Supplementary Materials and Methods

Cell culture

The mouse melanoma cell line (B16-F10) and the mouse monocyte/macrophage cell line (RAW 264.7) was purchased from the ATCC between 2011 and 2015. B16-F10 cells were maintained in PRMI-1640 (Wako) supplemented with 10% heat-inactivated FBS, 2 mmol/L L-glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL). RAW 264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) medium (Invitrogen) supplemented with 10% heat-inactivated FBS, 2 mmol/L L-glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL). Frozen aliquots of cells were prepared upon receipt. Cell lines were used within 3 months of testing negative for mycoplasma using a PCR kit (TaKaRa).

Mice

MFG-E8 KO C57BL/6 mice were generated, and genotyped as described previously (1, 2). MFG-E8 KO mice were generated by interbreeding homozygous animals carrying the targeted MFG-E8 allele. Interbreeding homozygous C57BL/6 mice and C57BL/6-Tg (CAG-EGFP) mice were purchased from Japan SLC. Eight- to twelve-week-old mice were used for all experiments. Mice were bred and maintained at

the Institute of Experimental Animal Research of Gunma University Graduate School of Medicine under specific pathogen-free conditions. Mice were handled in accordance with the animal care guidelines of Gunma University.

Antibodies

The following antibodies were used: rat anti-mouse CD31 monoclonal antibody (mAb) (MEC13.3; BD Biosciences), rabbit anti-mouse NG2 polyclonal antibody (pAb) (Millipore), rat anti-mouse CD68 mAb (Bio-Rad Laboratories), rabbit anti-mouse arginase-1 mAb (Thermo Scientific), rabbit anti-mouse Ym1 pAb (Abcam), rat anti-mouse CD206 mAb (Abcam), rabbit anti-human CD31 pAb (Abcam), rabbit anti-human αSMA pAb (Abcam), mouse anti-human MFG-E8 pAb (R&D Systems) and rat anti-GFP Ab (NACALAI TESQUE). Alexa 488- and Alexa 568-conjugated secondary Abs were obtained from Invitrogen. Rabbit anti-mouse MFG-E8 pAb was generated and characterized in our laboratory as described (1, 2).

Isolation and characterization of bone marrow-derived MSC

MSC were obtained as previously described (3). Bone marrow (BM) cell suspensions were obtained from MFG-E8 WT and KO C57BL/6 female mice between 6-10 weeks of

age, and cultured in αMEM medium supplemented with 20% heat-inactivated FBS, 2 mmol/L L-glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL). When adherent cells reached 70-90% confluence, nonadherent cells were removed, and adherent cells were harvested and expanded. Magnetic-activated cell sorting (MACS) (Miltenyi Biotec) was performed to remove $CD11b^+$ cells according to the manufacture's instructions. For examination of surface expression of MSC markers, BM-derived MSC were washed and incubated consecutively at 4°C with Alexa 488-conjugated anti-human Sca-1, CD105, CD44, CD45, CD11b Ab or isotype control Ab (BioLegend) before flow cytometric analysis with a FACS Calibur instrument and CellQuest software (BD Biosciences).

MSC differentiation assay

To analyze the differentiation potential of MSC, we used Mouse Mesenchymal Stem Cell Functional Identification Kit (R&D systems) according to the manufacture's instructions. For adipogenic differentiation, MSC were incubated in adipogenic differentiation medium composed of α-MEM supplemented with 10% FBS, hydrocortisone, isobutylmethylxanthine and indomethacin (R&D systems). The medium was changed every 3 days for 2 weeks. The cells were fixed with 4% formalin for 10

minutes and stained with oil-red-O for 15 minutes at room temperature. For osteogenic differentiation, MSCs were incubated in osteogenic differentiation medium composed of α-MEM supplemented with 10% FBS, ascorbate-phosphate, β-glycerolphosphate and recombinant human BMP-2. The medium was changed every 3 days for 2 weeks. After fixation with cold 70% ethanol, cells were stained with 40 mM alizarin red S (pH 4.2) for 15 minutes. For quantification, cells were incubated with 10% cetylpyridinium chloride in 10 mM sodium phosphate for 15 minutes with shaking. The concentrations of alizarin red S were analysed by measurement of absorbance at 562nm using a microplate reader. For chondrogenic differentiation, MSCs were incubated in chondrogenic differentiation medium composed of DMEM supplemented with dexamethasone, ascorbate-phosphate, proline, pyruvate and recombinant human TGF-β3. The medium was changed every 3 days for 3 weeks. After fixation with formalin, sections were stained with anti-mouse collagen II Ab (R&D systems).

RNA extraction and real-time RT-PCR

Total RNA was isolated using RNeasy Mini Kits (Qiagen) and was subjected to reverse transcription with a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. Quantitative RT-PCR

was performed via the TaqMan system (Applied Biosystems) using a 7300 Real-Time PCR machine (Applied Biosystems). TaqMan probes and primers for MFG-E8, fatty acid binding protein 4 (FABP4), iNOS, arginase-1, CD206, Ym1, vascular endothelial growth factor (VEGF), angiopoietin, endothelin-1 (ET-1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18s rRNA were purchased from Applied Biosystems. As an internal control, levels of 18s rRNA or GAPDH were quantified in parallel with target genes. Normalization and fold changes were calculated using the comparative Ct method.

