Latexin regulation by HMGB2 is required for hematopoietic stem cell maintenance

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MATERIALS AND METHODS

Animals

Young 8- to 12-week old female C57BL/6, DBA2, 129X1/SvJ, A/J and CD45.1 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were kept in the animal facilities of the University of Kentucky under pathogen-free conditions according to NIH-mandated guidelines for animal welfare. They were fed with acidified water and food *ad libitum*.

Cell culture

293TA and NIH 3T3 cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS), 80 U/mL penicillin, and 80 mg/mL streptomycin. EML cells was cultured in Iscove's Modified Dulbecco Medium (IMDM, Stemcell technology) supplemented with 20% FBS (Stemcell technology) and 200 ng/mL murine stem cell factor (Peprotech). These cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. FBMD-1 cells were maintained in DMEM with 20% horse serum (HS), 0.1 mM 2-mecaptoethanol, 10 mg/mL hydrocortisone (HC, Sigma), 80 U/mL penicillin, and 80 mg/mL streptomycin, in a 33°C incubator with a humidified atmosphere of 5% CO_2. All cell lines used in this study were purchased from American Type Culture Collection.

Preparation and isolation of stem/progenitor-enriched hematopoietic cells

Bone marrow (BM) cells were harvested after crunching the bilateral iliac, femora and tibia and used for isolation of primitive HSCs. The Lin- cells was sorted using MACS mouse Lineage Cell Depletion Kit (Cat# 130-090-858) following the instructions. The Lin- cells were labelled with lineage antibodies including CD5 (clone 53–7.3), CD8a (clone 53–6.7), CD45R/B220 (clone RA3-6B2), CD11b/MAC-1 (clone M1/70), LY-6G/GR-1 (clone RB6-8C5), and TER119/Ly-76 (clone TER-119)-APC-Cy7, and stem cell markers, Sca-1-PE (clone E13-161.7) and c-KIT-APC (clone 2B8). The viable cells were distinguished by their ability to exclude 7-AAD. All monoclonal antibodies were purchased from BD Pharmingen (San Diego, CA). The LSK (Lin- Sca-1+ c-KIT+) cell sorting and flow cytometry analysis were performed on FACS LSRII (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Generation of lentiviral vectors

The mouse *Lxn* promoter sequence was isolated from the genomic DNA of Lin- cells of C57/BL6 strains by PCR using the following primers: forward (from -341 to -327nt): 5'-TCGACTCGAGTCCAGGTTCTCCCTC-3', and reverse (from +13 to +27nt): 5'-

AGCCAAGCTTCAGCATAGTGGGTGG-3’. The PCR products were digested with XhoI and HindIII and cloned into the pGL3-basic vector (Promega, Madison, WI). The G in pGL-3-Lxn promoter vector was mutated to C using a QuickChangeII sited-directed mutagenesis kit (Stratagene, La Jolla, CA) following manufacturer’s instructions. Hmgb2 shRNA plasmids were purchased from GeneCopoeia (Catlog# MSH027321-LVRU6GP). *Lxn* shRNA plasmids were purchased from Sigma-Aldrich (Lxn Mission shRNA). Crimson fluorescence marker replaced the puromycin resistance gene and was used for fluorescent cell sorting. Negative control was the scramble shRNA. Scramble sequence was amplified from mouse Lxn CDS zone using the following primers: forward 5’ TCGACTCGAGTGGCGGAGAACTGCATT, and reverse 5’ AGCCAAGCTTTAAGTGCTGGACTGGCTT , and digested with XhoI and HindIII and cloned into the PGL3-basic vector. HshRNA and LshRNA plasmids are both lentivirus in this study. The lentiviral vectors were packaged using 293TA cells by co-transfection with ∆8.9 and VSVG plasmids through calcium-phosphate precipitation. After culturing the respective cell lines for 48 h and 72 h, virus supernatant was collected and viral titers were determined.

