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Latexin regulation by HMGB2 is required for hematopoietic stem cell maintenance

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Ferrata Storti Foundation

Haematologica 2020
Volume 105(3):573-584

ABSTRACT

Hematopoietic stem cells provide life-long production of blood cells and undergo self-renewal division in order to sustain the stem cell pool. Homeostatic maintenance of hematopoietic stem cell pool and blood cell production is vital for the organism to survive. We previously reported that latexin is a negative regulator of hematopoietic stem cells in mice. Its natural variation in the expression is inversely correlated with hematopoietic stem cell number. However, the molecular mechanisms regulating latexin transcription remain largely unknown, and the genetic factors contributing to its natural variation are not clearly defined. Here we discovered a chromatin protein, high-mobility group protein B2, as a novel transcriptional suppressor of latexin by using DNA pull-down and mass spectrometry. High-mobility group protein B2 knockdown increases latexin expression at transcript and protein levels, and decreases hematopoietic stem cell number and regeneration capacity *in vivo*. Concomitant blockage of latexin activation significantly reverses these phenotypic changes, suggesting that latexin is one of the downstream targets and functional mediators of high-mobility group protein B2. We further identified a functional single nucleotide polymorphism, rs31528793, in the latexin promoter that binds to high-mobility group protein B2 and affects the promoter activity. G allelic variation in rs31528793 associates with the higher latexin expression and lower hematopoietic stem cell number, whereas C allele indicates the lower latexin expression and higher stem cell number. This study reveals for the first time that latexin transcription is regulated by both trans-acting (high-mobility group protein B2) and cis-acting (single nucleotide polymorphism rs31528793) factors. It uncovers the functional role of naturally occurring genetic variants, in combination with epigenetic regulator, in determining differential gene expression and phenotypic diversity in the hematopoietic stem cell population.

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Received: September 17, 2018.

Accepted: June 5, 2019.

Pre-published: June 6, 2019.

doi:10.3324/haematol.2018.207092

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/105/3/573

Introduction

Stem cells are key to the homeostatic maintenance of mature and functional cells in a variety of tissues and organs. They have the unique ability to perpetuate themselves through self-renewal and to replenish dying or damaged cells through multilineage differentiation. The balance between self-renewal and differentiation is critical for tissue homeostasis, and any disruption in this balance could lead to serious problems such as tissue degeneration and development of cancer.[1] Probably the best-studied adult stem cells are hematopoietic stem cells (HSC), which are responsible for life-long production of all hematopoietic lineages.[2-4] The total number of HSC is kept constant under steady-state conditions, but it also changes in response

to stress or injury. The flexibility of stem cells to adapt to physiological needs is achieved by precise regulation of self-renewal and differentiation. Many molecules and signaling pathways have been found to be involved in this process.[3] However, identification of the collection of genes contributing to critical stem cell functions is far from complete.

Hematopoietic stem cell number and function exhibit natural variation among humans as well as among different mouse strains.[5-8] The natural variation is largely attributed to DNA variants in the genome that function as regulatory elements to control gene expression.[9] The genetic diversity is a powerful but underused tool for unraveling the critical gene networks in stem cell regulation. Using genome-wide association studies, increasing numbers of gene regulatory variants have been strongly implicated in hematologic phenotypes and diseases in humans, such as fetal hemoglobin-associated genetic variants in patients with sickle cell disease (SCD) and β-thalassemia.[10,11] Recently, several reports have revealed an important yet previously unrecognized role of genetic variants in regulating epigenetics.[9,12-15] For example, DNA variations may affect the recruitment and binding affinity of transcription factors, which in turn lead to histone tail modifications.

