The SKI proto-oncogene enhances the *in vivo* repopulation of hematopoietic stem cells and causes myeloproliferative disease

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ABSTRACT

The proto-oncogene SKI is highly expressed in human myeloid leukemia and also in murine hematopoietic stem cells. However, its operative relevance in these cells remains elusive. We have over-expressed SKI to define its intrinsic role in hematopoiesis and myeloid neoplasms, which resulted in a robust competitive advantage upon transplantation, a complete dominance of the stem and progenitor compartments, and a marked enhancement of myeloid differentiation at the expense of other lineages. Accordingly, enforced expression of SKI induced a gene signature associated with hematopoietic stem cells and myeloid differentiation, as well as hepatocyte growth factor signaling. Here we demonstrate that, in contrast to what has generally been assumed, the significant impact of SKI on hematopoiesis is independent of its ability to inhibit TGF-beta signaling. Instead, myeloid progenitors expressing SKI are partially dependent on functional hepatocyte growth factor signaling. Collectively our results demonstrate that SKI is an important regulator of hematopoietic stem cell activity and its overexpression leads to myeloproliferative disease.

Introduction

The proto-oncogene SKI, a v-ski avian sarcoma viral oncogene homolog,[1] is highly expressed in patients with chronic myeloid leukemia (CML) and acute myeloid leukemia (AML).[2,3] More recently, recurrent mutations that cluster in the SKI homologous region of SETBP1 have been reported in patients with atypical CML, chronic neutrophilic leukemia (CNL) and chronic myelomonocytic leukemia (CMML).[4] SKI is expressed at low levels in all normal adult and embryonic tissues including hematopoietic cells,[5] and is highly expressed in murine T cells and hematopoietic stem cells (HSC).[6-8] Furthermore, SKI was shown to enhance HSC activity *ex vivo* in a non-cell-autonomous manner in a gain-of-function screen.[9,10] Deletion of Ski in mice results in death around birth due to defects in neurulation and skeletal muscle development. While SKI-deficient mice suffer from blood loss, no hematologic differences were reported between wild-type and mutant embryos.[11]

SKI is a transcriptional co-repressor inhibiting transcription of target genes downstream of the Transforming Growth Factor-β (TGF-β) superfamily[12] which includes TGF-β, Activin and Bone Morphogenetic Protein (BMP). Generally, Smad2, 3 and 4 act downstream of the TGF-β and Activin receptors, whereas Smad1, 5, 8 and 4 primarily mediate BMP signals.[13] SKI does not bind to DNA directly,[14] but when associated with active Smad-complexes it blocks their ability to initiate gene expression.[15-21] We have recently demonstrated that the Smad-signaling circuitry is intimately linked to HSC regulation,[22-25] suggesting that SKI may play a role in controlling hematopoiesis. To generate a model for investigation of the intrinsic role of SKI in hematopoiesis and its potential role in myeloid neoplasms, we have over-expressed human SKI in adult murine hematopoietic stem and progenitor cells. Here we show that enforced expression of SKI results in activation of HSC transcriptional programs and an HSC autonomous repopulating advantage that favors myeloid development from an early progenitor stage, ultimately resulting in a myeloproliferative disorder. It has been assumed that effects of SKI are determined through its role as a transcriptional co-repressor downstream of the TGF-β superfamily. Importantly, we demonstrate here that the role of SKI in regulating adult murine hematopoiesis is independent of its ability to inhibit TGF-β signaling. Instead, myeloid progenitor cells expressing SKI are reliant on functional hepatocyte growth factor (HGF) signaling.

Methods

Virus and infections

The SKI[15] and ARPG mutant[26] constructs were cloned into the murine stem cell (MSCV)-based MIG vector, and virus was produced.[27] Pre-stimulated stem and progenitor cell-enriched bone marrow (BM) was cultured on retronectin-coated plates (Takara Bioscience, Clontech) pre-loaded with viral supernatant (1-2 hits).

