SUPPLEMENTARY APPENDIX

Targeting of plasminogen activator inhibitor-1 activity promotes elimination of chronic myeloid leukemia stem cells

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Supplemental Methods

Generation of gene-modified CML-like cell lines

A murine myeloid cell line, 32D cells purchased from the American Type Culture Collection (ATCC), was used to establish gene-modified cells. Cells were transduced with the human BCR/ABL1-*ires* GFP retrovirus. The BCR/ABL-transduced 32D cells were transformed using a lentivirus vector with murine PAI-1 cDNA that was cloned into a pCSII-GFP construct. At 48 hours post-infection, GFP-expressing cells were sorted and plated clonally by limiting dilution. PAI-1-expressing clones were verified by real-time quantitative reverse-transcription PCR (qRT-PCR). For genomic deletions of PAI-1, pairs of chimeric single guide RNAs (sgRNAs)-specifying oligos were selected using online tools available to public (crispr.mit.edu). sgRNA-specifying oligos were phosphorylated, annealed, and cloned into spCas9 plasmids (pX459; Addgene plasmid ID: 48139). Cells were electroporated with the paired pX459-sgRNA plasmids. Transfected cells showing resistance to puromycin (2 μg/mL) were selected and cloned by limiting dilution. Monoallelic and biallelic deletion clones were validated by conventional polymerase chain reaction (PCR) and by Sanger sequencing. The sequence of PCR primers were: PAI-1, 5'-GGGTTCACTTTACCCCTCCG-3' and 5'-TATCGCAGCACCAGAGTCAC-3'. Downregulations of the target gene expression were confirmed by qRT-PCR analyses using appropriate TaqMan primers/probes: PAI-1, Mm00435860_m1, and 18S rRNA, Hs99999901_s1 (Thermo Fisher Scientific). The relative amounts of target genes were determined in reference to 18S rRNA. A comparative threshold cycle (CT) was used to quantify transcripts. The value was calculated by the expression $2^{-\Delta CT}$. All reactions were performed in triplicate.

Trans-Matrigel chemotaxis Assay

BCR/ABL-GFP$^+$Lin$^-$c-kit$^+$ cells (1×10^5) isolated from BM MNCs were seeded onto transwell inserts of 5 μm pore size Transwell chambers (Costar), precoated with a thick layer (60 μg/insert) of trans-reconstituted basement membrane (Matrigel®, BD Bioscience). One hundred ng/mL SDF-1α (Sigma-Aldrich) was added to the bottom well, anti-MT1-MMP neutralizing Ab (50 μg/mL; Merck Millipore), or non-immune species- and isotype-matched control Abs were added to the transwell insert. The number of cells migrated to the bottom well through the Matrigel coating during the 16-hour incubation period was evaluated.

Antibodies

Antibodies used in this study are listed with relevant information in **supplemental Table 1**.

Flow cytometry

Flow cytometry was performed on the FACS LSRFortessa instrument equipped with the FACSDiva software program (BD Bioscience). Data were analyzed with FlowJo® software (Tree Star). The proportion of designated cell fraction was determined by collecting 1,000,000 events. Dead cells stained with propidium iodide (PI) were excluded from data collection. For identification of LSK, cells were stained with a biotinylated lineage cocktail (CD5, CD11b, CD45R, Gr-1, 7-4 and Ter119; Miltenyi Biotec) followed by PerCP-Cy5.5-conjugated streptavidin together with APC-conjugated anti-mouse c-kit (CD117), PE-Cy7-conjugated anti-mouse Sca-1 (Ly6A/E), and FITC-conjugated anti-mouse CD34 (all from Biolegend). The following antibodies (Abs) were used to identify

expressions of TGF-β–iPAI-1 signaling molecules: rabbit anti-mouse MT1-MMP Ab (Merck Millipore), rabbit anti-PAI-1 Ab (Abcam), and rabbit anti-phospho-Smad3 (Ser423/425) Ab (Cell Signaling Technology). For intracellular staining, Cytofix/Cytoperm buffer (BD Biosciences) was used in accordance with manufacturer's instructions. Cells were stained with corresponding isotype-matched Abs to determine the baseline staining for analyses.