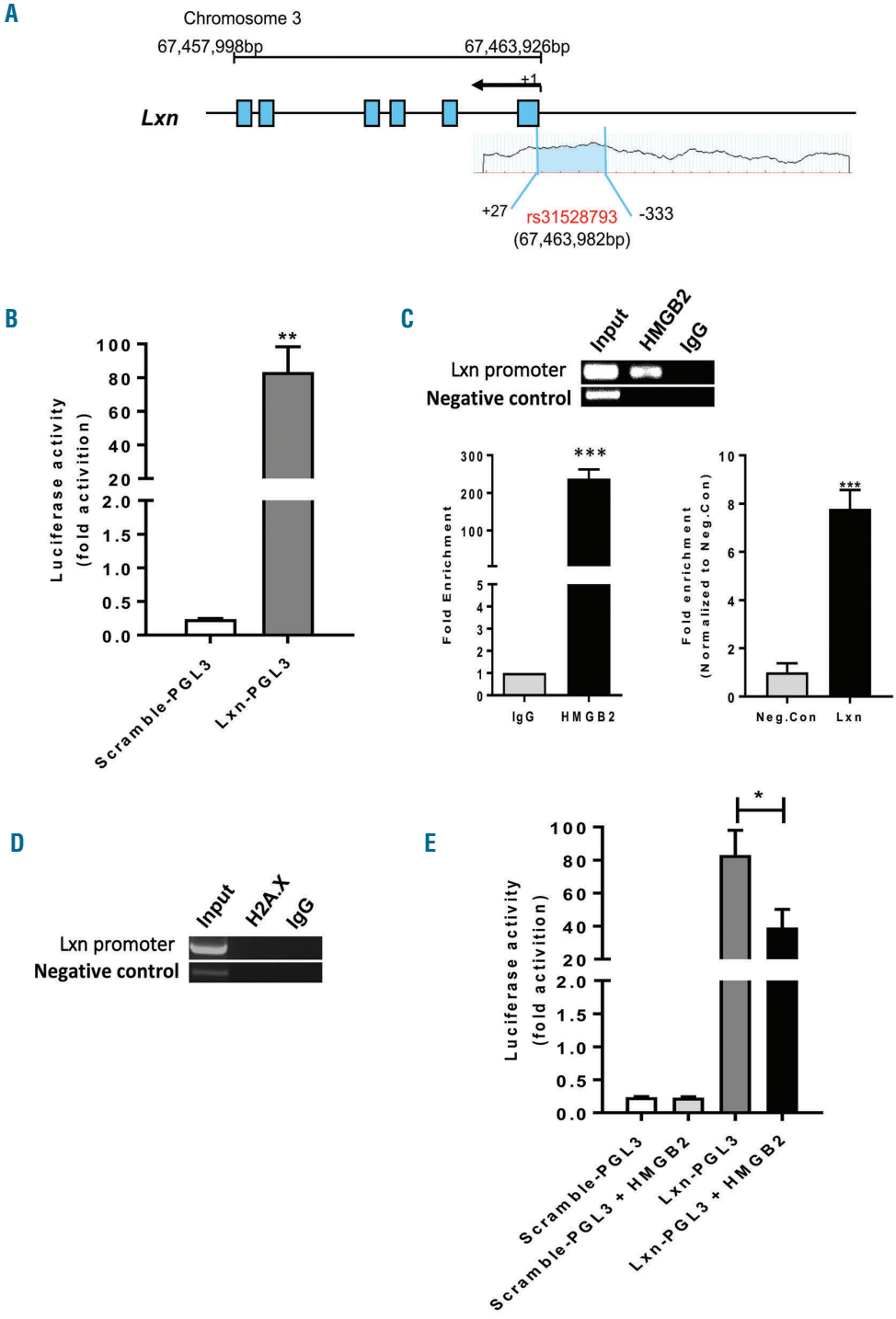

Figure 1. HMGB2 suppresses *Lxn* promoter activity. (A) *Lxn* promoter sequence spans from 333 nucleotides upstream of the transcription start site (+1) of *Lxn* gene to 27 nucleotides into the first exon. The chromosomal positions for *Lxn* gene and SNP rs31528793 are indicated. (B) Lxn promoter sequence has strong promoter activity. Luciferase activity was determined in HEK cells transduced with luciferase reporter construct containing either *Lxn* promoter sequence (Lxn-PGL3) or control vector (PGL3). (C) HMGB2 specifically binds to *Lxn* promoter sequence. Cromatin immunoprecipitation (ChIP) assay was performed with an HMGB2 polyclonal antibody (HMGB2) or IgG control (IgG). The genomic sequence in the 500 base pairs downstream of the *Lxn* promoter region were used as the negative sequence control to determine the HMGB2 binding specificity (Negative control). *Lxn* promoter sequence was amplified and quantified by real-time polymerase chain reaction (PCR) (top). The fold enrichment of HMGB2 in the *Lxn* promoter was quantified by normalization to either IgG control (bottom left) or negative sequence control (bottom right). (D) H2A.X does not bind to *Lxn* promoter. ChIP assay was performed with an H2A.X polyclonal antibody (H2A.X) or IgG control (IgG). The genomic sequence in the 500 base pairs downstream of the *Lxn* promoter region were used as the negative sequence control to determine the H2A.X binding specificity (Negative control). *Lxn* promoter sequence was amplified and quantified by real-time PCR. (E) HMGB2 suppresses *Lxn* promoter activity. Luciferase activity was determined in HEK cells transduced with luciferase reporter construct containing either Lxn promoter sequence (Lxn-PGL3) or control vector with scramble sequence (Scramble-PGL3) without or with HMGB2 plasmid (Scramble-PGL3 + HMGB2, and Lxn-PGL3 + HMGB2). Data are the average of three independent experiments with triplicates in each experiment (n=9). **P*<0.05; ***P*<0.01; ****P*<0.001.

Such variations in the epigenetic environment result in significant variations in gene expression, which collectively manifest as a phenotypic trait. Despite these advances, the precise molecular mechanisms underlying the association between the genetic variants and hematopoietic phenotypes remain largely unknown.

Inbred mouse strains provide a model system for exploring the myriad of regulatory gene network contributing to hematologic diversity.[10,16] We carried out a comparative study of two inbred strains, C57BL/6 (B6) and DBA/2 (D2), in which we documented large natural variation in a number of stem cell traits.[5-8] One of the most significant traits is the natural size of the HSC population, i.e. young B6 mice have 3- to 8-fold fewer stem cells in bone marrow (BM) than D2 mice, depending on the assay used for stem cell quantification. We further identified *Lxn* as the regulatory gene whose expression is negatively correlated with HSC number.[17,18] *Lxn* regulates HSC in a cell-autonomous manner through concerted mechanisms of decreased self-renewal and increased apoptosis. Even though we identified several genetic variants that might be associated with the differential expression of *Lxn* in B6 and D2 stem cells, there is no direct evidence of how these variants regulate *Lxn* transcription and whether they have any functional effects.

In this study, we report for the first time that a chromatin protein, HMGB2, binds to *Lxn* promoter and plays an important role in the transcriptional regulation of *Lxn*. Knockdown of HMGB2 increases *Lxn* expression at both transcript and protein levels, suggesting a suppressive role of HMGB2 in *Lxn* transcription. HMGB2 knockdown decreases the number of functional HSC by promoting apoptosis and reducing proliferation. Concomitant knockdown of *Lxn* reverses these functional effects, suggesting that *Lxn* is one of the downstream targets of HMGB2. Moreover, we discovered that a functional polymorphism, SNP rs31528793, is associated with the differential expression of *Lxn* in different mouse strains, including B6 and D2. This study, for the first time, reveals the genetic and epigenetic regulation of *Lxn* transcription, suggesting that both trans- and cis-elements (HMGB2 and SNP, respectively) contribute to the differential gene expression and phenotypic diversity in the HSC population

Methods

Luciferase reporter assay

Lxn promoter activity and HMGB2 transcription activity were measured by luciferase reporter assay with a Tropix TR717 luminometer using a dual luciferase assay kit.

Identification of *Lxn* promoter binding proteins

Lxn promoter binding proteins were isolated by µMACSTM FactorFinder Kit (Miltenyi Biotec Inc., Auburn, CA, USA). The high purity double-strand DNA oligonucleotides containing SNP rs31528793 was used as the DNA bait for protein pull-down. The associated proteins were determined by mass spectrometry at the Mass Spectrometry and Proteomics Facility at Ohio State University.

Protein-DNA binding assays

Chromatin immunoprecipitation: chromatin immunoprecipitation (ChIP) assay was performed on LK (Lin- c-KIT+) cells using ChIP assay kit (Sigma Aldrich, #CHP1) with HMGB2 polyclonal antibodies (ab67282), H2A.X antibody (ab11175), or Rabbit IgG control (ab171870) (Abcam, Cambridge, MA, USA). HMGB2 binding affinity was determined by SYBR green quantitative polymerase chain reaction (qPCR).

Electrophoretic mobility shift assay: electrophoretic mobility shift assays (EMSA) were performed in 293T cells transduced with HMGB2 lentivirus using the LightShift™ Chemiluminescent EMSA Kit (Thermo Scientific™).

Table 1. Lxn promoter binging protein.

Lxn promoter sequence containing SNP rs31528793	
Histone H2A type 1- F	(H2A1F)
Histone H2A type 2- A	(H2A2A)
Protein S100-A9	(S10A9)
Protein S100-A8	(S10A8)
Histone H2A.X	(H2A.X)
High mobility group protein B2	(HMGB2)
Histone H2B type 1- H	(H2B1H)
Histone H2AV	(H2AV)
Histone H1.3	(H1.3)
Peptidyl-prolyl cis-trans isomerase A	(PPIA)
Eosinophil cationic protein 1 precursor	(ECP1)
Myeloperoxidase precursor	(PERM)
Histone H1.1	(H11)
Histone H1.5	(H15)
Coronin-1A	(COF1)

Double-strand DNA oligonucleotides containing single nucleotide polymorphism (SNP rs31528793) were used as "bait" to capture associated proteins from bone marrow cell lysate of C57BL/6 mouse. Proteins binding to Lxn promoter sequences were isolated by µMACSTM FactorFinder Kit (Miltenyi Biotec Inc., Auburn, CA, USA) and identified by Mass Spectrometry. 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 considered significant.

Gene knockdown and expression measurement

EML or c-KIT$^+$ (LSK) cells were transduced with HMGB2 shRNA (MSH027321-LVRU6GP, GeneCopoeia), *Lxn* Mission shRNA (Sigma-Aldrich) virus. Gene expression was measured by real-time PCR with commercially available primer/probe mix for *Hmgb2* or *Lxn* in ABI PRISM 7700 (Applied Biosystems, Foster City, CA, USA). Protein expression was measured by western blot with anti-Hmgb2 antibody (ab67282), goat polyclonal anti-*Lxn* antibodies (ab59521, Abcam), or mouse monoclonal anti-β-actin antibody (A5441, Sigma).

Immunostaining and flow cytometry

Hematopoietic stem cells and hematopoietic progenitor cells: young (8-12 week) female C57BL/6, DBA2, 129X1/SvJ, A/J and CD45.1 mice (Jackson Laboratories, Bar Harbor, ME, USA) were used for HSC/hematopoietic progenitor cell isolation (HPC). HSC/HPC were defined as Lin$^-$, Sca-1$^+$ (clone E13-161.7) and c-KIT$^+$ (LSK) cells. Long-term HSC (LT-HSC) were identified as LSK plus CD34 and FLT3 negative cells.

Cell cycle: cell cycle was analyzed by BrdU incorporation using BrdU Flow Kit.

Apoptosis: apoptosis was evaluated by Annexin V staining.

