ARTICLE

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Real-Time *in Vivo* Imaging of Stem Cells Following Transgenesis by Transposition

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Previous studies have identified *Sleeping Beauty* transposons as efficient vectors for nonviral gene delivery in mammalian cells. However, studies demonstrating the usefulness of transposons as gene delivery vehicles into adult stem cells are lacking. Multipotent adult progenitor cells (MAPC) are nonhematopoietic stem cells with the capacity to form most, if not all, cell types of the body and as such hold great therapeutic potential. The whole-body biodistribution and persistence of MAPC are unknown, and such data would help direct clinical applications. We have nucleofected murine MAPC with two plasmid-based *Sleeping Beauty* transposons encoding the red fluorescent protein (DsRed2) and firefly luciferase. Transgenic euploid MAPC clones maintained their characteristic multilineage differentiation potential *in vitro.* DsRed2 and luciferase expression allowed for MAPC detection *in vivo* and in tissue sections. To confirm that transgenesis occurred by transposition into the genome of MAPC, we mapped *Sleeping Beauty* transposon integration sites in two MAPC clones using splinkerette PCR. This novel dual-reporter imaging approach based on the transgenesis of MAPC with *Sleeping Beauty* transposons sheds light on the homing patterns of MAPC and paves the way for quantification of MAPC engraftment in real time *in vivo.*

Key Words: multipotent adult progenitor cell, *Sleeping Beauty*, DsRed2, luciferase, stem cell homing, whole-body imaging, biophotonic imaging

INTRODUCTION

The real-time distribution of stem cells after infusion in recipients is largely unknown. The evidence of engraftment has historically been obtained only later, either in the form of an analysis of peripheral blood or from postmortem tissue evaluation. As the goal of novel cellular therapies is their application to human disease, it has become increasingly important to complement tissue studies with the *in vivo* assessment of infused cells in live mice [1]. For these reasons, we have used an *in vivo* imaging approach to study a novel class of progenitor cells, termed multipotent adult progenitor cells (MAPC).

MAPC are nonhematopoietic stem cells derived from adult bone marrow, muscle, and brain of several species, including rodents and humans [2–4]. *In vitro* MAPC can be grown indefinitely (unlike related mesenchymal stem cells) and can be induced to form cell types with cell surface characteristics and gene profiles of cells derived from endoderm, ectoderm, and mesoderm. MAPC display broad differentiation potential *in vivo,* contributing to nearly all somatic tissues when injected into blastocyst and to hematopoietic cells as well as to epithelial cells in liver, lung, and intestine when injected into irradiated NOD-SCID mice [5–8].

For gene transfer into MAPC we have chosen the *Sleeping Beauty* (SB) transposase/transposon system. SB is a synthetic *Tc1/mariner*-type DNA transposon from salmonid fish that has been functionally restored by site-directed mutagenesis after more than 10 million years of inactivity in the salmonid genome [9,10]. SB is functional in eukaryotic cells and recognizes specific inverted/direct repeat sequences flanking a sequence of interest (e.g., therapeutic gene) to mediate a "cut-and-paste" mechanism of transposition, which results in the

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excision of the intervening DNA sequence and its reinsertion into a TA target dinucleotide elsewhere in the genome. Importantly, integration by transposition does not significantly change adjacent host DNA sequences except for the duplication of the target TA dinucleotide [11]. Transposons have been used successfully for both germ line and somatic transgenesis and may have several potential advantages compared with viral-based vectors [10,12–19]. Transposons are relatively easy to produce and may be less immunogenic, since no viral proteins are present, which may be relevant for clinical applications.

Here we report for the first time the transgenesis of adult stem cells using the SB transposase/transposon system. SB-mediated transposition resulted in stable, long-term expression of two different marker genes, those encoding luciferase and the DsRed2 fluorescent protein. Additionally, the real-time, *in vivo* biodistribution of MAPC is assessed by imaging expression of two reporters with distinct and complementary properties.

