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INVESTIGATION OF ANGIOGENESIS BY IN VIVO MULTIPHOTON MICROSCOPY DURING BONE FORMATION IN MURINE CALVARIAL CRITICAL BONE DEFECT REPAIRED BY GENETICALLY MODIFIED 3D-PLGA/nHAp SCAFFOLD

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Objective: Reconstruction of critical bone defects remains a major orthopaedic clinical challenge because of poor bone regeneration, especially angiogenesis. Implant failure of critical bone defect is often due to avascular necrosis. A satisfactory vascularisation is a prerequisite for the survival and integration of new tissue, with existing host tissue. Genetic modification of biological scaffolds to enhance angiogenesis is one of the effective methods for bone regeneration. Present work investigates angiogenesis by *in vivo* multiphoton microscopy during bone-formation in murine calvarial critical bone defect, repaired by a genetically modified 3D-printed PLGA/nHAp scaffold.
Methods: In this study, we attempt to design lentivirus-mediated genetic modification of three-dimensional porous PLGA/nHAp scaffolds, which can deliver a recombinant lentivirus carrying the cytokine gene-*pdgfb*. Lentivirus can be time-released from the scaffold via a surface of lyophilisation immobilised strategy. After implanting the PLGA/nHAp scaffolds, modified with lentivirus, to the critical-sized calvarial bone defect model, we studied how the genetically modified 3D scaffolds affect the angiogenesis and bone formation by multiphoton microscopy, RT-qPCR, microCT, and histomorphological methods, respectively.
Results: We constructed vectors needing to be tested and produced lentiviral vector particles by using 293FT cells. We successfully fabricated the 3D porous PLGA/nHAp scaffold to fit the circular bone defect. The *in vitro* coating, release, titre, and bioactivity of the lentivirus with the scaffold were tested. Based on what we did, the lentivirus could be loaded onto the PLGA/nHAp scaffold and survive. The modified scaffolds could continuously release bioactive LV-*pdgfb* particles for up to 5 days *in vitro*. In scaffold implanted critical calvarial bone defect mouse model after eight weeks post-implantation, blood vessel areas (BVA) were 9428±944 μm^2, 4090±680.3 μm^2 and none in the PLGA/nHAp/LV-*pdgfb* (PHp), PLGA/nHAp (PH), and empty group, respectively. At each observed time point, BVA in PHp scaffolds was significantly higher than in PH scaffolds. Additionally, expression of *pdgfb* and angiogenesis related genes *vWF* and *VEGFR2* increased correspondingly. MicroCT analysis indicated that the new bone formation in the PHp group dramatically improved compared to the other groups, the parameters including bone mineral density (BMD), the ratio of bone volume to tissue volume (BV/TV), trabecular separation (Tb.Sp), and trabecular number (Tb.N) were significantly higher than the other groups.
Conclusion: The results shown in this work demonstrate that a genetically modified scaffold with *pdgfb* gene significantly improved angiogenesis and further enhanced bone regeneration in a mouse critical-size calvarial bone defect model. By using a considered clinically bio-safe viral vector, for example Adeno-associated virus (AAV) vectors, this strategy and this gene could be a potential therapy for critical bone defect.
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MIR-X SUPPRESSES TENOGENIC DIFFERENTIATION OF HUMAN TENDON DERIVED STEM CELLS THROUGH TARGETING EGR1

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Objective: Transcription factor Egr1 (early growth response-1) regulates tendon differentiation and promotes tendon repair. The function of miRNA that regulate Egr1 expression remains largely unknown. In our preliminary study, hsa-mir-X was predicted to target Egr1 by bio-informatic screening. Further investigation also demonstrated that Egr1 was indeed a direct target of mir-X in tendon derived stem cells (TDSCs). According to this preliminary data, we hypothesise that anti-mir-X-3p could promote human TDSC differentiation through regulating the expression of Egr1.
Methods: The human TDSCs were isolated from a donor's patellar tendon following digestion in 2 mg/ml collagenase I. The stemness of TDSCs were confirmed by Oil red O and Alizarin red staining after adipo-induction and osteo-induction, respectively. To test the function of mir-X in tendon lineage differentiation, we used low-glucose DMEM containing 10ng/ml TGFb-1 as induction medium in all experiments. A luciferase assay was used to confirm the binding between mir-X and the promoter reign of Egr1. Collagen formation was measured by Sirus red staining. Expression of tendon marker genes was verified by qPCR. Western blot was used to quantify the expression of Egr1 at the protein level. Synthesised mimic of mir-X and siRNA of Egr1 were both purchased from a commercial company and transfected into human tendon cells using lipo3000.
Results: 10ng/ml of TGF-β was able to induce the tendon lineage differentiation of TDSCs. Real-time qPCR results showed increased expression of several tendon marker genes, including Fmod and Col1a1. Whereas expression of mir-X dramatically decreased with the tenogenic differentiation. The potential binding site between mir-X and the Egr1 promotor was predicted using TARGET SCAN. The luciferase assay demonstrated mir-X mimic significantly suppressed luciferase activity of the WT reporter. On the contrary, the mutant reporter successfully abolished the suppression effect. The expression level of Egr1 was confirmed in the protein level by western blot. Furthermore, the inhibitor of mir-X upregulated the expression of Egr1 at the protein level too. Mir-X mimic transfected TDSCs exhibited low expression of Fmod and Collagen staining by Sirus red, were totally blocked. mir-X inhibitor transfection showed the opposite effects. siRNA of Egr1 was co-transfected with a mimic and inhibitor of mir-X. The qPCR and Sirus red staining showed the inhibitory effects of mir-X on Egr1 expression were partially abolished.
Conclusion: Taken together, mir-X could inhibit TDSCs' tenogenic differentiation by directly suppressing the expression of Egr1. mir-X inhibitors may be developed as a potential therapeutic agent for promoting tendon repair.
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