Supplementary Material (ESI) for Molecular BioSystems

Electronic supplementary information (ESI)

Induction of Neurogenesis in Rat Bone Marrow Mesenchymal Stem Cells Using Purine Structure-Based Compounds

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Experimental Procedures

Preparation of small molecule solution

CMP, MMP and RA were dissolved in DMSO at the concentration of 100 mM and diluted in culture medium to make desirable concentrations (1 – 100 μM). These final concentrations of small molecules render a final concentration below 0.02% DMSO to maximally eliminate the effect of DMSO. Control cells were also incubated under experimental condition of 0.02% DMSO to compare the effect of only small molecules.

rBMSC isolation

The rBMSCs were harvested from the femurs and tibias of four week-old female Fischer rats. The rats were housed in sterilized cages with sterile food and water and filtered air, and were handled under a laminar flow hood following aseptic techniques. All animals were treated and all surgical procedures followed protocols approved by in accordance with the Korea Research Institute of Chemical Technology's Council on Animal Care Guidelines. Briefly, the rats were subjected to ether euthanasia and the bones of the hind limbs were aseptically excised. The soft tissues were removed, and the femurs and tibias were placed in 50 mL PBS. The proximal end of each femur and the distal end of each tibia were removed using sterile scissors. A hole was then created in the knee joint end of each bone, using a 26-gauge needle, and PBS (pH 7.4) was used to flush the marrow from the shaft. The flushed marrow was fully suspended in PBS, and the cell suspensions from all bones were combined and centrifuged at 2000 rpm for 5 min. The resulting pellet was resuspended in fresh primary medium [DMEM supplemented

with 10% fetal bovine serum (FBS; Gibco BRL, USA), 100 U/mL penicillin, and 100 g/mL streptomycin] and seeded to tissue culture flasks at 1 x 10^5 cells. After 5 days of expansion, the cultures were rinsed 2 times with PBS for removal of non-adherent cells. The medium was exchanged every two days throughout the studies. For use in the differentiation experiments, adherent cells were rinsed thoroughly with PBS and then detached by trypsinization.

For characterization, rBMSCs of 1 x 10^6 were washed with PBS and incubated with fluorescent-conjugated antibodies (1 μg/10^6 cells) for CD44 and CD45 (BD Pharmingen, San Diego, USA) in PBS [supplemented with 1% bovine serum albumin (BSA) in pH 7.5 PBS] for 30 min on ice, then washed with PBS and fixed in 1% paraformaldehyde (R & D system, Minneapolis, USA) solution. Cells were analyzed using a FACScan (Becton Dickinson, Franklin Lakes, USA) flow cytometry system. Cell analysis was performed using at least 10,000 events per sample. Data acquisition and analysis were then performed by Becton Dickinson cell quest software.

Cytotoxicity tests and neurogenesis of rBMSCs

The rBMSCs were seeded in a 24-well plate at a density of 3 x 10^4 cells/well in culture media [Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin]. After incubation for 24 h, the culture media were replaced with media with DMEM supplemented by 10 ng/mL bFGF and 20% FBS and incubated overnight. Then the media were replaced with chemical induction media (500 μL). Chemical induction media were prepared at final chemical concentration of 1, 10, and 100 μM in cell culture. For control experiment, three wells were treated with culture media. At 1 day and 4 days, the viability of rBMSCs in the chemical media was determined by using water-soluble enzyme substrate MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) which was converted to purple water-insoluble product formazan accumulated in the cytoplasm of only alive rBMSCs. rBMSCs viability of three well plates performed individually and then calculated as average value. In brief, 100 μL of PBS solution of the MTT tetrazolium substrate (5 mg/mL) was added at 1 day and 4 days after induction. After incubation for 4 h at 37 ^oC, the resulting violet formazan precipitate was solubilized by the addition of 1 mL of

DMSO and shaken for 30 min. The solutions were placed on 96 well plates and then read using a plate reader of an ELISA (E-max, Molecular Device, USA). The optical density of each well determined at 590 nm. All experiments were performed at least 3 times and the results were presented with mean ± standard deviation (SD). To detect neural differentiation, digital images of the rBMSCs in the chemical induction media were captured from an inverted phase contrast microscope at 5h, 1 day and 4 days after induction. CMP and MMP (10 μM), and RA (2 μM) with neurogenesis-inducing activity were identified based on this treatment.