Real-time PCR

To examine the expression of BCR/ABL in LSCs of SCLtTAxBCR/ABL double Tg mice, LSK cells were collected from BM using a cell sorter (FACS Melody) and real-time quantitative PCR for BCR/ABL gene was done using a fluorogenic probe designed using the appropriate TaqMan primers/probes (Hs03024541_ft).

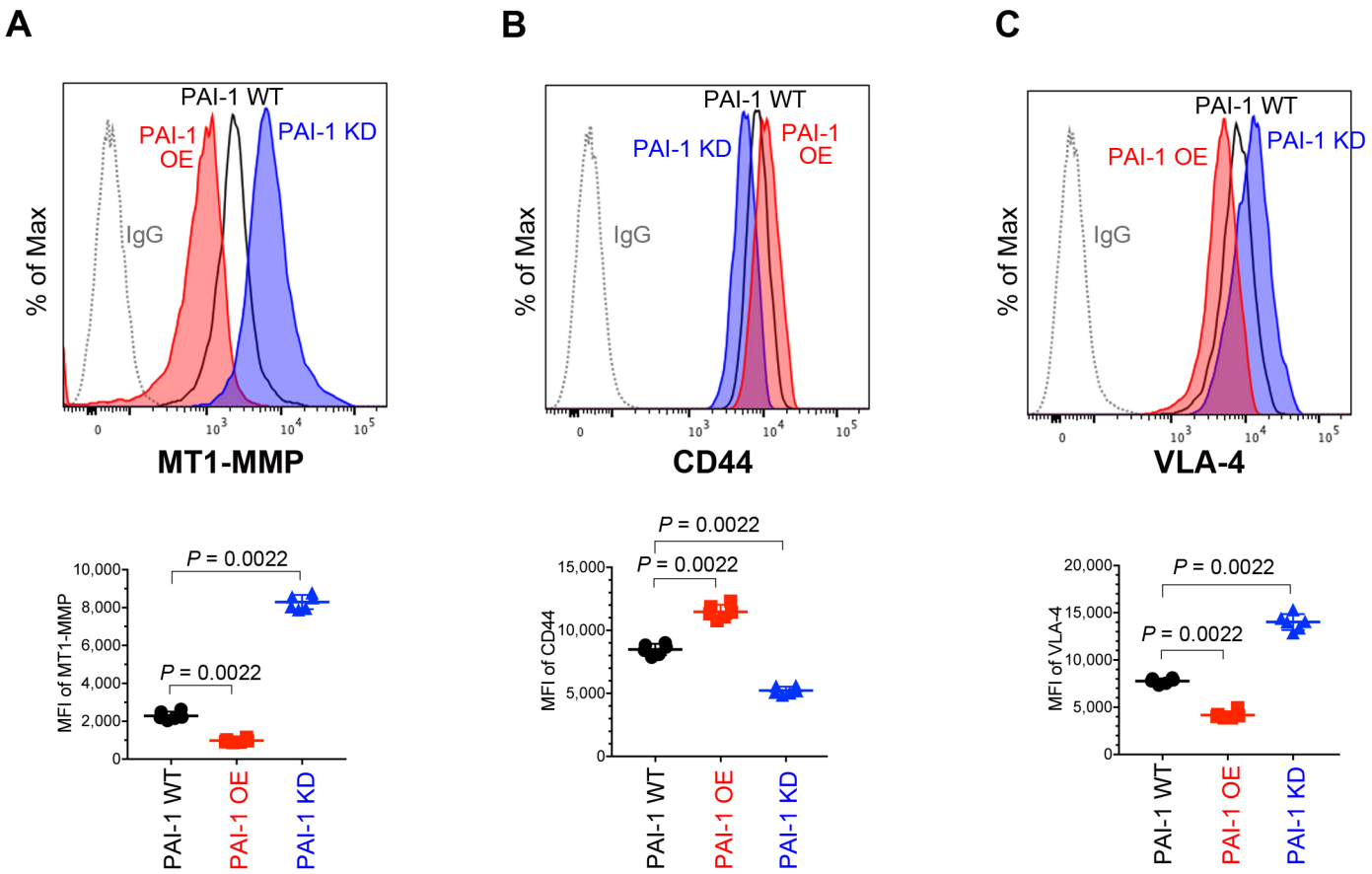
Immunohistochemistry

Isofluorane-anesthetized mice were perfused with 4% paraformaldehyde in PBS through the left ventricle. Femur and tibia were removed, decalcified, embedded in OCT compound, and frozen in liquid nitrogen. The bone sections were stained with rat anti-TGF-β Ab (R&D), followed by co-staining with PE-conjugated anti-rat IgG Ab (BD BioSciences). After washing, the bone sections were further stained with Pacific blue-conjugated anti-CD150 (Biolegend), FITC-conjugated anti-CD41 (eBiosciences), biotin-conjugated lineage cocktail including CD3, B220, Gr-1, Mac-1, Ter-119, and CD48 (Biolegend), and then incubated with Cy5-conjugated avidin for visualization of lineage marker positive cells. Fluorescent images were captured using the HS All-in-one Fluorescence Microscope Biorevo 9000 (Keyence Corporation) and analyzed with the BZ II analyzer software program (Keyence Corporation).

