# Supporting Information

# Functionalized Hydrogel-Microsphere Composites Stimulating Neurite Outgrowth for Vascularized Bone Regeneration

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**Characterization of alginate composite hydrogels**

Alginate hydrogels containing GMs were soaked in phosphate buffered saline (PBS, 37°C) for 7 days, and after freeze-drying. The macroporous structure produced by the degradation of GMs was observed by scanning electron microscopy (SEM; Hitachi, Japan).

QK/GM was prepared using FITC-labeled QK peptide, and the QK/GM-encapsulated alginate hydrogel was immersed in PBS (37 °C) for up to 35 days. The peptide concentration in the supernatant was measured by a fluorescence microplate reader (Molecular Devices, USA), and QK peptide release profile was plotted.

**Quantitative detection of alkaline phosphatase (ALP)**

After the cells were washed with PBS and lysed with 1% Triton X-100, the supernatant was collected. ALP activity was quantified with the Alkaline Phosphatase Assay Kit (Nanjing Jiancheng, China). For normalization, total protein concentration was measured with the Bicinchoninic Acid Protein Assay Kit (Thermo, USA).

**Alizarin red staining (ARS)**

After the cells were washed with PBS, they were fixed with 4% paraformaldehyde for 30 min. Alizarin red solution (2%, pH=4.2) was added after washing with distilled water, and the reaction was continued for 30 min. After washing with distilled water for several times, the staining of calcium nodules was observed under a microscope (Olympus, Japan).

**RNA extraction and quantitative reverse-transcription polymerase chain reaction (qRT-PCR)**

The cells were lysed with Trizol reagent (Invitrogen, USA), and RNA was extracted. cDNA was obtained using a reverse transcription kit (Thermo, USA). The gene expression was analyzed by qRT-PCR with fluorescence quantitative PCR instrument (Roche, Switzerland). Data were normalized to 28S rRNA expression, and the primers are presented in Table S1.

**Table S1. Primer sequence**

| Gene | Forward primer （5' - 3'） | Reverse primer（5' - 3'） |
|---|---|---|
| 28S rRNA | CCCAGTGCTCTGAATGTCAA | AGTGGGAATCTCGTTCATCC |
| ALP | CAACCCTGGGGAGGAGAC | GCATTGGTGTTGTACGTCTTG |
| Col1a1 | AGACACTGGTGCTAAGGGAGAG | GACCAGCAACACCATCTGCG |
| Runx2 | CCGCCTCAGTGATTTAGGGC | GGGTCTGTAATCTGACTCTGTCC |
| OCN | CCTGAAAGCCGATGTGGT | AGGGCAGCGAGGTAGTGA |
| MAP2K6 | GAAGCATTTGAACAACCTCAGAC | CCTGGCTATTTACTGTGGCTC |
| MAPK9 | GAAACTAAGCCGTCCTTTTCAGA | TCCAGCTCCATGTGAATAACCT |
| MAPK14 | CCCGAGCGTTACCAGAACC | TCGCATGAATGATGGACTGAAAT |
| ERK | GACAAGGGCTCAGAGGACTG | AGGACCAGGGGTCAAGAACT |
| PI3K | CCACGACCATCATCAGGTGAA | CCTCACGGAGGCATTCTAAAGT |
| Akt | AGCGACGTGGCTATTGTGAAG | GCCATCATTCTTGAGGAGGAAGT |