RESULTS

Nucleofection of *Sleeping Beauty* Transposons

The purpose of this study was to investigate the efficacy of SB-mediated gene transfer and the *in vivo* migration patterns and biodistribution of MAPC. We used two marker genes: one encoding DsRed2, a red fluorescent fluorophore from *Discosoma* coral [20], and the other encoding luciferase, a bioluminescent protein from the North American firefly *Photinus pyralis*. DsRed2 and luciferase genes were cloned individually into SB transposons (pT/CAGGS-DsRed2 and pT/CAGGS-Luciferase; Fig. 1A) and conucleofected into MAPC with a plasmid encoding an enhanced SB transposase mutant (p/CMV-HSB2; Fig. 1A). Nucleofection reportedly delivers DNA directly to the nucleus, thus circumventing nuclear transport of plasmid DNA in an attempt to increase the efficiency of gene transfer [21–23]. Seven days postnucleofection, flow cytometry analysis demonstrated that approximately 20% of MAPC were positive for DsRed2 expression (Fig. 1B).

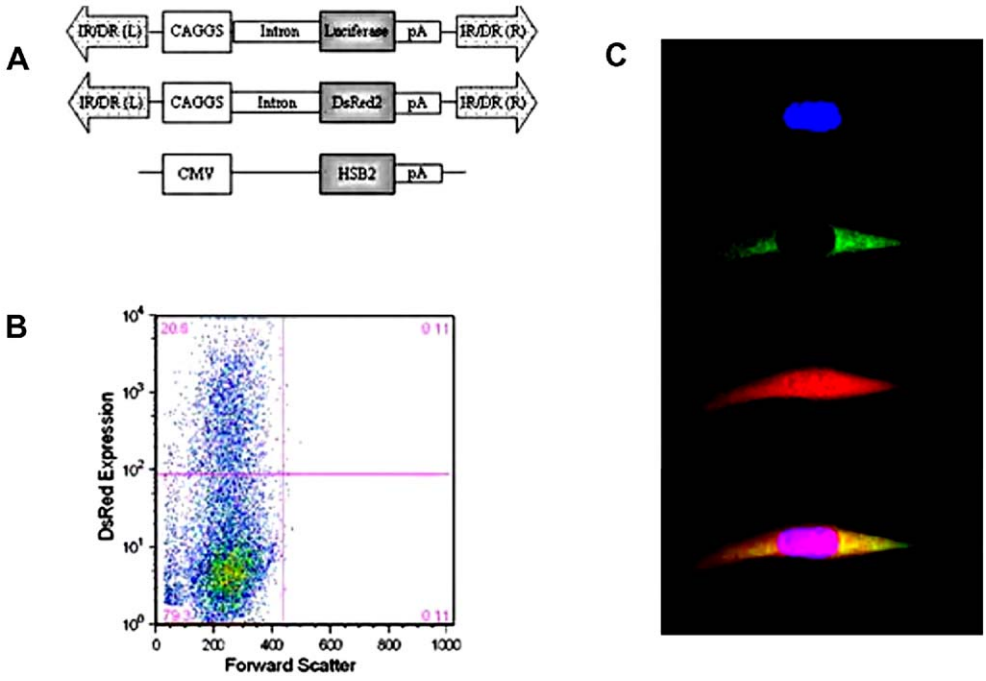

FIG. 1. (A) Transposase plasmid p/CMV-HSB2 is a hyperactive SB mutant expressed from the cytomegalovirus promoter (CMV) [33]. The cassette of pT/CAGGS-DsRed2 and pT/CAGGS-Luciferase is flanked by the SB transposase recognition sequences (inverted/direct repeats, IR/DR (L) left and IR/DR (R) right). CAGGS is a strong composite promoter that is a hybrid of the CMV immediate-early enhancer and the chicken β-actin promoter followed by a chicken β-actin/β-globin intron. Polyadenylation signals in pT/CAGGS-DsRed2 and pT/CAGGS-Luciferase vectors were from the rabbit β-globin gene and simian virus 40, respectively. (B) MAPC were nucleofected and then FACS sorted for DsRed2 expression. Single-cell clones expressing the highest level of DsRed2 were isolated and expanded. (C) Approximately 5000 MAPC were added to each well of 8-well chamber slides (Falcon) and allowed to attach overnight. Cells were incubated sequentially in primary goat anti-Luciferase-Biotin antibody and secondary donkey anti-goat-FITC antibody and then cover slipped with Anti-Fade DAPI solution. From top to bottom: DAPI; anti-Luciferase; DsRed2; merged image. No fluorescence was observed in unmanipulated cells (data not shown). Magnification, 400×.

