Electronic Supporting Information

Bacterial flagella as an osteogenic differentiation nano-promoter

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Methods

Flagella Display and purification of flagella: Genetically displaying a RGDEEEEEEEE peptide on flagella was conducted following a protocol in our earlier publication.[1] Briefly, the synthesized oligonucleotides (Invitrogen), which encoded the fusion peptide (RGD-E8) with sticky ends of *Xho* I and *Bgl* II sites at each end, respectively, were inserted in a vector (containing the gene of flagellin with a multicloning site at the central region) by T4 ligase and transformed into competent cell of flagellin deficient salmonella. All the recombinant vectors were verified via DNA sequencing at MC LAB (San Francisco, CA). Only the recombinant flagellin gene located on the vector could be expressed. The bioengineered bacterial flagella were detached from the bacterial cells by vigorous shaking through vortex with highest speed for 3 times (30 sec/each time). The supernatant containing the sheared flagella was separated by centrifugation at 12,000 g for 20 min. Flagella were further washed and purified by centrifugation at 100,000 g for 2 h at 4 ℃. Finally, highly concentrated flagella were dissolved in distilled water.

Layer-by-layer preparation of flagella based substrates: Flagella substrates were prepared by the layer-by-layer (LBL) method reported in our earlier publication,[2] which utilized electrostatic interactions between different layers. Briefly, pre-cleaned glass coverslips were placed at the bottom of 24-well cell culture plate. Poly-lysine solution (0.01%) (Sigma-Aldrich) was added to the wells containing coverslips (350 μl/well) and incubated for 30 min at room temperature,

allowing the adsorption of the first cationic polyelectrolyte layer onto the slides. Then, the slides were washed in ultra-pure water (5 min, continuous water flow) and dried under pressurized air stream. Flagella (150 μl, 4.264 μg/μl) in water or mineralized in supersaturated HAP solution were added into the well and incubated at room temperature for 20 min. The slides were then washed with ultra-pure water (5 min) and dried under pressurized air stream. The previous steps were repeated three times and the final layer was deposited as flagella (Figure 1B). Coverslips with one layer of poly-lysine were used as a control.

MSCs isolation and culture: Primary Bone marrow derived MSCs were isolated from the bone marrow of a young Fisher 334 adult female rat femur (Harlan) based on a previously described method.[2] Briefly, the isolated bone marrow cells were washed several times with Dulbecco's Modified Eagle Medium (DMEM; GIBCO BRL, Grand Island, NY, USA). After cell viability was tested and the density of the cells confirmed using trypan blue staining, cells were maintained in DMEM (low glucose) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), penicillin G 100 U/ml, streptomycin 100 μg/ml, amphotericin B 0.25 μg/ml. The cells were incubated in humidified atmosphere containing 5% CO_2 at 37 °C. The non-adherent cells were removed after three days by changing the culture media. Before use, the MSCs were subcultured no more than three times after isolation. This animal work was approved by Institutional Animal Care and Use Committee of the University of Oklahoma and performed under the guideline of National Institutes of Health.

Methylthiazoletetrazolium (MTT) test: The cells were plated at a density of 4×10^3 cells/well in 96-well plates coated with flagella in DMEM for 72 h. The proliferation of the cells was then

tested by a 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay following the manual of the kit (Sigma-Aldrich). MTT (5 mg/ml, 20 µl /well, Sigma-Aldrich, USA) was added to the cell cultures for 4 h at 37 °C. The supernatant was then discarded, followed by the addition of dimethyl sulfoxide (DMSO, 150 µl/well, Sigma-Aldrich, USA) and agitated for 30 seconds to dissolve the crystal completely. The absorbance was measured at 490 nm using a Biotek spectrophotometric microplate reader.

Scanning electron microscopy (SEM) and Transmission electron microscopy (TEM) characterization: BMSCs were seeded on flagella film at a density of 1×10^4 cells/ml. After 24 h in culture, the cells were washed with 1× PBS and fixed with 2.5% glutaraldehyde in 0.1× PBS for 1 h. The cells were washed again with 1× PBS and then dehydrated in a graded series of ethanol (50%, 70%, 90%, and 100%) for 30 min each. The samples were further dehydrated with a supercritical point CO_2 dryer. The dried samples were then sputter-coated with very thin gold for SEM characterization (XL30, FEI Corporation). Cell surface area was calculated by the computer-assisted planimetry from at least forty five cells per sample in the SEM micrographs using the automated measurement function of Image J (downloaded from the National Institute of Health, Bethesda, MD, USA, and free download available at http://rsb.info.nih.gov/ij/). After 6 days, a drop of aqueous solution was mounted on the carbon TEM grid. After being carefully rinsed with double distilled water and dried at room temperature, these grids were subjected to TEM (Zeiss 10) measurements operated at 80 kV.

