Stem Cell Research 17 (2016) 93–96

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Lab Resource

A human *MIXL1* green fluorescent protein reporter embryonic stem cell line engineered using TALEN-based genome editing

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ARTICLE INFO

Article history:
Received 16 May 2016
Accepted 20 May 2016
Available online 26 May 2016

ABSTRACT

We have generated a *MIXL1*-eGFP reporter human embryonic stem cell (hESC) line using TALEN-based genome engineering. This line accurately traces endogenous MIXL1 expression via an eGFP reporter to mesendodermal precursor cells. The utility of the *MIXL1*-eGFP reporter hESC line lies in the prospective isolation, lineage tracing, and developmental and mechanistic studies of MIXL1^{+} cell populations.

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Resource table: *MIXL1*-eGFP hESC.

Name of Stem Cell construct	*MIXL1*-eGFP reporter human embryonic stem cell line
Institution	Icahn School of Medicine at Mount Sinai
Person who created resource	Vera Alexeeva, Sunita L. D'Souza, Christoph Schaniel
Contact person and email	Sunita L. D'Souza, sunita.d'souza@mssm.edu; Christoph Schaniel, christoph.schaniel@mssm.edu
Date archived/stock date	May 22, 2013
Origin	Human embryonic stem cell line WA09 (H9; NIH registration number 0062)
Type of resource	Biological reagent; human embryonic stem cell line; genetically modified
Sub-type	Cell line
Key transcription factors	MIXL1
Authentication	Identity and purity of cell line confirmed (Fig. 1F)
Link to related literature (direct URL links and full references)	N/A
Information in public databases	N/A

1. Resource details

A *MIXL1*-eGFP reporter human embryonic stem cell (hESC; clones 4–21) line was generated using TALEN-based genome targeting of WA09 (H9, NIH registration number 0062) hESCs replacing the stop codon of endogenous *MIXL1* with a 2A-eGFP cassette, thus, creating a *MIXL1-2A-eGFP* allele, (Fig. 1A).

Correct integration was confimed by sequencing of PRC products obtained from genomic DNA using specific primers (see Table 1) as well as Southern blot analysis (Fig. 1B).

Prior to deciding to use WA09 to create the MIXL1 reporter line, we sent the line for G-banded karyotype analysis. Initial results showed a normal karyotype of parental WA09 (Fig. 1C, left panel). The reporter was created and then both the reporter and the parental line were resent for karyotype analysis. However, upon high resolution chromosomal analysis of the *MIXL1*-eGFP hESC line as well as the parental WA09 hESCs, a clonal abnormality, an interstitial duplication, resulting in partial trisomy of the long (q) arm of chromosome 1 was identified in both lines, which is a recurrent acquired abnormality in human pluripotent stem cell cultures (Na et al. 2014) (Fig. 1C, right panels). In addition, the long (q) arm of chromosome 1 is partially duplicated and translocated to the end of the short arm of chromosome X in the *MIXL1*-eGFP hESC line (10 of 20 cells examined). Seven of 20 *MIXL1*-eGFP hESCs were found to have no abnormality. Short tandem repeat analysis confirmed the WA09 hESC origin of the generated *MIXL1*-eGFP reporter hESC line (Fig. 1D).

To confirm the pluripotency of the *MIXL1*-eGFP hESCs, the expression of several pluripotency markers was analyzed by quantitative real-time PCR as well as immunocytochemsitry. Endogenous expression