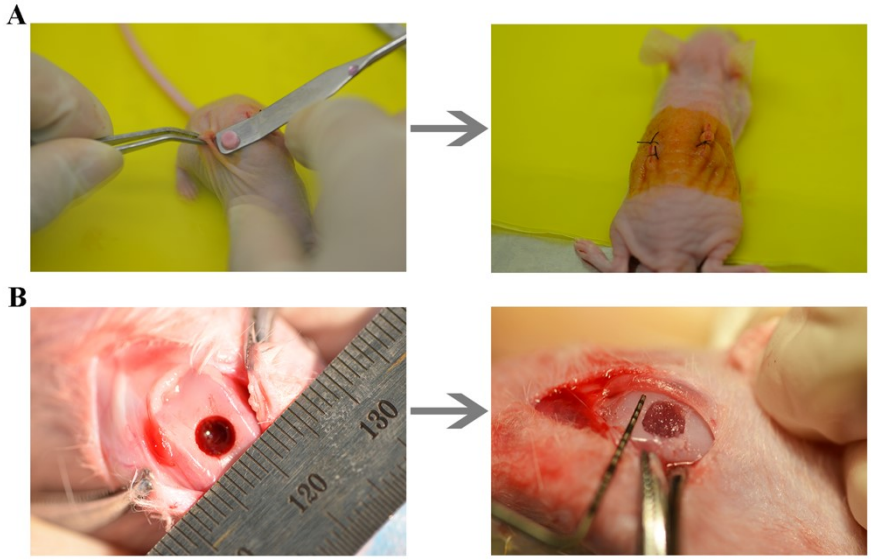


**Figure S1.** Photographs of animal experiments. (A) Subcutaneous transplantation experiments in mice. (B) Rabbit femoral defect experiment.

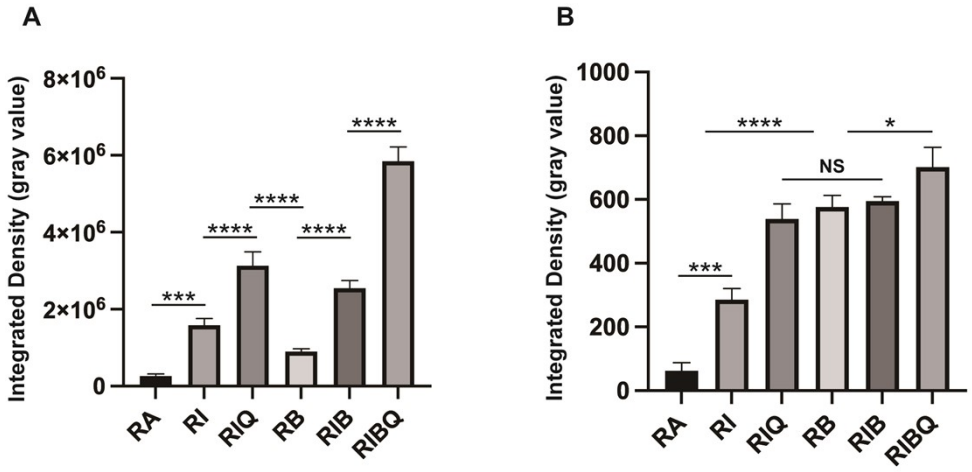


**Figure S2.** The expression of osteogenic and neurogenic related markers after 4 weeks of subcutaneous transplantation in mice. (A) Analysis of β3-tubulin expression in the different groups by fluorescence intensity. (B) Analysis of OCN expression in the different groups by fluorescence intensity. The p-value was calculated by Tukey's post-hoc test (*$p < 0.05$, ***$p < 0.001$, and ****$p < 0.0001$). All data represent means± SD (n = 3).