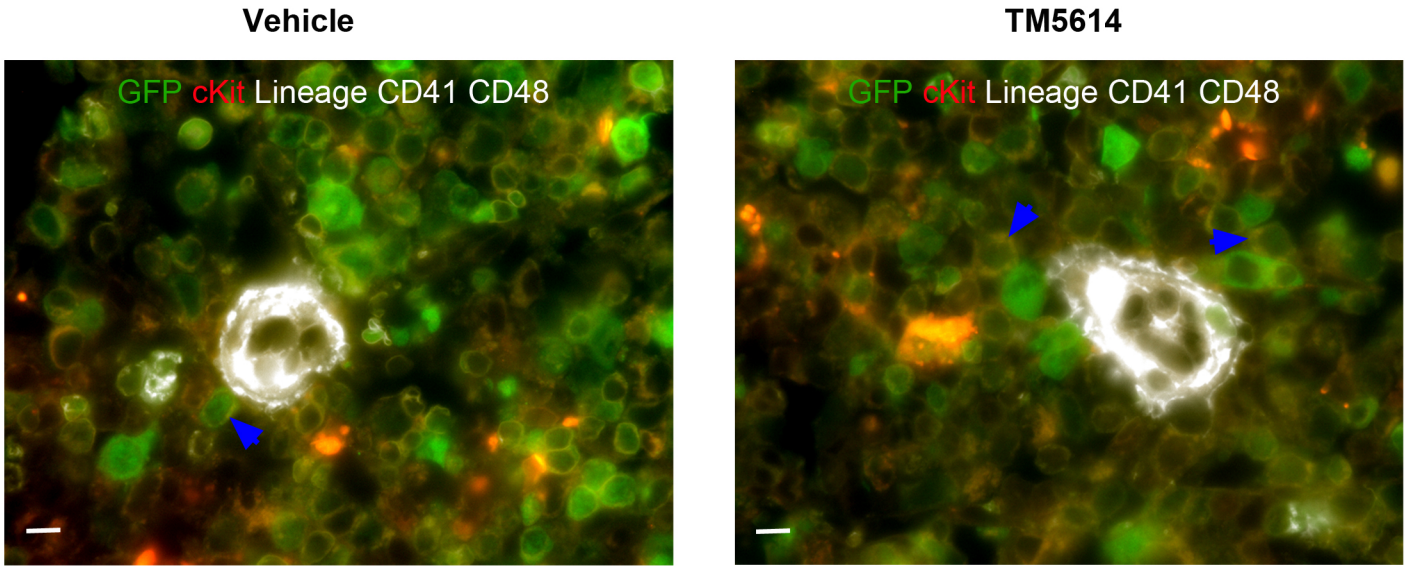
Statistical analyses

All data were pooled from at least three independent experiments. No randomization or blinding was used to allocate experimental groups and no animals were excluded from analysis. All statistical analyses were conducted with GraphPad Prism, version 7.0 (GraphPad Software). Data are expressed as means ± SD of three to five independent experiments. Mann-Whitney unpaired t-test was used to determine the significance of the difference between the means of two groups. Statistical differences were also determined using a log-rank non-parametric test for survival curves. A Kruskal-Wallis test was used to compare the means among three or more independent groups. A value of $P < .05$ was considered significant.

Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure Legends

Supplemental Figure 1. iPAI-1 modulates cellular motility-related molecules.
Representative flow cytometric profiles and MFI (n = 5) for MT1-MMP (A), CD44 (B), and VLA-4 (C) expressions in PAI-1-overexpressed (OE) or PAI-1-knockdown (KD) CML cell lines.

Supplemental Figure 2. iPAI-1 regulates CML-LSCs localization in the BM.
Representative pictures in lower magnification of the BM cavity of vehicle- or TM5614-treated mice. BM sections were stained with anti-c-kit (red) and anti-lineage markers (white) antibodies. Blue arrow heads indicate BCR/ABL-GFP^{+}Lin^{-}c-kit^{+} CML cells. Bars represent 100 μm.

Supplemental Table 1. Antibody List