Histological analysis

The small molecules treated rBMSCs were stained with DAPI (6-diamino-2-phenylindoadihydrochloride, Sigma, USA), NSE (mouse anti rat neuron specific enolase, serotec, UK), Tuj1 (mouse anti-tubulin β III isoform, Chemicon International, USA), CNPases (monoclonal anti CNPase, sigma, USA), and GFAP (polyclonal rabbit anti glial fibrillary acidic protein, DAKO, Denmark).

For Tuj1 staining, the small molecules treated rBMSCs was performed using ABC kit (Immunothch, France). The typical staining process is as follows. At 1 and 4 days, the small molecules treated rBMSCs were fixed with 10% formalin (Sigma, USA) for 10 min. The fixed cells were washed two times with PBS for 5 min. The endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 min at 37 ^oC. After washing two times with PBS, the cells were blocked with blocking solution for 30 min at 37 ^oC. The cells were incubated with mouse anti-tubulin β III isoform (1:300 dilution ratio, Chemicon, International, Temecula, CA) for 90 min at 37 ^oC. After washing two times with PBS, the cells were incubated with biotinylated secondary antibody for 20 min at room temperature and washed two times with PBS. The cells were incubated with streptavidin for 30 min at room temperature. After washing two times with PBS, chromogen was applied for 15 min at room temperature, followed by counter staining with hematoxylin.

For NSE, CNPases and GFAP staining, the small molecules treated rBMSCs were fixed with 10% formalin at 1 and 4 days after chemical treatment. The cells were washed two times with PBS-T (0.05%

Tween 20 in PBS) and blocked with buffer of 5% bovine serum albumin (BSA, Roche, Germany) and 5% horse serum (HS, GIBCO™, Invitrogen) in PBS for 1 h at 37 ^{o}C. Sections were incubated with primary antibody (NSE, CNPase and GFAP) overnight at 4 ^{o}C. After washing with PBS-T, the cells were incubated with the secondary antibody (rat anti-mouse Alexa Fluor®594, Invitrogen) for 3 h at room temperature in the dark condition and then washed again with PBS-T, counter stained with DAPI, and then mounted with fluorescent mounting diluent solution (DAKO, Denmark). Immunofluorescence images were visualized under an Olympus IX81 fluorescent microscope (Olympus, Japan) equipped with the Meta Image Series software (MetaMorph, Molecular Devices Corporation, USA).

RNA extraction and RT-PCR

At each time point, cells treated under small molecules were washed with PBS, mixed with 1 mL of TRIzol reagent (Invitrogen Life Technologies Co., Groningen, Netherlands), incubated for 5 min at 37°C, and collected in 1.5 ml tubes. The solution was mixed with 200 μL of chloroform and centrifuged at 12,000 × g for 15 min at 4°C. The upper aqueous phase was collected, mixed with 500 μL of isopropanol, and the RNA pellet was collected by centrifugation at 12,000 × g for 10 min at 4°C. The concentration and purification of RNA was determined by the ratio of the absorbance at 260/280 nm. The final concentration of total RNA was adjusted to 3 μg/μL. DNAse/RNAse-free water (Gibco BRL), oligo dT Primer (Invitrogen, Carlsbad, CA, USA), 5× first strand buffer (Invitrogen, USA), dNTPs (Gibco), RNAse inhibitor (Invitrogen), and SuperScript II RNase H^{-} reverse transcriptase (Invitrogen) were sequentially added to the mixture, after which it was incubated at 42 °C for 15 min. The PCR reaction was carried out with the SYBR green PCR kit (Bio-Rad) and operated using Chromo 4 real time system (Bio-Rad). All reactions were done in a 20 μL volume. Each primer sequence for the individual rat genes was listed at table 1. The following primer pairs were designed from Primer Premier 5.0 software (Biosoft International, CA, USA) and were purchased from GenoTech. (Daejeon, Korea). Data analysis was done using comparative Ct method ($2^{-\Delta\Delta Ct}$) for relative quantification. All samples were analyzed in triplicate. Their expression values were normalized by GAPDH expression of rBMSCs without and with small molecules and compared with neuronal and glial gene expression.