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Expression of Transgenes in Single-Cell Clones

The MAPC were analyzed by FACS, and cells with the highest expression of DsRed2 were sorted into a 96-well plate (one cell per well). Approximately one-third of the cells underwent cell division. Ten clones with a spindle-shaped or triangular-shaped morphology, the normal morphology of MAPC, were expanded in culture. Four of these DsRed2-positive clones were randomly selected and found to be positive for luciferase activity, whereas unmanipulated MAPC controls were completely devoid of any detectable luciferase activity (data not shown). High-level, persistent gene expression of both transgenes was further confirmed in cultured cells by staining with antibodies specific for luciferase and DsRed2 (Fig. 1C). While about 8 percent of MAPC expressed DsRed2 and luciferase at 4 weeks after conucleofection with transposase, less than 1 percent of MAPC expressed both proteins when no transposase plasmid was conucleofected with the transposons (data not shown), suggesting that stable reporter gene expression in MAPC is SB-dependent.

Transgenic MAPC Retain the Capacity to Widely Differentiate

As no definitive phenotypic profile of MAPC currently exists [3,24], MAPC are defined functionally by their ability to differentiate into multiple cell types. Cells derived from the four selected MAPC clones were cultured in the presence of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), or fibroblast growth factor 4 with hepatocyte growth factor (HGF), and their differentiation into endothelium, astrocytes, and hepatocytes, respectively, was evaluated by testing for the expression of specific cell type surface markers [3]. Specifically, MAPC were induced *in vitro* to express the endothelial markers von Willebrand factor and CD31 (when cultured with VEGF), the astrocytic marker glial fibrillar acidic protein (when cultured with bFGF), and hepatocyte nuclear factor and albumin (when cultured with HGF). Two of four clones displayed a proper MAPC morphology and differentiation pattern (i.e., cellular morphology and ability to differentiate into each of the three germ layers; Fig. 2) and were cytogenetically normal as determined by metaphase spread/G-banding. After expansion of nonnucleofected MAPC from single cells under these conditions, less than 50 percent of clones maintained trilineage differentiation capacity; therefore, nucleofection does not appear to negatively affect the ability of MAPC to differentiate.

Integration Site Analysis

SB transposons integrate into the genome in TA sites via a cut-and-paste fashion in a transposase-catalyzed reaction. To verify that transgene integration into MAPC chromosomes occurred by transposition, junction sequences were amplified from genomic sites of transposon integrants using the splinkerette PCR technique (Table 1). This approach enabled the isolation of unknown sequences flanking known sequences as a result of PCR amplification between a transposon inverted repeat and a splinkerette that was ligated to digested genomic DNA [13,14].

In the genome of MAPC clone 1, SB transposition occurred at two identified TA sites on chromosome 5. One site (5qA3) was located within an intron of the activin receptor-interacting protein 1 (Acvrinp1) gene. Acvrinp1 is a member of the membrane-associated guanylate kinase factors (MAGUK), which interact with the intracellular domain of the Notch ligand, Delta 1, in the evolutionarily conserved cell-cell signaling pathway [25]. In clone 2, the SB element transposed into an intron of the SHPRH gene, which encodes a putative protein