Luciferase reporter assay

Human embryonic kidney (HEK) cells were transfected with 290ng of luciferase reporter constructs including pGL-3-*Lxn* promoter vector (G Lxn-PGL3) and the vector containing SNP rs31528793 (C Lxn-PGL3), together with 10ng of pRL-null (thymidine kinase promoter-Renilla luciferase) plasmid using FuGene® 6 (Roche, Indianapolis, IN). To test HMGB2 and H2A.X activity, cells were contransfected with pcDNA-HMGB2 and pcDNA-H2A.X plasmids (10ng, 50ng, 100ng, 200ng or 500ng). Cell lysate was made 48 h later and the luciferase activities were measured with a Tropix TR717 luminometer using a dual luciferase assay kit and were normalized to Renilla luciferase values. Except as indicated, all vectors and assay kit were purchased from Promega (Madison MI). All assays were performed 2 to 3 times with 4 to 6 replicates in each time.

Microbeads-magnetic extraction of *Lxn* promoter binding proteins

Lxn promoter binding proteins were isolated by µMACSTM FactorFinder Kit (Miltenyi Biotec Inc. Auburn, CA). In brief, a high purity double-strand DNA oligonucleotides containing SNP rs31528793 (5’ GGTAGGCGGGCACCTCCCG(C)GAGGAAAGCTC 3’) was modified with biotin and incubated with the bone marrow cell lysate. µMACSTM streptavidin microbeads, which bind to biotinylated DNA with extremely high affinity, were added to capture the DNA (oligoes)-protein complex. The associated proteins were separated by MACS® µ Columns in

which MicroBeads-attached proteins were retained on the strong magnetic field of column. The associated proteins were eluted and analyzed by gel electrophoresis and visualized by silver staining (SilverSNAP Stain Kit II, PIERCE, Rockford, IL).

Identification of binding proteins by mass spectrometry

To identify components of eluted proteins, mass spectrometry was performed on the samples obtained above at the Mass Spectrometry & Proteomics Facility at Ohio State University. Briefly, proteins were digested by trypsin or chymotrypsin. Capillary-liquid chromatography-nanospray tandem mass spectrometry (Nano-LC/MS/MS) was performed on a Thermo Finnigan LTQ mass spectrometer equipped with a nanospray source operated in positive ion mode. The LC system is an UltiMateTM Plus system from LC-Packings A Dionex CO (Sunnyvale, CA) with a Famous autosampler and Switchos column switcher. 5 ul of each sample was first injected on to the trapping column (LC-Packings A Dionex Co, Sunnyvale, CA), and washed with 50 mM acetic acid. The peptides were eluted off the trap onto the column and then into the LTQ system using a gradient of 2-80% acetonitrile over 50 minutes, with a flow rate of 300 nl/min. The MS/MS was acquired by using a nanospray source operated with a spray voltage of 3 KV and a capillary temperature of 200°C. The analysis was programmed for a full scan recorder between 350-2000 Da, and a MS/MS scan to generate product ion spectra to determine amino acid sequence in consecutive instrument scans of the ten most abundant peaks in the spectrum. Sequence information from the MS/MS data was processed by converting the raw data files into a merged file. The resulting mgf files were searched using Mascot Daemon by Matrix Science Version 2.2.1 (Boston, MA) and the database searched against the full SwissProt database version 54.1 (283454 sequences; 104030551 residues). Proteins with a Mascot score of 100 or higher with a minimum of two unique peptides from one protein having a –b or –y ion sequence tag of five residues or better were accepted.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays kit (Sigma Aldrich, #CHP1) was used for this purpose. FACS sorted LK (Lin- c-KIT+) cells cross-linked with 1% formaldehyde were lysed and sonicated. The protein-DNA complex was immunoprecipitated with HMGB2 polyclonal antibodies (Abcam, Catalog# ab67282) or H2A.X antibody (Abcam, Catalog# ab11175). PCR specific for amplifying *Lxn* promoter region or for 500 base pairs downstream of the promoter region was performed on the DNA recovered after reverse cross-linking. The primers for amplifying the *Lxn* promoter

sequence are 5' GGAATGCCCGAGCTTTTCT 3' (forward) and 5' TCCTTCCCTCTTCCTCCTTCA 3' (reverse). The primers for control downstream 500 base pairs are 5' GAGGCAGAGGACTTGAGTTTGGT 3' (forward) and 5' GCTTAATGAGCGTGAACCACC 3' (reverse). Immunoprecipitation using HMGB2 or H2A.X antibody related normal Rabbit IgG (Abcam, Catalog# ab171870) and amplification using the primer for downstream 500 base pairs serve as negative control. SYBR green was used for the quantitative real-time PCR.