Table 1. Primer for real time PCR.

Name		Primer	Size (mer)
GAPDH	Forward	CAAGTTCAACGGCACAGTCAAGG	123
	Reverse	ACATACTCAGCACCAGCATCA CC	
NF-M	Forward r	AGACGCCCTCACAGTCATTGC	137
	Reverse	CTTCCTCCTCTTCCTTAGC CTCAG	
MBP	Forward	TCGGCTCACAAGGGATTCAAGG	145
	Reverse	GGGCAGGATTCGGGAAGG C	
rGFAP	Forward	TGGGCAGGTGAGGAAGAAATGG	125
	Reverse	TGAAGGTTAGCAGAGGTG ACAAGG	
rNeuroD	Forward	GCCGCCACACGCCTACAG	121
	Reverse	AGAGAAGTTGCCATTGATGCT GAG	

Statistical analysis

Cytotoxicity data were obtained from independent experiments in which three wells per control and chemical treated groups were examined, with data given as the mean and standard deviation (SD). The results were analyzed with one way-ANOVA, using the Prism 3.0 software package (GraphPad Software Inc., San Diego, CA, USA).

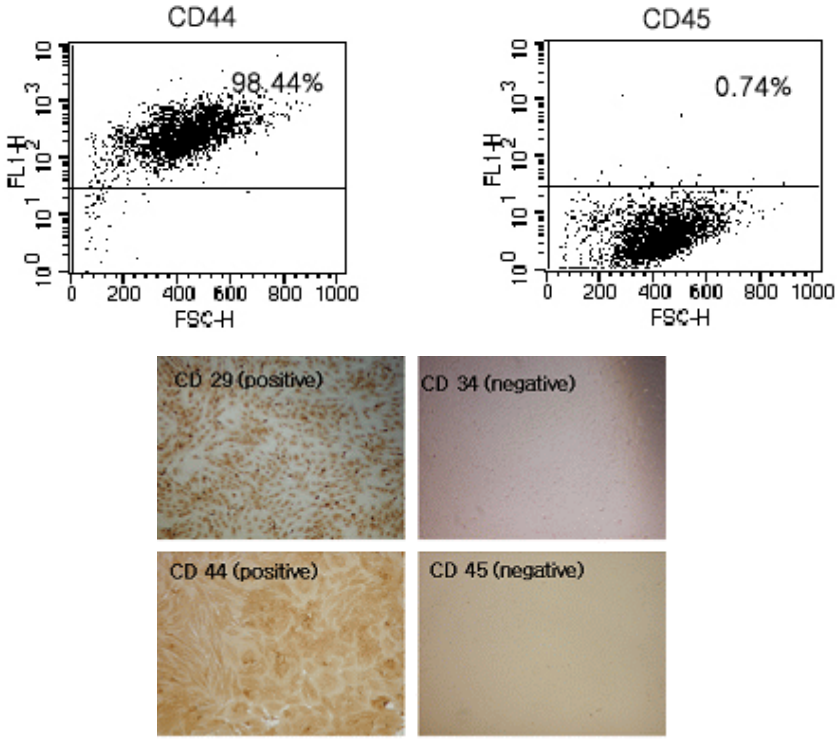

Figure S1. FACS and immunocytochemical analysis of rBMSCs. rBMSCs showed positive to CD29 and CD44, and negative to CD34 and CD45.

