Supplementary Data

Materials and Methods

Differentiation capacity of BMSCs

Cells were seeded at a density of 1×10^4 cells/well in 24-well tissue culture plates for adipogenic and osteogenic differentiation. After 24 h, the culture media were replaced with the corresponding differentiation media.

Adipogenic differentiation. In vitro adipogenesis was induced by using complete MEM (10% FBS, 1% P/S) supplemented with 5 μg/mL human insulin, 0.5 mM isobutylmethylxanthine (IBMX), 200 μM indomethacin, and 0.5 mM hydrocortisone (all Sigma-Aldrich). The adipogenic induction medium was changed every 3 days. On day 10, the induction medium was replaced with an adipogenic maintenance medium, which consisted of complete MEM (10% FBS, 1% P/S) supplemented solely with 5 μg/mL insulin. Cells treated with complete MEM were used as control. After 3, 7, 14, and 21 days of differentiation, the cells were washed twice with DPBS and fixed with 4% paraformaldehyde (Sigma-Aldrich) for 30 min at RT. After washing the fixative from the cultures, 2 mL of 60% isopropanol (Sigma-Aldrich) was added, and the cultures were incubated for 5 min. Subsequently, the cells were stained with three volumes of oil red O (3 mg/mL in 100% isopropanol, Sigma-Aldrich) and two volumes of bidistilled water for 15 min at RT. Before microscopic observation, the stained cells were washed with bidistilled water until the solution rinsed off clear. Adipogenically differentiated cells were identified by their round shape and presence of lipid droplets stained red. For quantification of triglyceride accumulation, oil red O was extracted from the cultures by 100% isopropanol for 30 min at RT. One hundred microliters of each culture were transferred to a 96-well plate and the optical density was determined with a microplate reader at 502 nm.

Osteogenic differentiation. For the evaluation of *in vitro* osteogenesis, the cells were treated with MEM containing 2% FBS and 1% P/S and supplemented with 10 nM dexamethasone, 10 mM β-glycerol phosphate, and 200 μM ascorbic acid (all Sigma-Aldrich). Cells treated with MEM (2% FBS and 1% P/S) were used as control. After 3, 7, 14, and 21 days, cells were carefully washed twice with DPBS and fixed with 100% ethanol (Sigma-Aldrich) for 15 min at RT. After removing the fixative and washing the cells twice with DPBS, 500 μL of alizarin red solution (5 mg/mL in DPBS) was added to each well and plates were incubated for 15 min. Stained cells were washed extensively with bidistilled water to remove unspecific staining and/or possible precipitates. Mineralized nodules and calcium deposits stained as red spots indicated osteogenesis. For quantification, the alizarin red dye was subsequently extracted with 100 mM cetylpyridinium chloride (Sigma-Aldrich) at RT for 3 h. Absorbance was measured at 570 nm.

Chondrogenic differentiation. To evaluate the chondrogenic differentiation capacity, a pellet culture system was used. 2.5×10^5 cells at each of the six passages were pelleted by centrifugation at 400 *g* for 10 min. The pellets were carefully transferred into a 15-mL conical bottom polypropylene tube (Corning, Tewksbury, MA) and cultured in chondrogenic differentiation medium under standard culture conditions for up to 35 days. For chondrogenic differentiation medium, MEM was supplemented with 1% P/S, 1 mg/mL bovine serum albumin, 100 μg/mL sodium pyruvate, 40 μg/mL proline, 50 μg/mL L-ascorbate, 10 nM dexamethasone, 10 ng/mL transforming growth factor-β1 (TGF-β1) and 6.25 μg/mL 1X ITS+1 (insulin, transferrin, and selenium). All supplements were purchased from Sigma-Aldrich except for TGF-β1, which was acquired from Peprotech (London, United Kingdom). In addition, control pellets cultured under the same conditions, but in the presence of complete MEM, were used for each point. Samples were collected after 7, 14, 21, and 35 days of culture. Each sample was washed twice with DPBS carefully, fixed with 4% paraformaldehyde, dehydrated through grade series of ethanol and acetone, and embedded using Technovit 8100 (Heraeus Kulzer, Hanau, Germany). Five-micrometer-thick sections were stained with toluidine blue and nuclear fast red as counterstain to identify proteoglycans and glycosaminoglycans indicating chondrogenic differentiation.

In order to evaluate the extent of chondrogenesis, proteoglycans and glycosaminoglycans were quantified by using Blyscan assay for sulfated glycosaminoglycans (Biocolor, County Antrim, United Kingdom). The assay was performed according to the manufacturer's instructions. Cell pellets were washed twice with DPBS and subsequently digested with papain digestion buffer (pH 6.4, 0.2 M sodium phosphate buffer containing 0.1 M sodium acetate, 0.01 M Na_2EDTA, 0.005 M cysteine HCl, and 0.2 mg/mL papain). All reagents were purchased from Sigma-Aldrich. Once 2 mL digestion buffer had been added to each pellet, the falcon tubes were placed on a thermomixer at 65°C allowing gentle mixing overnight. Papain-digested samples were centrifuged at 10,000 *g* for 10 min and the supernatant was collected for the Blyscan assay. Absorbance was measured at 656 nm. Experiments were performed in triplicate and results were reported in comparison to pellets culture in complete MEM. Total GAG values were normalized to total DNA content using the PicoGreen dsDNA quantification assay (Molecular Probes).