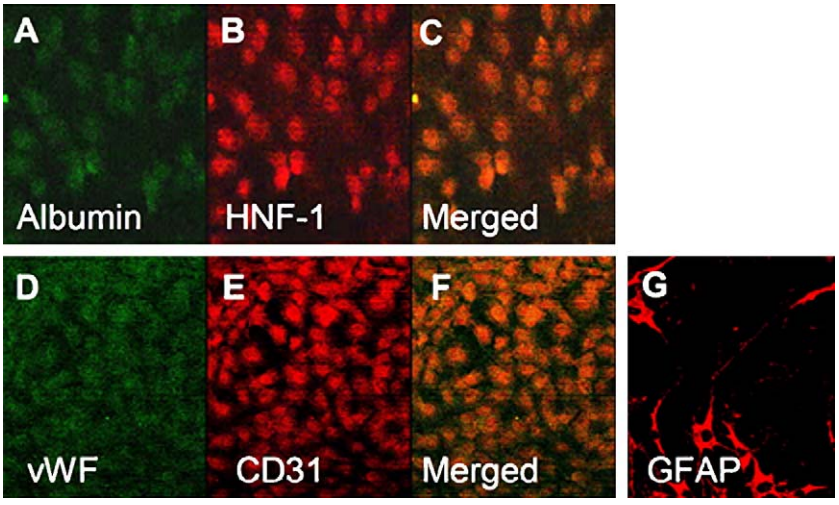

FIG. 2. MAPC were cultured for 14 days in the presence of fibroblast growth factor 4 and hepatocyte growth factor (A, B), or vascular endothelial growth factor (D, E), or basic fibroblast growth factor (G, H). Cell cultures were stained with anti-albumin labeled with FITC (A), anti-hepatocyte nuclear factor 1 labeled with Cy3 (B), anti-von Willebrand factor labeled with FITC (D), anti-CD31 labeled with Cy3 (E), or anti-glial fibrillar acidic protein labeled with Cy3 (G). Overlay images are shown in (C) and (F). FITC, green; Cy3, red; double positive for FITC and Cy3, yellow. Magnification, 100×.

TABLE 1: Genomic sites of transposition recovered by splinkerette PCR

ID	Flanking sequence	Position	Gene
Plasmid	CAACTG**TA**TAGGGATCCGAGCTCCAATTCGCCCTATAGTGAGTCGTATTACGCGC	NA	NA
Clone 1	CAACTG**TA**TGTTTTGACTAACTTTTTGTGAGAGTATTTTTTATTCGCCTGATATA	5qA3	Acvrinp1
Clone 1	CAACTG**TA**TATACATATAAATATATATATACACACAACATATACAAACATACACA	5qG1.1	ND
Clone 2	CAACTG**TA**CATAAAGAGTTCAGGATAGGGCTACTTAGAGACTGGATTTCAAACCA	10qA1	SHPRH

The last six nucleotides of the transposon inverted repeat sequence are underlined, followed by the genomic sequence recovered. Position is the chromosomal location of integration followed by the gene in which integration occurred. Sites of integration were mapped using both the NCBI Mouse Genome Database and the UCSC Mouse BLAT Search Database. Acvrinp1, activin receptor-interacting protein 1; SHPRH, a putative protein with SNF2/helicase and PHD-finger domains; NA, not applicable; ND, no open reading frames were determined in this location.

with domains characteristic of transcription factors and DNA repair proteins [26] (Table 1).

DsRed2-Luciferase Dual Reporter Imaging

We next sought to determine whether MAPC biodistribution can be followed in real time *in vivo*. T-, B-, and NK-immunodeficient mice (Rag2/IL-2Rγc$^{-/-}$, H2^{b}) were used as recipients to minimize the likelihood that the host would reject donor MAPC (H2^{b}). For DsRed2 *in vivo* tracking, we injected 10^6 MAPC (clone 2, Table 1) via the facial vein into a 3-day-old Rag2/IL-2Rγc$^{-/-}$ mouse and conducted imaging 1 h postinjection using a Magnafire

