Development of a highly efficient messenger RNA-activated scaffold for enhanced healing of large weight-bearing bone defects

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INTRODUCTION: The treatment of non-union bone fractures and critical-sized bone defects remains a significant clinical challenge, underscoring the need for novel therapeutic approaches. The standard clinical practice relies on the delivery of osteogenic growth factors or recombinant proteins, such as bone morphogenetic proteins (e.g. BMP-2), but these can be associated with numerous side effects and high costs. The COVID-19 pandemic highlighted the potential of mRNA-based therapeutics, paving the way for their application beyond vaccines, including in regenerative medicine. Our group has developed collagen-nanohydroxyapatite (coll-nHA) scaffolds with proven efficacy *in vivo* in healing bone tissue (1), which can deliver gene therapeutics locally, reducing uncontrolled release and protein production (2-3). This system is particularly advantageous because the scaffold provides structural support to the developing bone tissue whilst the delivery of genes activates endogenous cells to produce protein in a controlled fashion. Utilising our experience in scaffold-based gene delivery, and capitalising on the growing opportunity for mRNA therapeutics, this study aimed to develop an mRNA-activated scaffold capable of promoting both blood vessel ingrowth and bone formation by combining coll-nHA scaffolds (2,3) with nanoparticles carrying the most potent angiogenic (messenger RNA encoding for VEGF) and osteogenic (messenger RNA encoding for BMP-2) therapeutics (4).

METHODS: *Development and physicochemical characterisation of mRNA nanoparticles and mRNA-activated scaffolds:* Luciferase-encoding mRNAs were combined with MessengerMax®, a lipid non-viral vector, and assessed in terms of zeta potential, polydispersity index, and stability. The coll-nHA scaffolds were prepared using freeze-drying techniques (2), followed by the incorporation of mRNA-BMP or mRNA-VEGF nanoparticles (the dual-loaded mRNA scaffold). The scaffolds were assessed in terms of the mRNA NPs distribution and release, morphology, and compressive modulus. *In vitro evaluation with rat mesenchymal stem cells:* The biological validation of nanoparticles with mRNA-BMP or mRNA-VEGF, and mRNA-activated scaffolds included assessment of: DNA content, transfection efficiency, BMP-2, and VEGF release, angiogenic tubule formation and mineralisation (Ca^{2+} quantification and Alizarin Red staining). *In vivo evaluation in a weight-bearing femoral defect in rats:* The *in vivo* efficacy of mRNA-activated scaffolds complexed with non-viral jetPEI vector ware tested in 5 mm femoral defect in female Sprague Dawley Rats (n=8). The bone volume and density were assessed by Micro-Computed Tomography (µCT), and samples were harvested at week 8 and assessed histomorphometrically (H&E, Masson-Goldner) and quantifying the presence of blood vessels.

RESULTS SECTION: The mRNA nanoparticles encapsulated both genetic cargoes, demonstrating effective transfection of rat mesenchymal stem cells in vitro. Transfections with 1µg and 2µg of mRNA-BMP-2 resulted in the production of ~700 pg/mL and ~1000 pg/mL on days 1 and 2 of the study, respectively (Fig 1A). This translated into enhanced calcium deposition evidenced through Alizarin Red staining (Fig 1B). The rat MSCs transfected with mRNA-VEGF produced ~200 ng/mL and ~250 ng/mL on days 1 and 2 for 1µg and 2µg dosages, respectively (Fig 1C). The mRNA-VEGF also enhanced tubule formation in the Matrigel assay, demonstrating its angiogenic potential. After incorporating mRNA cargoes into the coll-nHA scaffold and confirming its osteogenic potential *in vitro*, we assessed the regenerative potential of the dual-loaded mRNA scaffold *in vivo* in a load-bearing femoral defect in female rats. Notably, the *in vivo* assessment showed that the dual-loaded mRNA scaffold promoted bone healing in the femoral defect (Fig 2A), resulting in 30% higher bone volume at 8 weeks compared to the gene-free scaffold. Both gene-free and dual-loaded mRNA scaffolds demonstrated good infiltration by host cells. Importantly, the implantation of the dual-loaded mRNA scaffold increased the number of blood vessels (Fig. 2C-D), highlighting their potential to foster highly vascularised bone tissue.

DISCUSSION: This study demonstrated the potential of using mRNA complexed nanoparticles encoding for strong osteogenic and angiogenic molecules in healing of large bone defects. We demonstrated that both therapeutic targets effectively transfected rat MSCs, even at the low dosage of 1 µg. This, in turn, led to increased calcium deposition and mineralisation at later time points (day 28) and the formation of vascularised tube-like structures. Furthermore, we showed that the coll-nHA scaffold system is an optimal matrix for the *in vitro* and *in vivo* delivery of these mRNA therapeutics. Importantly, our research highlighted that simultaneous delivery of mRNA-BMP-2 and mRNA-VEGF is particularly advantageous, resulting in significantly enhanced mineralisation of rat MSCs. This scenario was reflected *in vivo* when the dual-loaded scaffolds were implanted into the challenging weight-bearing femoral defect. The delivery of therapeutic cargoes led to highly mineralised and vascularised bone tissue, suggesting that the scaffolds foster the creation of superior-quality new tissue, indicating a promising therapeutic result.

SIGNIFICANCE/CLINICAL RELEVANCE: The dual-loaded scaffold system proposed here, delivering therapeutic mRNAs, has the potential to serve as next-generation therapeutics for the repair of large bone defects. It offers precise and transient gene expression with minimal immunogenicity. The mRNA-activated scaffold described in this study illustrates successful target gene manipulation and promising effects in producing osteogenic and angiogenic proteins at physiological levels *in vitro*, along with enhancing the formation of high-quality bone tissue *in vivo*. With this research, we introduced the concept of mRNA activated scaffolds for regenerative medicine applications, providing results that could significantly impact the field of gene therapy. This system has the potential for a range of applications, including beyond orthopaedics thereby potentially improving the quality of life for many patients worldwide.

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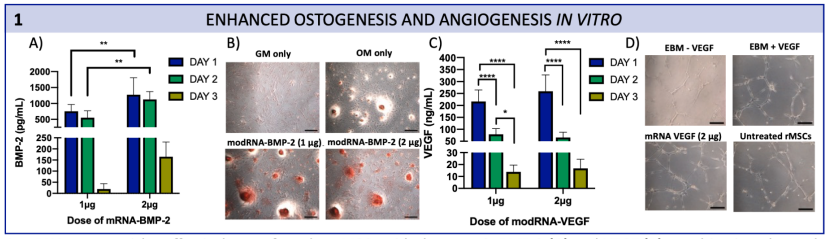
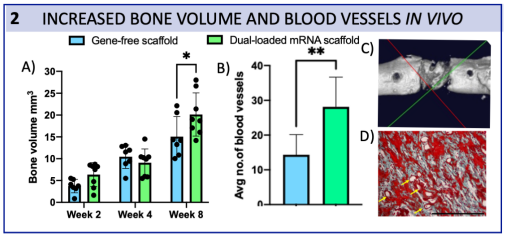
IMAGES AND TABLES:



1. mRNA nanoparticles effectively transfected rat MSCs with therapeutic BMP-2 **(A)** and VEGF **(B),** resulting in enhanced calcium deposition **(C)** and tube formation **(D)**.



2. Dual-loaded scaffold containing mRNA BMP-2 and mRNA VEGF accelerated bone repair in critical size weigh bearing defect at 8 weeks resulting in higher bone volume **(A-B)** and better vascularisation **(C-D)**