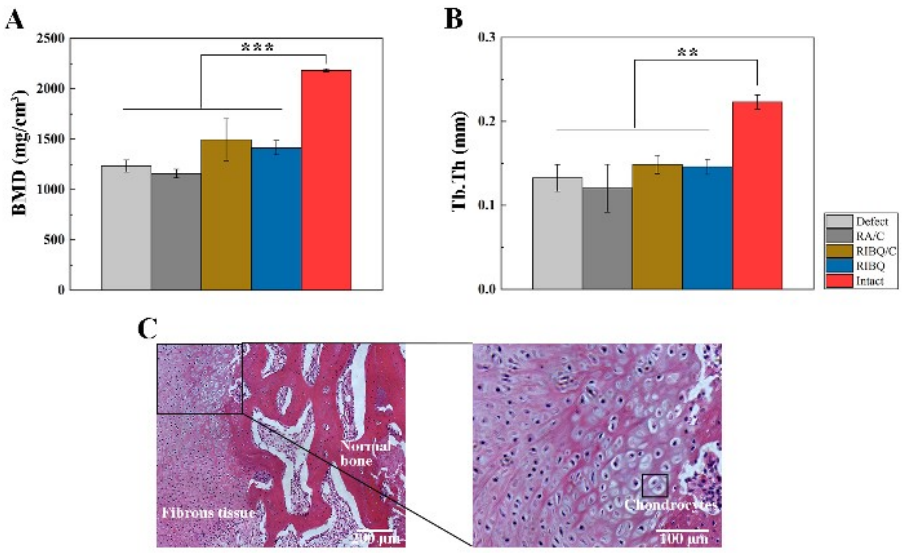


**Figure S3.** Evaluation of bone repair in the rabbit femoral defect. Micro-CT quantitative analysis of (A) bone mineral density and (B) trabecular thickness. (C) H&E staining images at the edge of bone defect, scale bar, 200 μm.

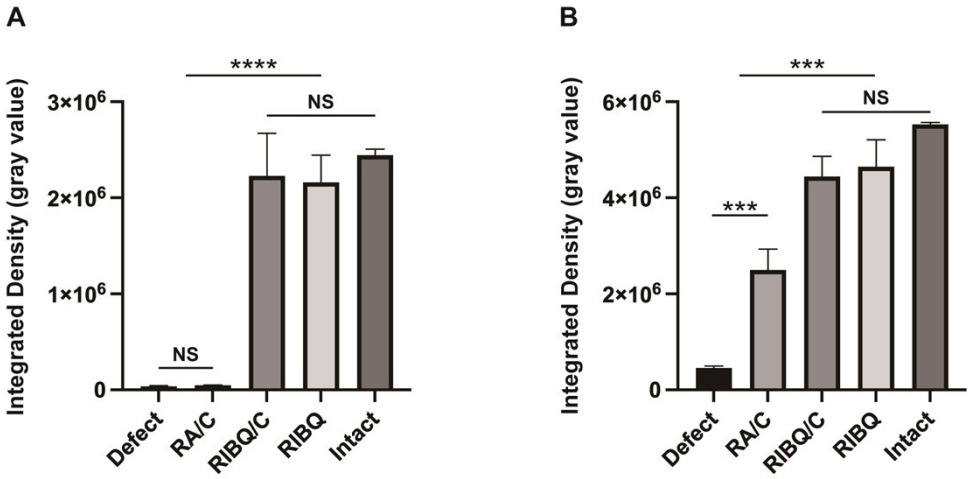


**Figure S4.** The expression of angiogenic and neurogenic related markers in the rabbit femoral defect after 4 weeks. (A) Analysis of β3-tubulin expression in the different groups by fluorescence intensity. (B) Analysis of CD31 expression in the different groups by fluorescence intensity. The p-value was calculated by Tukey's post-hoc test (***$p < 0.001$, and ****$p < 0.0001$). All data represent means± SD (n = 3). Defect represents defect alone with no treatment; RA/C represents RMSCs -laden RA; RIBQ/C represents RMSCs -laden RIBQ; RIBQ represents RI loaded with BFP-1@MSNs and QK/GM.