Immunofluorescence of Osteopontin and Osteocalcin: The cell culture on the flagella substrates was terminated on 7 and 21 days in osteogenic media. The cells on the flagella substrates were

fixed using 70% ethanol in 1× PBS for 30 min at room temperature. After washed with PBST buffer (1× PBS containing 0.05% Tween-20), the samples were permeabilized for 5 min (0.1% Triton X-100 in 1× PBS solution). Afterwards, the samples were blocked in 5% bovine serum albumin (BSA, Sigma-Aldrich) for 1 h at room temperature. Primary antibodies, either anti-osteopontin (anti-OPN) (1: 500, Abcam Biotechnology) or anti-osteocalcin (anti-OCN) (1:1000, Abcam Biotechnology) diluted in 5% BSA were incubated with cells overnight at 4 °C. After 3 washes for 5 min with 1× PBST, secondary antibodies of Goat anti-rabbit IgG-TRITC (1: 500, Santa Cruz Biotechnology) at 1: 100 dilutions in blocking buffer were then added for 1 h at room temperature. Filamentous actin was stained with FITC-conjugated phalloidin (1: 40, Invitrogen) in 1× PBS and nuclei were stained with DAPI (1: 1000, Chemicon). The coverslips with samples were then inverted onto glass slides, mounted, and the images were collected by a fluorescence microscope.

Quantitative Real-time PCR: The cells are harvested at 7 and 21 days. Total RNA isolation with reverse transcription to cDNA was prepared with Ambion® Cells-to-cDNA™ II Kit (Invitrogen). Power SYBR Green PCR master mix (Applied Biosystems) was applied in this study for monitoring the changes of DNA during qPCR process. Quantitative examination of the samples was done by a mini Fast real-time PCR system (Bio-rad Laboratories) using 10 μL SYBR Green I mastermix, 3 pmol/mL of each forward and reverse primers and 5 μL cDNA templates in a final reaction volume of 50 μL. The PCR reaction involved 45 cycles was performed as follows: 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. In our study, acidic ribosomal phosphoprotein P0 (Arbp) was used as the housekeeping gene. Data collection and analysis was performed using the

software supplied with the instrument. The specificity of the PCR amplification was confirmed by agarose gel electrophoresis. The primer sequences for qPCR were: Osteopontin (OPN),

Forward primer 5'-GACGGCCGAGGTGATAGCTT-3'

Reverse primer 5'-CATGGCTGGTCTTCCCGTTGC-3'

Osteocalcin (OCN),

5'-AAAGCCCAGCGACTCT-3'

5'-CTAAACGGTGGTGCCATAGAT-3'

Runx2,

5'–GCTTCTCCAACCCACGAATG–3'

5'–GAACTGATAGGACGCTGACGA–3'

Type I collagen,

5'-TCCTGCCGATGTCGCTATC-3'

5'-CAAGTTCCGGTGTGACTCGTG-3'

Arbp (Housekeeping gene)

5' -CGACCTGGAAGTCCAACTAC–3'

5'–ATCTGCTGCATCTGCTTG–3'.

Calcium assay: The cells were plated at a density of 4×10^3 cells per well in 96-well plates and coated with flagella and mineralized flagella samples and grown in osteogenic media for 2 weeks. The cells were fixed in 4% paraformaldehyde at room temperature for 40 min and the mineralized nodules were stained with 0.1% solution of alizarin red S (Sigma-Aldrich) at pH 4.1-4.5 for 30 min. The number of calcified nodules was counted in each well. To prevent bias, each experimental group was calculated by averaging the five counted wells.

Statistical analysis: All experiments were conducted at least 3 times. Data is represented as mean ± standard deviation in all the figures. Student t tests were carried out to determine significant difference. Significant difference was set as p-values less than 0.05 or 0.01 in all analysis.

