Total body irradiation causes long-term mouse BM injury via induction of HSC premature senescence in an Ink4a- and Arf-independent manner

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SUPPLEMENTAL MATERIALS

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Supplemental Methods

Animals. Male C57BL/6J (or CD45.2) mice and B6.SJL-*Ptprc*^{a}*Pep3*^{b}/BoyJ (or CD45.1) mice were purchased from Jackson Lab (Bar Harbor, MA). Breeding pairs of *p16*$^{-/-}$ (*Ink4a* Δexon 1α) mice were provided by Dr. Sharpless (University of North Carolina at Chapel Hill, Chapel Hill, NC), and those of *Arf*$^{-/+}$ (*Ink4a* Δexon 1β) and *p16Arf*$^{-/+}$ (*Ink4a* Δexon 2 & 3) mice were obtained from the NCI Mouse Models of Human Cancers Consortium. All of them were backcrossed to C57BL/6J mice for more than 10 generations before being used in our study. Mice were housed at the Medical University of South Carolina (MUSC) and University of Arkansas for Medical Sciences (UAMS) AAALAC-certified animal facilities. They received food and water *ad libitum*. All mice were used at approximately 8-12 weeks of age. The Institutional Animal Care and Use Committees of MUSC and UAMS approved all experimental procedures used in this study.

Isolation of BM-MNCs, lineage-negative hematopoietic cells (Lin^{-} cells) and HSCs. The femora and tibiae were harvested from mice immediately after they were euthanized with CO_2. BM cells were flushed from the bones into HBSS containing 2% FCS using a 21-gauge needle and syringe. Cells from three to ten mice were pooled and centrifuged through Histopaque 1083 (Sigma, St. Louis, MO) to isolate BM-MNCs. For the isolation of Lin^{-} cells, BM-MNCs were incubated with biotin-conjugated rat antibodies specific for murine CD5, Mac-1, CD45R/B220, Ter-119, and Gr-1. The labeled mature lymphoid and myeloid cells were depleted twice by incubation with goat anti-rat IgG paramagnetic beads (Dynal Inc, Lake Success, NY) at a bead:cell ratio of approximately 4:1. Cells

binding the paramagnetic beads were removed with a magnetic field. The negatively isolated Lin- cells were washed twice with 2% FCS/HBSS and resuspended in complete medium (RPMI1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 10 µM HEPES buffer, and 100 U/ml penicillin and streptomycin) at $1x10^6$ cells/ml. HSCs ($CD150^+CD48^-LSK^+$ cells) were sorted with an Aria II cell sorter (BD Biosciences, San Jose, CA) after Lin^- cells were preincubated with anti-CD16/32 antibody to block the Fcγ receptors and then stained with anti-Sca1-PE, c-Kit-APC-Cy7, CD150-APC and CD48-Pacific blue antibodies. Dead cells were excluded by gating out the cells stained positive with propidium iodide (PI).

Analysis of the frequencies and numbers of different hematopoietic cell populations by flow cytometry. BM-MNCs were preincubated with biotin-conjugated anti-CD3e, anti-CD45R/B220, anti-Gr-1, anti-CD11b, and anti-Ter-119 antibodies and with anti-CD16/32 antibody to block the Fcγ receptors. They were then stained with streptavidin-FITC and anti-Sca1-PE-Cy7, c-Kit-APC-Cy7, CD150-APC, CD48-Pacific blue, CD135-PE and CD34-Alex-700 antibodies. The frequencies of HPCs ($Lin^-Sca1^-c\text{-}kit^+$ cells), LSK cells ($Lin^-Sca1^+c\text{-}kit^+$ cells), MPPs ($CD150^-CD48^-LSK$ cells), HSCs ($CD150^+CD48^-LSK$ cells), ST-HSCs ($CD34^+CD150^+CD48^-LSK$ cells) and LT-HSCs ($CD34^-CD150^+CD48^-$ LSK cells) were analyzed with an Aria II cell sorter. For each sample, approximately 5 x 10^5 to 1 x 10^6 BM-MNCs were acquired and the data were analyzed using BD FACSDiva 6.0 (BD Biosciences) and FlowJo (FlowJo, Ashland, OR) software. The numbers of different hematopoietic cell populations in each mouse were calculated by multipling the

total numbers of BM-MNCs harvested from the two hind legs of each mouse with the frequencies of each population in BM-MNCs.

