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Supplemental Information

The Distinct Metabolic Profile of Hematopoietic Stem Cells Reflects Their Location in a Hypoxic Niche

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Supplemental Experimental Procedures:

Isolation mouse bone marrow cells:

Mouse bone marrow was extracted from the femurs and tibias of C57BL/6 mice (Jackson Labs) following euthanasia by flushing the marrow space with PBS. Following RBC lysis, the cells were pelleted, washed and re-suspended at 2-3 x 10^6 cells/ml in DMEM media with 10% fetal bovine serum in preparation for flowcytometric analysis.

ABCG2 blocking:

To exclude the possibility that dye efflux plays a role in the mitotracker profile observed, we used Fumitremorgin C (FTC), which is a specific blocker of the ABCG2 transporter. Cells were pretreated with 10 μM FTC prior to staining with mitotracker dyes. The percentage of cells in the low mitotracker gate were compared +/- FTC.

Oxygen consumption:

Oxygen consumption was measured according to the Stern-Volmer method ($I_0 / I = 1 + Ksv * [O_2]$) using the BD Oxygen Biosensor System (BD, CA, USA) according to manufacturer's recommendations. Equal numbers of cells (2-3x10^5 cells/well) were incubated for 2 hours in the provided 96 well plate prior to measurement. Three wells were used for each experimental condition. Culture media lacking cells was used as a negative control and sodium sulfite (100 mM) was used as a positive control. Oxygen consumption is presented as relative units.

Measurement of ATP Concentration:

2-3x10^5 cells/well were used for ATP assays. ATP standard curves were prepared using ATP concentrations between 10^{-6}-10^{-12}M. Then, 50ul of ATP standards and 50ul cell lysates were quantified using ATP Bioluminescence Assay Kit HS II (Roche) using Fluostart Optima plate reader (BMG Labtech). Finally, data were normalized to cell count.

Measurement of ^{13}C Lactate Production:

2-3x10^5 cells/well were cultured in DMEM (Sigma D5030) supplemented with L-glutamine (4 mM), $NaHCO_3$ (42.5 mM), HEPES (25 mM), dialyzed fetal calf serum (10% v/v), Penicillin/Streptomycin and no glucose. The

medium was then supplemented with 10 mM D-[1-^{13}C]-glucose (Cambridge Isotope Labs) to allow up to half of the glucose-derived lactate pool to be labeled on C-3. After the culture, the cells were pelleted and an aliquot of 25 μL of the medium was transferred to a glass test tube with the internal standard (17.9 nmol of Sodium L-[$^{13}C_3$]-Lactate, Cambridge Isotope Labs). Lactate was extracted by sequential addition of 1 mL of methanol, chloroform and water followed by vortexing and centrifugation at 2000 rpm for 5 minutes. The aqueous phase was evaporated, derivatized with 100 μL Tri-Sil reagent (Pierce) for 30 minutes at 42^{o}C, and analyzed by gas chromatography-mass spectrometry. A three-point standard curve was also prepared using mixtures of un-enriched lactate and L-[3-$^{13}C_3$]-lactate (Cambridge Isotope Labs). Lactate abundance was determined by monitoring m/z at 117 (un-enriched), 118 (lactate containing ^{13}C from glucose) and 119 (internal standard). The areas of 117 and 118 were summed and corrected against the 119 area to calculate total lactate abundance. To determine the atom percent excess (APE), the 117 and 118 areas were first corrected against the 119 abundance to account for inter-sample variability of extraction. Then the corrected ratio of 118/(117+118) was determined and compared to the standard curve. Finally, the APE was multiplied by the total nmoles lactate to determine the nmoles of ^{13}C-lactate produced. The final results were corrected for total cellular ATP concentration.

Methocult assay:

Colony forming cell (CFC) assays were performed on mouse cells separated based on their flow cytometric mitochondrial profile. Equal numbers of mouse high and low MP cells (3×10^4 cells) were used in each methocult plate (MethoCult, Stem Cell Technologies, USA). Viability assessment was carried out prior to final cell count and the corrected number of viable cells was used (this is important as the low MP gate can include dying cells in addition to healthy cells with low mitochondrial activity). The tissue culture plates were precoated with 1% agarose and allowed to dry for 10 minutes prior to their use in methocult studies to minimize cell attachment. Twelve days following initiation of culture, the total number of colonies (per plate) was counted and the types of colonies were quantified as follows: BFU-E (erythrocyte), CFU-GM (granulocyte/macrophage) and CFU-GEMM (mixed).

