Factors that influence short-term homing of human bone marrow-derived mesenchymal stem cells in a xenogeneic animal model

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Supplementary Methods

Human MSC isolation, culture and characterization

Mononuclear cells (MNC), obtained by centrifugation over a Ficoll-Paque gradient (Amersham Biosciences, Piscataway, NJ, USA) at 1800 rpm for 30 minutes, were plated at a density of 2×10^5 cell/m^2, in six-well tissue culture plates in Mesencult culture medium (Stem Cell Technologies, Grenoble, France). Non-adherent cells were removed after 48 hours; and media were changed twice weekly until adherent cells reached confluence (usually between 10 to 14 days). Confluent cultures were detached using 0.25% trypsin-EDTA, and sub-cultured at a density of 1×10^4/cm^2. Cells were immunophenotyped and found to express SH2, SH4, CD13, CD44, AS02 and CD49e, and were weakly positive for CD54 but were negative for CD45, CD34, CD11a, CD38, CD49b, CD49d, CD184 and CD31.[2] Further characterization was performed by demonstration of multi-lineage differentiation.[1,2] Osteogenic differentiation was induced in confluent MSC using 1M beta-glycerophosphate, 50 mg/mL ascorbic 2-phosphate, 10 μM dexamethasone (all from Sigma Aldrich, Poole, UK) and 1 μg/mL basic fibroblast growth factor (PeproTech, London, UK) for 14-21 days, before staining for alkaline phosphatase and calcium (Alizarin red, Sigma Aldrich, Poole, UK). In addition, adipocytic differentiation was induced by culture with dexamethasone (1 μM), insulin (10 μg/mL), indomethacin (100 mM) and isobutylmethylxanthine (0.5 mM, all from Sigma Aldrich) and confirmed by direct staining of lipid formation with Nile Red. Immunophenotype and differentiation potential were maintained for multiple (up to 20) passages.

Labeling human MSC with PKH26

Briefly, cells were washed in serum-free medium, cooled and incubated with an equal volume of staining solution (16 ∝M of PKH-26) for 2 minutes on ice. An equal volume of cold FCS was added to the cell mixture and labeled cells were washed twice in HBSS containing 5% FCS. An aliquot of cells before and after PKH-26 staining was analyzed by flow cytometry to determine the staining efficiency and a small number of stained cells were re-plated and kept in culture to confirm viability. Cell growth was not altered following PKH-26 staining.

Analysis of murine tissues for human mesenchymal stem cell homing

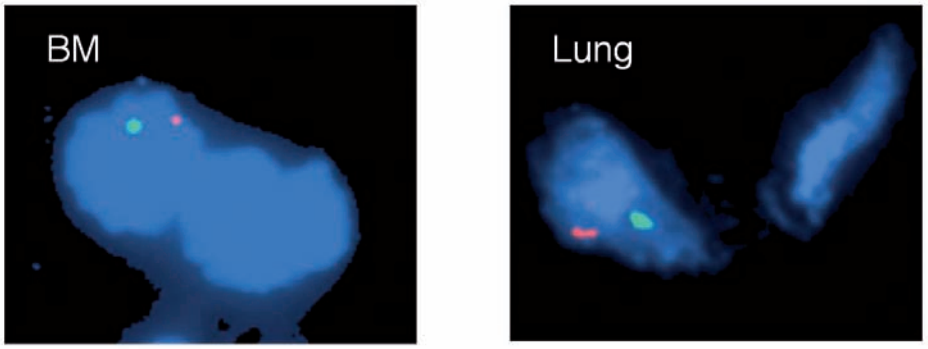
Bone marrow was flushed out and cell suspensions obtained following erythrocyte lysis (in cold buffer: 155 mM NH_4Cl, 20 mM $NaHCO_3$, 1 mM EDTA for 4 minutes on ice), and two washes in medium with 5% FCS. Tissues from spleen, lungs and liver were gently eased through a 40 micron filter, and treated similarly to yield single cell suspensions. Samples were analyzed on an Epics-Elite flow cytometer (Beckman-Coulter, High Wycombe, UK), and at least 500,000 events were acquired and analyzed using Elite Workstation Analysis Software Version 4.5. Homing results are expressed as number of PKH-positive cells per 100,000 events unless otherwise stated.