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http://dx.doi.org/10.1016/j.scr.2016.05.010
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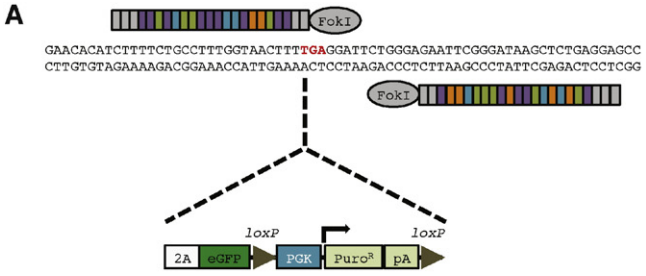
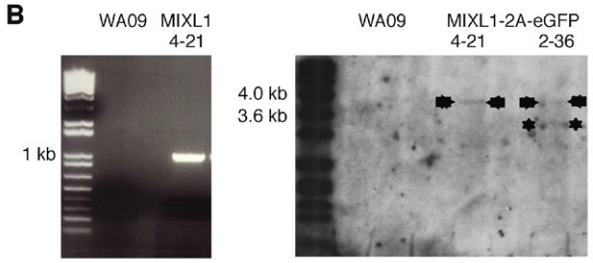
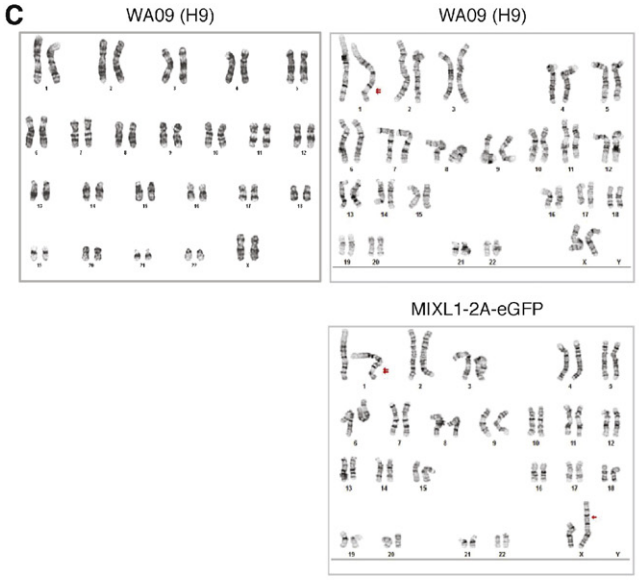
of NANOG, OCT4 and SOX2 was determined at the mRNA level by real-time PCR (Fig. 1C). Protein expression of OCT4, NANOG, and SOX2 was assayed by immunocytochemsitry (Fig. 1D).

Three germ-layer differentiation ability was demonstrated by spontaneous in vitro differentiation of embryoid bodies with subsequent replating and immunocytochemical detection of smooth muscle actin