Mice and transplantations

Mice were bred and maintained at BMC Lund University, Sweden, or obtained from A.R.C., WA, Australia, and kept

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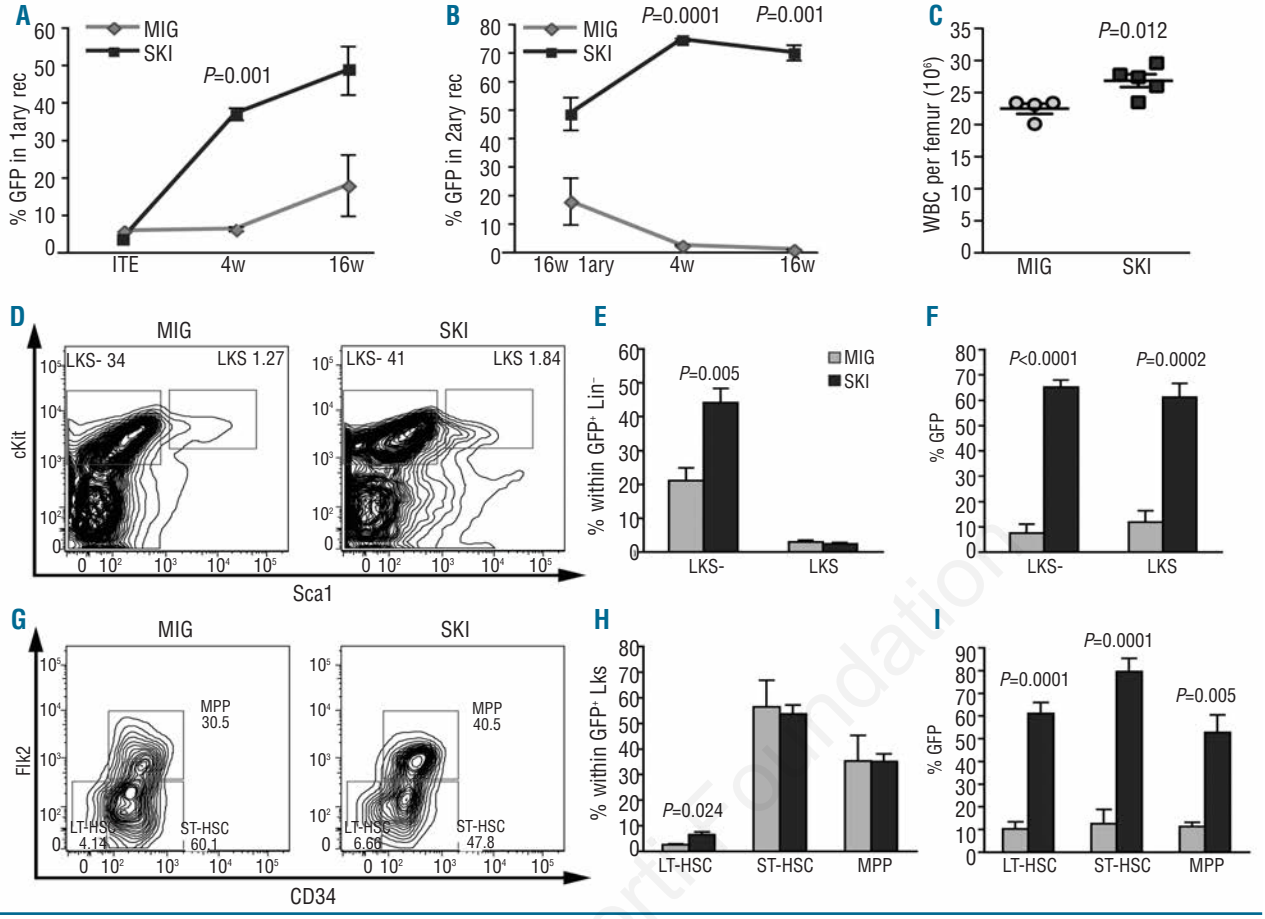