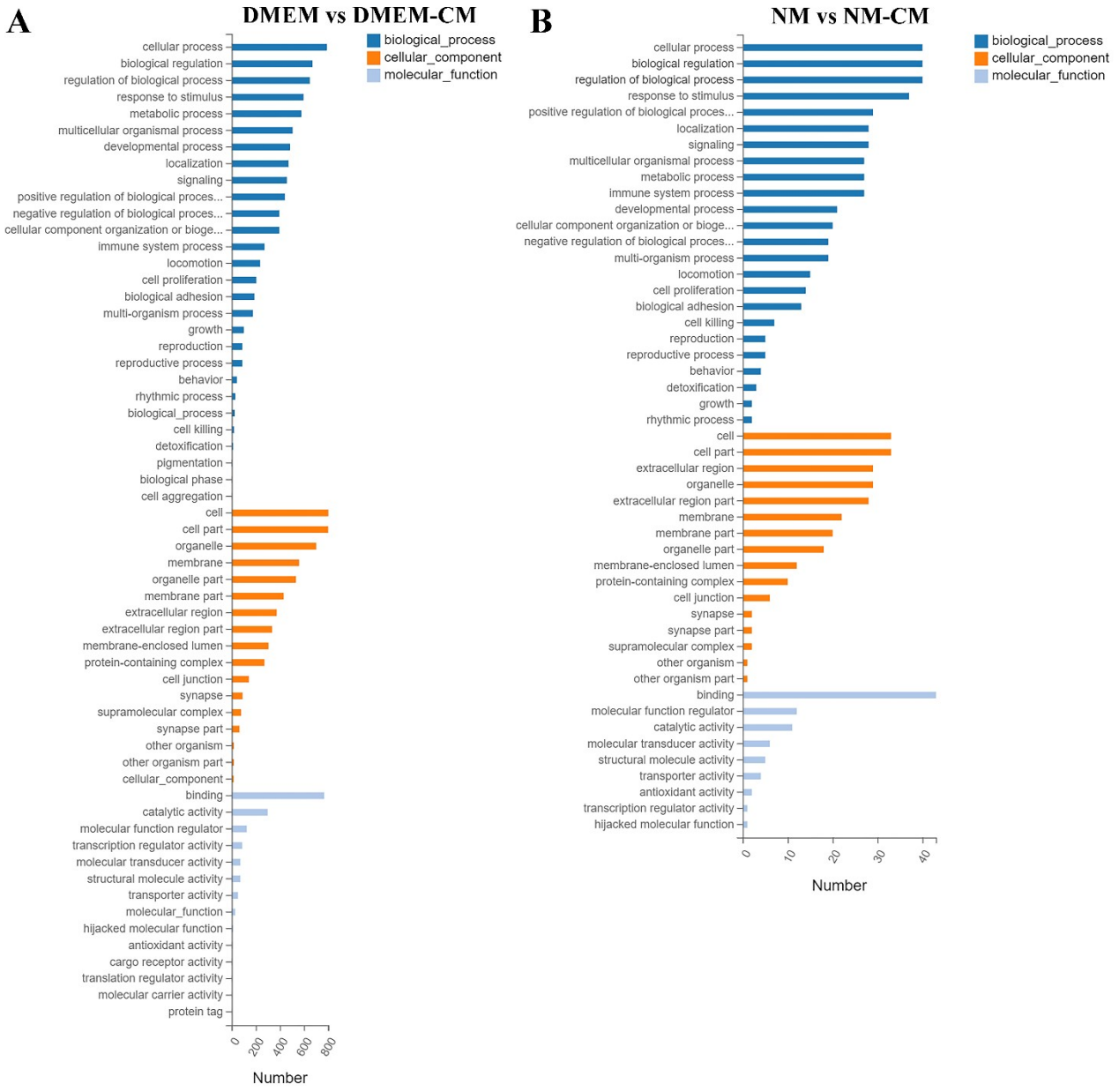


**Figure S5.** Histogram of Gene Ontology (GO) enrichment terms of differentially expressed genes. (A) GO between DMEM and DMEM-CM. (B) GO analysis between NM and NM-CM.