Cobblestone area forming cell (CAFC) assay. Feeder cell stromal layers were prepared by seeding 10^3/well FBMD-1 stromal cells in each well of flat-bottom 96-well plates (Falcon, Lincoln Park, NJ). One week later, BM cells resuspended in CAFC medium (Iscove's MDM supplemented with 20% horse serum, 10^{-5} M hydrocortisone, 10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin) were overlaid on these stromal layers in 6 dilutions and 3-fold apart. Twenty wells were plated for each dilution to allow limiting dilution analysis of the precursor cells forming hemopoietic clones under the stromal layer. Cultures were fed weekly by changing one-half of the media. The frequencies of CAFC were determined at weekly intervals. Wells were scored positive if at least one phase-dark hematopoietic clone (containing 5 or more cells) was seen. The frequency of CAFC was then calculated by using Poisson statistics as described previously.[1]

Competitive repopulation assay (CRA). BM cells (1×10^6) were pooled from four CD45.2 mice 8 weeks after they were exposed to a sublethal dose (6.0 Gy) of TBI or sham-irradiated. The cells were mixed with 2×10^5 competitive BM cells pooled from three CD45.1 mice and then transplanted into lethally irradiated (9.5 Gy TBI) CD45.1 recipients (6-8 recipients/group) via retro-orbital injection of the venous sinus. Donor cell engraftment in the recipients was analyzed at various times after transplantation as previously described.[2] In addition, we calculated the repopulating unit (RU) of donor

cells according to the original formula (e.g., 1 RU = Testing cells % / Competitors % x (nC)) proposed by Dr. Harrison's group.[3] Specifically, each RU is the relative repopulating ability of 10^5 fresh marrow cells from the same standard competitor pool. Numbers of RU from each testing donor cell population are calculated from the percentage of testing donor cells where the number of competitive donor cells used divided by C (1 C = 10^5 competitors). This formula has been widely used for evaluating the relative repopulating ability of donor cells in a competitive transplantation experiment without serial dilutions of donor cells.[4]

SA-β-gal activity analysis and isolation of SA-β-gal$^+$ and SA-β-gal$^-$ LSK cells for characterization, single cell culture, and transplantation. SA-β-gal activity in different hematopoietic cell populations was measured by flow cytometry using an ImaGene Green^{TM} C_{12}FDG lacZ gene expression kit from Molecular Probes (Carlsbad, CA), according to the manufacturer's instructions and the protocols reported previously with the following modifications.[5] Specifically, Lin$^-$ cells were first stained with antibodies against various cell-surface markers and then incubated with 25 μM chloroquine for 30 minutes at 37°C to induce lysosomal alkalinization and inhibit the basal levels of endogenous β-galactosidase activity in normal hematopoietic cells. After being washed with PBS, they were incubated with a β-galactosidase reaction buffer containing 16μM 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG) for 10 minutes at 37°C. The cells were washed again with PBS and were then analyzed immediately with an Aria II cell sorter. Dead cells were excluded from the assay by PI staining. In addition, SA-β-gal$^+$ and SA-β-gal$^-$ LSK cells were isolated from irradiated mice by cell sorting according to the mean fluorescent intensity of C_{12}FDG staining (Supplemental Figure 2A). LSK

cells from normal unirradiated mice were isolated as controls. SA-β-gal$^+$ and SA-β-gal$^-$ LSK cells were confirmed with the SA-β-gal enzymatic activity assay using a kit from Cell Signaling Technology (Beverly, MA) according to the manufacturer's instructions or with qRT-PCR to measure levels of p16 mRNA. In addition, SA-β-gal$^+$ and SA-β-gal$^-$ LSK cells from irradiated mice and control LSK cells from unirradiated mice were isolated and directly deposited by the cell sorter into wells of a 96-well plate at one cell/well and cultured in complete medium plus 50 ng/ml SCF, thrombopoietin (TPO), FLT3 ligand, G-CSF and GM-CSF, and 20 ng/ml IL-3, 5 U/ml erythropoietin (EPO) for 10 days. The cumulative production of the number of cells in a well confirmed with only single cells was numerated. Alternatively, 500 SA-β-gal$^+$ LSK cells or SA-β-gal$^-$ LSK cells isolated from irradiated mice or 500 control unirradiated LSK cells were transplanted into a lethally irradiated recipient in a way similar to that described above in the competitive repopulation assay.

Apoptosis assay. Lin$^-$ cells were incubated with anti-CD16/32 at 4^{o}C for 15 min to block the Fc-γ receptors and then stained with antibodies against various cell surface markers in the dark. After annexin V staining with a kit from BD Pharmingen (San Diego, CA) according to the manufacturer's instructions, apoptotic cells in different hematopoietic cell populations were analyzed with an Aria II cell sorter.

