Development of a scaffold-based platform for dual delivery of a microRNA mimic and a microRNA inhibitor for the treatment of large-volume bone defects

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INTRODUCTION: The treatment of large-volume bone defects, often resulting in delayed or non-union of tissue, remains one of the most significant challenges in orthopaedics. The delivery of microRNAs from biomaterial-based scaffolds has emerged as a promising strategy to tackle this issue, as they can control the expression of multiple genes while suppressing detrimental effects. While a scaffold provides structural support to bone tissue, the combination with controlled delivery of microRNAs (miRs) also offers the potential to induce endogenous cells to produce relevant therapeutic proteins with a physiological release profile and minimal side effects (1,2). In our lab, we have shown that the delivery of miR-133a inhibitor, using hydroxyapatite nanoparticles (NPs) as a non-viral vector, enhances both *in vitro* and *in vivo* osteogenesis by up-regulating Runx2-mediated bone formation (2). Another potential target is miR-26a which regulates bone formation through positive regulation of angiogenic-osteogenic signalling pathways (3). In this study, a collagen-nanohydroxyapatite (coll-nHA) scaffold (1,2), previously optimised for bone repair in our lab, was combined with the self-assembling, amphiphilic, cell-penetrating RALA peptide (4) as a delivery vector in order to develop a miR-activated scaffold system for the simultaneous delivery of both a miR-26a mimic (3) and a miR-133a inhibitor (2). The potential of this platform for the treatment of large-volume bone defects was then assessed in a critical-sized rat calvarial defect model.

METHODS: *Development and physicochemical characterisation of dual-miR scaffolds:* The coll-nHA scaffolds were prepared using a freeze-drying technique (2) followed by the incorporation of a mixture of miR-26a-RALA and antagomiR-133a-RALA nanoparticles at 1 µg and 3µg concentrations. The dual-miR scaffolds were assessed in terms of: the miR and antagomiR NPs distribution and release, morphology, weight loss, and calcium release. *In vitro evaluation with human mesenchymal stem cells:* The biological validation included assessment of: metabolic activity, DNA content, cell distribution (H&E staining), transfection efficiency, expression of osteogenic targets and mineralisation (Ca^{2+} quantification and Alizarin Red staining). *In vivo evaluation in a calvarial defect in rats:* The miR-activated scaffolds (0.5 µg of each of miR-26a-RALA and antagomiR-133a-RALA nanoparticles) were implanted into 7 mm defects; bone volume and density were assessed by µCT at 4- and 8 weeks post-surgery. The samples were harvested at 8 weeks and assessed histomorphometrically using H&E staining.

RESULTS: The dual-miR activated scaffolds showed a uniform distribution of miR and antagomiR NPs, and the incorporation of these NPs did not compromise the scaffold's optimal architecture and porosity. Notably, the scaffolds facilitated a controlled and prolonged release of the therapeutic cargo over 24 hours, with a retention of approximately 60% for the 1µg dosage and about 80% for the 3 µg dosage of NPs spanning the 28 days of the study. The dual-miR-activated scaffold effectively transfected human mesenchymal stem cells (hMSCs) with both therapeutic agents, resulting in a 20-fold increase in miR-26a expression and a 10-fold decrease in miR-133a expression (Fig 1A). hMSCs cultured on dual-miR activated scaffolds demonstrated increased mineralisation and calcium production (Fig 1B). Impressively, the dual-miR-activated scaffolds significantly accelerated bone repair in critical-sized calvarial defects of male rats (Fig 2), leading to greater than a 50% increase in bone volume compared to the miR-free scaffolds (Fig. 2A). The dual-miR activated scaffolds yielded a higher bone mineral density (Fig. 2C), underscoring their potential to foster the growth of tissue with superior quality.

DISCUSSION: This study describes the development of a miR-activated scaffold system capable of controlled dual delivery of both a microRNA mimic and a microRNA inhibitor- both of which have been shown to enhance osteogenesis. The dual-miR activated scaffold was capable of transfection of hMSCs with both the miR-26a mimic and the miR-133a inhibitor, which translated into an enhanced expression of targeted, osteogenic genes (ALP, BMP2). The increased level of ALP and enhanced calcium deposition shows that the miR-26a mimics and miR-133a inhibitor positively regulate osteogenesis. This in vitro success was then supported in the in vivo study, which demonstrated improved healing of critical-sized defects, yielding a highly mineralised bone matrix. This demonstrates that the miR-activated scaffold system developed herein accelerates bone healing and produces a new tissue of superior quality resulting in the repair of large-volume bone defects.

SIGNIFICANCE/CLINICAL RELEVANCE: In this study, collagen-hydroxyapatite scaffolds were effectively utilised as a delivery system for therapeutic microRNAs enabling the simultaneous dual transfection of hMSCs with a microRNA mimic and a microRNA inhibitor delivered using a cell-penetrating peptide. Scaffold-based delivery of microRNAs facilitates transient gene editing and encourages host cells to produce targeted proteins at physiological levels with minimal adverse side effects. As a result, this cell-free system holds potential as a new 'off-the-shelf' product, capable of enhancing bone healing as the therapeutic miRs allow enhancement of genes with a positive effect (miR-26a) and shut off aberrant effects, in this case suppressing miR-133a which is known to inhibit Runx2 and bone formation. By varying the targets and scaffold composition, this novel dual-microRNA activated scaffold system for combined delivery of both mimics and inhibitors of microRNA expression could be tailored for a myriad of applications beyond bone repair.

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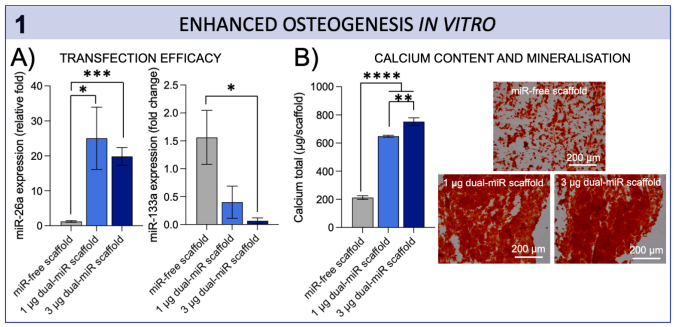
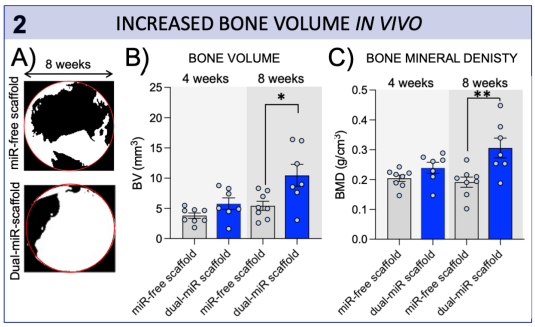
IMAGES AND TABLES:



1. The dual-miR scaffolds effectively transfected the hMSCs with both therapeutic cargos at day 3 **(A)** resulting in enhanced calcium production and mineralization **(B)**



2. The dual-miR scaffolds accelerated bone repair in critical-sized calvarial defects at 8 weeks resulting in higher bone volume **(A,B)** and density **(C)**