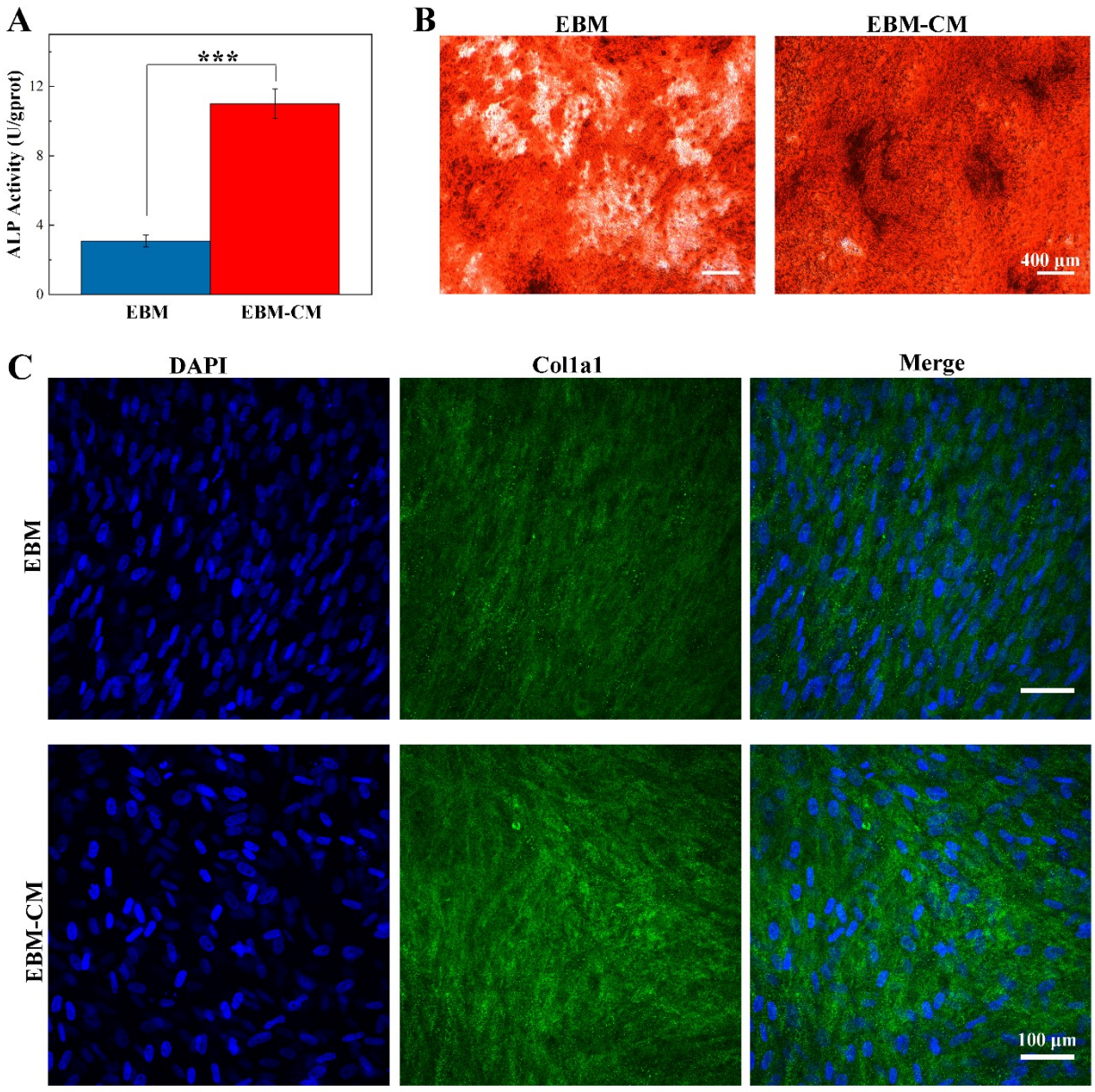


**Figure S6.** Preliminary study on the effect of HUVECs on the osteogenic differentiation of hMSCs. (A) Quantitative analysis results of ALP activity of the hMSCs osteogenic induction for 14 days, ***$p<0.001$. (B) ARS images, scale bar, 400 μm. (C) Immunofluorescence staining images for Col1a1 (green) and DAPI (blue), scale bar, 100 μm.