Figure 1. Enforced expression of SKI results in an extensive competitive advantage and increased numbers of phenotypic HSCs. (A-B) Donor contribution in PB in primary and secondary recipients at the indicated time points after transplantation of one representative transduction (for additional transductions see *Online Supplementary Figure 1*). ITE: initial transduction efficiency. (C) White blood cell count (WBC) in BM at 16 weeks post transplantation. (D-I) Phenotypic analysis of hematopoietic stem and progenitors using FACS and the indicated surface markers. (D-F) Fractionation of lineage- BM into the lin- c-kit$^+$ Sca1$^+$ (LKS) population enriched for stem cells, and LKS$^-$ cells containing myloerythroid progenitors. (G-I) Further subdivision of LKS into long-term HSCs (LT-HSC), short-term HSCs (ST-HSC) and multipotent progenitors (MPP). (F, I) The contribution of GFP$^+$ cells to each individual stem and progenitor populations. The data are presented as mean ± S.E.M. (n=4 for MIG and 5 for SKI).

at B.R.C. St Vincent's Hospital, Australia. All experiments were performed under the ethical guidelines of the Lund University or St Vincent's Health Melbourne animal ethics committees. 0.1-5x10^5 unsorted transduced cells from C57Bl/6 mice (BalbC in *Online Supplementary Figure S3*) were injected intravenously (IV) with 1-2x10^5 fresh whole BM cells into lethally irradiated recipients (8-12 weeks old, tracked using CD45.1/CD45.2). Cells from multiple transductions (n>8 for MIG and SKI; n=3 for ARPG) were transplanted into 4-6 recipients/transduction. Recipients were sacrificed after 16 weeks and the equivalent of half a femur of BM was transplanted into secondary and tertiary recipients. One cohort of mice was kept for 18 months.

Cell preparations and flow cytometry

Peripheral blood (PB), BM and spleen were analyzed on a Sysmex (Boule Medonice CA 530-16, or Sysmex KX-21N, Roche Diagnostics, Australia). FACS analysis was performed on a FACS Calibur or LSRFortessa, while cell sorting was performed on a FACS Diva or FACS Aria (BD, San Jose, CA, USA). Results were analyzed with FlowJo software, v.8.8.6 (Tree Star, Ashland, OR, USA).

Hematopoietic progenitor assays

To analyze TGF-β sensitivity, 200 GFP+ cells/mL were seeded in methylcellulose (M3231, Stem Cell Technologies), supplemented with 50 ng/mL mSCF, 10 ng/mL mIL-3, and 10 ng/mL hIL-6, with or without 10 ng/mL TGF-β (all from PeproTech, Rocky Hill, NJ, USA) (n=2 transductions). Unsorted BM cells from 16 weeks post-transplantation were plated for colonies; 30,000 cells/mL as above for myeloid and 50,000 cells/mL for pre-B-cell colonies (M3630). For HGF-analysis 250 sorted GFP+ cells/mL were seeded in methylcellulose with 50 ng/mL mSCF, 10 ng/mL mIL-3, 50 ng/mL hIL6, and 3 U/mL rhEpo (Jansen-Cilag) together with 0, 100 nm or 300 nm of MET Inhibitor III (Calbiochem cat. n. 448105) (n=3 separate transductions). All colonies were scored on Day 7.

Morphology

PB smears and BM cytospins were stained with May Grünwald (Merck) and Giemsa (BDH). Megakaryocytes were scored in paraffin-embedded sections of decalcified tibiae stained with Mayer's hematoxylin and eosin (n=4 for MIG; n=5 for SKI). Images were taken using an