Active caspase 3 analysis: active caspase 3 analysis was analyzed using PE Active Caspase-3 Apoptosis Kit. All kits are from BD Pharmingen™. Flow cytometry was performed on a FacsAria II (Becton Dickinson) and the data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

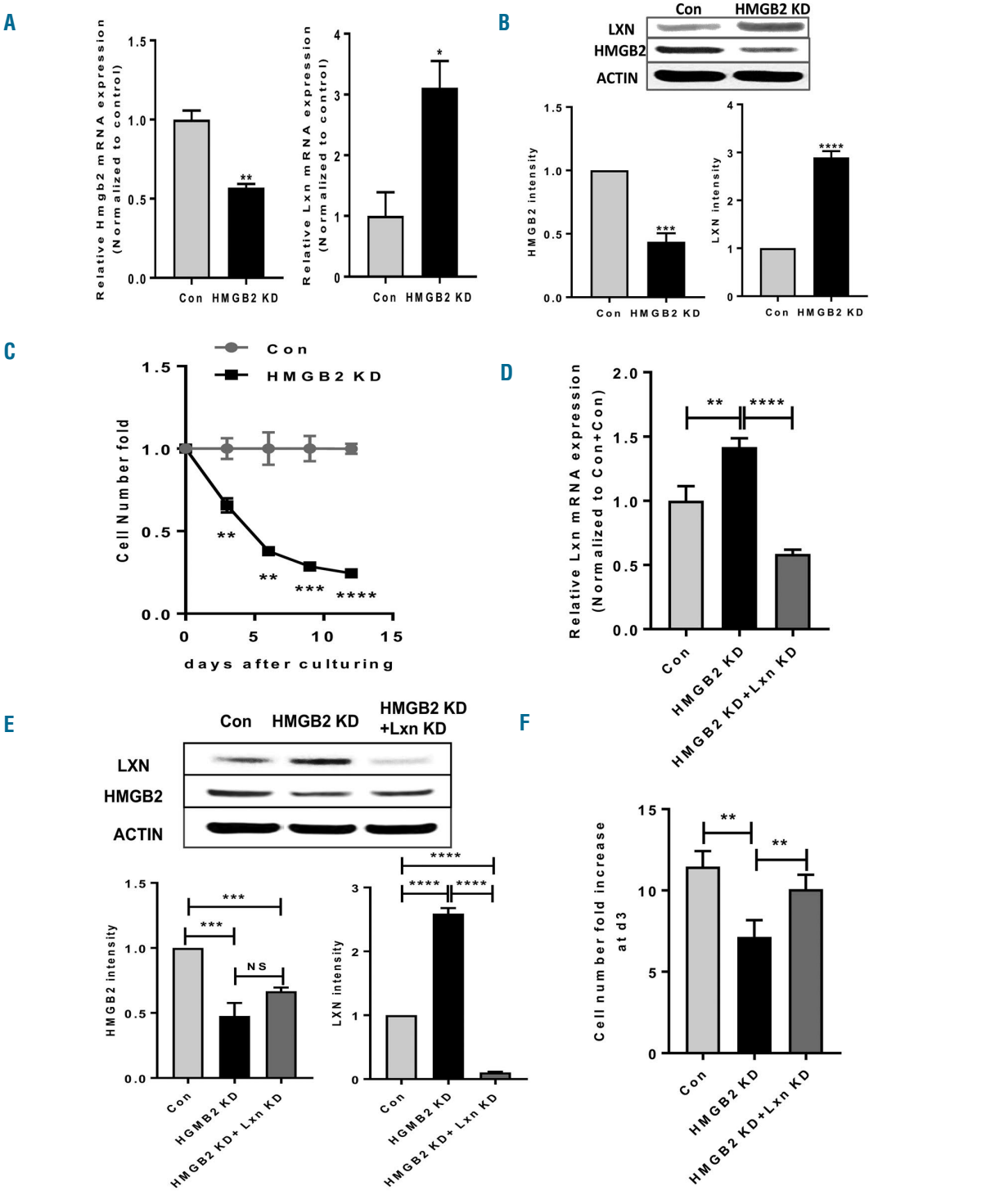

Figure 2. HMGB2 knockdown increases *Lxn* expression and decreases the number of hematopoietic stem cell (HSC) cell line. (A) Knockdown of HMGB2 in EML cells increases *Lxn* mRNA expression. EML cells were infected by control lentivirus (Con) or HMGB2 knockdown shRNA (HMGB2 KD). HMGB2 and *Lxn* mRNA levels were measured by quantitative real-time polymerase chain reaction (PCR). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the endogenous control for mRNA expression normalization. (B) Knockdown of HMGB2 in EML cells increases *Lxn* protein expression. EML cells were infected by control lentivirus (Con) or HMGB2 knockdown shRNA (HMGB2 KD). HMGB2 and *Lxn* protein levels were measured by western blot. Actin was the normalization control. (Top) Representative western blot out of three independent experiments. (Bottom) Quantification of intensity of HMGB2 (left) and *Lxn* (right) proteins. (C) HMGB2 knockdown decreases EML cell number. EML cells infected with empty (Con) or HMGB2 shRNA (HMGB2 KD) were cultured for 12 days and counted at different time points. (D) *Lxn* mRNA was decreased in HMGB2-knockdown EML cells with simultaneous knockdown of *Lxn*. HMGB2-knockdown EML cells (HMGB2 KD) were co-transfected with *Lxn* shRNA lentiviral vector (HMGB2 KD + *Lxn* KD). *Lxn* mRNA and protein was measured by real-time PCR and western blot. (E) *Lxn* protein was decreased in HMGB2-knockdown EML cells with simultaneous knockdown of *Lxn*. HMGB2-knockdown EML cells (HMGB2 KD) were co-transfected with *Lxn* shRNA lentiviral vector (HMGB2 KD + *Lxn* KD). HMGB2 and *Lxn* protein levels were measured by western blot. Actin was the normalization control. (Top) Representative western blot out of three independent experiments. (Bottom) Quantification of intensity of HMGB2 (left) and *Lxn* (right) proteins. (F) *Lxn* knockdown restores the number of HMGB2-knockdown EML cells to a level comparable to control group. Data shown are EML cell number at day 3 of cell culture. All data are the average of three independent experiments with triplicates in each experiment (n=9). **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001.

