Enhancing effects of the microRNA-181a/b-1 cluster on endochondral bone fracture repair

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INTRODUCTION: MicroRNAs (miRNAs) are highly conserved non-coding epigenetic factors that regulate many cellular pathways due to their ability to target multiple mRNAs. We previously reported miRNA expression profiles in chondrocytes of human embryonic developing long bones (1). From this study, we found miR-181a-1 to be more highly expressed in hypertrophic chondrocytes compared to less differentiated chondrocytes in the developing limb, suggesting a role in regulating endochondral ossification. In later studies, we demonstrated that over-expression of miR-181a-1 together with the closely clustered miR-181b-1 (miR-181a/b-1) enhanced osteogenesis *in vitro* (2). One of the mechanisms by which this miRNA cluster enhanced osteoblast differentiation was via targeting PTEN resulting in increased PI3K/AKT signaling and mitochondrial metabolism. To follow on from these studies, our goals are to determine how miR-181a/b-1 affects bone formation *in vivo* and to further decipher its effects on mitochondrial metabolism. In our previous ORS abstract submission (3), we presented preliminary data on miR-181a/b-1-mediated enhancement of endochondral ulnar fracture repair in male mice. To further explain the effects on osteogenesis and mitochondrial metabolism, we also identified pyruvate dehydrogenase kinase 4 (PDK4) as a potential target of this miRNA cluster and that a PDK4 inhibitor drug, diisopropylamine dichloroacetate (DADA), and PDK4 knockdown (via shRNA) could enhance *in vitro* osteogenesis (3). In this study, we now confirm the effects of miR-181a/b-1 on fracture repair in male and female mice and demonstrate that inhibition of PDK4 is one mechanism by which this miRNA cluster enhances fracture repair *in vivo*.

METHODS: Studies involving human cells and tissue were approved by the Washington University Human Research Protection Office and animal studies were approved by Washington University IACUC. Murine ulnar fractures were created in the left forelimbs of 12 wk C57BL/6 male or female mice (n = 8 per treatment group). Lentivirus over-expressing miR-181a/b-1 (LV-181) or non-silencing RNA (LV-NS) (20µl; ~10^7 pfu) was injected into the fracture site at day 1 and day 5 after fracture followed by harvest at day 14 for microCT scanning and analysis to quantify the total callus volume and bone volume within the callus region. In male mice, limbs were also harvested at day 21 for mechanical testing (uniaxial compression). An additional treatment group, 4 µM DADA (20µl) injection (day 3 and day 7) and 12 µM drinking water (daily from day 3), was included for microCT analysis at day 14. At day 7 or day 10 following fracture, repair callus tissue was collected for protein isolation or for generating paraffin tissue sections for histology and type X collagen immunostaining, respectively. Computational analysis (TargetScan, miRTarBase) was used to identify PDK4 as a potential target of miR-181a/b and confirmed by luciferase reporter assays using a pmirGLO vector containing the PDK4 3'-UTR wild type sequence or a sequence containing mutations at the miRNA binding site. Multipotent cartilage progenitor-like cells (CPCs) from human OA cartilage (2) were used for *in vitro* experiments to investigate the effect of PDK4 inhibition on osteogenesis by: i) miR-181a/b-1 overexpression, ii) PDK4 inhibitor drug (DADA) treatment (2 µM), or iii) PDK4-shRNA transduction. Appropriate controls were included for each treatment. Similar treatments were used to determine effects on mitochondrial metabolism (using the Agilent Seahorse XF Cell Mito Stress Test Kit) as well as the activity of pyruvate dehydrogenase (PDH) using the PDH assay kit (Abcam). Wherever applicable, a Student's t-test was used to calculate statistically significant differences between treatment and control groups.