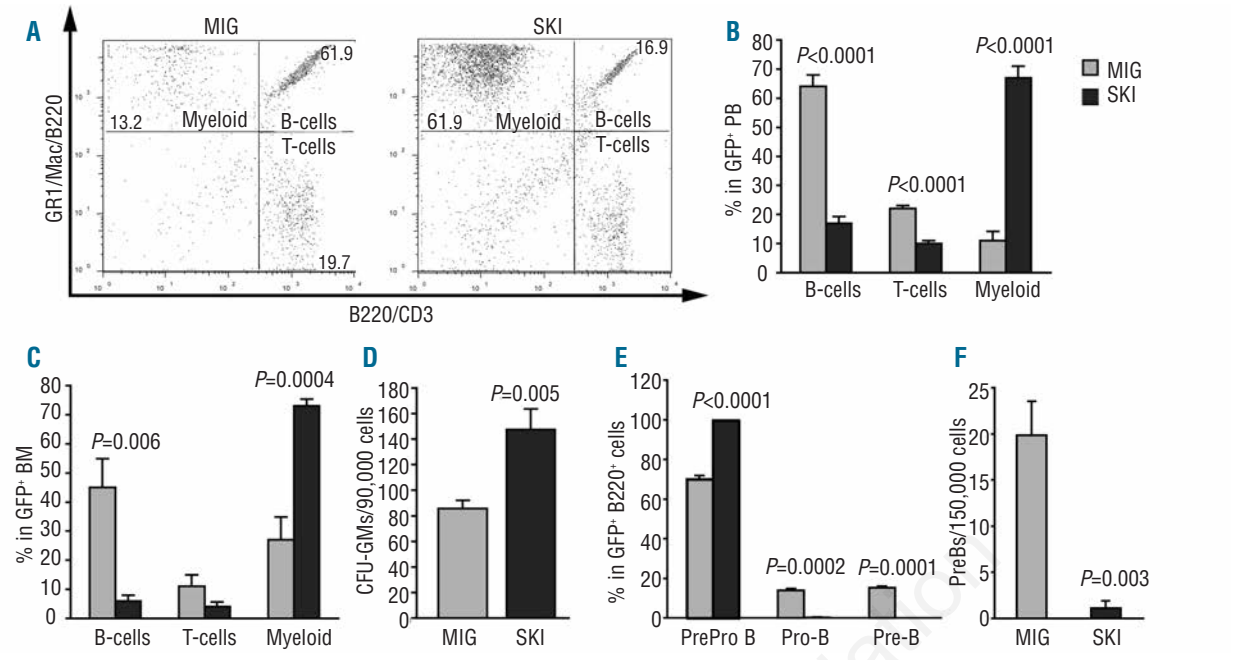

Figure 2. Increased levels of SKI result in skewing towards myeloid differentiation at the expense of lymphoid development. FACS analysis of lineage distribution in (A-B) PB and (C) BM, using the indicated markers. (D) Myeloid colony formation using unsorted BM cells from primary recipients. (E) FACS analysis of B-cell development in GFP$^+$ B220$^+$ BM cells from secondary recipients (PreProB; CD43$^+$CD19$^-$, ProB; CD43$^+$CD19$^+$, PreB; CD43$^-$CD19$^+$, n=4 for MIG and 5 for SKI). (F) Pre-B-cell colony formation using unsorted BM from primary recipients. Data were collected 16 weeks post transplantation and are presented as mean ± S.E.M. (n=8 for MIG and 9 for SKI if not stated otherwise).

Olympus BH2 microscope and a MagnaFire-SP Optronics Digital Camera.

Gene expression profiling

Quantification of RNA was made on GFP+ BM cells using the Taq-Man™ System (Applied Biosystems, Foster City, CA, USA). For microarray, total RNA was extracted from GFP+ LKS cells using the RNeasy Micro kit (Qiagen), and hybridized to the Illumina Mouse WG-6 v.2.0 Expression BeadChip at the Ramaciotti Centre for Gene Function Analysis, University of NSW, Australia (n=6 independent transductions). For Illumina data analysis see the *Online Supplementary Methods*, differentially expressed genes in *Online Supplementary Appendix File 1*, enriched gene sets in *Online Supplementary Appendix File 2*, and raw and processed microarray data in GEO (*http://www.ncbi.nlm.nih.gov/geo/accession GSE39457*).

Statistical analysis

All data are presented as mean±SEM. Significance was determined using two-tailed Student's t-test.

Results

Enforced expression of SKI results in an extensive competitive advantage and increased numbers of phenotypic HSCs

To investigate the role of SKI in murine hematopoiesis we over-expressed human SKI or an empty vector (MIG) using retrovirus in BM enriched for hematopoietic stem and progenitor cells (*Online Supplementary Figure S1A*). SKI mRNA and protein was readily detected, and demonstrated a 3.1-fold increase over endogenous at the protein level (*Online Supplementary Figure S1B and C*). Furthermore, sensitivity to TGF-β-induced inhibition of colony formation was significantly impaired, demonstrating that the over-expressed SKI was functional (*Online Supplementary Figure S1D*). When transplanted competitively into lethally irradiated recipients, SKI-transduced cells demonstrated a significant repopulating advantage compared to vector control cells, apparent as early as four weeks post-transplant (Figure 1A and *Online Supplementary Figure S1E*). This competitive advantage was further enhanced in secondary recipients (Figure 1B and *Online Supplementary Figure S1F*) and maintained in tertiary recipients (*Online Supplementary Figure S1G*), indicating that overexpression of SKI enhances the self-renewal capacity of HSCs. Additionally, SKI overexpression resulted in a trend toward increased numbers of white blood cells (WBC) in the peripheral blood (PB) (*Online Supplementary Figure S1H*), as well as increased BM (Figure 1C) and spleen (*Online Supplementary Figure S1I*) cellularity. The spleen weight of SKI over-expressing mice was 2-fold that of control mice (*Online Supplementary Figure S1J*).