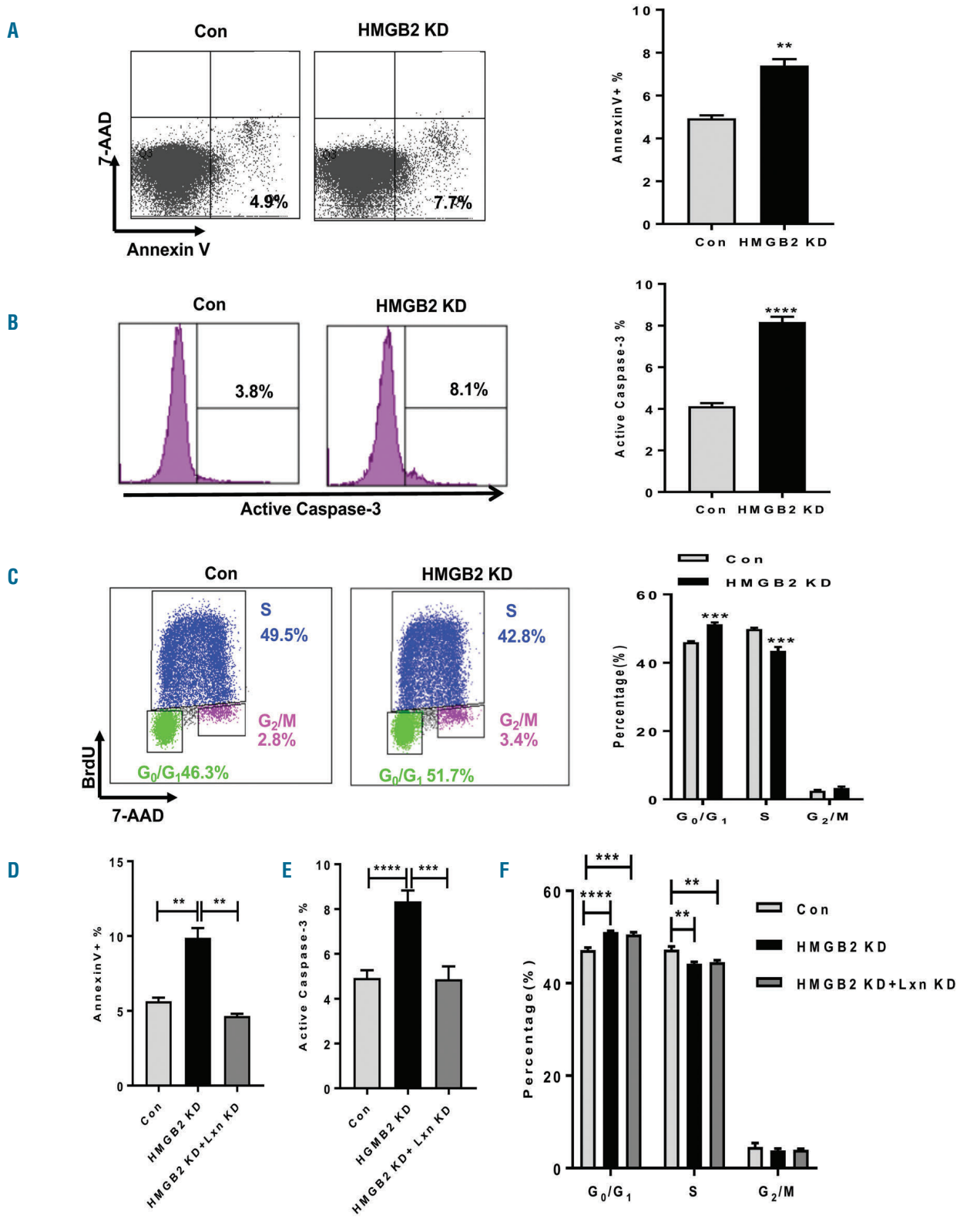

Figure 3. HMGB2 knockdown increases apoptosis and decreases proliferation. (A) HMGB2 knockdown increases apoptosis of EML cells. Representative FACS plots (left) and quantification (right) of Annexin V+ and 7AAD- apoptotic EML cells transduced with control-shRNA (Con) or HMGB2 knockdown shRNA lentivirus (HMGB2 KD). (B) HMGB2 knockdown increases the percentage of active caspase 3 positive EML cells. Representative flow cytometry profile (left) and quantification (right) of active caspase 3 immnuofluorescence signal in EML cells. (C) HMGB2 knockdown decreases proliferation of EML cells. Representative FACS plots showing the G0/G1 (BrdU- and 7AAD-), S (BrdU+), and G2/M (BrdU- and 7AAD+) phages of cell cycle in EML cells (left). (Right) Frequencies of each phase. (D) *Lxn* knockdown (HMGB2 KD + *Lxn* KD) restores the percentage of apoptotic (Annexin V+) HMGB2-knockdown EML cells (HMGB2 KD) to a level comparable to control group (Con). (E) *Lxn* knockdown (HMGB2 KD + *Lxn* KD) restores the percentage of active caspase-3 positive HMGB2-knockdown EML cells (HMGB2 KD) to a level comparable to control group (Con). (F) *Lxn* knockdown (HMGB2 KD + *Lxn* KD) did not restore the cell cycle status of HMGB2-knockdown EML cells (HMGB2 KD) to control group level (Con). Data presented as the average±Standard Deviation of six measurements from two independent experiments. **P*<0.05; ***P*<0.01; ****P*<0.001.

Functional analysis of hematopoietic stem cells and hematopoietic progenitor cells

Colony forming cell assay: colony forming cell (CFC) assay was performed in complete MethoCult media (Stem Cell Technologies, Vancouver, Canada), and colony was counted on day 14.

Cobblestone area forming cell (CAFC) assay: cobblestone area forming cell (CAFC) assay was performed as described previously.[17] The most primitive HSC showed cobblestones at day 35 of culture, and their frequency was calculated by using L-Calc Limiting Dilution Analysis Software (Stem Cell Technologies, Vancouver, Canada).

In vivo transplantation assay: *in vivo* transplantation assay, $3x10^5$ transduced cells (GFP+ cells) plus $2x10^5$ competitor B6.SJL/BoyJ BM cells were injected into B6.SJL/BoyJ mice after 24 hours of transduction, and GFP+ chimerism in peripheral blood (PB) and BM was measured at 16 weeks post transplantation.