Bone marrow reconstitution:

High and low MP cells were isolated from 8-10 week old C57BL/6 CD45.2 mice. Equal numbers of high and low MP CD45.2 donor cells (5×10^4) were mixed with 1×10^5 freshly isolated CD45.1 competitor bone marrow cells, and the mixture was injected intravenously via the retro-orbital route

into each of a group of 6-9 week old CD45.1 mice previously irradiated with a total dose of 10 Gy. To measure reconstitution of transplanted mice, peripheral blood was collected at the indicated times post-transplant and the presence of $CD45.1^+$ and $CD45.2^+$ cells in lymphoid and myeloid compartments were measured. Briefly, for analyzing repopulation of mouse HSCs, peripheral blood cells of recipient CD45.1 mice were collected by retro-orbital bleeding, followed by lysis of red blood cells and staining with anti-CD45.2-FITC, and anti-CD45.1-PE, and anti-Thy1.2-PE (for T-lymphoid lineage), anti-B220-PE (for B-lymphoid lineage), anti-Mac-1-PE, anti-Gr-1-PE (cells co-staining with anti-Mac-1 and anti-Gr-1 were deemed to be of the myeloid lineage). The "Percent repopulation" shown in all Figures was based on the staining results of anti-CD45.2-FITC and anti-CD45.1-PE. In all cases FACS analysis of the above listed lineages was also performed to confirm multilineage reconstitution.

Real time PCR for *Meis1*, *Hif1–α:*

Total RNA was isolated using TRIzol reagent (Invitrogen). cDNA was generated by following the recommended protocol for SuperScript II Reverse Transcriptase (Invitrogen) using 2ug total RNA. Real time PCR was performed with SyberGreen (Applied Biosystems) on ABI Prism 7700 Sequence Detector (Applied Biosystems) using primers as follows:

M-*Hif-1* α-F AAA CTT CAG ACT CTT TGC TTC G

M-*Hif-1*α-R CGG GCA GAA CGA GAA GAA

M-*Meis1*-F ACG CTT TTT GTG ACG CTT TT

M-*Meis1*-R TCA CAC AGT GGG GAC AAC AG

Hypoxic and anoxic stress:

In order to evaluate susceptibility to hypoxic and anoxic stress, high and low MP cells were cultured in DMEM media (supplemented with 10% FBS and penicillin and streptomycin) under severe hypoxia (1% oxygen for 12 hours) or anoxia (0% oxygen for 12 hours). Viability was assessed immediately using trypan blue. Three separate culture plates were used for each cell type for each experimental condition. Viability was calculated as percent of total cell count.

Western Blot:

Whole protein extracts (15 μg/well) were used in all assays. Primary monoclonal mouse anti Hif1-α antibody (BD Transduction Laboratories #610958) was used at 1:1000 dilution (overnight incubation 4°C). Secondary anti- mouse IgG HRP linked antibody (Cell Signaling Technology #7076) was used at 1:2000 dilution (3 hours incubation at

4°C). Relative Optical Density analysis was carried out using Adobe Photoshop software (corrected for beta actin).

Generation of *Hif-1α* Reporter vectors

An 818 bp long DNA fragment from the first intronic region of *Hif1-α* containing the conserved Meis1 binding site (+1799 bp from ATG start site, chr14:61,232,973-61,233,790) was amplified from mouse genomic DNA using 5`-CGTGCGGGTTTGGTTGTAATCT-3` and 5`-GCCCATCATTTCATCAAAAGCA-3` primers and subcloned into pCR2.1-TOPO vector (Invitrogen). Both the TOPO vector with PCR fragment and pGL2-Elb vector were digested with XhoI and KpnI. Then the PCR fragment was cloned into Elb-pGL2 to generate Hif1-α-pGL2 luciferase reporter vector. To test Meis1 site specificity, the Meis1 binding site (TGAC) was mutated using QuikChange® II Site-Directed Mutagenesis Kit (Stratagene) from Hif1-α-pGL2 vector with the following primers: Sense: 5'-CAT AAC GTCTGGGCTCTCCTAAAAGGTGATGCAAACACA A-3' and Antisense: 5'-TTGTGTTTGCATCACCTTTTAGGAGAGCCCAGACGTTATG-3'and mut-TGAC-Hif1-α-pGL2 was generated.