Phenotypic characterization of the stem and progenitor cell compartments following SKI overexpression demonstrated normal numbers of the stem and progenitor enriched lineage-cKit+Sca1$^+$ (LKS) population, while the LKS$^-$ population containing more mature progenitors was expanded 2-fold (Figure 1D-F). Further subdivision of GFP$^+$ LKS cells into long-term HSCs, short-term HSCs, and multi-potent progenitors using CD34 and Flk2 revealed a 2.4-fold increase in the frequency of phenotypic long-term HSCs (Figure 1G-I), consistent with the extensive repopulating advantage

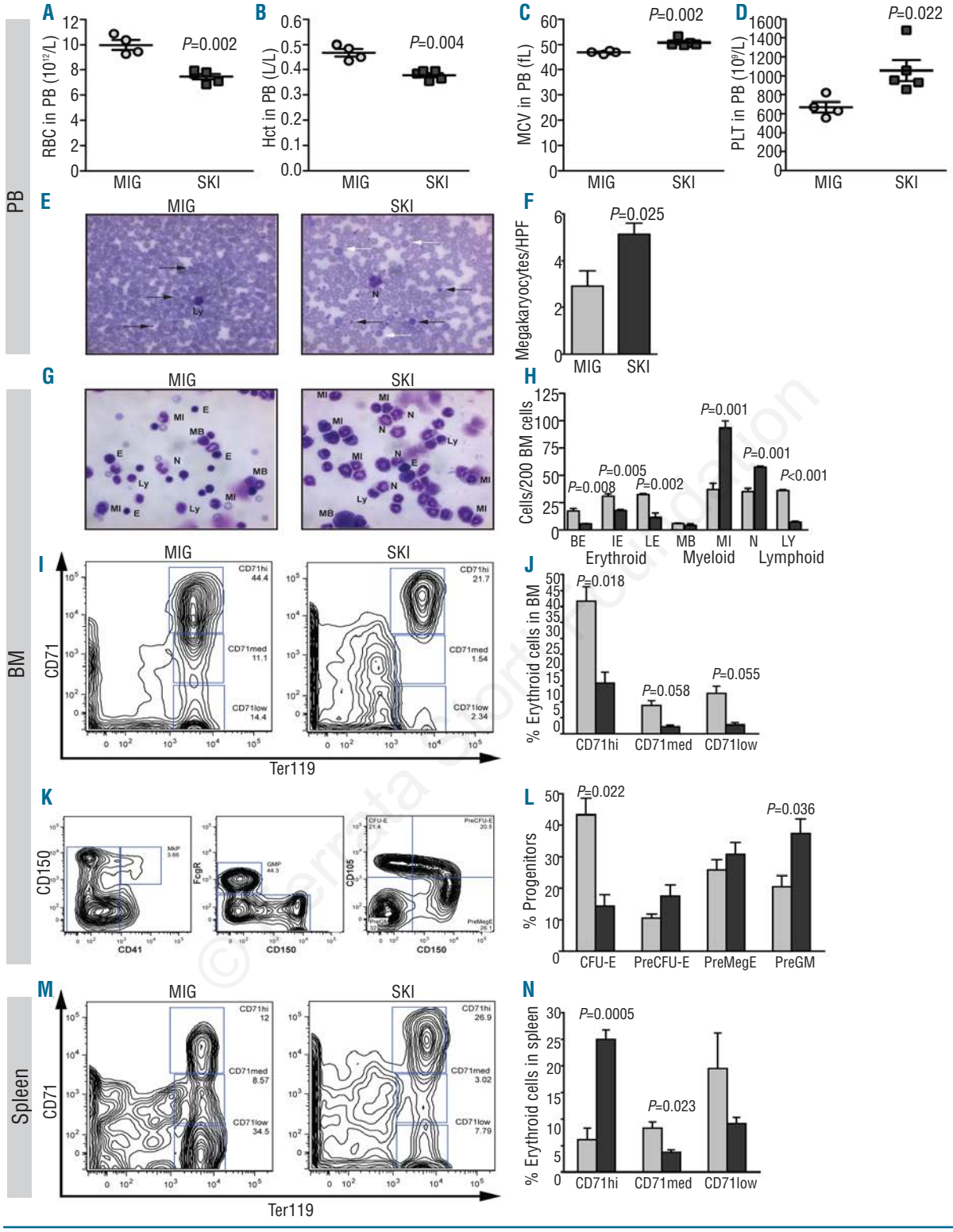

Figure 3. SKI overexpression results in impaired BM erythropoiesis and compensatory stress erythropoiesis in the spleen. (A) Red blood cell count (RBC), (B) hematocrit (Hct), (C) mean cellular volume (MCV) and (D) platelet count (PLT) in PB at 14 weeks. Morphological analysis of (E) blood smears; black arrows: platelets; white arrows: polychromatic erythrocytes (original magnification 400x). (F) Differential count of megakaryocytes in hematoxylin and eosin stained BM sections (original magnification 200x). (G-H) Cytospins of unsorted BM from primary recipients (original magnification 400x). E: erythroid; M: myeloid; N: neutrophil; Ly: lymphocyte; B: blast; I: intermediate; L: late; HPF: high power field. (I-L) FACS analysis of erythroblasts in GFP+ BM. (K) Gating strategy for fractionation of early myeloerythroid progenitor subsets in BM into megakaryocyte progenitors (MkP), myeloid progenitors (GMP), and (L) CFU-Es, pre-CFU-E, PreMegE and PreGM progenitors. (M-N) FACS analysis of erythroblasts in GFP+ spleen. Data were collected 16 weeks post transplantation if not stated otherwise and are presented as mean ± S.E.M (n=4-5 for MIG and 5 for SKI).