Statistical analysis

Data were examined for homogeneity of variances (F-test), then

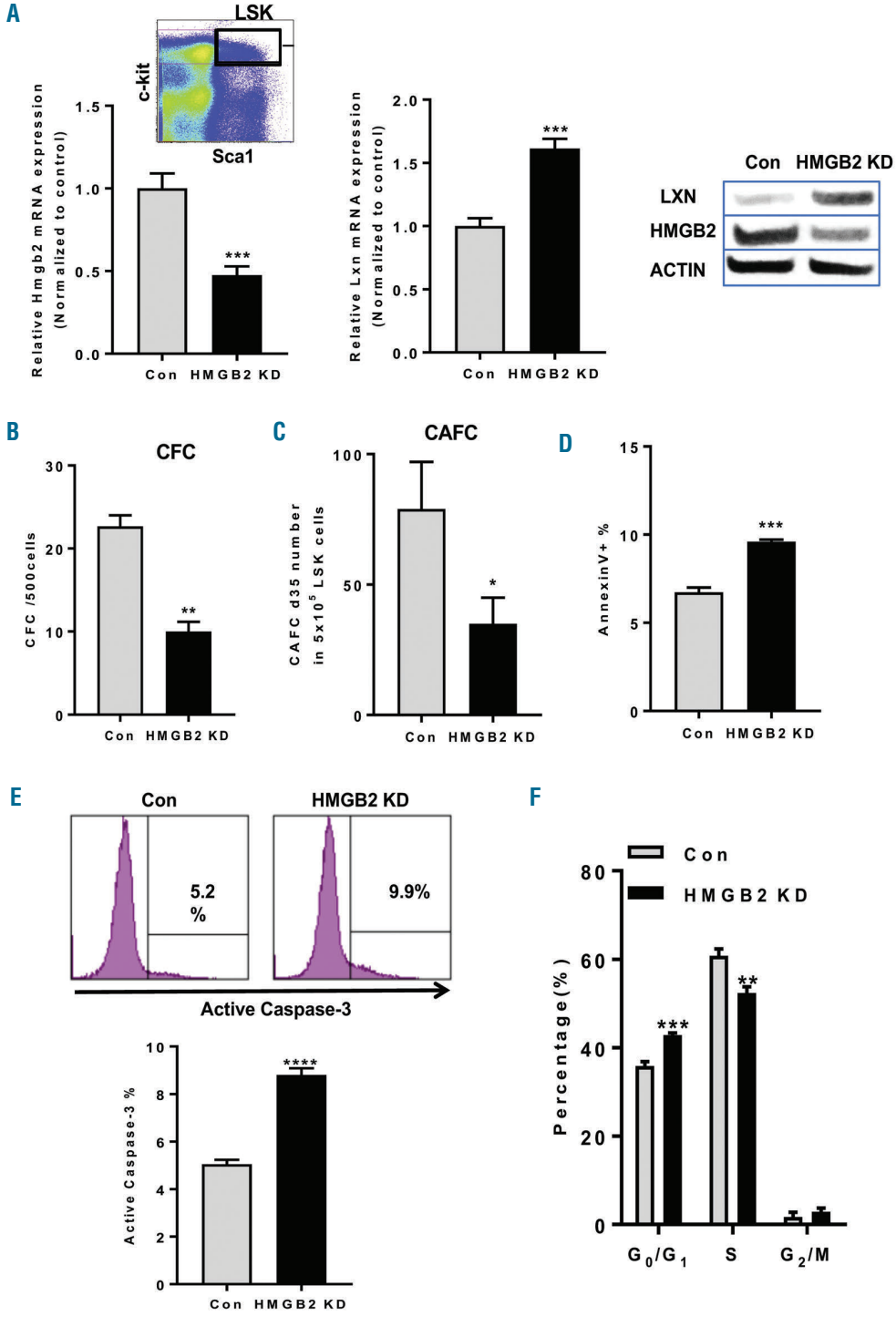

Figure 4. HMGB2 knockdown increases *Lxn* expression and decreases the number and function of bone marrow hematopoietic stem cells. (A) HMGB2 knockdown increases *Lxn* expression in hematopoietic stem and progenitor cells. HMGB2 knockdown (HMGB2 KD) in bone marrow LSK cells (left) increases *Lxn* mRNA (middle) and protein expression (right) compared to control group (Con). *Lxn* mRNA and protein levels were measured by quantitative real-time polymerase chain reaction and western blot. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the endogenous control for mRNA expression normalization. Actin was the control for protein normalization. (B) HMGB2 knockdown decreases the number of clonogenic and functional hematopoietic progenitor and (C) stem cells. LSK cells transduced with control and knockdown lentivirus were sorted, and the numbers of progenitor and stem cells were measured by colony forming cell (CFC) and cobblestone area forming cell assay (CAFC), respectively. (D) HMGB2 knockdown increases apoptosis (Annexin V+) of LSK cells. (E) HMGB2 knockdown increases the proportion of active caspase-3 positive LSK cells. The representative histogram of active capase-3 flow cytometry profile (top) and the quantification of positive cell proportion (bottom) are shown. (F) HMGB2 knockdown decreases proliferation of LSK cells. The apoptosis and proliferation were determined with the same way as in EML cells. Values are the mean±Standard Deviation from three independent experiments. **P*<0.05; ***P*<0.01; ****P*<0.001.

analyzed by Student's *t*-test or One-way ANOVA using Tukey's test. $P<0.05$ was considered statistically significant. All statistical analyses were conducted with Graphpad Prism.[7]

All animal work and experiments were performed under the guideline of approved Institutional Review Board and Ethics Committee, Biosafety Committee, and Animal Care and Use Committee protocols at the University of Kentucky.

Results

HMGB2 binds to *Lxn* promoter and suppresses its activity

The transcriptional regulation of the *Lxn* gene remains largely unknown. We used two criteria to identify the potential promoter in the upstream regulatory region of *Lxn*. First, we looked for the regions containing SNP because the natural variation of *Lxn* expression is mainly caused by genetic variants. Secondly, we and others have shown that promoter hypermethylation is involved in the downregulation of *Lxn* in several types of cancer cells, including leukemia stem cells.[19-24] This prompted us to search for regions enriched with CG dinucleotides (CpG island). We thus analyzed the mouse *Lxn* upstream putative promoter sequence (*http://www.methprimer.com*) and identified a CG-enriched region that contains a SNP rs31528793 (Figure 1A). Using the NCBI SNP database (*https://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=rs31528793*) search, we confirmed the presence of this polymorphism. This region spans from the canonical 5' promoter (-333 nucleotide, nt) to the transcription start site (+1 nt), and extends through the first exon (+27 nt). We next amplified and sequenced this region, confirming the existence of this SNP (*data not shown*). To determine whether it has promoter activity, we performed *in vitro* luciferase reporter assay and found that this sequence in *Lxn* upstream regulatory region had a strong promoter activity (Figure 1B).

We next performed *in silico* analyses to search for the potential transcription factors in the *Lxn* promoter region by using the transcription factor prediction program, TRANSFAC (*www.cbrc.jp/research/db/TFSEARCH.html*). Results from this analysis showed that SNP rs31528793 falls within the consensus binding motif for the transcription factors, *Adr-1* and *Ets-1*. Our previous microarray data showed that only *Ets-1* was expressed in HSC (*data not shown*). We thus only evaluated the binding of *Ets-1* to *Lxn* promoter with chromatin immunoprecipitation (ChIP)

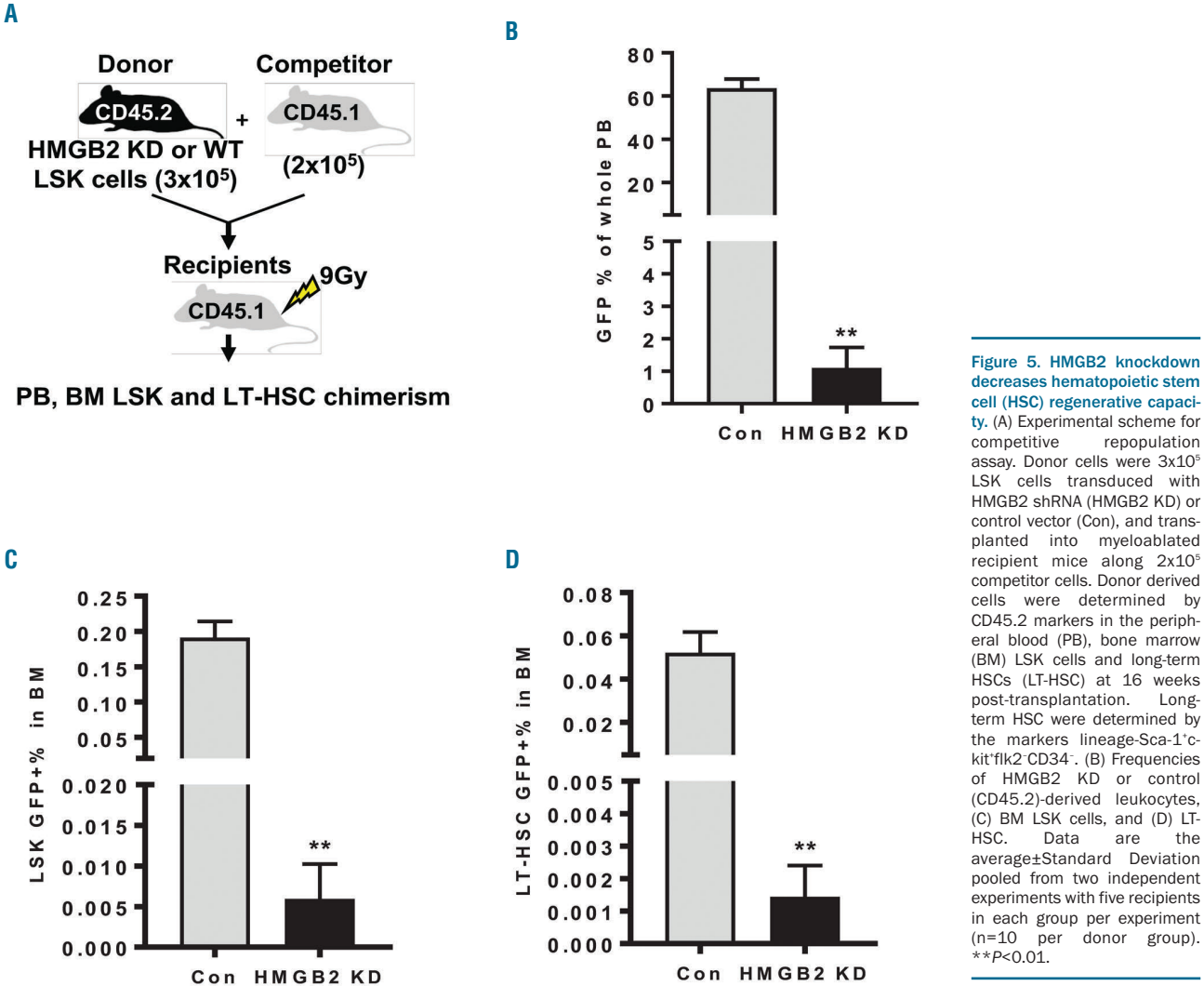

Figure 5. HMGB2 knockdown decreases hematopoietic stem cell (HSC) regenerative capacity. (A) Experimental scheme for competitive repopulation assay. Donor cells were $3x10^5$ LSK cells transduced with HMGB2 shRNA (HMGB2 KD) or control vector (Con), and transplanted into myeloablated recipient mice along $2x10^5$ competitor cells. Donor derived cells were determined by CD45.2 markers in the peripheral blood (PB), bone marrow (BM) LSK cells and long-term HSCs (LT-HSC) at 16 weeks post-transplantation. Long-term HSC were determined by the markers lineage-Sca-1$^+$c-kit$^+$flk2$^-$CD34$^-$. (B) Frequencies of HMGB2 KD or control (CD45.2)-derived leukocytes, (C) BM LSK cells, and (D) LT-HSC. Data are the average±Standard Deviation pooled from two independent experiments with five recipients in each group per experiment (n=10 per donor group). $^{**}P<0.01$.