Luciferase reporter assays:

Transcriptional activation of *Hif-1α* by Meis1 was evaluated using Hif-1α-pGL2 and del-TGAC-Hif1-α-pGL2 vectors. 0.8 μg of Hif1-α-pGL2 was co-transfected with 50ng, 100ng, 200ng and 400ng of the *Meis1* expression vector pCMV-SPORT6-Meis1 (OpenBiosystems) and 0.2 μg of pCMV-LacZ (internal control) into COS cells using lipofectamine transfection reagent (Invitrogen). At 48 h after transfection, cell lysate was prepared and quantified for firefly luciferase activity using a luciferase reporter system (Promega). Luciferase measurements were calculated as firefly luciferase units versus β-gal units. Transcriptional activation was compared to basal luciferase levels in cells transfected with Hif1-α-pGL2 and empty pGL2-Elb.

Meis1 Knockdown:

LT-HSCs were incubated in HSC media as previously described (Zhang et al., 2006; Zhang et al., 2008) during siRNA knockdown for 20 hours.

siRNA sequence:

Sense sequence (5`->3`): GAUUUGGUGAUAGACGAUAtt

Antisense sequence (5`->3`): UAUCGUCUAUCACCAAAUCga

Chromatin immunoprecipitation assay (ChIP)

Chip assays were performed to evaluate the in vivo binding of Meis1 to its consensus sequence in the *Hif-1*α gene. The assays were done using the ChIP kit (Upstate, cat#17-295) according to the manufacturer's instructions. 1.5×10^6 Kasumi-1 cells were crosslinked in 1% formaldehyde for 10 min at 37^{o}C. After two washes with PBS, cells were spun down for 4 min at 2000g. Then, cell pellet were lysed in 200 µl of SDS Lysis Buffer with 1mM PMSF (Sigma P7626) and 1X Protease inhibitor cocktail (Roche, 11836170001). Sonication was carried out in 1.5 ml tubes with the settings; 5 s on, 5 s off and repeated for 4 times on ice (550 Sonic Dismembrator, Fisher Scientific). The resulting DNA lengths were 200–1000 bp in length. 200 µl of sonicated sample was used for each assay. Meis1 antibody (Santa Cruz Biotechnology, sc-10599) and normal goat IgG (Santa Cruz Biotechnology, sc-2028) were used. The DNA isolated from input chromatin fragments and from the precipitated chromatin fragments by anti-Meis1 antibody or control IgG was subjected to real time PCR using primers flanking the consensus Meis1 binding site (TGAC): 5'-TTGAAAAACTTGCCTAGACTGAGAGT-3' and 5'-CAGTATCATTAAAACTGCCTAAATTT-3'.

Animal Care

All experiments using animals were previously approved by the Institutional Animal Care and Use Committee at UT Southwestern Medical Center.

Supplemental References:

Zhang, C.C., Kaba, M., Ge, G., Xie, K., Tong, W., Hug, C., and Lodish, H.F. (2006). Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells. Nature Medicine *12*, 240-245.

Zhang, C.C., Kaba, M., Iizuka, S., Huynh, H., and Lodish, H.F. (2008). Angiopoietin-like 5 and IGFBP2 stimulate ex vivo expansion of human cord blood hematopoietic stem cells as assayed by NOD/SCID transplantation. Blood *111*, 3415-3423.

