Experimental

Mesenchymal stem cell isolation and culture

Rat BMSCs were isolated from the femurs and tibia bone marrow of 30-day-old neonatal male Wistar rats (90-100 g) as described earlier with modification [1]. The epiphyses were removed. The bone-marrow mononuclear cells were obtained by Percoll (1.073 g/ml) density gradient centrifugation. After 3 washes in phosphate-buffered saline (PBS) at centrifugation 300 × g, the cells were seeded in low-glucose Dulbecco's modified Eagle's medium (DMEM-LG, Gibco, USA) supplemented with 20% fetal bovine serum (FBS; Hyclone, USA) and fibroblast growth factor 2 (FGF-2), 5 ng/mL, at 37 °C in humidified air with 5% CO_2. At 24 h after plating, nonadherent cells were removed by replacing medium. The medium was changed every 2–3 days and cells were passaged in 0.05% trypsin with 1 mM EDTA. Cells from 5 to 15 passages were used.

Cell labelling with MNPs

The 3 kinds of magnetic nanoparticles (MNPs) were prepared as reported previously, including the naked uncoated iron oxide (Fe_3O_4) nanoparticles, Fe_3O_4@3-glycidoxypropyltrimethoxysilane@glycine (MNPs@Gly) and Fe_3O_4@3-glycidoxypropyltrimethoxysilane@Lysine (MNPs@Lys) [2]. MNPs were sterilized at 121 ◦C for 30 min, then suspended in DMEM-LG plus 20% FBS. Stock solutions (1 mg/ml) were sonicated for 5 min to achieve good suspension before they were diluted with DMEM-LG supplemented with 20% FBS and FGF-2 to final concentrations of 0.1, 0.5, 1, 1.5 and 2 μg/ml. For cellular labelling, BMSCs were seeded in 6- or 24-

well plates and incubated with MNPs@Gly, MNPs@Lys or uncoated Fe_3O_4 MNPs at different concentrations for 24 and 48 h, respectively. Control cells were seeded without the addition of NPs. For microscopy observations, treated cells were washed 3 times with PBS solution. The morphological changes were observed by phase-contrast microscopy (Nikon, Japan). Five optical fields from each well were selected randomly for imaging by use of NIS-Elements F3.0. Three independent experiments were run in duplicate. Representative images were chosen for figures.

Prussian blue staining

To visualize the labelling efficiency and bio-distribution of MNPs in cells, Prussian blue staining was performed according to manufacturer's instruction [3]. Cells were divided into 3 groups: control (not treated with MNPs), MNPs@Gly (treated with 1 μg/ml MNPs@Gly) and MNPs@Lys (treated with 1 μg/ml MNPs@Lys). Cells seeded in 24-well plates for 24 h were incubated with the 2 MNPs (1 μg/ml) for 24 h, then repeatedly washed with fresh PBS to remove loosely attached or extracellular particles until the PBS appeared free of particles under light microscopy. The images before staining were captured by phase-contrast microscopy and use of NIS-Elements F3.0. Then, cells in the 3 groups were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min, washed twice with PBS and incubated with Perls reagent (20% potassium ferrocyanide and 20% hydrochloric acid; Sigma-Aldrich) for 20 min at room temperature. Cultures were then washed once in deionized water. Samples were observed by light microscopy. Three independent experiments were run in duplicate. The MNP labelling efficiency was determined by

manual counting of Prussian blue-stained and unstained cells. For quantification of labelling, the percentage of labelled cells was determined from the average of 5 high-powered fields by using a 20x objective.

Cell viability assay

Cell viability was determined by the WST-8 assay with the Cell Counting Kit-8 (Sigma Chemical Co., USA, 96992). Cells were seeded into 96-well plates and divided into 6 groups: normal (not treated with any MNPs) and MNP groups (treated with 0.1, 0.5, 1, 1.5 and 2 μg/ml MNPs@Gly or MNPs@Lys). Every group had 6 parallel wells. After cells were treated with MNPs for 44 h, 10 μl of WST-8 solution was added and the plate was further incubated for 4 h. The aggregated particles were removed by centrifugation at 1500 rpm for 10 min. The proportion of living cells was calculated by the ratio of OD at 450 nm (Tecan, San Jose, CA, USA). In every group, the highest and lowest OD numbers were deleted and the average value of the remaining 4 OD numbers was calculated. The value for the normal group was set to 100%. The cell viability of other MNP treatment groups was calculated by the ratio of the value of every group to the normal group. Three independent experiments were performed.