Single cell PCR analysis of p16 and p21 mRNA expression. Single-cell gene expression analysis was performed using M48 (48.48) Dynamic Array integrated fluidics chips (Fluidigm, South San Francisco, CA) on the Fluidigm's BioMark HD platform. Briefly,

single HSCs were sorted by FACS directly into individual wells of 96-well plates containing 2.5 µl CellsDirect 2× reaction mix (Invitrogen, Grand Island, NY), 0.75 µl nuclease free water, 0.5 µl Superscript III/Platinum Taq mix (Invitrogen), and 1.25 µl 0.2× TaqMan assay mix containing a pool of 1:100 diluted TaqMan assays for p16, p21, and HPRT (Invitrogen). Reverse transcription and specific target amplification were performed in the same plates immediately after sorting as follows: 50 °C for 15 min, 95 °C for 2 min, 22× (95 °C for 15 s and 60 °C for 4 min). cDNA was diluted 1:10 with nuclease free water before quantitative PCR (qPCR) using the BioMark HD system. For qPCR, 3 µl of each TaqMan assay was mixed with 3 µl Gene Expression Assay Loading Reagent (Fluidigm). Then, 2.7 µl of diluted cDNA was mixed with 3 µl 2× TaqMan Universal Mastermix (Invitrogen) and 0.3 µl Gene Expression Sample Loading Reagent (Fluidigm). Next, 5 µl of each sample and assay were loaded into individual sample and assay inlets on the M48 Dynamic Array. Samples and assays were then loaded into the reaction chambers of the Dynamic Array using the IFC Controller MX (Fluidigm), and transferred to the BioMark HD system for qPCR (95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 60 s). We set the detect-limitations of Biomark HD system by excluding the cycle threshold (Ct) values that are less than 7, greater than 27. Basing on Fluidigm recommendation, Ct value 24 is considered as the limit of detection (Lod) of the system and the expression threshold (Et) = Lod-Ct. If $Ct \geq 24$, then Et = 0. Therefore, if a cell has a Ct value < 24 for both the housekeeping gene *HPRT* and a target gene, it is considered as a target gene expressing cell; if a cell has a Ct value < 24 for housekeeping gene (HPRT) but ≥ 24 for a target gene, it is considered as a target gene non-expressing

cell; and if a cell has a Ct value ≥ 24 for housekeeping gene (HPRT), it is considered as no cell in the assay.

Analysis of the levels of intracellular reactive oxygen species (ROS). After stained with appropriate cell surface marker antibodies, Lin^- cells (1×10^6/ml) were suspended in PBS supplemented with 5 mM glucose, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$ and 5 mg/ml BSA and then incubated with 10 μM 2',7'-dichlorofluorescin diacetate (DCFDA, Invitrogen, Carlsbad, CA) for 30 minutes at 37 ^oC. The levels of ROS in various populations of BM hematopoietic cells were analyzed by measuring the mean fluorescence intensity (MFI) of 2',7'-dichlorofluorescein (DCF) with an Aria II cell sorter. For each sample, a minimum of 200,000 Lin^- cells was acquired and the data were analyzed as we previously described.[6]

Telomere length measurements by quantitative PCR (qPCR). Average telomere lengths in T cells, myeloid cells, LSK cells and HSCs were measured by qPCR as previously described.[7] Briefly, genomic DNA was extracted from sorted T cells, myeloid cells, LSK cells, and HSCs with a PureLink™ Genomic DNA Mini kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For telomeric qPCR, each reaction included 12.5 μl Syber Green PCR Master Mix (Applied Biosystems, Foster City, CA), 300 nM each of the forward (5' CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT 3') and reverse (5' GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT 3') telomeric primers, 20 ng genomic DNA, and distilled H_2O to yield a 25-μl reaction. For single copy gene qPCR (e.g., 36B4 qPCR which was used as an

internal control), each reaction consisted of 12.5 μl Syber Green PCR Master Mix, 300 nM forward (5' ACT GGT CTA GGA CCC GAG AAG 3') and 500 nM reverse (5' TCA ATG GTG CCT CTG GAG ATT 3') 36B4 primers, 10 ng genomic DNA, and distilled H_2O to a final volume of 25-μl. The thermal cycling profile for telomeric qPCR was denature at 95°C for 15 s, anneal/extend at 56°C for 60 s, with data collection, 30 cycles; and that for 36B4 qPCR was denature at 95°C for 15 s, anneal at 52°C for 20 s, extend at 72°C for 30 s, with data collection, 35 cycles. All reactions were run in triplicate on an ABI StepOne Plus Real-Time PCR System (Applied Biosystems). The ratio of telomere:36B4 was calculated. The average of these ratios was reported as the average telomere length.