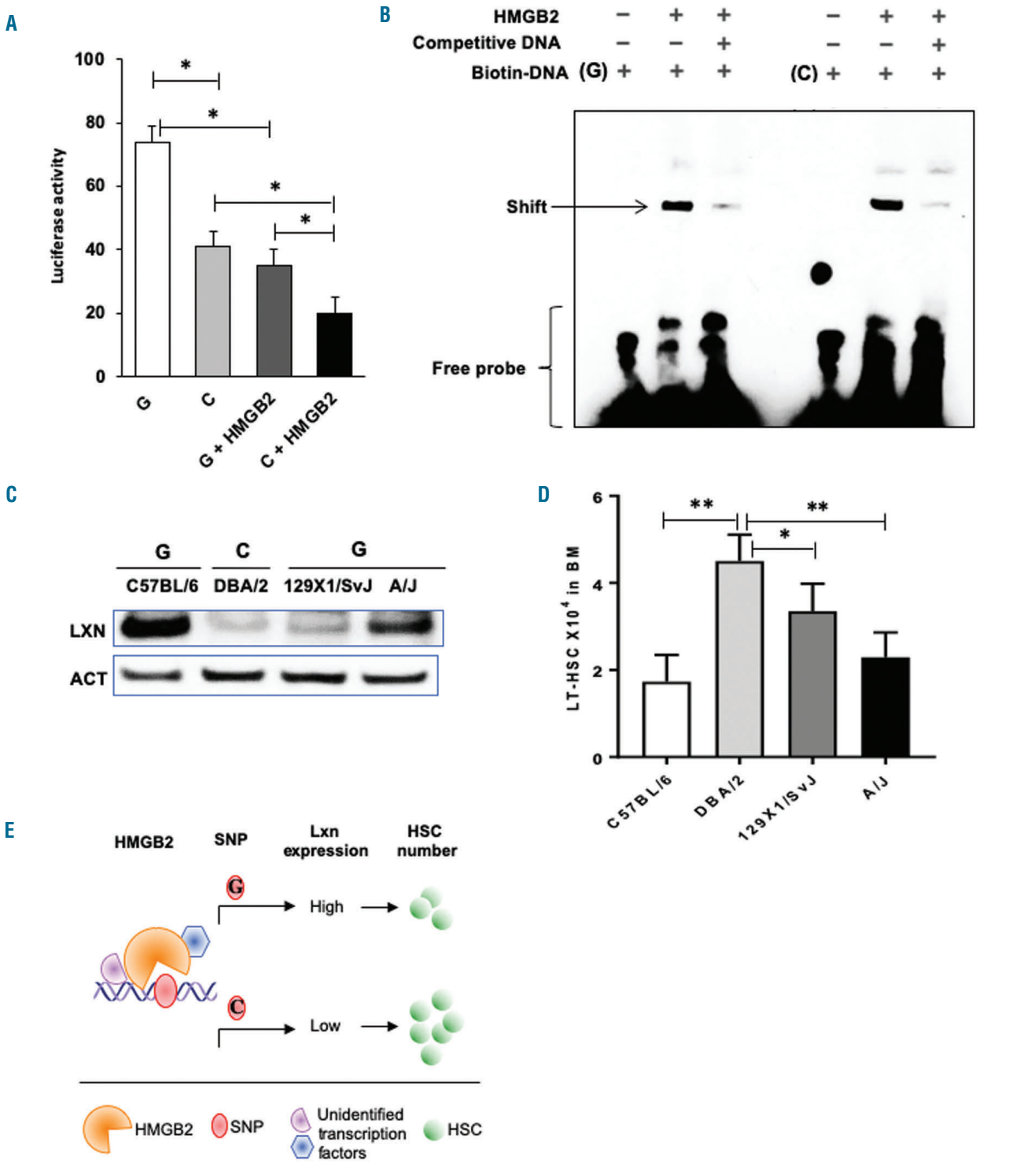

Figure 6. SNP rs31528793 associates with *Lxn* promoter activity and hematopoietic stem cell (HSC) number. (A) SNP rs31528793 affects *Lxn* promoter activity. G allele is associated with the higher promoter activity whereas C allele lowers its activity. HMGB2 has transcription suppressor activity in both alleles. The luciferase reporter assay was performed on G or C containing vectors (G or C) and on the vectors co-transfected with HMGB2 plasmids (G+ HMGB2; or C+ HMGB2). The average values of three independent experiments are shown with standard deviation (SD) (n=12). (B) HMGB2 binds to G/C containing *Lxn* promoter. Electrophoretic mobility shift assay (EMSA) was performed with HMGB2 polyclonal antibody and biotin labeled oligonucleotides containing G or C allele. One representative experiment shown out of three independent experiments. (C) G/C allele in SNP rs31528793 indicates LXN protein level. Western blot measured LXN protein in bone marrow cells of different mouse strains carrying either G or C allele. One representative experiment shown out of three independent experiments. (D) G/C allele in SNP rs31528793 indicates HSC number. Long-term HSC and frequency (%) were determined by the markers lineage-Sca-1+c-kit+flk2-CD34-. Results are average (± 1 Standard Deviation) of nine mice from three independent experiments. *P<0.05; **P<0.01. (E) Model of transcriptional regulation of *Lxn* by HMGB2 and the effect of SNP rs31528793 on the natural variation of *Lxn* expression and HSC number. HMGB2 binds to the *Lxn* promoter and acts as a transcriptional suppressor. SNP rs31528793 in the *Lxn* promoter sequence causes the differential promoter activity in which G allele is associated with higher *Lxn* expression and lower HSC number whereas C allele is associated with higher *Lxn* expression and lower HSC number. HMGB2 is involved in *Lxn* transcriptional regulation either by itself or by modifying chromatin structure in the *Lxn* promoter region, thereby facilitating access of other transcription factors to the region.

and found that *Ets-1* did not bind to *Lxn* promoter (*data not shown*). These results prompted us to use the "DNA-pull down" and mass spectrometry to directly look for proteins that bind to this region. We found 15 candidate binding proteins (Table 1). HMGB2 and H2A.X are of particular interest because of the involvement of their family members in the regulation of stem cell function and differentiation as the chromatin modifiers.[25-34] We thus focused on these two proteins and examined their role in *Lxn* transcriptional regulation.

We performed ChIP-qPCR assay to measure the binding and occupancy of HMGB2 and H2A.X to the *Lxn* promoter. The result showed that HMGB2, but not H2A.X, were significantly enriched at the *Lxn* promoter region in comparison to IgG control and to the control region at the downstream 500 bps of the promoter (Figure 1C and D). This result supports the idea of the specific binding of HMGB2 to *Lxn* promoter. We next performed luciferase assay, and found that HMGB2 suppressed *Lxn* promoter activity (Figure 1E). Altogether, to our knowledge, this is the first time HMGB2 has been identified as a novel transcription suppressor of *Lxn* gene.

