SUPPLEMENTARY MATERIAL

Image-guided Pro-angiogenic Therapy in Diabetic Stroke Mouse Models Using a Multi-modal Nanoprobe

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Supplementary Methods

Characterization of the Nanoprobes

The molar ratios of the dendrimer, PEG, cRGDyk peptide, and DTPA in the nanoprobes were quantified *via* proton nuclear magnetic resonance (^{1}H NMR, Varian Mercury 400 spectrometer, Palo Alto, USA). The hydrodynamic radius of the nanoprobes was determined using a Malvern Zetasizer dynamic light scattering instrument (Malvern Instruments Inc., UK). The Gd^{3+} concentration of the nanoprobe was determined using inductively coupled plasma mass spectrometry (ICP-MS, PerkinElmer, USA).

Cytotoxicity and Cellular Uptake Studies

U87MG human glioblastoma cells (ATCC, USA) were utilized as $\alpha_v\beta_3$ integrin positive-cells. The cells were maintained in DMEM medium (Life Technologies, USA), supplemented with 10% fetal bovine serum (Life Technologies, USA), and incubated in a fully humidified atmosphere at 37°C with 5% CO_2.

The cytotoxicity of the nanoprobes was detected using a MTT assay. In brief, the U87MG cells were seeded in 96-well plates (Corning Incorporated, USA) at a density of 1×10^4 cells per well in 200 μL of culture medium and were maintained for 24 h. Six duplicated wells were set for each concentration. The cells were then incubated with the medium containing nanoprobes for 4 days. Twenty microlitre of MTT solution (Sigma-Aldrich, USA) was added to each well and incubated for 4 h. The medium was removed, and 150 μL of dimethyl sulfoxide (Sigma-Aldrich, USA) was added to each well to dissolve the crystals. The optical densities at a wavelength of 570 nm (OD570 nm) of the dissolved formazan were detected using a microplate spectrophotometer (Thermo Fisher Scientific, USA).

To demonstrate the targeting specificity of the nanoprobes *in vitro*, the U87MG cells were cultured with 5 μM of Den-RGD or Den-PEG. For competitive inhibition of probe binding, the cells were pretreated with a 100-fold concentration of c(RGDyk) 30 min prior to Den-RGD incubation. After incubation with the nanoprobes for 2 h at 37°C, the cells were washed twice with phosphate-buffered saline (PBS) and then harvested for cell phantom

imaging and immunofluorescence staining.

Characterization of Endothelial Progenitor cells (EPCs)

The cells were characterized by double staining with Dil-acetylated low-density lipoprotein (Dil-acLDL, Life Technologies, USA) and FITC-ulex europaeus agglutinin (FITC-UEA, Life Technologies, USA) and by flow cytometric analysis (FACSCalibur, BD Biosciences, USA) of the specific surface markers of EPCs (CD133, VEGFR2, and CD34, Abcam, USA) on day 14.

Behavioral tests

The modified neurological severity score (mNSS) is a composite of motor, sensory, reflex, and balance tests. Neurological function is graded on a scale of 0 to 18 (normal score, 0; maximal deficit score, 18). In the severity scores of injury, one score point is awarded for the inability to perform the test or for the lack of a tested reflex. Thus, the higher the score, the greater the severity of the injury.

For locomotor assessment, mice were tested for a placement dysfunction of the forelimbs with the foot-fault test. Each mouse was individually placed on the top of an elevated wire grid and was allowed to freely walk for a period of 5 min. A video was located beneath the apparatus to assess the stepping errors of the animals. With each weight-bearing step, the paw may fall or slip between the wire. This fall was recorded as a foot fault. The percentage of foot-faults of the left paw to total steps was determined.

The behavioral tests were performed prior to photothrombotic stroke and on days 1, 7, 10, 14, and 21 after photothrombotic stroke by an investigator who was blinded to the experimental groups.

