Therapeutic Use of Runx3-Targeting Micro-RNA to Improve Bone Fracture Healing

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Introduction: In previous studies, we determined that deletion of transcription factor Runx3 in periosteal mesenchymal stem cells or chondrogenic precursors accelerated bone fracture healing in genetically modified mice. *Runx3* deletion in these cells led to earlier transition of cartilaginous to bony fracture callus and increased strength of healed bone, indicating that Runx3 suppresses osteogenesis and may be a suitable target for therapeutic intervention to improve fracture healing. In the present study, we tested the efficacy of localized delivery of a *Runx3*-targeting microRNA to the fracture callus and observed increased de novo bone formation, demonstrating the therapeutic effects of *Runx3* inhibition on fracture healing.

Methods: We initially performed in silico target prediction analysis using programs Target Scan, microT-CDS and miRanda to identify putative *Runx3*-targeting micro-RNAs. These were further prioritized by expression in the fracture callus. We tested the efficacy of a subset of these miRNAs on Runx3 knockdown in vitro and observed that *miR194-5p* mimic (50nM) reduced Runx3 protein expression by ~90% in cultured mesenchymal cells and is predicted to bind 2 sites within the 3'-UTR of *Runx3* (Figure 1C, D). For in vivo studies, 3 month old male C57BL/6 mice underwent mid-diaphyseal fracture surgery followed by fracture callus injection of *Runx3*-targeting (*miR194-5p* mimic) or negative control miR on days 2, 5 and 7 post-fracture. Injections were performed under anesthesia with 10µg *miR194-5p* mimic, 20µg *miR194-5p* mimic, 10µg control miRNA, 20µg control miRNA, or PBS (in 50µl), using a Hamilton syringe fitted with a 29-gauge needle. Fracture calluses were phenotypically assessed on day 21 post fracture by radiographic scoring, µCT analysis and histomorphometric analysis. Additional cohorts of mice were subjected to the same procedures using only 20µg *miR194-5p* mimic or 20µg control miRNA and sacrificed on day 14 or day 21 post-fracture. All experimental procedures were approved by the Atlanta VAMC Institutional Animal Care and Use Committees and were conducted in accordance with all applicable state and federal guidelines.

Results: Immunohistochemical analysis of miR-injected fracture callus on day 14 post-fracture confirmed in vivo reduction of Runx3 protein expression in callus cartilage by *miR194-5p* mimic. Scoring of radiographic images from miRNA-injected fracture calluses for the following criteria: periosteal and endosteal reaction, callus opacity, and cortical remodeling and bridging, revealed accelerated healing in the *miR194-5p* mimic experimental groups at 21 days post-fracture. 3-dimensional analysis by µCT showed a trend toward increased % callus bone in the 10µg *miR194-5p* mimic-treated group and significantly increased % callus bone in the 20µg *miR194-5p* mimic-treated group compared to negative control miRNA-treated fractures. We have corroborated these data with static histomorphometric analysis and observe significantly increased % callus bone and decreased % callus cartilage in the 10ug *miR194-5p* mimic-treated group and increased % callus bone in the 20ug *miR194-5p* mimic-treated group. For subsequent cohorts, we treated mice with either 20µg control miRNA, or 20µg *miR194-5p* mimic and analysed fracture healing at 14 or 21 days post-fracture. We didn't observe any effect of *miR194-5p* mimic on fracture callus composition at day 14, however confirmed our earlier observation that miR194-5p mimic promotes fracture healing by day 21 (Figure 1).

Discussion: In the present study, we observed that delivery of a microRNA targeting *Runx3* to the fracture callus increased callus bone composition, supporting our genetic studies that Runx3 suppression promotes endochondral ossification in the context of fracture healing. Although we have shown that *miRNA-194-5p* significantly reduces Runx3 expression in fracture callus, we cannot exclude the possibility that the ensuing phenotype may, in part, be due to other targets. Nevertheless, we demonstrate the *miRNA-194-5p* promotes osteogenesis during fracture healing.

Significance: These data demonstrate the potential for using miRNA targeting *Runx3* as a novel therapeutic for fracture healing.

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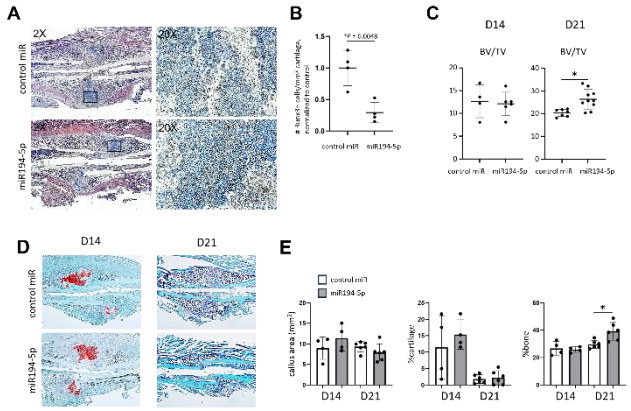

Figure 1. *miRNA-194-5p* increases callus bone composition. (A) Runx3 immunostaining of miR control and *miR194-5p* injected fracture callus, 14 days post-fracture. (B) Quantification of Runx3 immunostaining showing reduced Runx3 expression in *miR194-5p*-treated callus. (C) µCT analysis of day 14 and 21 post-fracture callus indicates a significant increase in BV/TV in *miR194-5p*-treated callus at day 21. (D, E) Histological sections stained with Safranin O (red: cartilage) and fast green (green: mineralized tissue) indicate that injection of miR194-5p increased callus bone % at day 21 post-fracture. Data are expressed as mean ± SD. *p < 0.05.