Cell cycle analysis. Lin^- cells were first stained with antibodies against various cell-surface markers and fixed and permeabilized using the Fixation/Permeabilization Solution from BD Biosciences (San Diego, CA). Subsequently, they were stained with anti-Ki67-FITC antibody and 7-AAD and then analyzed by flow cytometer.

BrdU incorporation assay. Lin^- cells were incubated with BrdU (10 μM in 10%FBS-DMEM medium) for 2.5 h. BrdU incorporation in different hematopoietic cell populations was measured by flow cytometry using the FITC BrdU Flow Kit from BD Biosciences (San Diego, CA) according to the protocol provided by the manufacturer after the cells were stained with antibodies against various cell-surface markers.

Statistical analysis. The data were analyzed by analysis of variance (ANOVA). Differences among group means were analyzed by Student-Newman-Keuls multiple comparisons test after one- or two-way ANOVA. For experiments in which only single experimental and control groups were used, group differences were examined by unpaired Student's *t* test. Differences were considered significant at $p < 0.05$. All analyses were done with GraphPad Prism from GraphPad Software.

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Supplemental Tables

Table 1. List of antibodies used to stain various types of hematopoietic cells

Markers	Clone	Antibody isotype	Conjugate
CD45R/B220[1]	RA3-6B2	IgG_{2a}	purified
CD3e[1]	145-2C11	IgG_1	purified
CD11b[1]	M1/70	IgG_{2b}	purified
Gr-1[1]	RB6-8C5	IgG_{2b}	purified
Ter-119[1]	Ter-119	IgG_{2b}	purified
CD45R/B220[1]	RA3-6B2	IgG_{2a}	biotin
CD3e[1]	145-2C11	IgG_1	biotin
CD11b[1]	M1/70	IgG_{2b}	biotin
Gr-1[1]	RB6-8C5	IgG_{2b}	biotin
Ter-119[1]	Ter-119	IgG_{2b}	biotin
CD16/CD32[1]	2.4G2	IgG_{2b}	Purified
CD45.2[1]	104	IgG_{2a}	FITC
CD45R/B220[1]	RA3-6B2	IgG_{2a}	APC
CD45R/B220[1]	RA3-6B2	IgG_{2a}	PE
CD90.2[1]	53-2.1	IgG_{2a}	APC
CD11b[1]	M1/70	IgG_{2a}	PE
Gr-1[1]	RB6-8C5	IgG_{2a}	PE
Streptavidin	Streptavidin		FITC
Sca-1[1]	E13-161.7	IgG_{2a}	PE

Sca-1[1]	E13-161.7	IgG_{2a}	PE-Cy™ 7
CD135[1]	4G8	IgG_1	PE
c-kit[1]	2B8	IgG_{2b}	APC-H7
c-kit[2]	2B8	IgG_{2b}	APC-eFluor® 780
CD150[2]	9D1	IgG_{2a}	APC
CD34[2]	RAM34	IgG_{2a}	Alexa Fluor® 700
Ki-67[2]	20Raj1	IgG_1	FITC
CD48[3]	HM481	IgG_{2a}	Pacific blue

Footnotes: [1]BD Biosciences, San Jose, CA; [2]eBioscience, San Jose, CA; [3]Biolegend, San Diego, CA.

Table 2. Sequences of the primers used for qRT-PCR

Genes	Forward sequences	Reverse sequences
p15	5-AGGCGCCCAATCCAGGTCATGATG-3	5-GAGCTGCGTCGTGCACAGGTCTG-3
p16	5-CGGTCGTACCCCGATTCAG-3	5-GCACCGTAGTTGAGCAGAAGAG-3
p18	5-CTGTCATTCATGATGCTGCCAGAG-3	5-GAACTCCACCACAGGGAGGTGGC-3
Arf	5-TGAGGCTAGAGAGGATCTTGAGAAG-3	5-GTGAACGTTGCCCATCATCATC-3
p21	5-AATCCTGGTGATGTCCGACC-3	5-AAAGTTCCACGGTTCTCGG-3
p27	5-GGTCTCAGGCAAACTCTGAGGAC-3	5-GCGAAGAAGAATCTTCTGCAG-3
p57	5-GTGGCGCTGTGGGACACAAAGCAC-3	5-ACCGGATAGAGCTGTCACCCTTGC-3
HPRT	5-AGCAGTACAGCCCCAAAATGGTTA-3	5-TCAAGGGCATATCCAACAACAAAC-3