HMGB2 knockdown increases *Lxn* expression and changes the function of a HSC cell line

We next examined the regulatory role of HMGB2 in *Lxn* transcription and its effects on EML cells. EML is the only known hematopoietic cell line with both lympho and myelo-erythroid differentiation potential, and is considered to represent HSC.[35-37] Knockdown HMGB2 in EML cells significantly increased *Lxn* expression at both mRNA and protein level (Figure 2A and B, respectively), reinforcing the finding of transcriptional suppression of HMGB2 on *Lxn* expression. We previously reported *Lxn* as a negative regulator of HSC number,[17] we thus hypothesized that knockdown of HMGB2 would decrease EML cell number *via* upregulation of *Lxn*. We therefore monitored the growth of EML cells with HMGB2 knockdown for two weeks, and found that HMGB2 knockdown led to a dramatic decrease in the cell number compared to control group (Figure 2C). Since HMGB2 is a chromatin binding protein, its effect on EML number may not act solely through *Lxn* upregulation. We simultaneously knocked down *Lxn* in HMGB2 shRNA-transduced cells, and determined whether blocking *Lxn* upregulation could attenuate HMGB2-induced growth inhibition. We confirmed that the expression of *Lxn* mRNA and protein in HMGB2-shRNA transduced EML cells was reduced by the co-transduction with *Lxn*-shRNA (Figure 2D and E). Figure 2F shows that HMGB2 knockdown significantly decreased EML cell number, and the concomitant *Lxn* knockdown reversed this change, resulting in an increase in the cell number to the level comparable to control group. These data imply that *Lxn* is one of the downstream transcriptional targets of HMGB2, and that HMGB2 suppresses *Lxn* transcription which in turn increases EML number.

We previously reported that *Lxn* negatively regulates HSC function through increasing apoptosis and decreasing proliferation,[23,38,39] we hypothesized that HMGB2 inhibition may have similar effects on EML cells. Indeed, we found that knocking down HMGB2 significantly increased the percentage of apoptotic EML cells (Figure 3A). The change was further confirmed by the increased proportion of active caspase 3 positive cells (Figure 3B). Moreover, knocking down HMGB2 significantly decreased the percentage of cells in the S phase in the cell cycle (Figure 3C). The concomitant increase in apoptosis and decrease in proliferation by HMGB2 inhibition may contribute to the decreased cell number (Figure 2B). We next tested whether apoptosis and proliferation could be rescued by blocking *Lxn* upregulation in HMGB2 knockdown condition (see also Figure 2E and F). The results showed that *Lxn* knockdown on the top of HMGB2 knockdown decreased apoptosis (Figure 3D) and the proportion of active caspase 3 positive cells (Figure 3E), and restored their changes to the level of control group. However, the cell cycle changes were not fully restored (Figure 3F), suggesting that *Lxn* may be a major player in regulating apoptosis, and other downstream targets of HMGB2 might be involved in cell cycle regulation that counteract *Lxn* function. Overall, these data suggest that HMGB2 positively regulates HSC function *via* the suppression of *Lxn* expression.

HMGB2 knockdown increases *Lxn* expression in bone marrow hematopoietic stem cells and decreases their number and regenerative function

Because of the observed effect of HMGB2 on *Lxn* expression and EML function, we next asked whether HMGB2 plays a similar role in primary HSC. We knocked down HMGB2 in bone marrow lineage- Sca-1$^+$ c-Kit$^+$ (LSK) cells (Figure 4A, left), which are enriched with HSC and hematopoietic progenitor cells (HPC), and then determined the effect of HMGB2 knockdown on *Lxn* expression and HSC and HPC cell numbers, apoptosis and cell cycling. We found that knockdown HMGB2 in LSK cells also led to a significant increase in *Lxn* expression at both transcript and protein levels (Figure 4A, middle and right). We next performed *in vitro* short-term CFC and long-term CAFC assays to determine functional HPC and HSC, respectively. The result showed that the numbers of HPC and HSC in HMGB2-knockdown cells were nearly 2-fold lower than those in control cells (Figure 4B and C). Moreover, HMGB2 knockdown led to an increase in apoptosis (Figure 4D) and the proportion of active caspase 3 positive cells (Figure 4E), and a decrease in proliferation in LSK cells (Figure 4F), similar to those seen in the EML cells. These data confirm that HMGB2 also regulates *Lxn* transcription in primary HSC, and thereby affects the number and clonality of HSC and HPC.

We next performed a more stringent transplantation experiment to determine the effect of HMGB2 inhibition on HSC regenerative capacity *in vivo*. Donor cells are LSK cells that were transduced with either HMGB2 shRNA or control shRNA. They were next transplanted into the myeloablated recipient mice with helper cells, and blood and BM regeneration were examined at 16 weeks post transplantation (Figure 5A). The results showed that HMGB2 knockdown resulted in significant decreases in the regeneration of blood cells, BM HSC/HPC-enriched LSK cells, and the most primitive long-term HSC with unlimited self-renewal capacity (Figure 5B-D). These results suggest that HMGB2 inhibition impairs HSC regenerative functionality. Altogether, our data obtained from the EML cell line and primary HSC strongly support the idea that the HMGB2 suppresses *Lxn* expression, which in turn affects HSC and HPC number and function.

SNP rs31528793 affects *Lxn* promoter activity and hematopoietic stem cell number

The level of a given mRNA transcript is controlled by trans-acting factors and/or cis-acting modulators. Our data suggest that HMGB2 might act as a trans-acting modulator to regulate *Lxn* transcription. Since we previously reported that several SNP identified by us may contribute to the natural variation of *Lxn* expression,[17] we next asked whether any of these SNP is associated with *Lxn* expression as a cis-acting regulator. SNP rs31528793 is the only genetic variant in the *Lxn* promoter region (Figure 1A), we thus asked whether it affects the *Lxn* promoter activity. We made a G to C mutation in the luciferase reporter construct containing the *Lxn* promoter sequence and performed the luciferase reporter assay. The G to C change decreased the promoter activity by more than 2-fold (Figure 6A), suggesting a potential suppressive role of this polymorphism in *Lxn* transcription (Figure 6A, left two columns). Since HMGB2 binds to this region, we next examined whether G/C variant affects HMGB2 binding. The result showed that HMGB2 further suppresses *Lxn* promoter activity, and C allele still causes nearly 2-fold decrease of the promoter activity. We next performed the EMSA assay and further confirmed the interaction of HMGB2 with the *Lxn* promoter containing SNP rs31528793 (Figure 6B). These results indicate that SNP rs31528793 influences *Lxn* promoter activity, with the G allele conferring a high activity, while the C allele is associated with a low activity. Therefore, the genetic variants of the *Lxn* promoter add another layer of regulatory mechanism of *Lxn* transcription.

We previously reported that *Lxn* is differentially expressed in HSC of C57BL/6 (B6) and DBA2 (D2) mice, and its expression level is inversely correlated with HSC number.[17] It is known that B6 mice carry G allele whereas D2 mice have C allele. We therefore hypothesized that the G allele is associated with the higher promoter activity, high *Lxn* expression and low HSC numbers, whereas the C allele has the opposite effect. Next, we examined *Lxn* expression and HSC numbers in B6, D2 and the other two mouse strains, 129X1/SvJ and A/J that carry G allele at the SNP rs31528793 position (*http://www.informatics.jax.org/snp/rs31528793*). We found that D2 mouse strain had the lowest expression of *Lxn* and highest HSC number, whereas all the other three strains showed higher *Lxn* expression and lower HSC number (Figure 6C and D), suggesting that G/C allelic variant could be indicative of *Lxn* expression level and HSC number variation. It is noted that *Lxn* expression level varies in strains carrying G allele suggesting that other SNP outside of the *Lxn* promoter region may contribute to such variation.

Discussion

Lxn plays an important role in regulating HSC function.[17,38] It was originally identified *via* the natural variation of HSC numbers between B6 and D2 inbred mouse strains in which B6 mice have fewer HSC than D2 mice at a young age. The expression of *Lxn* is inversely correlated to the size of HSC population, i.e. its level in B6 is higher than that in D2 cells. Therefore, *Lxn* is a negative regulator of HSC number and its mode of action is primarily through increasing HSC apoptosis and decreasing HSC regenerative capability and proliferation. However, nothing is known about how *Lxn* is transcriptionally regulated in HSC and other stem cells, or why it is differentially expressed in different inbred mouse strains.