Hoechst 33258 staining

To examine whether MNPs could induce cell apoptosis, Hoechst 33258 staining was performed. Cells were fixed in 4% formaldehyde for 10 min, then incubated with Hoechst 33258 (2 μg/ml) (Sigma, St. Louis, MO, USA) for 60 min at 37 ℃. Stained cells were washed with PBS twice, and then viewed under an Olympus inverted

fluorescence microscope. Cells were scored as apoptotic if nuclei were much brighter or exhibited condensation of chromatin and nuclear fragmentation. Five optical fields from each well were selected randomly for images. Three independent experiments were run in duplicate. Representative images were chosen for figures.

Cell necrosis assay

Cell necrosis was determined by measuring the release of lactic dehydrogenase (LDH); the assay involved use of the LDH kit (Nanjing Jiancheng Chemical Industrial, China). The OD values were read at 440 nm by using a microplate reader (Tecan). The value for the control group was set to 1. The value for the MNP treatment groups was calculated by the ratio to the normal group. Three independent experiments were run in duplicate.

Flow cytometry of the cell cycle by PI staining

Cells were divided into groups as follows: control (not treated with any MNPs), MNPs@Gly (treated with 1 and 2 μg/ml MNPs@Gly), MNPs@Lys (treated with 1 and 2 μg/ml MNPs@ Lys) and uncoated group (treated with 1 μg/ml uncoated Fe_3O_4 MNPs). Cells were seeded at 3×10^5 per well in 6-well plates and cultured until they reached subconfluence. Each group of cells was washed 3 times with PBS, then collected by centrifugation at 400 g for 5 min at 4℃. The cell pellets were suspended in 500 μl 70% ice-cold ethanol at 4℃ overnight. Then the fixed cells were centrifuged at 400 g for 5 min at 4℃, washed twice with PBS, and resuspended in propidium iodide (PI) staining solution (Sigma-Aldrich) containing 50 μl/ml PI and 250 μg/ml RNase A (Sigma-Aldrich). The cell suspension, which was maintained in

darkness, was incubated for 30 min at 4˚C. Cells were centrifuged at 400 g for 5 min at 4℃ and suspended in 50 μl PBS, then analyzed by using a FACS system (ImageStreamX MarkII, Amnis, USA) and IDEAS Application v6.0 (Amnis, USA). Three independent experiments were run in duplicate.

Statistical analysis

All experiments were repeated at least 3 times independently. Data are expressed as mean ± SEM and were analyzed by one-way ANOVA with use of SPSS v11.5 (SPSS Inc., Chicago, IL). Images were processed by use of Graphpad Prism 5 (GraphPad Software, La Jolla, CA, USA) and Adobe Photoshop CC (Adobe, San Jose, USA). $P<0.05$ was considered statistically significant.

References

1. L. Su, H. Zhao, C. Sun, B. Zhao, J. Zhao, S. Zhang, H. Su and J. Miao, *ACS chemical biology*, 2010, **5**, 1035-1043.
2. Q. H. Wu, N. Meng, Y. R. Zhang, L. Han, L. Su, J. Zhao, S. L. Zhang, Y. Zhang, B. X. Zhao and J. Y. Miao, *Nanoscale Res Lett*, 2014, **9**.
3. M. Babic, D. Horak, P. Jendelova, V. Herynek, V. Proks, V. Vanecek, P. Lesny and E. Sykova, *International journal of nanomedicine*, 2012, **7**, 1461-1474.

Supplementary figures:

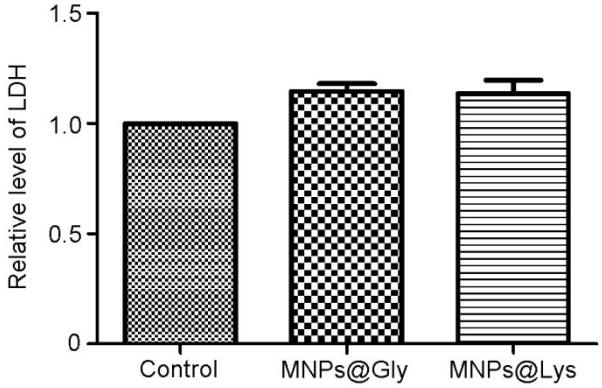

Fig. S1 1 μg/ml MNPs did not induce bone-marrow mesenchymal stem cell (BMSC) necrosis. Cells were labeled with 1 μg/ml MNPs for 24 h. Control: cells not treated with any MNPs; MNPs@Gly: cells treated with 1 μg/ml MNPs@Gly; MNPs@Lys: cells treated with 1 μg/ml MNPs@Lys. Lactate dehydrogenase (LDH) release was analyzed. Data are mean±SEM from 3 experiments.