Small interfering RNAs (siRNAs) specific for mouse MFG-E8 mRNA were designed by Qiagen. The targeting sequences were as follows: mouse MFG-E8 siRNA, 5'-AAGCGGTGGAGACAAGGAGTT-3'. siRNAs and AllStars negative control siRNA were purchased from Qiagen. To inhibit MFG-E8 production, 5 x 10^5 MSC were transfected with 10 nmol/L siRNA using HiPerFect transfection reagent (Qiagen). At 24 hours after siRNA transfection, cells were incubated under hypoxic condition (1% O_2, 5% CO_2 and 94% N_2) for 24 hours, and then, RNA was extracted.

Enzyme-linked immunosorbent assay

The supernatant levels of MFG-E8, IL-10 and IL-4 were quantified via a

Quantikine ELISA kit according to the manufacturer's instructions (R&D Systems).

Melanoma xenograft model

B16F10 melanoma cells (2×10^5 cells) and MFG-E8 WT/KO MSC (2×10^5 cells) were implanted subcutaneously into the flanks of MFG-E8 WT and KO mice. MSC was incubated under hypoxic condition for 24 hours prior to implantation. Tumor sizes (width x length; mm^2) were determined with calipers every 2 or 3 days. Tumor bearing mice were euthanized when maximal tumor diameters reached 2 cm or humane endpoints incorporated into the protocol prospectively were reached. To analyze the localization of implanted MSC in melanomas, MSC derived from BM of green fluorescent protein (GFP) transgenic mice were incubated under hypoxic condition for 24 hours, and then GFP-MSC (2×10^5 cells) were implanted with B16F10 melanoma cells (2×10^5 cells) into MFG-E8 KO mice. MSC was labelled with CM-DiI (Molecular Probe) as previously described (4). MSC was incubated under hypoxic condition for 24 hours prior to labelling. MSC was labelled with 4 μM CM-DiI for 5 minutes at 37°C. CM-DiI-labelled MSC (2×10^5 cells) were implanted with B16F10 melanoma cells (2×10^5 cells) into mice. To analyze the effect of macrophages depletion on tumor growth, 200 μl clodronate liposomes (Clophosome-A, FormuMax) or placebo control

liposomes (FormuMax) was injected intraperitoneally at day 0 and then 100 μl clodronate liposomes or control was injected twice per week. To confirm the depletion of macrophages, melanoma tumor was resected at 15 days after inoculation, and the amount of $CD68^+$ macrophages were analyzed by immunofluorescence staining.

Immunofluorescence staining

Tumors (100 mm^2) were excised from flank skin and fixed in 4% paraformaldehyde for 24 hours and 30% Sucrose/H_2O for 24 hours. Frozen sections (4 μm thick) were fixed in 4% paraformaldehyde in PBS for 30 minutes. After blocking with 3% dry milk-PBS supplemented with 5% normal goat serum for 1 hour at room temperature, sections were stained with the antibody of interest, followed by the secondary antibody conjugated with Alexa Fluor 488 or 568. Sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI) to visualize nuclei mounted in ProLong Gold antifade reagent (Life Technologies). All immunofluorescence images were collected and visualized with a FV10i-DOC confocal laserscanning microscope (Olympus). The positive area was determined by Image J (version1.48, NIH, Bethesda, MD) in the field (x600) as previously reported (2).

Human melanoma tissues were used after surgical resection in the patients

treated at the Department of Dermatology, of Gunma University hospital. The study was approved by the institutional review board of Gunma University. All patients provided written informed consent before participation. This study was conducted according to the Declaration of Helsinki principles.

Macrophage differentiation assay

MFG-E8 WT/KO MSC (5×10^5 cells) were incubated in 2.5 ml αMEM medium supplemented with 20% FBS for 24 hours under hypoxic conditions, and then conditioned media was collected. Mouse RAW 264.7 cells (1×10^6 cells) were incubated in 1 ml of MFG-E8 WT/KO MSC-conditioned medium. After 48 hours incubation, RNA was extracted. To assess the effect of recombinant MFG-E8 (rMFG-E8) on macrophage differentiation, RAW 264.7 cells (1×10^6 cells) were incubated with or without rMFG-E8 (500 ng/ml) (R&D Systems) for 48 hours, then RNA was extracted.

Proliferation assay

Cell proliferation was measured using the MTS assay. MSC were treated with trypsin-EDTA and plated at a density of 5,000 cells per well in 96 well plates. Cells were treated with or without hypoxic conditions. After 48 hours at 37°C, 20 μl of

CellTiter 96 AQueous One Solution Reagent (Promega) was added. After an additional incubation at 37°C for 4 hours, the absorbance at 490 nm was measured using an ELISA plate reader.

BM chimeric mice and tumor implantation

C57BL/6 MFG-E8 WT/KO BM cells were collected from the femurs of mice by aspiration and flushing. Recipient C57BL/6 mice were irradiated with 12 Gy and then 5 x 10^6 MFG-E8 WT/KO BM were injected intravenously. Eight weeks after BM cell injection, B16F10 melanoma cells (2×10^5 cells) were implanted subcutaneously into the flanks of mice.

Statistical analysis

P values were calculated using the Student's t-test (two-sided) or by analysis of one-way ANOVA followed by Bonferroni's post test as appropriate. Error bars represent standard errors of the mean, and numbers of experiments (n) are as indicated.

Supplementary References

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