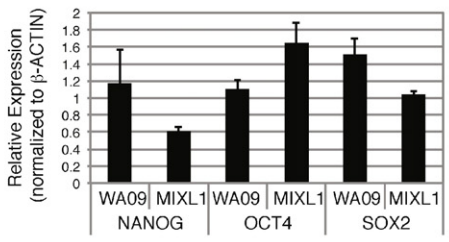


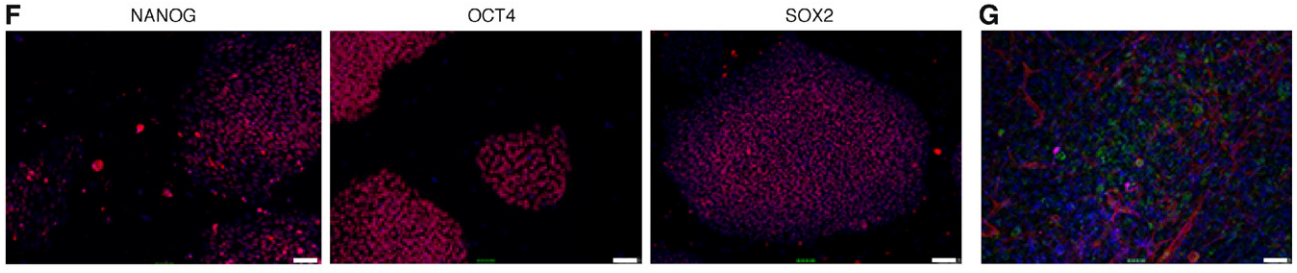


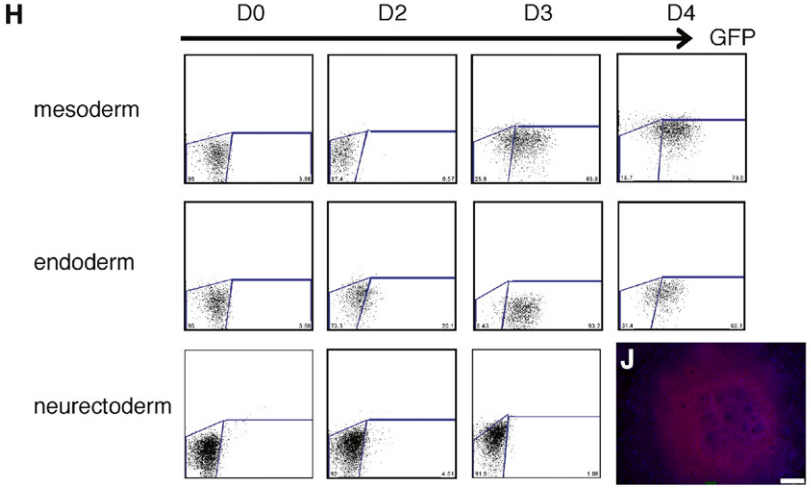


D

STR Locus	STR Genotype Repeat #	STR Genotype
FGA	16–18,18.2,19,19.2,20,20.2,21,21.2,22, 22.2, 23, 23.2, 24, 24.2, 25, 25.2, 26–30, 31.2, 43.2, 44.2,45.2, 46.2	26,28
TPOX	6-13	10,11
D8S1179	7-18	8,14
vWA	10-22	17,17
Amelogenin	X,Y	X,X
Penta_D	2.2, 3.2, 5, 7-17	9,13
CSF1PO	6-15	11,11
D16S539	5, 8-15	12,13
D7S820	6-14	9,11
D13S317	7-15	9,9
D5S818	7-16	11,12
Penta_E	5-24	11,14
D18S51	8-10, 10.2, 11-13, 13.2, 14-27	13,13
D21S11	24,24.2,25,25.2,26-28,28.2,29,29.2, 30, 30.2,31, 31.2,32.32.2,33,33.2, 34,34.2,35,35.2,36-38	30,30
TH01	4-9,9.3,10-11,13.3	9.3,9.3
D3S1358	12-20	13,16







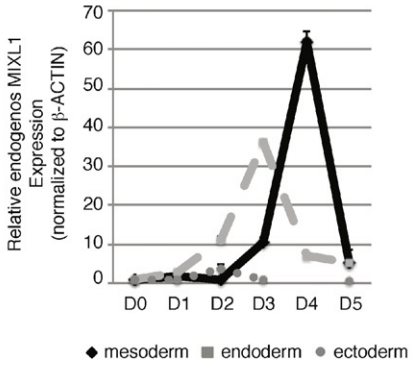


Table 1
Primer sets used.

Gene or locus	5′ primer sequence [5′ → 3′]	3′ primer sequence [5′ → 3′]
Amplification of homologous arms		
MIXL1 upstream arm	GACCTGCAGGCATGAGCAAAATACCTC[a]	GCGCTAGCAAAGTTACCAAAGGCAGAAAAGA
MIXL1 downstream arm	GAGGCGCGCCAATTCGGGATAAGCT	GAGGCCGGCCGTGCCCAGCTAAGTTTTA
Targeted integration		
MIXL1 locus	GGTAAGGAGAAGCCTGCCTTT	AAGTCGTGCTGCCTTCATGTG[a] [PMID: 21738127]
Southern blot probe		
GFP probe	ACGTAAACGGCCACAAGTTC	CTGGGTGCTCAGGTAGTGGT
Gene expression by real-time PCR		
hOCT4	AACCTGGAGTTTGTGCCAGGGTTT	GCTGATTAGGCTCCAACCATAC
hNANOG	CCTGAAGACGTGTGAAGATGAG	GCTGATTAGGCTCCAACCATAC
hSOX2	AGAAGAGGAGAGAGAAAGAAAGGGAGAGA	GAGAGAGGCAAACTGGAATCAGGATCAAA
hMIXL1 (endogenous)	CTGTGCTCCTGGAACTGAAACGAAAT	ACCTTGGGAGCTAGAGTCAGAGATG
hβ-ACTIN	TTTTTGGCTTGACTCAGGATTT	GCAAGGGACTTCCTGTAACAAC

[a] Hockemeyer at al. (2011). Genetic engineering of human pluripotent cells using TALE nucleases. Nat. Biotechnol. 29, 731–734.

(SMA) for mesoderm, alpha-feto protein (AFP) for endoderm and beta-III tubulin (TUJI) for ectoderm (Fig. 1G).

Correlation of eGFP expression with endogenous *MIXL1* expression was confirmed by flow cytometry and real-time PCR during specific in vitro differentiation to the 3-germ layers (mesoderm, endoderm and neuroectoderm) (Fig. 1H & I). Expression was dynamic and restricted to specific times during differentiation that correlate with the emergence of mesodermal or endodermal precursors. Immunocytochemistry corroborated the flow cytometry and PCR results (Fig. 1J and data not shown).