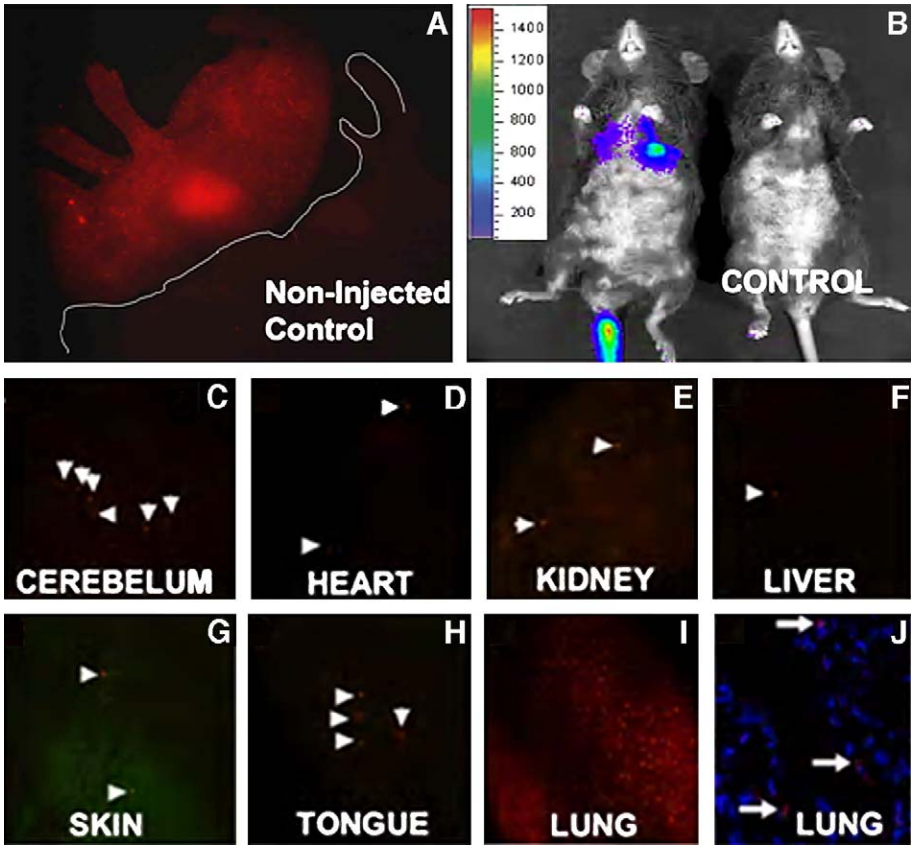

FIG. 3. (A) *In vivo* DsRed2 signals were observed after the transfer of 10^6 of MAPC (clone 2, Table 1). Cells were injected into a 3-day-old Rag2/IL-2Rγc$^{-/-}$ mouse and imaged at 1 h postinjection using a Magnafire color camera (Optronics) mounted onto a Leica MZFLIII stereomicroscope. DsRed2 signals were observed throughout the whole body of the animal and a bright spot was observed in the upper thorax, corresponding to the lungs. An uninjected littermate is shown on the right, outlined with a white line. (B) *In vivo* imaging of luciferase expression (left, at 10 days after infusion of 5×10^6 of MAPC, clone 2, into 8-week-old Rag2/IL-2Rγc$^{-/-}$ mice; animal supine) was performed using the IVIS100 Imaging System. Signals were apparent over the regions corresponding to the lungs and the tail injection site. No bioluminescence was observed in mice injected with unmanipulated cells (animal on the right). (C–I) DsRed2- and luciferase-positive MAPC (5×10^6, clone 2, Table 1) were injected intravenously into 8-week-old Rag2/IL-2Rγc$^{-/-}$ mice ($n = 3$). At 10 weeks after MAPC infusion, a midline incision was made on euthanized animals and a whole-organ image was taken with a Magnafire color camera (Optronics) mounted onto a Leica MZFLIII stereomicroscope. One representative animal (of three injected) is shown. Cells expressing DsRed2 were detected mesoscopically in cerebellum, heart, kidney, liver, skin, tongue, and lung (as indicated with arrowheads; transfer lens 0.63, zoom 8.0). (J) Cryosections of lung tissue specimens were examined by confocal fluorescence microscopy (magnification, 100×). Arrows point to the DsRed2-positive donor MAPC-derived cells. No DsRed2 fluorescence was observed in mice injected with unmanipulated cells (data not shown).

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color camera (Optronics, Goleta, CA, USA) mounted onto a Leica MZFLIII stereomicroscope (Fig. 3A). An uninjected littermate pup is shown for direct comparison (Fig. 3A, right, outlined with a white line).

Using *in vivo* bioluminescence imaging (BLI) in live animals we were able to see the distribution of the luciferase-marked MAPC at 10 days and at 10 weeks in the same recipient (data not shown and Fig. 3B). For that purpose, 5×10^6 MAPC (clone 2, Table 1) were injected via the tail vein into 8-week-old Rag2/IL-2R$\gamma c^{-/-}$ mice. Bioluminescence signals from MAPC were predominantly detected over regions corresponding to the lungs with even, bilateral distribution in all animals in the mesoscopic (organ-level imaging) and macroscopic (whole-body) images. Fluorescent signals from cells expressing DsRed2 were detected mesoscopically in multiple organs including lung, cerebellum, heart, kidney, liver, skin, and tongue (Figs. 3C–3I). In addition, DsRed2-positive cells were also found by confocal microscopy (microscopic imaging) in lung cryosections (Fig. 3J), further indicating persistent transgene expression *in vivo*.