Infection of EML cell line and measurement of growth of virally-transduced cells

EML cells were transduced with HMGB2 shRNA or control (Con) viral supernatant supplemented with 200 ng/mL murine stem cell factor (Peprotech) and 8 μg/ml of polybrene for 6 hours. Fresh culture medium replaced viral supernatant and the cells were recovered for another 42 hours. The infected (GFP+) cells were sorted and cultured for 12 days. GFP+ EML cells were counted on a hemacytometer using trypan blue dye exclusion and 100,000 cells were seeded into 24-well non-tissue culture plates (Costar). The cell numbers were subsequently measured at different time-points for 12 days. At each time point, cells were split and maintained at a concentration of 100,000 cells per well. The cumulative cell number was calculated from the cell counts and the dilutions made at each culture split. *Lxn* knockdown vector transduction, Crimson+ cell sorting and cell growth measurement were performed similarly as described above.

Quantitative real-time PCR

To measure the expression of *Hmgb2* and *Lxn* mRNA in virally transduced cells, quantitative real-time PCR was performed. Total RNA was extracted from EML cells or primary bone marrow cells using RNeasy Mini kit (QIAGEN) according to the manufacture's protocol. cDNA was synthesized from total RNA by MultiScribe reverse transcriptase (Applied Biosystems) following the manufacturer's instruction with random primers (Applied Biosystems). In real-time PCR reactions, primer and probe mixes for mice *Hmgb2* and *Lxn* were purchased from Applied Biosystems. TaqMan rodent glyceraldehyde-3-phosphate dehydrogenase (Gapdh) served as an endogenous control for normalization of *Hmgb2* and *Lxn* expression. The PCR amplification was carried out on an ABI PRISM 7500™ real-time PCR machine using the TaqMan reagent standard curve method.

Western blot

Total protein extraction was performed as previously reported[17]. For Western blot, protein lysates were thawed and mixed with running buffer and a reducing agent (Novex, LifeTechnologies) according to the manufacturer's instructions and heated at 95°C for 5 minutes. Samples were then separated on a denaturing 10% bis-Tris PAGE gel (Novex) using the equivalent of 1×10^5 cells per lane at 200 V for 35mins. Following electrophoresis, samples were then transferred onto a PVDF membrane (Millipore) by electro-blotting at 30 V for 1 h, which were subsequently blocked in 5% skimmed milk and probed with rabbit monoclonal anti-Hmgb2 antibody (Abcam, Catalog# ab67282), goat polyclonal anti-Lxn antibodies (Abcam, Catalog# ab59521), and mouse monoclonal anti-β-actin antibody (Sigma, Catalog# A5441), respectively. Primary antibodies were detected using HRP-linked secondary antibodies (Cell Signaling Technology) and Chemiluminescen reagent (Pharmacia Biotech) according to the manufacturer's instructions.

Infection of primary bone marrow cells

Flow cytometry sorted LSK cells from C57/BL6 mice were stimulated with cytokines including 100 ng/mL FMS-like tyrosine kinase-3 ligand, 50 ng/mL mouse stem cell factor, 20 ng/mL interleukin-3 (IL-3), and 20 ng/mL TPO in StemSpan SFEM (STEMCELL Technologies). After 24 hr, the cells were transduced with lentiviral particles encoding either HMGB2 shRNA, or its related scramble control vector, at an MOI of 100 along with 8 µg/ml of polybrene for 6 hr. After 48 hr, the GFP-positive cells were sorted for real-time PCR, western blotting, CAFC assay. In transplantation assay, 3×10^5transduced cells (GFP+ cells for both groups) plus 2×10^5 competitor B6.SJL/BoyJ BM cells were injected into B6.SJL/BoyJ mice after 24 hurs of transduction, and GFP+ chimerism in PB and BM was measured at 16 weeks post transplantation.