RESULTS: By increasing the group size of ulnar fractures performed per treatment (n = 8) we confirmed enhanced fracture repair in the LV-181 group compared to LV-NS group as shown by formation of a larger total callus volume as well as higher bone levels within the callus of male mice (Fig. 3A). However, this enhancement effect was not apparent in female mice (results not shown). Uniaxial mechanical testing of male fractured forelimbs at day 21 showed that LV-181 treatment led to increased callus stiffness (results not shown). Examination of tissue sections from day 10 male fractured limbs showed an apparent increase in hypertrophic chondrocytes (by safranin-O staining and type X collagen immunostaining) in the LV-181 group compared to LV-NS (results not shown). Following over-expression of LV-181 in CPCs *in vitro*, western blotting revealed reduced expression of PDK4 protein (Fig. 1A-C). Findings from the luciferase reporter assay confirmed PDK4 as a target of miR-181a/b (Fig. 1D, E). PDK4 is an enzyme that functions inside the mitochondria to inactivate the pyruvate dehydrogenase complex (PDC) by phosphorylating pyruvate dehydrogenase (PDH), a component of the PDC (Fig. 2A). Therefore, by suppressing PDK4, we expect PDC activity to increase, as measured by PDH activity assays. As expected, we found higher PDH activity in CPCs following either miR-181a/b over-expression, PDK-4 inhibition (DADA) or shRNA-PDK4 treatment (Fig. 2B,C,D). We then investigated if these results translated to higher mitochondrial metabolism and, indeed, we found increased oxygen consumption rates and respiration-linked ATP production following treatment of CPCs with DADA or sh-PDK4 (Fig. 2E-L). This was similar to what we reported for LV-181 treatment (2). To attempt to link *in vitro* findings to *in vivo* bone repair, we detected lower levels of PDK4 protein in day 7 fracture calluses following LV-181 injection compared to LV-NS. Importantly, DADA-treated male mice also showed enhanced fracture callus formation (Fig. 3B).

DISCUSSION: These studies have demonstrated that a major mechanism by which miR-181a/b-1 enhances osteogenesis as well as fracture repair in male mice is by suppressing PDK4 and increasing mitochondrial metabolism. Additional mechanisms involving induction of cartilage hypertrophy may also contribute to enhanced fracture repair and is worth further investigation. The finding that female mice do not respond to LV-181 treatment is intriguing and lays the foundation for future work to decipher these sex-dependent effects on fracture repair.

SIGNIFICANCE/CLINICAL RELEVANCE: This work has identified potential novel targets to enhance bone fracture repair. These findings have important implications in designing new strategies to repair non-healing endochondral bone fractures.

REFERENCES: 1) McAlinden, *et al.* 2013 PMID: 24040378. 2) Zheng, *et al.* 2019 PMID: 30898695. 3) Zheng, *et al.* ORS 2022 Abstract #0176

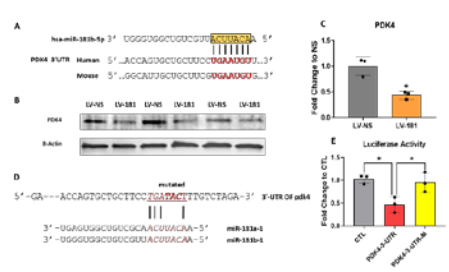

Fig. 1 miR-181a/b-1 targets and suppresses PDK4. n=3 *p < 0.05

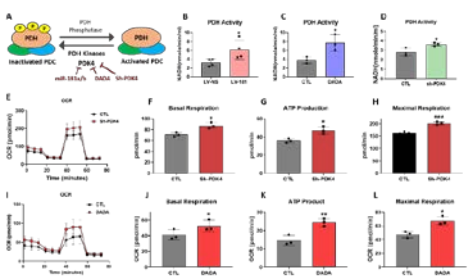

Fig. 2 Suppression of PDK4 increases PDH activity and mitochondria respiration. n=4 (B-D) n=3 (E-L) *p < 0.05, **p < 0.01

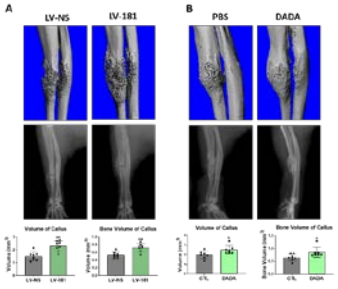

Fig. 3 LV-181 and DADA enhances ulnar fracture healing n=8 *p < 0.05 **p < 0.01