenic effects triggered by PGRN. Comprehensive characterization of 14-3-3ε KO mice and the requirement of 14-3-3ε for PGRN/TNFR2 pathways in various conditions, particularly fracture healing, are ongoing. **Conclusions:** This study identifies 14-3-3ε as a novel inhibitor of osteogenesis and a crucial regulator of PGRN/TNFR2 signaling in fracture healing. **Significance/Clinical Relevance:** This study not only presents a previously unknown negative regulator of osteogenesis and a critical mediator of TNFR2 regenerative pathway, thus significantly advancing our understanding the process of bone regeneration, but also holds the potential to unveil novel targets for improving the treatment of fracture healing after injuries.

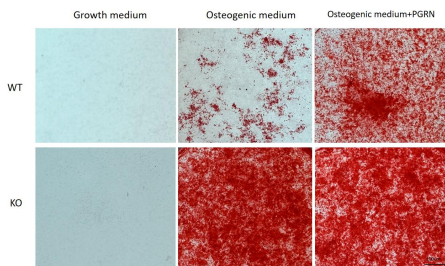


Figure 1. Alizarin red staining BMSCs treated with growth medium, osteogenic medium or osteogenic medium+PGRN. In WT group, Alizarin red staining demonstrated that the presence of PGRN increased mineral deposition, leading to the development of clustered nodules over a 21-day incubation period. Conversely, among KO group, the absence of 14-3-3ε notably intensified mineral deposition, resulting in the formation of nodular aggregates. Depletion of 14-3-3ε appeared to mitigate PGRN-induced mineral deposition in the KO group.

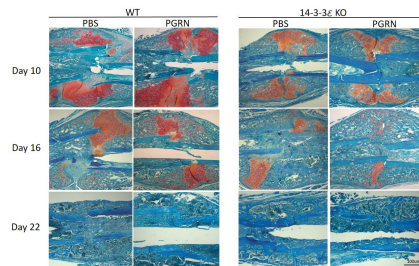


Figure 2. Safranin O/Fast Green staining of Bonnarens and Einhorn fractures at the indicated time point after surgery. Longitudinal sections of the fracture callus area at postoperative day10, 16 and 21 are shown by Safranin O/Fast green staining images for WT (control), and 14-3-3ε KO mice treated with PBS and PGRN. Cartilaginous matrix is specifically marked in a reddish color while mineralized tissue is observed as blue. Endochondral ossification was enhanced in the WT+PGRN mice and KO mice group treated with PBS and PGRN compared with the WT-PBS group.

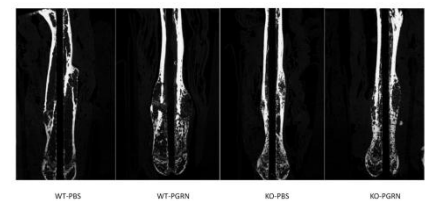


Figure 3. Micro-computed tomography (MicroCT) analysis. MicroCT analysis indicates that callus formation and the subsequent bone remodeling process accelerated in WT-PGRN mice and KO mice compared to WT-PBS mice during fracture healing. Representative microCT longitudinal section images of femurs reveal the state of healing of the bone fractures after 22 days in PBS, and PGRN groups. The images were used to qualitatively assess callus size and healing state.