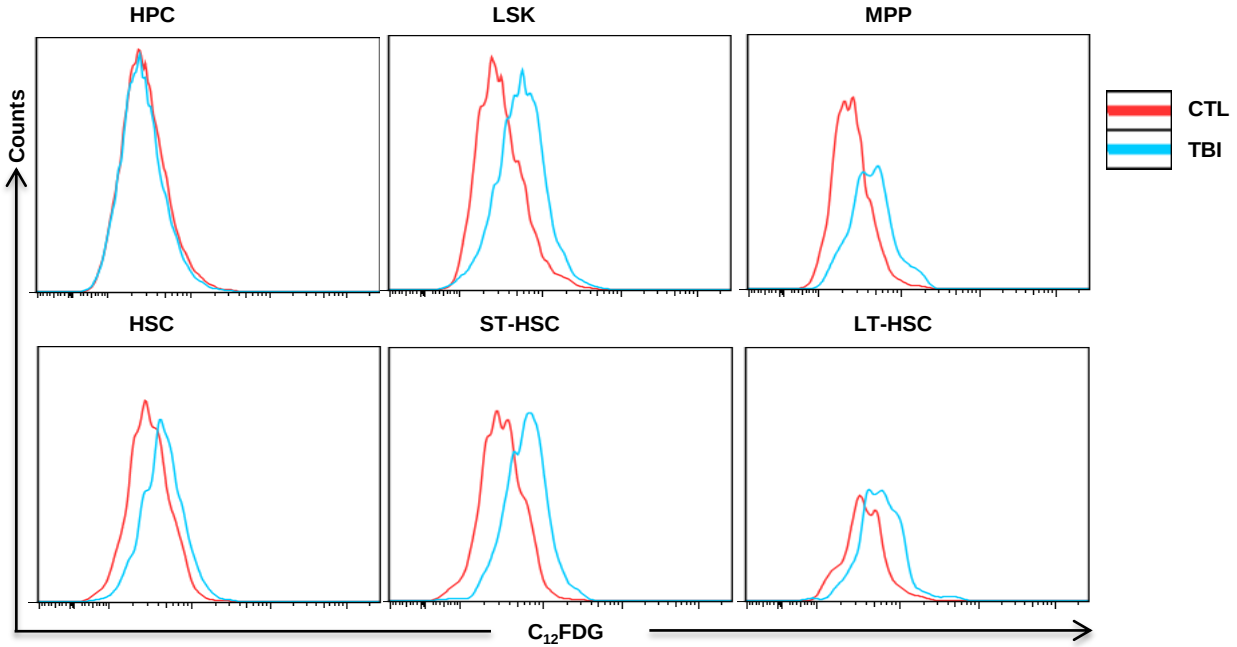

Figure 1. Flow cytometric analysis of SA-β-gal activity. BMCs were harvested from control (CTL) and irradiated (TBI) mice 2 months after 6 Gy TBI as described. Representative flow cytometric analyses of SA-β-gal activity in various populations of BM hematopoietic cells using C_{12}FDG as a substrate are shown.

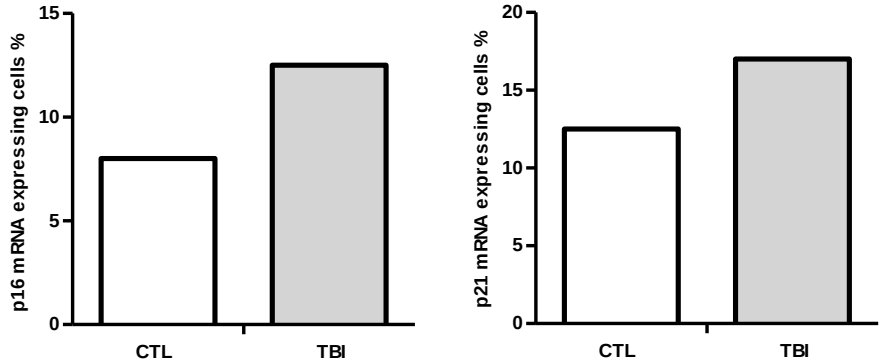

Figure 2. Single cell analysis of p16 and p21 mRNA expression in HSCs. The expression of p16 and p21 mRNA in HSCs from control (CTL) and irradiated (TBI) mice 2 months after 6 Gy TBI was analyzed by single cell PCR using the Fluidigm's BioMark HD system. Percentages of p16 and p21 mRNA expressing cells from a representative assay with 48 individual HSCs isolated from BM hematopoietic cells pooled from 3 to 4 animals are shown.