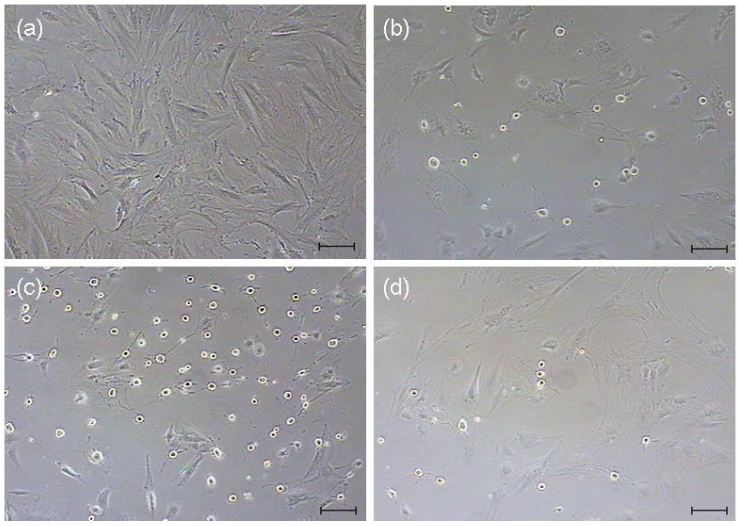

Figure S2. Morphology of rBMSCs at 24 h after treatment without (a) and with chemical CMP, (b) 1 μM, (c) 10 μM, and (d) 100 μM. Magnification is X100 and scale bar represents 100 μm.

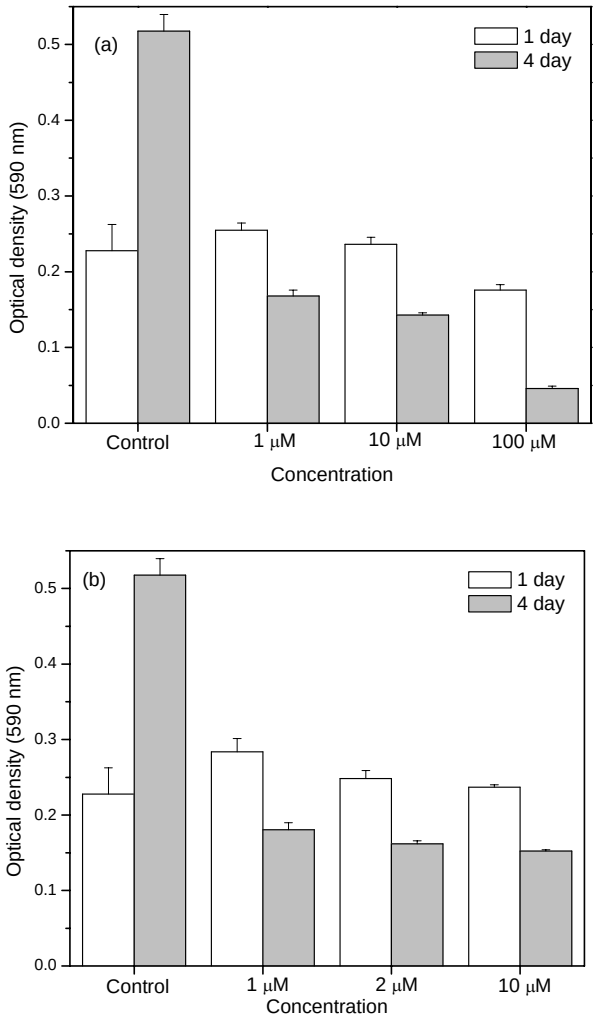

Figure S3. Cytotoxicity measured by MTT assay after rBMSCs treated with (a) CMP (1-100 μM) and (b) RA (1-10 μM) for 1 and 4 days. Control represents rBMSCs without chemical.