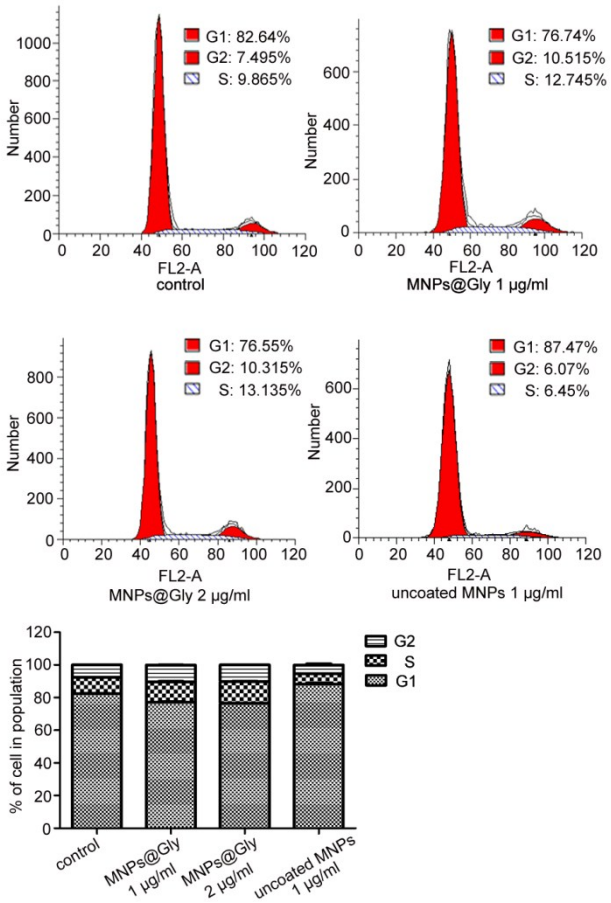

Figure S2 Effect of 1 μg/ml and 2 μg/ml MNPs@Gly and uncoated Fe_3O_4 MNPs on cell cycle progression. Control: cells not treated with any MNPs for 24 h; MNPs@Gly 1 μg/ml: cells treated with 1 μg/ml MNPs@Gly for 24 h; MNPs@Gly 2 μg/ml: cells treated with 2 μg/ml MNPs@Gly for 24 h; Uncoated Fe_3O_4 MNPs 1 μg/ml: cells treated with 1 μg/ml uncoated Fe_3O_4 MNPs for 24 h. Flow cytometry of proportion of cells at the S phase.

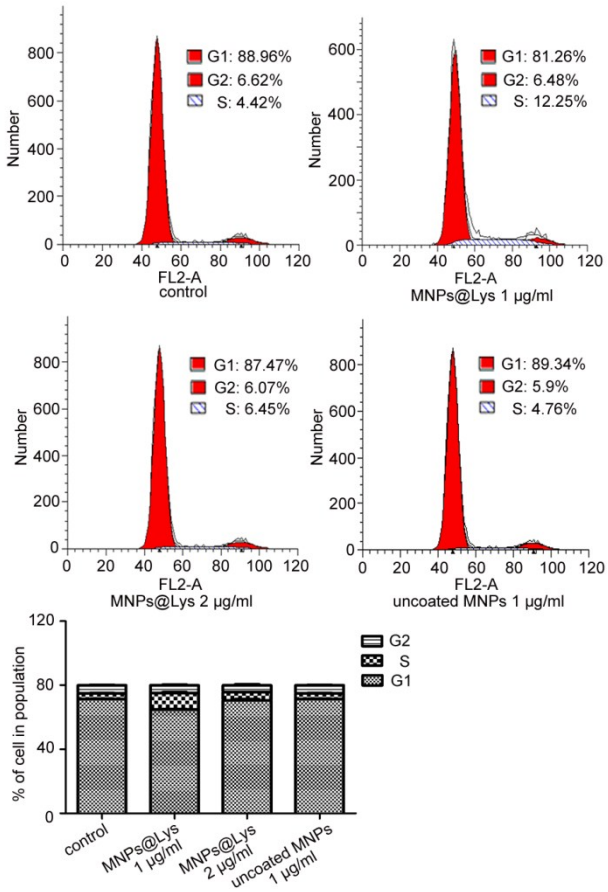

Figure S3 Effect of 1 μg/ml and 2 μg/ml MNPs@Lys and uncoated Fe_3O_4 MNPs on the cell cycle progression. Control: cells not treated with any MNPs for 24 h; MNPs@Lys 1 μg/ml: cells treated with 1 μg/ml MNPs@ Lys for 24 h; MNPs@Lys 2 μg/ml: cells treated with 2 μg/ml MNPs@Lys for 24 h; uncoated Fe_3O_4 NPs 1 μg/ml: cells treated with 1 μg/ml uncoated Fe_3O_4 NPs for 24 h. Flow cytometry analysis of

the proportion of cells at the S phase.