Here, we identified a *Lxn* upstream regulatory sequence with a strong promoter activity. More importantly, the SNP (rs31528793) in this region significantly affects its promoter activity, and the G allele carried in B6, 129X1/SvJ and A/J mouse strains confers the promoter a stronger activity than the C allele in D2 strain. Genetic variants have been recently identified to play an important role in transcriptional regulation and thereby resulting in gene expression and phenotype variation.[9,13-15,40] We thus proposed that the G/C containing promoter might be involved in *Lxn* transcriptional regulation. Using DNA pull-down and mass spectrometry, we, for the first time, identified a chromatin binding protein, HMGB2, as a novel transcriptional suppressor of *Lxn* expression. HMGB2 binding was validated by ChIP-qPCR assay in which the endogenous HMGB2 demonstrated a stronger affinity to the *Lxn* specific promoter sequence. HMGB2 knockdown increases *Lxn* expression and decreases HSC numbers in both HSC cell line and BM-derived primary LSK cells. This effect was abrogated when the increased level of *Lxn* was blocked, indicating that *Lxn* is one of the downstream targets and functional mediators of HMGB2 in HSC. Altogether, these results suggest that both cis- and trans-factors are involved in the regulation of *Lxn* transcription (Figure 6E). In trans-regulating mode, HMGB2 acts as a suppressor for *Lxn* transcription. In *cis*-regulating mode, G allele at SNP rs31528793 is associated with stronger promoter activity, a high level of *Lxn* expression, and a small size of HSC pool. In contrast, the C allelic variant attenuates these effects and *Lxn* transcription is less responsive to HMGB2, which leads to a lower *Lxn* expression and an increased stem cell number. Therefore, our work not only identified HMGB2 as a novel transcription regulator of *Lxn*, but also provides a potential functional significance of SNP rs31528793 in contributing to natural variations in *Lxn* expression and HSC number. Despite these findings, how H2MGB2 regulates *Lxn* transcription requires further investigation. We cannot exclude the possibility that HMGB2 directly regulates *Lxn* transcription as the transcription factor. But it is also likely that HMGB2 acts as a chromatin adaptor or modifier to recruit other transcription factors for the initiation of the transcription process (Figure 6E). This mode of action was shown in the GFI1b transcription during erythroid differentiation process in which the binding of HMGB2 to GFI1b promoter enhances the binding of other factors, such as Oct-1, GATA-1 and NF-Y, which collectively activates Gfi1b transcription.[32] In addition, the relationship of HMGB2 binding site to SNP rs31528793, and how they co-ordinately or independently regulate *Lxn* transcription requires further investigation. However, our current data provide more support for the independent regulatory mechanism because of the following observations. Firstly, the suppression extent of HMGB2 on G-containing promoter (ratio of "G+ HMGB2" to "G" is 0.47) is similar to that on C-containing promoter (ratio of "C+ HMGB2" to "C" is 0.48) (Figure 6A). These data suggest that suppression of HMGB2 on *Lxn* promoter activity is independent of allelic variant. Secondly, results of EMSA also show the similar intensity of shifted bands, suggesting that G/C variant does not affect HMGB2 binding (Figure 6B). Lastly, to further confirm binding of HMGB2 to the *Lxn* promoter

and determine the effect of SNP rs31528793 on HMGB2 binding *in vivo*, we performed ChIP-qPCR assay on BM cells of C57BL/6 and DBA/2 mice which naturally carry the SNP. The binding affinity of HMGB2 was quantitatively measured by real-time PCR with primers spanning the promoter sequence containing G/C SNP (see also Figure 1C). We did not detect any difference in the binding affinity, suggesting that the G/C allelic variant does not cause differential binding of HMGB2 to *Lxn* promoter (*data not shown*). Altogether, this evidence strongly suggests that HMGB2 and SNP rs31528793 act independently to regulate *Lxn* transcription.

HMGB2 is a member of the high mobility group family proteins. It is a non-histone chromatin-binding protein that remodels chromatin architecture, therefore affecting gene expression. HMGB2 has been shown to play an important role in maintaining stem cell population in a tissue-specific manner. For example, in the nervous system, HMGB2 deletion leads to the increased neural stem/progenitor cells by increasing their proliferation.[41] However, in articular cartilage, loss of HMGB2 reduces the regenerative capacity of mesenchymal stem cells by increasing apoptosis.[42] Similarly, knockdown of HMGB2 decreased the number of muscle stem (satellite) cells by inhibiting proliferation and stimulating differentiation, thereby leading to the impaired muscle regeneration.[43] Our study showed that the functional effects of HMGB2 on HSC and the blood system are similar to those in mesenchymal and muscle stem cells. Knockdown of HMGB2 decreased HSC number and blood regeneration by increasing apoptosis and decreasing proliferation. These effects are mediated, at least in part, *via* the upregulation of *Lxn*, which is a negative regulator of HSC function. HMGB2 has also been shown to play an important role in cellular senescence and aging.[44,45] It binds to the chromosome loci of key senescence-associated secretory phenotype (SASP) genes and prevents their incorporation into transcriptionally repressive heterochromatin environment during senescence, thereby inducing *SASP* gene expression. Since we found that *Lxn* is one of the transcriptional targets of HMGB2, whether *Lxn* is involved in senescence and aging remains a subject of great interest and remains to be determined. Our unpublished data show that *Lxn* expression increases with aging, and old HSC with *Lxn* depletion have the increased regenerative capacity that is comparable to young HSC. Thus, inhibition of *Lxn* may rejuvenate old HSC.

Natural genetic variation is associated with a variety of hematologic phenotypes in humans. Genome-wide association studies have revealed DNA variants that are implicated in hematologic traits such as fetal hemoglobin levels, hematocrit, cell counts and sizes of different types of blood cells, as well as in disease susceptibility.[10] One of the best examples of the functional effect of genetic variation is a regulatory SNP that causes the blood disorder α-thalassemia. This SNP creates a new transcriptional promoter that interferes with normal transcription of α-globin genes and leads to disease development.[11] However, very few genes underlying the vast majority of these DNA variants have been uncovered and very little is known about how they contribute to the phenotypic diversity in the population.[9] *Lxn* is the first stem cell regulatory gene reported that accounts for the natural diversity of HSC function.[17] Here, also for the first time, we discovered that SNP rs31528793 is one of the DNA variants that are associated with the differential expression of *Lxn* in mouse. The *Lxn* gene is evolutionarily conserved. Since it is identified by the genetic diversity that arises through natural selection, it may physiologically regulate a function in other natural populations, such as humans. In fact, our preliminary data have indicated that there is also a negative correlation between *Lxn* level and the number of HSC and HPC in healthy humans (*C Zhang et al., 2019, unpublished data*). Therefore, *Lxn* may be involved in human hematopoiesis and there might be polymorphisms in human genome that are functionally similar to mouse SNP rs31528793. Interestingly, a recent report has shown that a SNP rs6441224 in *Lxn* promoter is associated with its expression level in humans.[46] So it would be very interesting to determine whether HMGB2 binds to this SNP-containing promoter region and regulates human *Lxn* and HSC function. These would become very useful genetic markers for screening of transplantation donors with a larger stem cell reservoir or for prediction of better recovery of cancer patients from the therapy-induced BM and stem cell suppression.

Acknowledgments

The authors are supported by the National Heart, Lung, and Blood Institute of the National Institutes of Health under awards R01HL124015(YL), R21HL140213 (YL), UKY-CCTS UL1TR001998 (UKY-CCTS pilot), and the Markey Cancer Center's Biostatistics and Bioinformatics Shared Resource Facility as well as the Flow Cytometry Shared Resource Facility (P30CA177558). We thank the Markey Cancer Center's Research Communications Office for editing and graphics support.

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