Regulation of HIF1 pathway enhanced osteogenic differentiation of mMSCs in MCNP scaffold

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Statement of Purpose: The hypoxia-inducible factor-1 (HIF-1) pathway is the central regulator of adaptive responses to hypoxia and is required for vasculogenesis. Osteogenesis and vasculogenesis are tightly coupled processes during bone development and formation. It is thus well known that the enhancement of vascularization is of great importance in bone tissue engineering. In this study, we generated MCNP (Micro-channels and Nano-pores)-scaffolds to improve cell viability in hypoxic conditions. Because oxygen levels will be increased following hypoxia-induced vasculogenesis in vivo, emerging nomorxia will influence the osteogenic differentiation. To explore the hypothesis, mMSCs were cultured in MCNP-scaffolds for 3 weeks. After 3 weeks, mMSCs were treated with chetomin (a HIF inhibitor) or increased oxygen concentrations via a bioreactor culture for the inactivation of HIF1.

Methods:

A. Preparation of MCNP and SS (single structure that has no micro-channels and nano-pores) *scaffolds*

Both scaffolds were prepared in dimensions of 8 mm (D) X 10 mm (H) cylindrical type. Polyurethane template coating and paraffin beads casting methods were employed to fabricate each scaffold.

B. Culture of MC3T3-E1 and mMSCs

The mMSCs were obtained from compact murine bone and mMSCs cultured in the scaffold. MC3T3-E1 and mMSCs were cultured in MEM-α and supplemented with 10% FBS, 1% penicillin-streptomycin solution. After 3 weeks, mMSCs cultured in MCNP-scaffold were treated with chetomin (HIF inhibitor) or incubated in bioreactor for 3 days.

B. Cell viability assay

To evaluate cell viability, we created a standard curve using optical density (O.D) from an MTT assay. We measured the O.D using the MTT assay and converted the O.D to a cell number relative to the standard curve.

C. Immunocytochemistry

For Immunocytochemistry (ICC) imaging after cultivation, the scaffolds were frozen using PBS buffer and cut by a cryosection method using the EXAKT cutting system.

D. Quantification of Osteogenic markers

Total RNA was harvested using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Real-time Quantitative PCR was performed with LightCycler FastStart DNA MasterPLUS SYBR Green I (Roche) using Mastercycler (Eppendorf).

Results: In this study, the effects on cellular responses by the scaffold's dual microenvironment comprised of micro-channels and nano-pores were compared to that of single structured microenvironments. After 9 days of incubation, the live/dead staining showed significant differences between the MCNP scaffold and the SS scaffold. A dramatic difference was noted in the center region of scaffold (Figure 1). The viable cell number significantly increased in the MCNP scaffold compared to the SS scaffold (+50%). To investigate HIF1's relation to osteogenic differentiation, we treated these scaffolds with chetomin or used a bioreactor culture to increase oxygen levels to inhibit the HIF1 pathway while mMSCs were cultured in the MCNP scaffold. Following 3 days of treatment with chetomin or bioreactor culture, VEGF and RUNX2 expression was dramatically reduced in the mMSCs whereas DMP1 translocate to extracellular matrix (Figure 2). Consistently, we observed reduction of gene expression of RUNX2 downstream targets as well as HIF1 downstream targets in both the chetomin treatments group and the bioreactor culture group.

Conclusion: Large synthetic bone grafts require a functional, structural microenvironment that allows cell ingress and homogeneous habitation for successful bone regeneration. Moreover, the constructs should provide an adequate vascular-like structure to supply oxygen and nutrients to the new tissue until vasculogenesis occurs. The MCNP scaffold is a three-leveled hierarchical structure: a porous trabecular network similar to that of human trabecular bones (300-400 um), micro-sized channels (25-70 um) within each trabecular septum, and nano-sized pores (100-400 nm) on its surface to allow immobilized cells to anchor. Here, we show results that hypoxia has effects on the osteogenic differentiation of mMSCs not through vasculogenesis. Interestingly, we observed that not only does the activation of the HIF1 pathway promote osteoblastogenesis, but also the subsequent inhibition of HIF1 pathway enhances osteocytogensis. These studies suggest a novel approach to promote in vitro bone tissue regeneration using MCNP-scaffolds to change physiological oxygen concentrations or bioreactor culture systems mimic in vivo models to help understand mechanisms of osteogenesis.

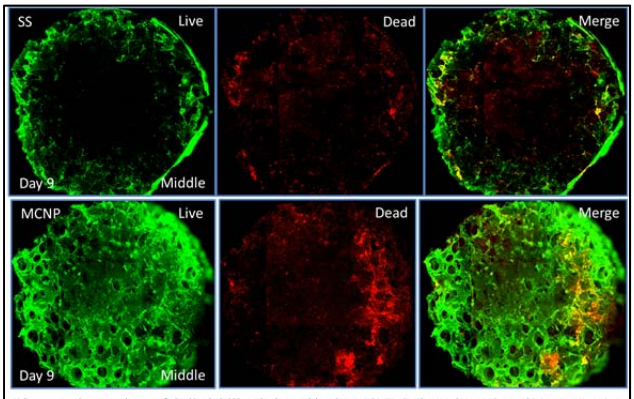

Figure 1. Comparison of Cell viability Cultured in the MCNP (Micro-channels and Nano-pores)-Scaffold and SS (Single scale)-Scaffold

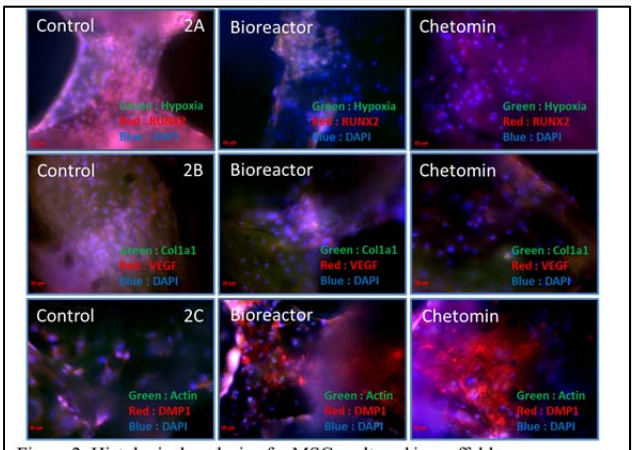

Figure 2. Histological analysis of mMSCs cultured in scaffold. Downregulated of RUNX2 (Fig 1A) and VEGF (Fig 1B) expression whereas DMP1 (Fig 1C) immunostained on extracellular matrix of surrounding cell in both chetomin treated mMSCs and mMSC cultured in perfusion bioreactor.

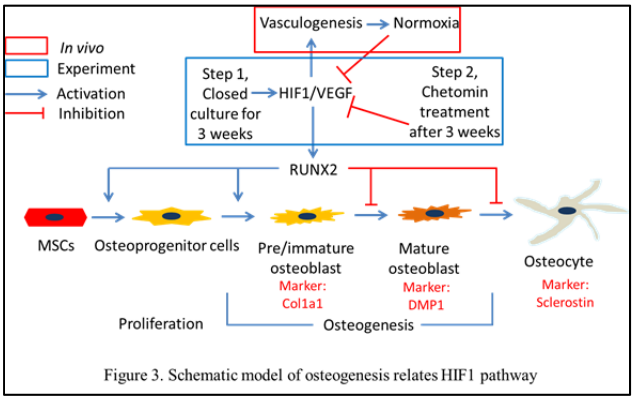

Figure 3. Schematic model of osteogenesis relates HIF1 pathway