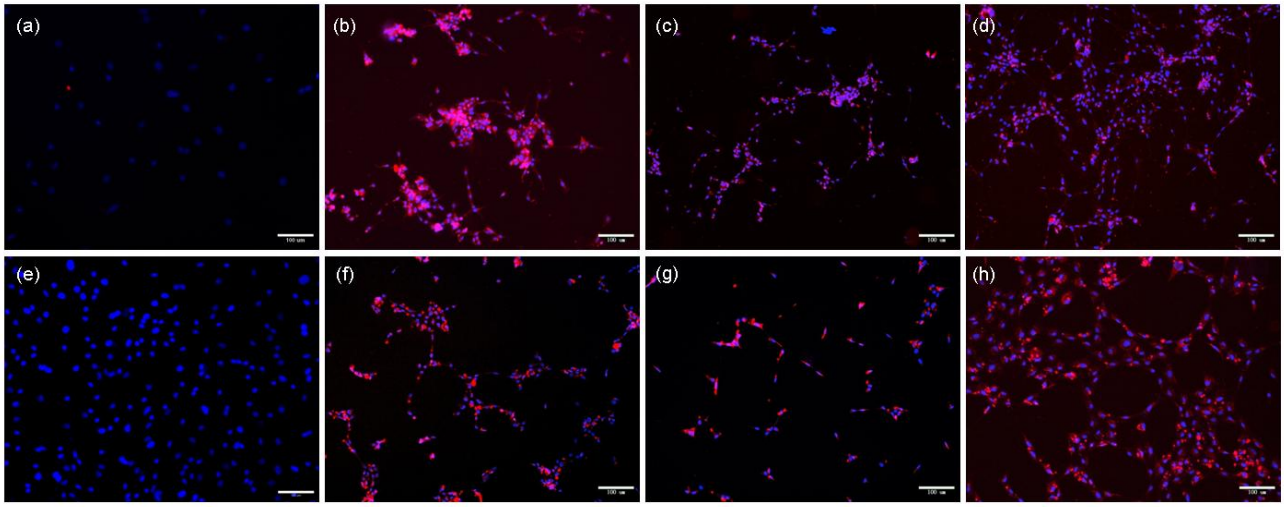

Figure S4. NSE immunostaining [DAPI (blue) and NSE (red)] of rBMSCs (a, e) without chemical treatment (control) and after treatment with (b, f) CMP, (c, g) MMP, and (d, h) RA for (a-d) 1 day and (e-d) 4 days. Magnification is X100. Scale bar represents 100 μm.