Colony Forming Cell (CFC) assay

CFC assay was performed in MethoCult™ GF M3434 medium (STEMCELL Technologies, Catalog # 03434) according to the manufacturer's protocol. Briefly, the sorted GFP+ bone marrow cells infected with HMGB2 shRNA lentivirus or control virus were seeded into 35 mm culture dishes, and colony formation was observed on days 12.

Cobblestone area forming cell (CAFC) assay

The CAFC assay was carried out as described previously[17]. Briefly, a confluent monolayer of FBMD-1 stromal cells was established in 96-well tissue culture plates (Costar). The sorted

GFP+ bone marrow cells infected with HMGB2 shRNA lentivirus or control virus were seeded on FBMD-1 containing plates at (3-fold) decreasing numbers. Twenty replicate wells were evaluated for each cell number. Individual wells were screened at day 35 for the presence of a cobblestone area, defined as a colony of at least five small, non-refractile cells growing underneath the stromal layer. The most primitive HSCs show cobblestones at day 35. Frequencies of CAFCs were calculated by using L-CaLc Limiting Dilution Analysis Software (Stem Cell Technology).

Cell cycle and apoptosis analysis

Cell cycle was analyzed by BrdU incorporation on sorted transduced GFP+ cells (after HMGB2 shRNA and control vector lentivirus transduction) or GFP+Crimson+ cells (after HMGB2 shRNA and Lxn shRNA with related control vector lentivirus transduction following the protocol provided by BD Pharmingen™ BrdU Flow Kit. Apoptosis was evaluated by flow cytometric determination of Annexin V (BD Pharmingen™) binding on transduced GFP+ EML cells or GFP+ primary LSK cells, or transduced GFP+ Crimson+ EML cells. For live cell isolation purpose, we used 7-aminoactinomycin D(Invitrogen) for dead cell exclusion. For active caspase 3 analysis, sorted transduced GFP+ EML or LSK cells (after HMGB2 shRNA and control vector lentivirus transduction) was analyzed using PE Active Caspase-3 Apoptosis Kit (BD Pharmingen™) following the instructions from the company.

Electrophoretic mobility shift assay

EMSAs were performed using the LightShift™ Chemiluminescent EMSA Kit (Thermo ScientificTM). Briefly, Hmgb2 lentiviral plasmid was transfected into 293T cells using calcium phosphate method. After 48 hs, 293T cells nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher Scientific) according to the manufacturer's protocol. EMSA binding reactions included 1× binding buffer, 1μg sperm DNA, 3 μl nuclear extract, and 20 uM biotin-labelled probes. One binding reaction without nuclear extract was as negative control. For competition assays, 4pM unlabeled oligonucleotides were allowed to bind the nuclear extracts (10 min at room temperature) before the addition of labeled probes. Binding reactions were incubated for 20 min at room temperature, size-separated on a 6% DNA retardation gel (ThermoFisher Scientific) in 0.5X TBE buffer, and transferred to a Biodyne B Nylon membrane (ThermoFisher Scientific) in 0.5xTBE buffer at 380mA for 45 minutes at 4 °C, and then crosslinked to the membrane using a TL-2000 UV Translinker (Ultra-Violet Products).Free or protein-bound biotin-labelled probes were detected

using streptavidin–horseradish peroxidase conjugates and chemiluminescent substrate according to the manufacturer's instructions. Probe sequences for promoter regions are listed below. (1) G 5' biotin-GGTAGGCGGGCACCTCCCGGAGGAAAGCTC (2) C 5' biotin - GGTAGGCGGGCACCTCCCCGAGGAAAGCTC

Statistical analysis

Data were examined for homogeneity of variances (F-test), then analyzed by student's t-test or One-way ANOVA using Tukey's test. Differences were considered significant at $P<0.05$. All statistical analyses were conducted with Graphpad Prism7.