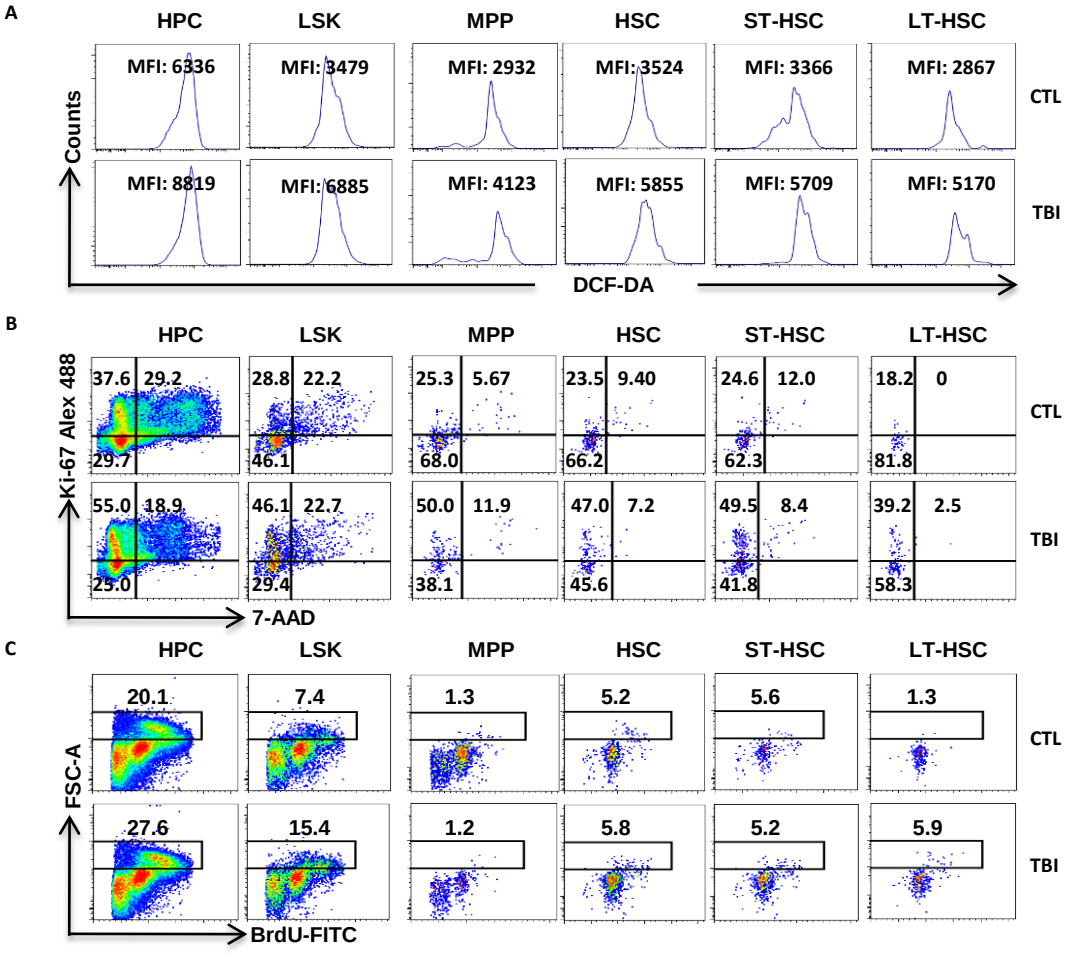

Figure 3. Flow cytometric analysis of ROS production, cell cycle distribution, and BrdU incorporation in various BM hematopoietic cells. C57BL/6 mice were exposed to a sublethal dose (6 Gy) of TBI or were sham irradiated as a control (CTL). Two months after TBI, BM cells (BMCs) were harvested from the two hind legs of individual mice for analysis. (A). Rrepresentative analysis of ROS production by flow cytometry using DCFDA in BM HPCs, LSK cells, MPPs, HSCs, ST-HSCs, and LT-HSCs from control and irradiated mice. The numbers presented in the histograms are DCF MFI from a representative experiment. (B). Representative flow cytometric analyses of cell cycle distribution of BM HPCs, LSK cells, MPPs, HSCs, ST-HSCs, and LT-HSCs from control and irradiated mice after Ki67 immunostaining. The numbers presented in the plots are the percentages of cells in the G_0 (left low quadrant), G_1 (left upper quadrant), and $S/G_2/M$ (right upper quadrant) phases of the cell cycle. (C). Representative flow cytometric analyses of DNA synthesis (or BrdU incorporation) in BM HPCs, LSK cells, MPPs, HSCs, ST-HSCs, and LT-HSCs from control and irradiated mice are shown. The numbers presented in the plots are the percentages of BrdU positive cells in the indicated cell populations.