2. Materials and methods

2.1. Maintenance of human embryonic stem cells

Human ESCs cells were generally maintained on an irradiated layer of mouse embryonic fibroblasts (MEFs) in hESC medium consisting of DMEM/F12 (Invitrogen) supplemented with 20% KnockOut Serum Replacement (Invitrogen), 2 mM L-Glutamine (Invitrogen), 100 units/mL Penicillin and 100 μg/mL of Streptomycin (Invitrogen), 1× Non-Essential Amino Acids, 0.1 mM 2-mercaptoethanol and 20 ng/mL bFGF (R&D Systems). For feeder-free culture, cells were grown on Matrigel (Corning)-coated surfaces in mTeSR1 medium (StemCell Technologies) supplemented with 2 mM L-Glutamine and 100 units/mL Penicillin and 100 μg/mL of Streptomycin.

2.2. Generation of a MIXL1-eGFP reporter H9 human embryonic stem cell line using TALEN-based genome engineering

Homologous arms were amplified using genomic DNA isolated from WA09 hESCs and primers to amplify the upstream homologous arm or the downstream homologous arm, respectively (see Table 1 for primer sequences). PCR products were run on a 1% agarose gel, gelpurified and digested with SbfI and NheI (upstream homologous arm), and AscI and FseI (downstream homologous arm), respectively, and ligated into the OCT4-2AeGFP-PGK-Puro vector (Addgene) replacing the OCT4 homologous arms.

TALENs targeting 5′-TTCTGCCTTTGGTAACTT-3′ upstream of the *MIXL1* Stop codon, and 5′-TCAGAGCTTATCCCGAAT-3′ downstream of the *MIXL1* Stop codon, respectively, were assembled using the Joung Lab REAL Assembly TALEN kit (Addgene) according to protocol (Sander et al. 2011).

To generate the *MIXL1*-eGFP reporter line, 10^7 WA09 hESCs were mixed with 40 μg of *MIXL1*-2A-eGFP-PGK-Puro donor plasmid and 5 μg of each TALEN encoding plasmid in 800 μL of PBS in 0.4 cm cuvettes. Electroporation was performed at 250 V and 500 μF using a GenePulser Xcell system (Bio-Rad). Cells were plated on irradiated DR4 MEFs and selected with 0.5 μg/mL puromycin starting on day 5 after electroporation. Resistant colonies were picked and expanded.

2.3. Southern blot analysis

Ten micrograms of genomic DNA from potential *MIXL1*-eGFP reporter clones were digested with NheI (NEB) overnight at 37 °C and run on a 1% agarose gel. The DNA was transferred from the gel to a positively charge nylon membrane using a standard protocol. The DIG DNA Labeling and Detection Kit (Roche) in combination with a GFP fragment (see Table 1 for primer sequences) was used for probe labeling, hybridization and signal detection according to the manufacturer's instruction.

2.4. PCR and quantitative real-time PCR

PCR on ~100 ng of genomic DNA was performed using Fusionflash High Fidelity PCR Master Mix (Finnzymes) at 98 °C for 1 min, 35 cycles of 98 °C for 1 min – 55 °C and 57 °C, respectively, for 5 s and 72 °C for 30 s, followed by a final extension at 72 °C for 1 min in a Veriti 96-well Thermal Cycler (Applied Biosystems). Quantitative real-time PCR on cDNA prepared from 500 ng RNA isolated from cells using TRIzol was performed using FastSybR Green Master Mix (Applied Biosystems) according to the manufacturer's instruction in a Lightcycler 480 (Roche)