Fluorescence in situ hybridization (FISH) assay

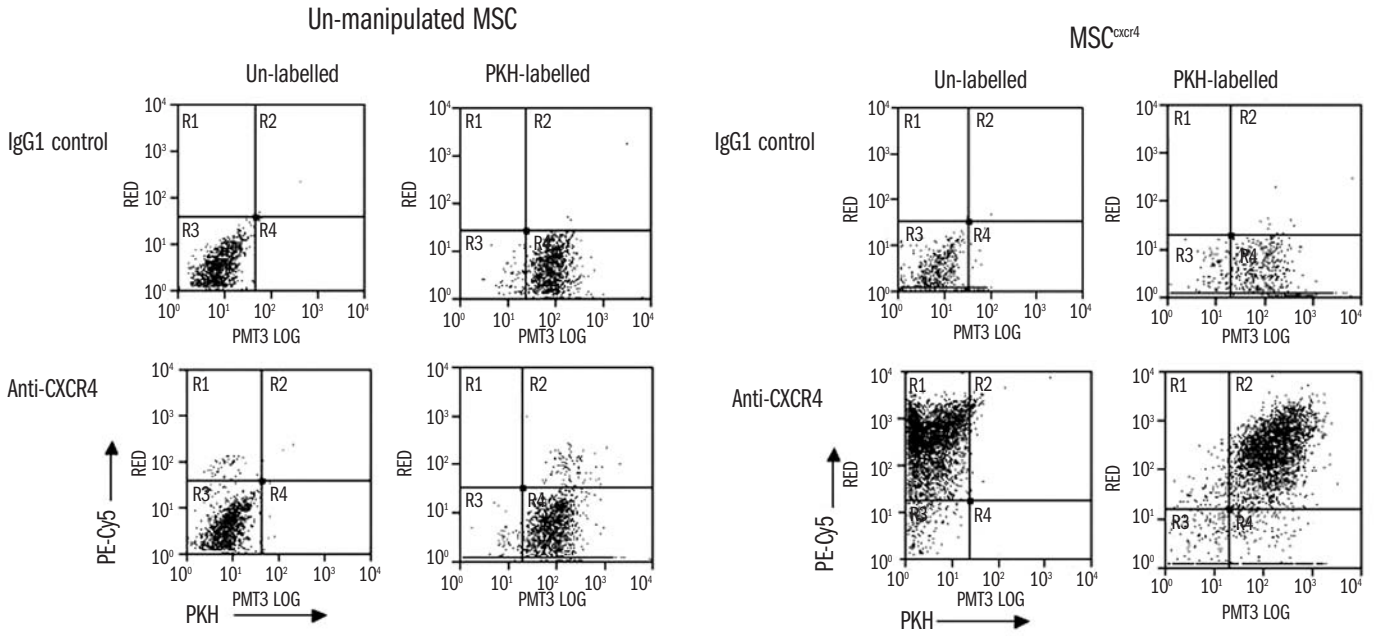
Cell suspensions from bone marrow, lungs, spleen and liver were fixed using a methanol/acetic acid (3:1) fixative solution. One drop of fixed cell suspension was placed in the center of the slide. The hybridization process was carried out over two consecutive days. Slide denaturation and probe preparation and hybridization were carried out on the first day. The slides were incubated at room temperature in 0.5% Tween/2xSCC for 1 hour and then passed through an alcohol series of 70%, 80%, 90% and 100% Industrial Methylated Spirit (IMS) and left to dry. Ten microliters of the probe mixture were applied onto the center of the target slide. For the post-hybridization wash a coplin jar of wash solution (40 mL 0.5xSCC) was placed in a 72°C water bath. Coverslips were removed from the slides which were immediately immersed in the wash solution and incubated for 5 min at 72°C and then transferred to a coplin jar containing PBD buffer at room temperature for at least 2 min. Caution was taken not to allow the slides to dry out after this stage. The slides were counterstained using 10 μL of 4,6-diamidino-2-phenylindole (DAPI) and then visualized using the epifluorescence microscope with filters appropriate to the fluorophores (X-green, Y-red).

Viral vector construction and transduction of human MSC

A bicistronic lentiviral vector was used in these studies. In this multiply attenuated pCL10.1EFIa-CXCR4-Ir-GFP vector, the elongation factor-1 enhancer-promoter element (EF1a) directs the expression of human CXCR4 and GFP which are separated by an internal ribosomal entry site (Figure 1) . The 5′ LTR was modified by replacing the U3 region with the CMV enhancer. A deletion in the U3 region of the 3′ LTR renders the vector self-inactivating. In addition, part of the R and U5 region was replaced with rabbit βglobin gene polyadenylation



Supplementary Figure S1 FISH analysis of human MSC. Cell suspensions from bone marrow (left panel) and lung (right panel) of animals 20 hours after infusion of MSC were analyzed for the presence of human cells by FISH using human specific X- and Y-probes.



Supplementary Figure S2. Surface CXCR4 expression on human MSC. (A) PE-Cy5-conjugated anti-CXCR4 monoclonal antibody stains surface CXCR4 on both unlabeled and PKH-labeled human MSC (one representative experiment of two) and (B) similarly for human MSC expressing CXCR4 by lentiviral transduction (MSC^{CXCR4}).

site. The central polypurine tract (flap) and central termination sequence (CTS) are also included. Self–inactivating vector stocks were produced by the four-plasmid transient transfection method we have previously described.[2] Biological titers of the supernatant were determined by limiting dilution on HeLa cells. P24 antigen levels were determined by an ELISA (Beckman Coulter (UK) Ltd., High Wycombe, UK). Early passage (≤ 3) human MSC were exposed to viral supernatants at a multiplicity of infection (MOI) of 2 for 6 hours, after which media were changed and fresh Mesencult added overnight. This process was repeated daily for 5 consecutive days, and cells expanded thereafter. Neither lentiviral transduction nor enforced CXCR4 expression altered the surface phenotype, proliferative rate, or differentiative potential of human MSC for up to eight passages after transduction.

Supplementary Results

In vivo *homing of MSC in unconditioned immunodeficient mice – optimization of cell dose*

We initially used a range of cell doses (0.5–3.5×10^6 cells/animal) for infusion into β2m/NOD/SCID animals. We found that at cell doses of 10^6 or less, the levels of homing were often at the limit of detection in our assay, and hence the data less reliable. At cell doses of 3.0×10^6 or higher, there was increased early mortality, perhaps due to large numbers of MSC lodging in the pulmonary circulation. We, therefore, selected a cell dose of 1.5 – 2.0×10^6 cells/animal.