Supplementary Figures and Figure Legends

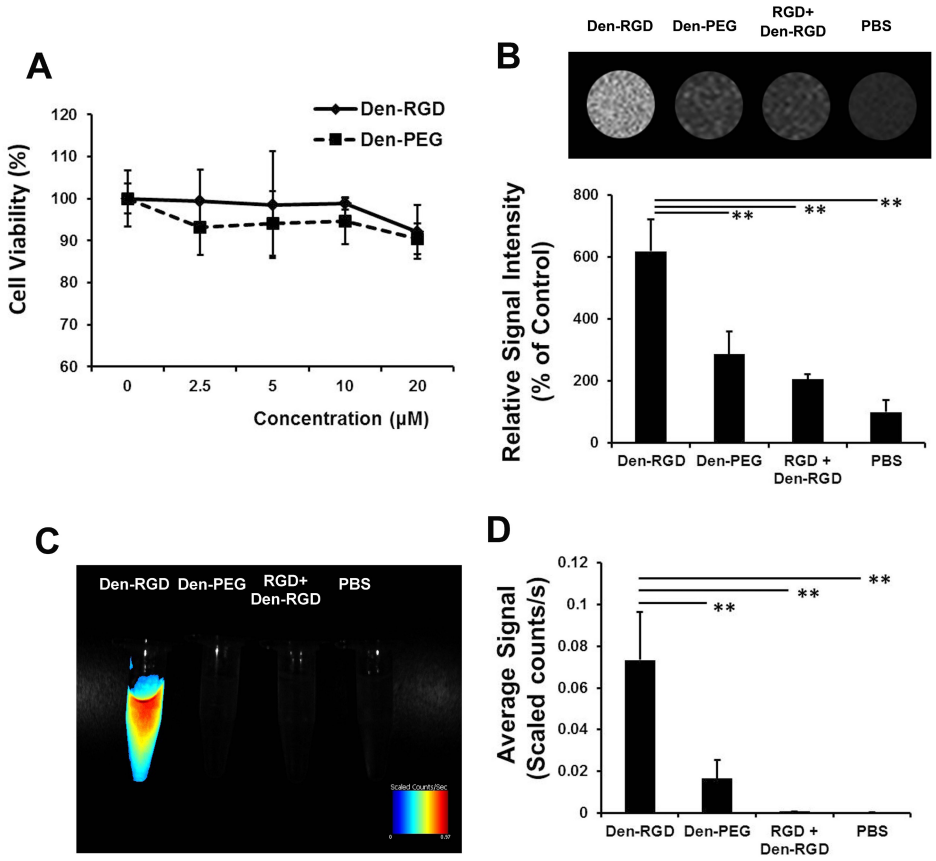

Figure S1: (A) Den-RGD and Den-PEG nanoprobes were incubated with U87MG cells for 4 days. The viabilities were normalized to the cells without any treatment (n=6). The nanoprobes were not toxic to U87MG cells in the tested concentration range (0-20 μM). (B) T_1-weighted images of the cells treated with 5 μM nanoprobes for 2 h or pretreated with 500 μM free c(RGDyk) for 30 min, followed by Den-RGD treatment. A significantly higher signal intensity was observed in the Den-RGD group compared with the other groups (n=3). (C, D) Near-infrared fluorescence images of the cells in different groups showed a remarkable fluorescence signal in the Den-RGD group, which indicated a higher cellular uptake efficacy of Den-RGD compared with the control nanoprobe in the U87MG cells (n=3). ** $p < 0.01$.

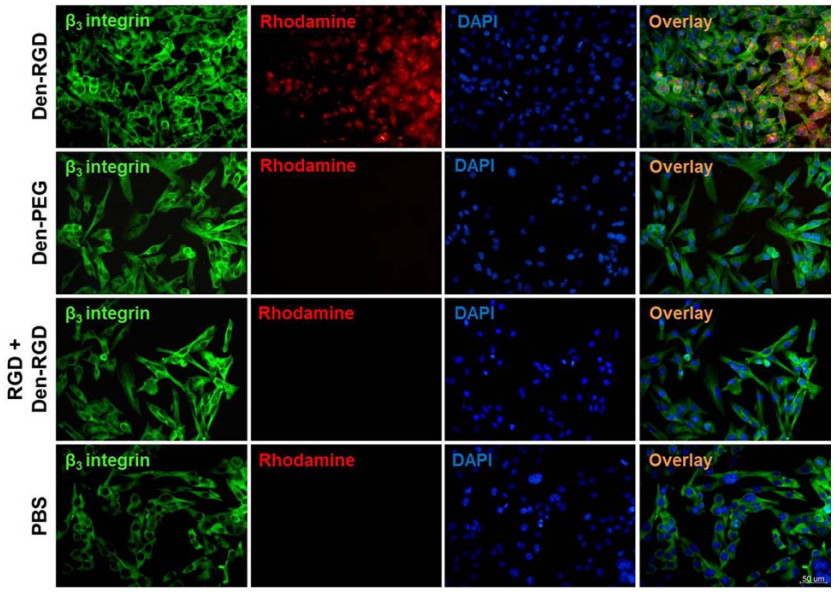

Figure S2: *In vitro* confocal fluorescence microscopic images of the nanoprobes uptake by U87MG cells. Using immunofluorescence staining, Den-RGD was confirmed to be attached to the cell membrane after incubation with the nanoprobes for 2 hours.