Fig. 1. Generation and characterization of engineered human *MIXL1-2A-eGFP* reporter embryonic stem cell. (A) Schematic of TALEN-based targeting of the *MIXL1* locus with a donor plasmid carrying 2A-eGFP and a floxed PGK promoter-driven puromycin resistance gene cassette. (B) PCR based integration analysis produced a band of ~1 kb, indicating correct integration (left). Southern blot analysis of NheI digested genomic DNA demonstrated a single GFP insert (arrow) in the correctly targeted clone 4–21 (right), while clone 3–16 had an additional random integration (asterisk). (C) G-band karyotype analysis of parental WA09 hESCs used for gene targeting (left, low resolution; right, high resolution) and the engineered *MIXL1*-eGFP reporter hESC line (right, lower panel, high resolution). (FD Short tandem repeat analysis of the *MIXL1*-eGFP line proved its WA09 hESC origin. (E) Quantitative real-time PCR analysis of the pluripotency factors NANOG, OCT4 and SOX2 in the targeted *MIXL1*-eGFP reporter hESC line and the original WA09 cells. Data are normalized to β-ACTIN and represented as mean ± SD ($n = 3$) relative to WA09. (F) Immunocytochemical analysis of NANOG, OCT4 and SOX2 protein expression in the *MIXL1*-eGFP reporter hESC line. Red, NANOG, OCT4 or SOX2, respectively; blue, DAPI (nucleus); scale bar, 75 μm. (G) *MIXL1*-eGFP reporter hESCs are pluripotent as demonstrated by immunocytochemistry on differentiated *MIXL1*-eGFP reporter hESCs. Purple, TUJ1 for neuroectoderm; green, AFP for endoderm and red, SMA for mesoderm. DAPI (blue) was used to stain the nucleus. Scale bar, 75 μm. (H) Flow cytometry analysis of eGFP expression at indicated time points after directed differentiation to the specific germ layer. (I) Relative MIXL1 mRNA expression by quantitative real-time PCR at indicated time points after directed differentiation to the specific germ layer. Data are normalized to β-ACTIN and represented as mean ± SD ($n = 3$) relative to the undifferentiated, pluripotent state (D0). (J) PAX6 expression in neuronal rosettes at day 10 of neuroectoderm differentiation. Red, PAX6; blue, DAPI (nucleus); scale bar, 250 μm.

at 95 °C for 1 min (initial denaturing step), followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Data were normalized to β-ACTIN. Primers are listed in Table 1.

2.5. Karyotype analysis and cell identity by short tandem repeat analysis

Chromosomal G-banding karyotype analysis was performed at the Cytogenetics Laboratory at Mount Sinai. High resolution karyotyping as well as short tandem repeat analysis for parental cell authentication was performed by the WiCell Research Institute (Madison, WI).

2.6. In vitro differentiation of MIXL1-eGFP reporter H9 embryonic stem cell line

For general 3-germ layer differentiation, *MIXL*1-eGFP cells grown feeder-free on Matrigel in mTeSR1 media (StemCell Technologies) to 60–70% confluence were dissociated with Accutase and replated into low attachment plates in DMEM/20% FBS media to enable embryoid body (EB) formation and random differentiation. On day 8 of differentiation, EBs were collected, replated onto Matrigel-coated 48-well plates and grown for an additional 14 days in DMEM/20% FBS with feeding every other day, and then processed for immunocytochemistry.

For mesoderm differentiation (Kennedy et al. 2012), *MIXL*1-eGFP cells, grown feeder-free on Matrigel in mTESR media (StemCell Technologies) to 60–70% confluency were collected with 0.5 mM EDTA in PBS, resuspended in StemSpan medium (Stemcell Technologies) supplemented with 2 mM L-Glutamine, 50 μg/mL ascorbic acid, 4×10^{-4} M monothioglycerol (StemSpan Complete), and containing 10 ng/mL BMP4 and 150 μg/mL Transferrin, and replated into low attachment plates for EBs formation (day 0). On day 1, medium was changed to StemSpan complete with 10 ng/mL BMP4 and 5 ng/mL bFGF. On day 2, medium was changed to StemSpan Complete supplemented with 10 ng/mL BMP4 and 1 ng/mL ActivinA. On day 4 medium was changed to StemSpan Complete supplemented with 5 ng/mL bFGF, 15 μg/mL VEGF, 150 ng/mL DKK-1, 10 ng/mL IL-6, 5 ng/mL IL-11 and 25 ng/mL IGF-1. Differentiation was continued until day 5. Cells were harvested daily for PCR and FACS analyses.

For endoderm differentiation, *MIXL*1-eGFP cells, grown feeder-free on Matrigel in mTESR media (StemCell Technologies) to 60–70% confluency were collected using Accutase and replated onto Matrigel-coated 24-well plates in mTESR media (D0). After 24 h, medium was replaced with STEMdiff Definitive Endoderm Basal Medium supplemented with Endoderm Supplement A and Endoderm Supplement B (1:100 dilution; StemCell Technologies). On Day 2, medium was changed to STEMdiff Definitive Endoderm Basal Medium supplemented with Definitive Endoderm Supplement B. On day 3 and day 4, medium was refreshed as on day 2. Differentiation was continued until day 5. Cells were harvested daily for PCR and FACS analysis.