DISCUSSION

The effectiveness of SB-mediated gene transfer into MAPC and the *in vivo* distribution of transgenic MAPC were demonstrated using MAPC nucleofected with transposon-based vectors coding for either DsRed2 or luciferase as reporter genes. Stable gene expression of both reporter genes was achieved and stable genomic integration by transposition was confirmed at the molecular level. The nucleofection procedure is rapid and efficient, and it did not affect the phenotypic characteristics of these MAPC cells.

In our previous studies, murine MAPC were derived from the bone marrow of C57BL/6J-rosa 26 mice transgenic for β-galactosidase and the neomycin phosphotransferase gene, which allowed the identification of donor cells in recipient tissue sections postmortem [3]. However, an assessment of tissue homing patterns and the distribution, persistence, and expansion of MAPC *in vivo* in real time had not previously been possible. We reasoned, therefore, that for clinical applications we would need a preclinical evaluation of the real-time kinetics of engraftment, migration, and tissue regeneration as well as persistence of gene expression in a MAPC population after transfer. The imaging approach used here enabled tracking of MAPC *in vivo* with the ability to observe the biodistribution of cells at micro-, meso-, and macroscopic levels. This approach utilizes the advantage of luciferase having a dynamic range that covers an extensive spectrum of signal intensities, both in culture and *in vivo*. The advantage of DsRed2 is its use as a marker with native fluorescence that obviates the need for staining using antibodies. Here we were able to monitor MAPC *in vivo* after a period of several months using BLI. Both DsRed2 and luciferase have previously been used successfully as marker genes [27–30], and, as recent examples, luciferase has been shown to be effective for monitoring xenografted human glioblastoma cells [31], hematopoietic stem cells [1], and immune therapies *in vivo* [32].

While the MAPC were transplanted into immunodeficient mice, the potential for immune-based clearance of the cells as a result of triggering of the immune response against the transgene products or the cells themselves is a distinct possibility in immune-competent organisms.

In addition to their tendency to integrate near transcriptionally active genes, long-term gene expression can also be difficult to achieve using viral vectors. The potential exists for diminished gene expression over time with transposon-based vectors as well. However, we have not noted significant changes in gene expression *in vitro* and *in vivo* over a period of more than 15 weeks (unpublished data), suggesting that SB-mediated gene delivery can result in sustained gene expression.

These data are the first to monitor the *in vivo* migratory properties of multipotent adult progenitor cells in real time as well as to establish the ability of SB to mediate adult stem cell gene transfer. MAPC are relatively easy to isolate and proliferate *in vitro* and are capable of broad differentiation, and therefore they are attractive candidates for cellular therapies. When coupled with the delivery of transposon-based gene vectors and *in vivo* tracking, future studies will seek to exploit the migratory properties and wide differentiation potential of MAPC as cellular-based therapies in clinical and preclinical models.

MATERIALS AND METHODS

Vector construction. Transposon (pT) and transposase (SB) constructs were originally derived from pT/neo and pSB10 plasmids [9]. For transposase pCMV/HSB2, the K33A and L91A mutations were introduced into the 5′ end of the SB108 gene using mutagenic PCR primers and then cloned under the control of the strong CMV promoter by *Bam*HI/*Eco*RI ligation into pc-N as described in [17], resulting in hyperactive transposase pCMV/HSB2.

The luciferase coding sequence was excised from pGL3C (Promega, Madison, WI, USA) as a *Hin*d3-*Xba*I fragment and cloned into the same restriction sites in pBlueScript SK(-) (New England Biolabs, Beverly, MA, USA). The luciferase cDNA was then cloned from pBlueScript SK(-) with *Not*I and *Xho* [15]. The *Xho*I site was filled in with Klenow large-fragment polymerase, and the resulting product was cloned into *Not*I and *Eco*R5 sites of pT/CAGGS. The final construct was pT/CAGGS-Luciferase.