Primary Antibody	**Clone / Cat. #**	**Manufacturer**	**Application**
Rat anti-mouse CD16/CD23 (Fc block™)	2.4G2 / 553142	BD Pharmingen	FC
Biotin anti-Lineage cocktail	130-090-858	Miltenyi Biotech	FC
APC anti-mouse cKit (CD117)	2B8 / 105812	Biolegend	FC
PE-Cy7 anti-mouse Sca-1 (Ly6A/E)	D7 / 108114	Biolegend	FC
FITC, PE-Cy5 anti-mouse CD34	RAM34 / 11-0341-85	eBioscience	FC
FITC, Alexa Fluor anti-mouse CD48	HM48.1 / 11-0481-82	eBioscience	FC
PE, PE-Cy5 anti-mouse CD150	TC15-12F12.2 / 115912	Biolegend	FC
Rabbit anti-mouse PAI-1	ab28207	Abcam	FC
Rabbit anti-human PAI-1	ab66705	Abcam	FC
Rabbit anti-mouse MT1-MMP	LEM-2.15.8 / MAB3328	Merck Millipore	Blocking
Rabbit anti-MT1-MMP	AB6005	Merck Millipore	FC
FITC, APC anti CD45.1	A20 / 553775	BD Pharmingen	FC
FITC, APC anti CD45.2	104 / 558701	BD Pharmingen	FC
FITC, PE anti Mac-1	M1-70 / 557397	BD Pharmingen	FC
PE-Cy7 anti Gr-1	RB6-8C5 / 553128	BD Pharmingen	FC
FITC anti-CD3e	145-2C11 / 108406	Biolegend	FC
FITC, PE-Cy7 anti B220	RA3-6B2 / 25-0452-81	eBioscience	FC
Rabbit anti-Phospho Smad3 (Ser423/425)	C25A9 / 9520	Cell Signaling Technology	FC
Secondary Antibody	**Clone / Cat. #**	**Manufacturer**	**Application**
PerCP/Cy5.5 Streptavidin	405214	Biolegend	FC
PE, Goat anti-Rat IgG	Poly4054 / 450406	Biolegend	FC
PE, Donkey anti-Rabbit IgG	Poly4064 / 406421	Biolegend	FC
Cy5 Streptavidin	405209	Biolegend	IF
PE, Alexa Flour647 anti-rabbit IgG	Poly4064 / 406421	Biolegend	FC

FC; FlowCytometry, IF; ImmunoFluorescence