Support Information

cAMP enhanced calvarial regeneration by bone marrow-derived mesenchymal stem cells on a hydroxyapatite/gelatin scaffold

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MATERIALS AND METHODS

Degradation. The *in vitro* degradation of the HA/Gel crosslinked scaffolds were assessed by immersing samples with a thickness of 2 mm and diameter of 5 mm in 15 ml of PBS at pH 7.4 and 37°C. The PBS was replaced weekly. At 1, 7, 14, 28 and 42 days, the scaffolds were transferred to a 24-well plate, rinsed five times with deionized water and freeze-dried for 48 hours before weighing with a precision balance using the following formula: $D = (W_0 - W_1)/W_0$, $n = 2$, where W_0 is the initial weight of the dried scaffolds and W_1 is the weight of the scaffolds at different time points after immersion and freeze-drying.

Establishment of rat BMSC culture. BMMSCs were isolated from the femurs of male Spregue-Dawley rats (100±5g, 4 weeks old, provided by AnHui medical university) as previously described. Animal protocols were carried out according to the international regulations. Briefly, rats were sacrificed by cervical dislocation and soaked in 75% alcohol for 15 minites. Then, the soft tissues were removed in the ultra-clean platform to expose the femur, tibia and epiphysis which were washed out with Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Life Technologies, Grand Island,

USA) containing 10% fetal bovine serum in order to obtain bone marrow. The primary cells were cultured with α-MEM medium (Invitrogen, Karlsruhe, Germeny) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Karlsruhe, Germeny)), 0.25 μg/ml Fungizone, and 1% (v/v) penicillin and streptomycin (Sigma-Aldrich, Steinheim, Germany) before incubating at 37 °C in 5% CO^2. The unattached cells were removed firstly by sterile phosphate buffer saline wash (PBS; pH 7.4) after inoculation for 72 hours. When the primary cells converge to 80%~ 90%, the cells were digested with 2.5g/L trypsin containing 0.02% EDTA, and then 1×10^6 cells were subculture after cell counting. BMSCs in passage 2 from rats were used for the in experiments.

Cell seeding. The Gel and HA/Gel scaffolds were first soaked in complete culture medium (CCM) containing 10% FBS at 37°C in a 5% CO_2, saturated humidity environment for three days. Most of the CCM was removed such that the scaffold could be inoculated with cells when the materials were slightly dry. Each scaffold was loaded with Passage 2 rBMSCs at a high concentration of 2×10^5 cells/20 μl. The scaffold was initially incubated at 37°C in a 5% CO_2 saturated humidity for 30 minutes, after which an appropriate amount of CCM was added. The CCM was changed every two days until the rBMSCs-seeded Gel (Gel) and rBMSCs-seeded HA/Gel scaffolds (HA/Gel) were obtained.

Assessment of cell proliferationt by CCK8. To obtain the extract of the Gel and HA/Gel scaffolds, they were added into DMEM containing 10% FBS in accordance with the ration of mass/medium=1g/10ml, respectively, and incubated for 72 hours at

37°C; then, it was centrifuged and the supernant was stored at 4°C. The proliferation of BMSCs on the composite scaffolds was investigated by CCK-8 assays. 5×10^3 BMSCs were taken into 96-well plates, and inoculated 150 ul of cell suspension per well, which were incubated at 37°C, 5% CO^2 with saturated humidity for 24 hours. After the cells adhered for 24 hours, removed the supernatant and add 100 μl of extract of Gel and HA/Gel scaffolds,respectively. Each group divided into 5 parallel wells were cultivated at a saturated humidity of 37°C, 5% CO^2. Then, add 10 μl of CCK-8 solution to each well on day 1, 3, 5 and 7, and continue to incubate for 1h at 37°C and 5% CO^2. The absorbance of each well were measured at 450nm wavelength with a microplate reader (Bio-tek, Vermont, USA).

Immunophenotypic profiles of rBMSCs cultures. rBMSCs in passage 2 were digested by 0.25% trypsin and collected cells to count. Then 2×10^6 BMSCs were made into single cell suspensions and labeled with 1.5 μl single-labeled flow antibodies: CD29, CD90, CD11b, CD45 (all from Biolegend, Fell, Germany). Resuspend and mix the cells, and incubate at room temperature in the dark for 45 minutes. Centrifuge at 100 rpm for 10 minutes and discard the supernatant. Resuspend in 3ml PBS, centrifuge at 1000 rpm for 10 minutes, and discard the supernatant. Finally, resuspend in 300 μl PBS, mix thoroughly, and detect the corresponding protein by flow cytometry (Merck Millipore, Billerica, USA).

RESULTS

Immunophenotypic profiles of BMSCs cultures. Based on dentification of BMSCs cultured in vitro by fluid cytometry, the cells higly expressed MSC surface markers: CD90, CD29; lower expression was observed for vascular endothelial cell markers: CD45, CD11b.

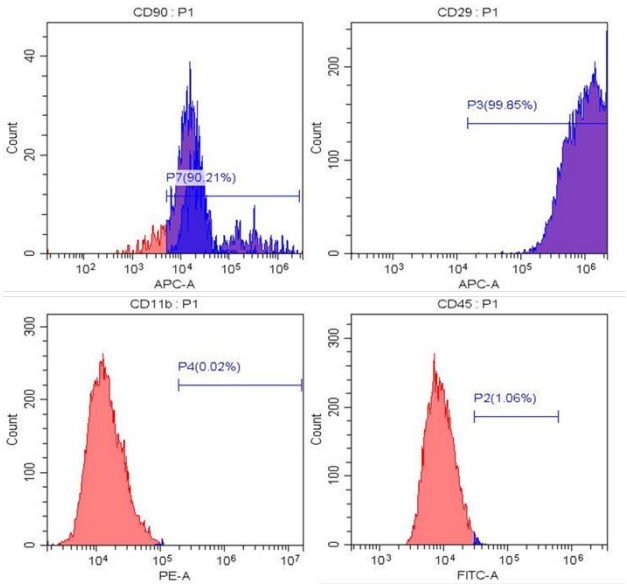

Figure. S1- Representative flow cytometry histograms showing the expression of MSC surface markers（CD90，CD29 and vascular endothelial cell markers(CD45,CD11b).