To create pT/CAGGS-DsRed2, the pCAGGS vector was digested with *Eco*RI (New England BioLabs) and blunt ended using the large (Klenow) fragment of *Escherichia coli* DNA polymerase I (Invitrogen, Carlsbad, CA, USA) followed by vector dephosphorylation using shrimp alkaline phosphatase (Promega). The coding sequence of DsRed2 (BD Biosciences/Clontech, Palo Alto, CA, USA) was excised by digesting with *Nco*I and *Eco*RI (New England BioLabs). This fragment was blunt ended using Klenow and ligated into the pCAGGS vector using T4 DNA ligase (Roche, Indianopolis, IN, USA). The resulting plasmid was digested with *Hin*cII and *Hin*dIII (New England BioLabs), which releases a fragment consisting of the promoter/enhancer/intron, DsRed2, and polyadenylation signal (Fig. 1). This fragment was blunt ended and ligated into the *Nhe*I- (New England BioLabs) digested, blunt-ended, alkaline phosphatase-treated pT vector.

Nucleofection. MAPC were derived from the bone marrow of C57BL/6J-rosa 26 mice ($H2^b$) transgenic for the β-galactosidase and neomycin phosphotransferase genes. MAPC were cultured at a low density in fibronectin- (Sigma Chemical Co., St Louis, MO, USA) coated flasks and induced to differentiate *in vitro* into neurons, hepatocytes, and endothelium as described [2,3]. Metaphase spread and G-banding were performed at the Cytogenetics Core Facility at the University of Minnesota, Minneapolis, MN (USA). MAPC (1×10^6 cells) were conucleofected (Amaxa, Gaithersburg, MD, USA; setting T-20, buffer T) with 5 μg of each plasmid (pT/CAGGS-DsRed2 and pT/CAGGS-Luciferase) and the SB transposase plasmid at a ratio of 1:50 of the DsRed2 and luciferase plasmids (0.1 μg of p/CMV-HSB2).

DsRed2 and luciferase expression. Nucleofected MAPC were single-cell sorted using fluorescence-activated cell sorting (FACSCalibur, BD Biosciences/Pharmingen, San Diego, CA, USA) for DsRed2 expression and expanded. MAPC DsRed2-positive clones were assayed for luciferase activity using bioluminescence as follows: the cells were harvested by centrifugation, resuspended in 100 μL of culture media with 10 μL of luciferin stock (30 mg/mL, Xenogen Corp., Alameda, CA, USA), and assayed immediately for bioluminescence activity on a Chameleon 425–100 Multi-label Counter (Hidex, Turku, Finland). Average relative luminescence values were expressed as counts/second.

Flow cytometry. Nucleofected cells were either evaluated for native DsRed2 fluorescence or stained with biotinylated goat polyclonal antibody for firefly luciferase (Ab Cam, Cambridge, MA, USA), followed by FITC-conjugated streptavidin (BD Biosciences, Pharmingen), and analyzed using a FACSCalibur (BD Biosciences).

Antibody staining and fluorescence in cultured cells. Approximately 5000 cells were added to each well of 8-well chamber slides (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) and allowed to attach overnight. The culture medium was removed and slides were washed, fixed in acetone for 5 min, and then coated with Normal Donkey Serum (Jackson ImmunoResearch, West Grove, PA, USA) for 2 h. Two hundred microliters/well of primary antibody [goat anti-Luciferase-Biotin (Ab Cam)] was added at 1:10,000 and incubated for 1 h at room temperature. Slides were washed twice with PBS, and then 200 μL/well of secondary antibody [donkey anti-goat-FITC (Jackson ImmunoResearch)] was added at a 1:1000 dilution and incubated for 1 h at room temperature. Slides were washed as before, cover slipped with Anti-Fade 4′,6-diamidino-2-phenylindole (DAPI) solution (Molecular Probes, Eugene, OR, USA), and examined using confocal fluorescence microscopy (Olympus AX70, Olympus Optical Co. LTD, Japan).