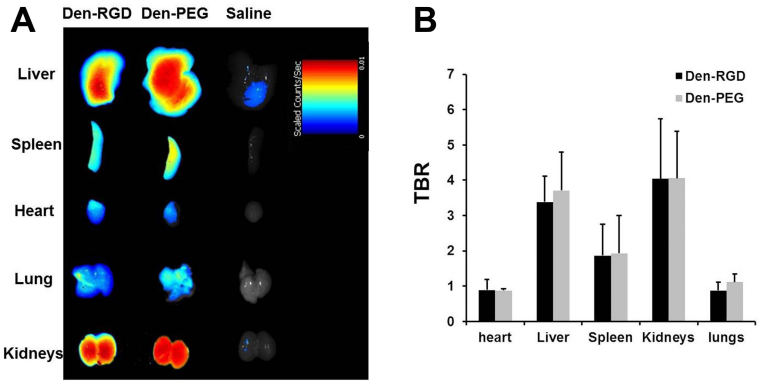

Figure S3: Biodistribution analysis of the nanoprobes. (A) *Ex vivo* near-infrared fluorescence imaging showed that Den-RGD and Den-PEG were primarily located in the livers, spleens, and kidneys of mice. (B) Quantitative data of the target-to-background ratio (TBR) in different organs (n=3).

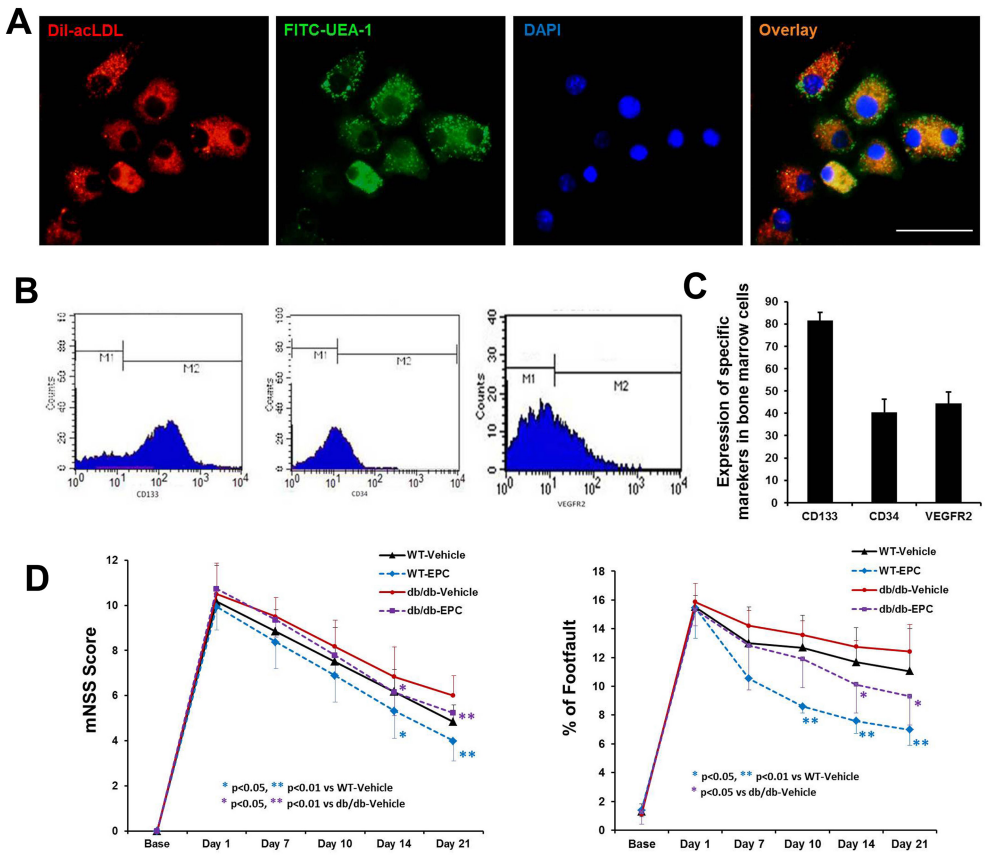

Figure S4: (A) Representative images showing cultured EPCs *via* double staining analysis. Scale bar: 30 μm. (B) Representative flow plot showing the percentage of CD133/CD34/VEGFR2 expression in EPCs. (C) At the end of EPC culture, 81.53 ± 3.61% of the cultivated cells expressed CD133, 40.31 ± 6.02% expressed CD34, and 44.64 ± 5.27% expressed VEGFR-2 (n=3). (D) EPC treatment improves functional outcome in wild-type and diabetic mouse models of ischemic stroke (n=6).