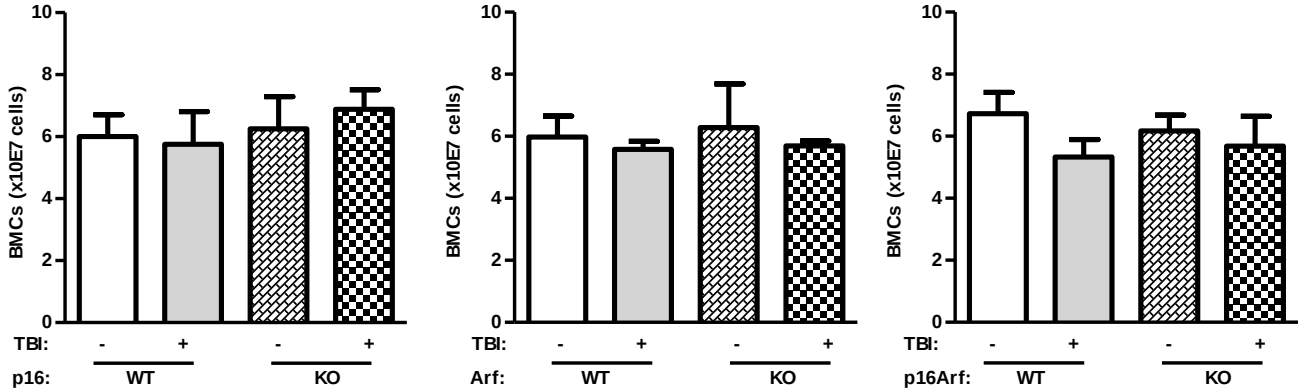

Figure 4. BM cell counts in TBI and control *p16* and/or *Arf* KO and WT mice. *p16*$^{-/-}$ (KO), *Arf*$^{-/-}$ (KO), *p16Arf*$^{-/-}$ (KO), and WT mice were exposed to a sublethal dose (6 Gy) of TBI or were sham irradiated as a control. Two months after TBI, BM cells (BMCs) were harvested from the two hind legs of individual mice and counted. Total numbers of BMCs from each mouse are presented as mean ± SD (CTL: n=4; TBI: n=6).