Splinkerette PCR of genomic transposon junctions. Genomic DNA was isolated from cultured MAPC using the DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). One microgram of DNA was digested at 37°C overnight with 5 U of *Sph*I and 20 U of *Bam*HI (New England BioLabs). Double-stranded oligonucleotides, splinkerettes [11], were created by heating equimolar amounts of the primerette long (5′-CCT CCA CTA CGA CTC ACT GAA GGG CAA GCA GTC CTA ACA ACC ATG-3′) with the splink-*Sph*I (5′-PO_4-GTT GTT AGG ACT GCT TGG AGG GGA AAT CAA TCC CCT-3′) at 80°C for 5 min and then cooling slowly to room temperature. Digested genomic DNA (25 ng) plus the splinkerette were ligated using T4 DNA ligase (Roche) at 16°C overnight. The ligation reaction was diluted 1:1000 and the primary PCR reaction was performed as follows: 25 μM each of the primerette short (5′-CCT CCA CTA CGA CTC ACT GAA GGG C-3′) and long inverted/direct repeats (IR/DR) (R) (5′-TCT CCT GAC TAC TCC CAG TCA TAG CTG TCC C-3′) was used in a reaction containing Platinum *Taq* DNA polymerase (Invitrogen) under the conditions of 95°C for 2 min, 10 cycles of 95°C for 5 s, 70°C for 2 min (−0.5°C/cycle), followed by 20 cycles of 95°C for 5 s and 65°C for 2 min, with a final 10-min extension at 70°C. The entire reaction was purified using the QIAquick PCR Purification Kit (Qiagen) and then digested with 40 U of *Bam*HI (NEB) at 37°C. The digested primary PCR reaction was diluted 1:50 and used in a second PCR containing 25 μM each of primerette nested (5′-GGG CAA GCA GTC CTA ACA ACC ATG-3′) and IR/DR (R) MJO1 (5′-GAC CCA CTG GAA TTG TGA TAC AGT GAA TTA TAA GTG-3′) and Platinum *Taq* DNA polymerase (Invitrogen) under the following conditions: 95°C for 2 min followed by 30 cycles of 95°C for 5 s, 61°C for 30 s, and 70°C for 90 s, with a final extension time of 10 min at 70°C. The PCR products were run on a 1% agarose gel (Invitrogen), and the bands were excised and purified using the QIAquick Gel Extraction Kit (Qiagen) and submitted to the University of Minnesota Advanced Genetics Analysis Center for direct sequencing. Sites of integration were mapped using both the NCBI Mouse Genome Database and the UCSC Mouse BLAT Search Database.

Mice. Immunodeficient mice carrying mutations in recombinase-activating gene 2 and the common cytokine receptor γ, Rag2/IL-2Rγc$^{-/-}$ ($H2^b$), were a gift from Dr. Stephen Jameson (University of Minnesota). Mice were maintained under specific pathogen-free conditions.

Cell infusion into immunodeficient mice. DsRed2- and luciferase-positive MAPC (clone 2, Table 1) were infused into Rag2/IL-2Rγc$^{-/-}$ mice either via the facial vein (3-day-old animals) or via the tail vein (8-week-old animals).

In vivo macroscopic imaging of luciferase activity. At 10 days and 10 weeks after MAPC infusion time, experimental mice were anesthetized with Nembutol (0.1 cc/10 mg body weight) and the abdomen and chest were shaved. Luciferin stock (30 mg/mL, Xenogen Corp.) was injected intraperitoneally into the mice at 150 mg/kg. A grayscale reference image was taken of the position of the mice prior to the assessment of luciferase activity. Bioluminescent signals were assessed at 5 min post-luciferin injection at an integration time of 2 min using an *in vivo* imaging system that utilizes a cooled charge-coupled device (CCD) camera (IVIS100, Xenogen Corp.). Pseudocolor images representing the bioluminescent signal intensity (blue is the least intense and red is the most intense) were superimposed over the grayscale reference image. The scales for the pseudocolor intensity plots are displayed with the images.

Mesoscopic imaging of DsRed2 in whole lung and in lung-tissue sections. A midline incision was made on euthanized mice 1 h and 10 weeks following MAPC injection; visceral organs and brain were removed and whole-organ (mesoscopic) images were taken with a Magnafire color camera (Optronics) mounted onto a Leica MZFLIII stereomicroscope. Next, lung-tissue specimens from the recipient and control animals were cryopreserved in OCT at −80°C. Six-micrometer-thick cryosections were mounted on glass slides, fixed in acetone for 5 min at RT. Cryosections were stained with DAPI (Molecular Probes) and examined by confocal fluorescence microscopy (Olympus AX70, Olympus Optical Co. LTD).

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