Figure S1

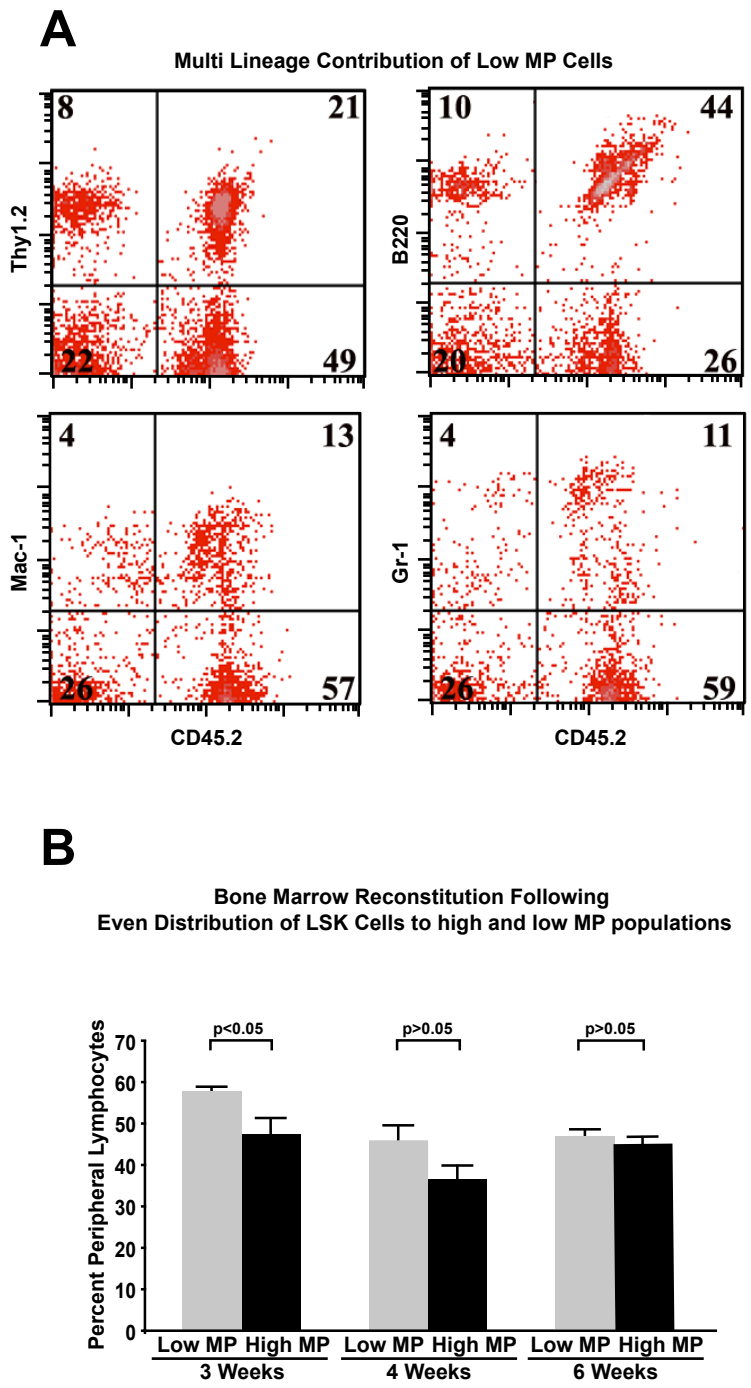

Figure S1 (Related to FIgure 4): A) Flowcytometry plots of peripheral blood mononuclear cells to demonstrate the multi-lineage contributions of HSCs in the low MP population in a mouse at 4-months post-transplant. **B)** Bone marrow reconstitution using high and low MP cells following depletion of LSK cells and equal distribution of LSK cells amongst high and low MP populations. Note the lack of significant difference in bone marrow reconstitution between the two populations following equal distribution of LSK cells. (n=3). Data presented as mean +/- SEM.

Figure S2:

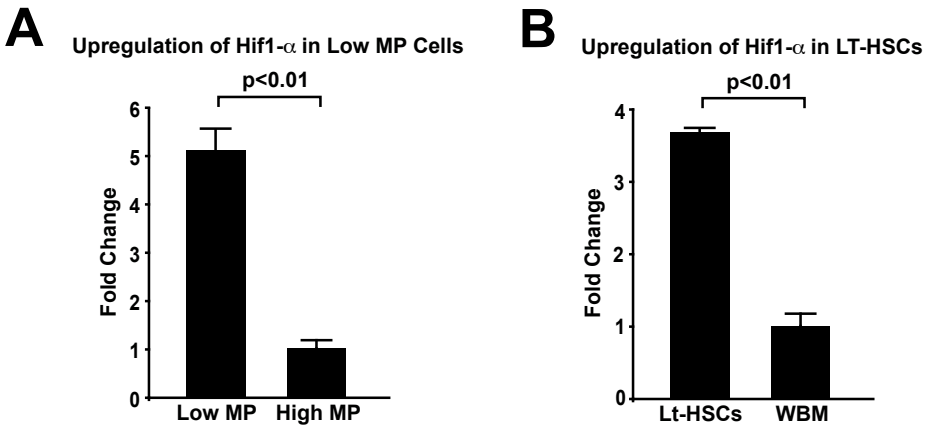

Figure S2 (Related to Figures 3 and 5): Real Time PCR demonstrating: **A)** Upregulation of Hif-1α in low MP cells, and **B)** Upregulation of Hif1-α in LT-HSCs cells compared to whole bone marrow. (n=3). Data presented as mean +/- SEM.