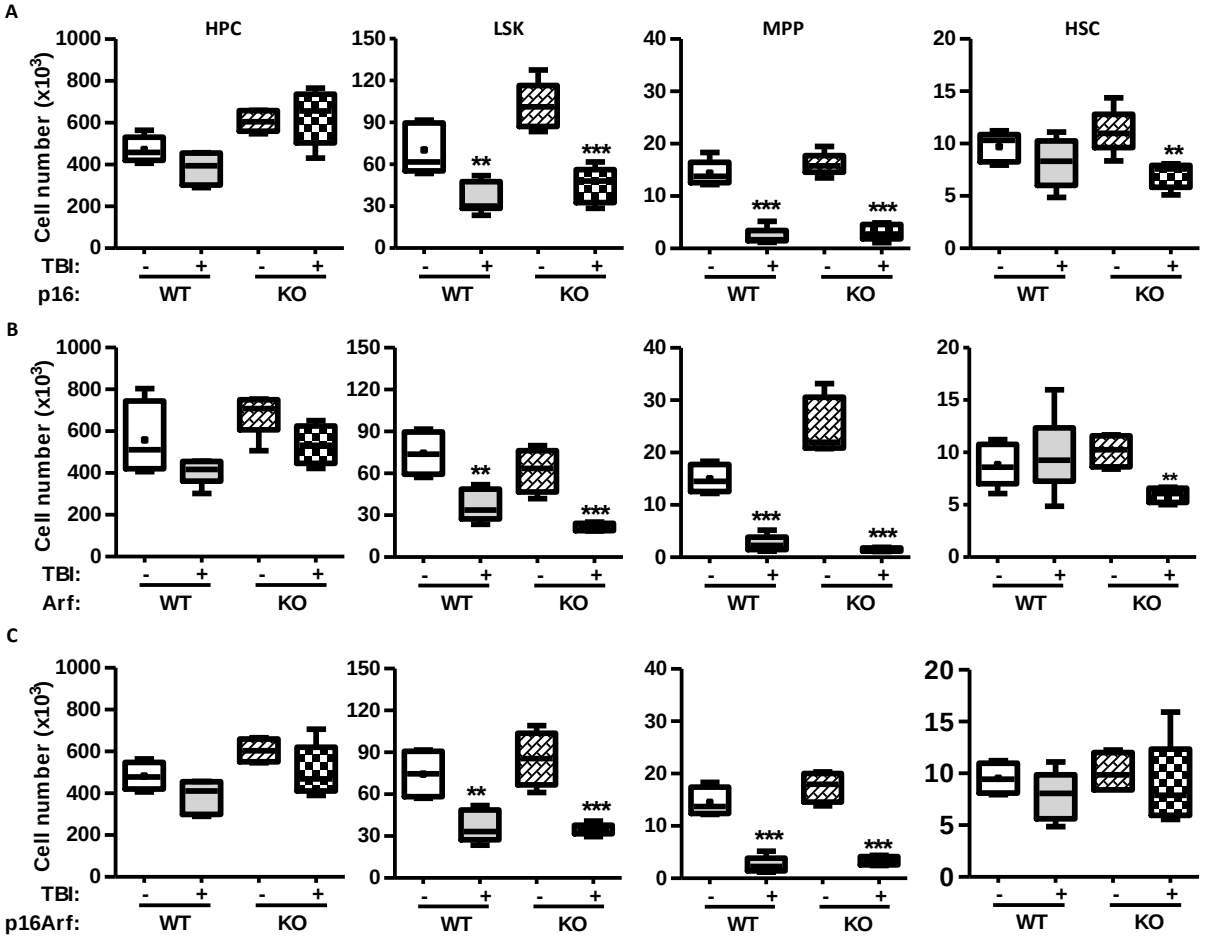

Figure 5. Numbers of various BM hematopoietic cells in *p16* and/or *Arf* KO and WT mice with or without TBI. *p16*$^{-/-}$ (KO), *Arf*$^{-/-}$ (KO), *p16Arf*$^{-/-}$ (KO), and WT mice were exposed to a sublethal dose (6 Gy) of TBI or were sham irradiated as a control. Two months after TBI, BM cells (BMCs) harvested from the two hind legs of individual mice were analyzed by flow cytometry for HPCs, LSK cells, MPPs, and HSCs. (A-C). Total numbers of HPCs, LSK cells, MPPs, and HSCs in BMCs from each mouse are presented as mean ± SD (CTL: n=4; TBI: n=6). **p<0.01 and ***p<0.001, TBI vs. control unirradiated mice.

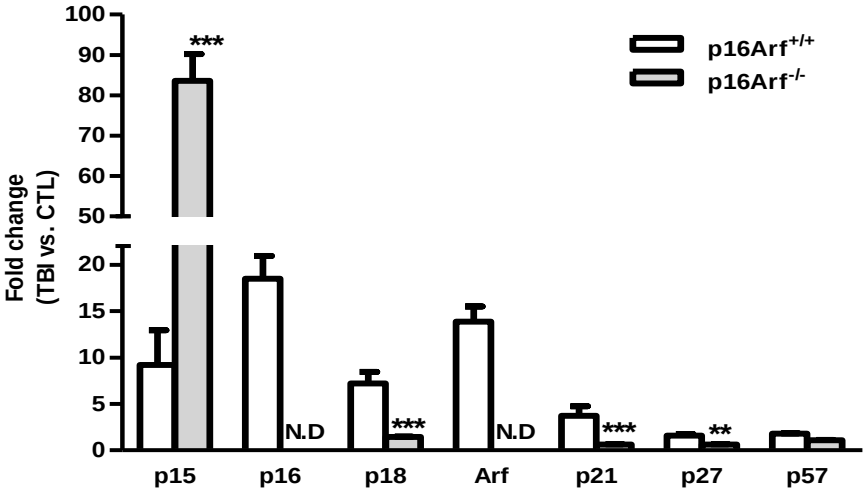

Figure 6. Gene expression for various CDK inhibitors in HSCs from *p16Arf* KO and WT mice with or without TBI. Fold increases in relative gene expression for various CDK inhibitors in sorted HSCs from control (CTL) and irradiated (TBI) *p16Arf*$^{-/-}$ and *p16Arf*$^{+/+}$ mice 2 months after TBI. Data from three independent experiments using sorted HSCs pooled from 3-4 mice per group are presented as mean ± SD (N.D., not detectable). **p<0.01 and ***p<0.001, *p16Arf*$^{-/-}$ mice vs. *p16Arf*$^{+/+}$ mice.