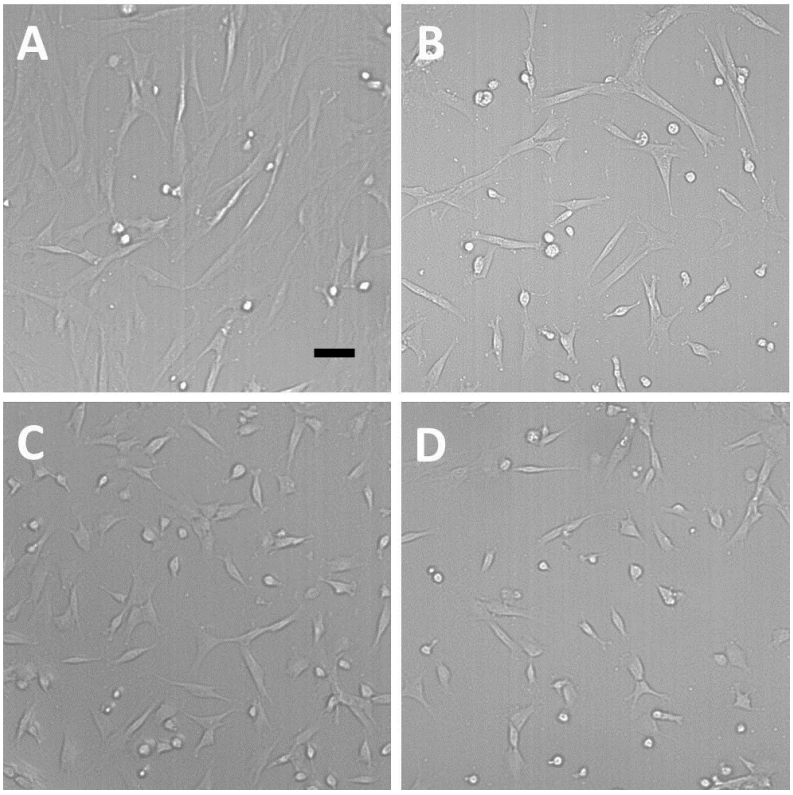

Figure S1 Bright field microscopy of BMSCs on flagella and polylysine coated substrates prepared by LBL at 24 h. A) BMSCs morphology on polylysine coated substrate (Control). The cells are totally spread on the substrate. B) BMSCs morphology on RGDE8 coated substrate. The cells are less spread. C) BMSCs on M-RGDE8 coated substrate. The cells are less spread compared to those on RGDE8 flagella. D) BMSCs on WT flagella coated substrate. The cells are much less spread on WT flagella and some of them only show round morphologies. (Scale bar: 100 μm)

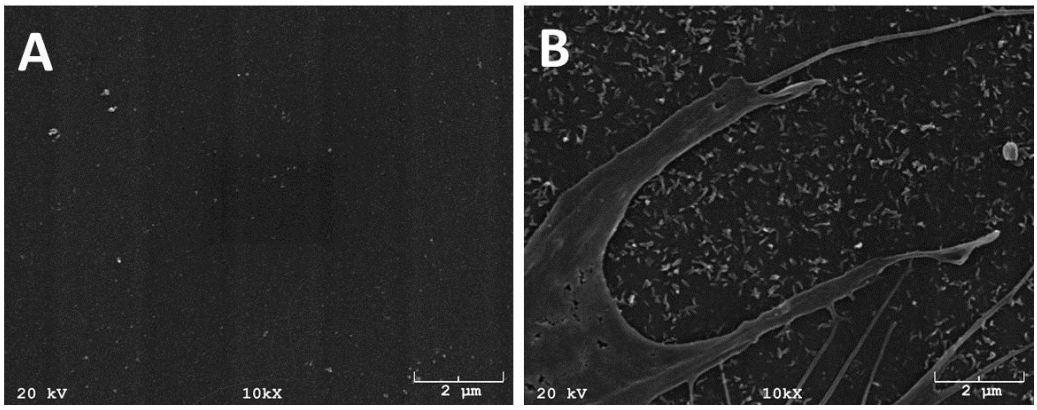

Figure S2 A) SEM topographies of polylysine coated substrate. The surface is very flat. B) SEM micrograph shows that MSCs anchor on biomineralized flagella substrate by filopodia-like extensions.

References:

1. F. Wang, D. Li and C. Mao, *Adv. Funct. Mater.*, 2008, **18**, 4007-4013.
2. H. Zhu, B. Cao, Z. Zhen, A. A. Laxmi, D. Li, S. Liu and C. Mao, *Biomaterials*, 2011, **32**, 4744-4752.