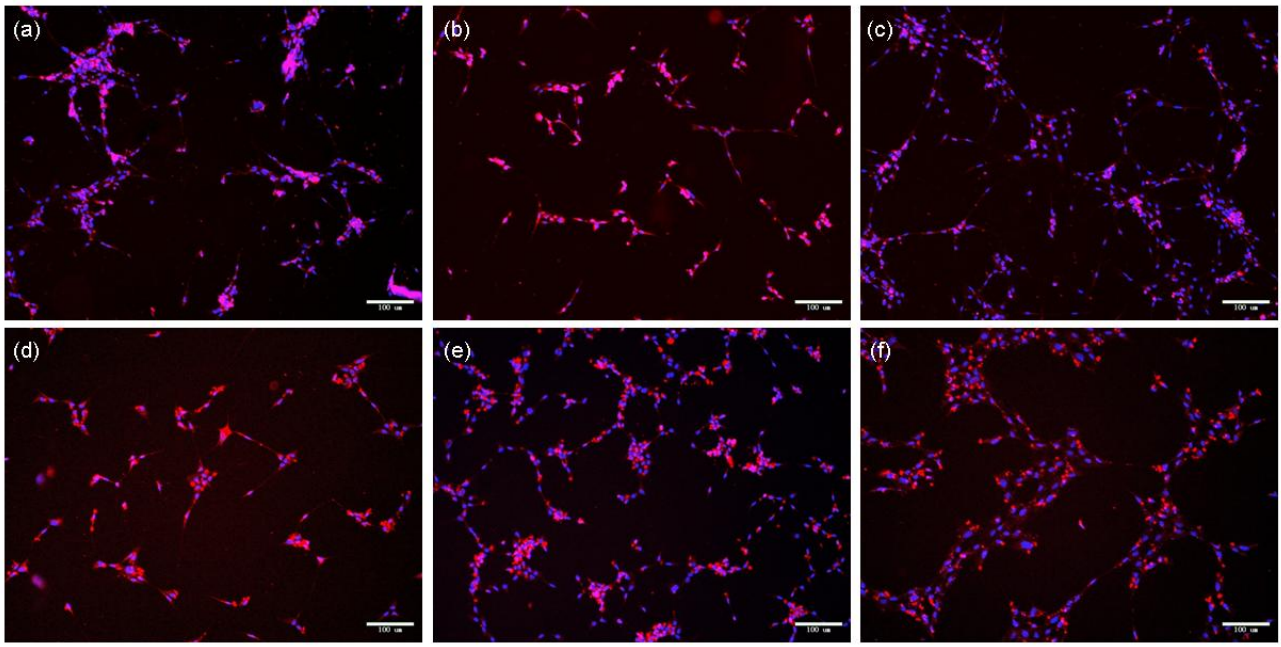

Figure S5. Tuj1 immunostaining [DAPI (blue) and CNPase (red)] of rBMSCs (a, d) CMP, (b, e) MMP and (c, f) RA for (a-c) 1 day and (d-f) 4 days. Magnification is X100. Scale bar represents 100 μm.

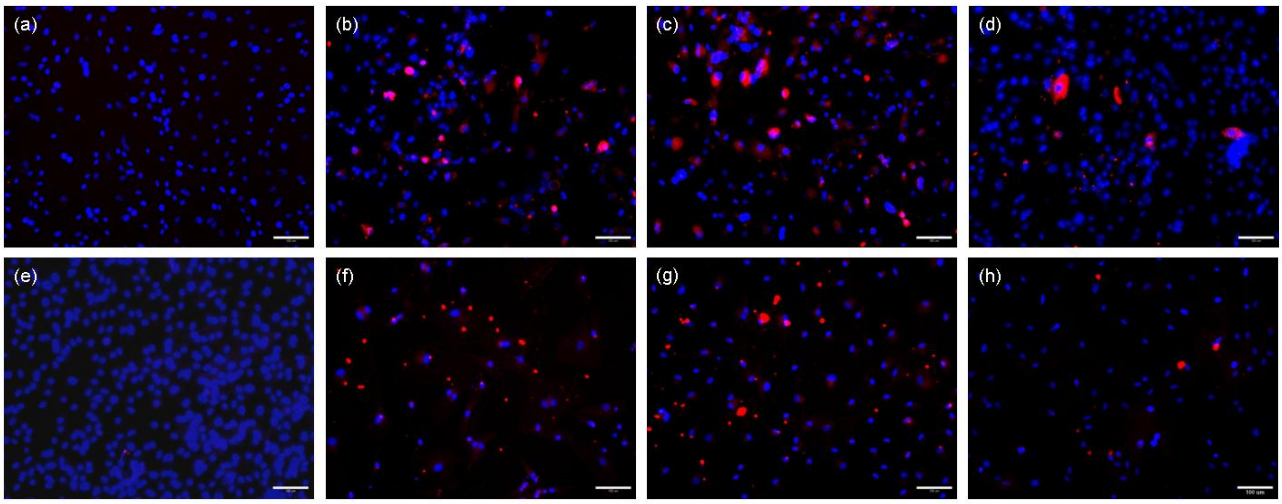

Figure S6. CNPase immunostaining [DAPI (blue) and CNPase (red)] of rBMSCs (a, e) without (control) and after treatment with (b, f) CMP, (c, g) MMP and (d, h) RA for (a-d) 1 day and (e-d) 4 days. Magnification is X100. Scale bar represents 100 μm.