For neuroectoderm differentiation, *MIXL*1-eGFP cells, grown feeder-free on Matrigel in mTESR media (StemCell Technologies) to 60–70% confluency were collected with 0.5 mM EDTA in PBS, and resuspended in STEMdiff Neural Induction medium (StemCell Technologies) and cultured in low attachment plates to enable EBs formation. On day 8 day, EBs were replated onto Matrigel-coated 12-well plates in STEMdiff Neural Induction medium for 2 days until neural rosettes were observed.

2.7. Immunocytochemistry

MIXL1-eGFP hESCs or its differentiated progenitors were fixed with 4% Paraformaldehyde in PBS for 15 min at RT, washed with PBS, permeabilized with 0.2% Triton X-100 for 10 min at RT and rewashed with PBS. Cells were blocked using a 0.05% saponin solution (PBS, 1% Glucose, 0.02% sodium azide, 5 mM HEPES, 0.05% Saponin) containing 10% of the serum from the animal in which the secondary antibodies were generated in.

For pluripotency marker detection, cells were incubated with the following primary antibodies at a 1:200 dilution overnight at 4 °C: rabbit-polyclonal anti-Oct4 (Stemgent), goat-polyclonal anti-Sox2 (Santa Cruz Biotechnologies), goat-polyclonal anti-Nanog (R&D Systems). Cells were washed three times in 0.05% saponin solution for 15 min each. Cells were then incubated with donkey anti-goat Alexa Fluor 647 or donkey anti-rabbit Alexa Fluor 647 (Life Technologies) secondary antibody, respectively, at a 1:500 concentration for 60 min. Cells were washed 3 times in 0.05% saponin solution to remove the unbound secondary antibody. DAPI at 1:1000 dilution was added for the last wash.

For detection of PAX6, cells were incubated with rabbit-polyclonal anti-Pax6 (BioLegend), followed by donkey anti-rabbit Alexa Fluor 555.

For 3-germ layer identification, fixed cells were processed using the 3-Germ Layer Immunocytochemistry Kit (Life Technologies) according to the manufacturer's instruction. Briefly, cells were incubated with the blocking solution for 1 h at RT, then incubated with primary antibodies (rabbit anti-TUJI (1:250), mouse IgG1 anti-AFP (1:250), and mouse IgG2 anti-SMA (1:100)) overnight at 4 °C. The next day, cells were washed 3 times for 15 min in 0.05% saponin solution followed by incubation with secondary antibodies (Alexa Fluor 647 donkey anti-rabbit; Alexa Fluor 488 goat anti-mouse IgG1, and Alexa Fluor 555 goat anti-anti mouse IgG2a; all at 1:250) in 0.05% saponin in PBS for 1 h at RT. After 3 washes with PBS, nuclei were stained with DAPI (1:1000).

Fluorescence was visualized using a Leica DMRA2 fluorescence microscope (Wetzlar, Germany) and images were recorded using a digital Hamamatsu CCD camera (Hamamatsu City-Japan).

2.8. Flow cytometry analysis

Expression of lineage-specific markers during mesoderm (CD34) or endoderm (CXCR4 and CD117) differentiation, respectively, was determined by flow cytometry. Briefly, cells/EBs were collected, dissociated with 0.25% Trypsin for 3 min at 37 °C. DMEM/10% FBS was added, cells centrifuged at 200 g for 2 min and resuspended in 400 μL DMEM. Cells were then incubated with the respective antibodies, CXCR4-APC (1:100, BD Pharmingen) and CD117 (c-Kit)-PE-Cy7 (1:100, eBioscience), or CD34-APC (1:100; Invitrogen), for 60 min on ice, washed, and resuspended in 400 μl DMEM/10% FBS. Stained cells were run on a FACS Canto II (BD Biosciences) and analyzed with FlowJo software program (TriStar).

Acknowledgment

This work was supported by the NY State Department of Health/Empire State Stem Cell Board (NYSTEM) grant (C024176) and the Black Family Stem Cell Institute at the Icahn School of Medicine at Mount Sinai. SLD and CS conceived the study. VA and SLD performed the experiments. VA, SLD and CS wrote the manuscript.

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