seen after transplantation. Fractionation of the LKS$^-$ population revealed no major shifts within the myeloerythroid progenitor population, although the percentage of granulocyte-monocyte progenitor (GMP) was slightly elevated (*Online Supplementary Figure S2A* and *B*). Furthermore, SKI overexpression resulted in a 50% increase in the common lymphoid progenitor (CLP) compared to control (*Online Supplementary Figure S2C and D*). Strikingly, SKI over-expressing cells gave rise to the vast majority of all stem and progenitor populations (Figure 1F and I). Taken together, these results indicate that SKI expressing HSCs and progenitors had a profound competitive advantage.

SKI favors myeloid differentiation at the expense of lymphoid development

Analysis of lineage distribution revealed a bias toward production of myeloid cells at the expense of lymphoid cells in mice over-expressing SKI that was observed in all hematopoietic organs analyzed including PB (Figure 2A and B), BM (Figure 2C) and spleen (*Online Supplementary Figure S2E*). This aberrant lineage distribution persisted in secondary and tertiary recipients (*data not shown*). Accordingly, there was a substantial increase in the number of myeloid colony-forming cells in SKI over-expressing BM (Figure 2D).

The percentages of both B- and T-lymphoid cells were significantly reduced following SKI expression. Further fractionation of B220$^+$ BM cells using the markers CD19 and CD43 revealed a profound impairment in B-cell differentiation after the pre-pro-B-cell stage (Figure 2E). Consistent with this, the numbers of pre-B-colony-producing cells in SKI over-expressing BM was greatly reduced (Figure 2F), demonstrating that SKI had a cell intrinsic inhibitory effect on B-cell differentiation. On the contrary, analysis of T-cell development in the thymus revealed that, although very few SKI over-expressing cells contributed to the thymus (Figure S2F), they were able to form all progenitor populations. SKI over-expressing cells even displayed a slight increase in the percentage of early double negative (DN) and DN1 populations, whereas they formed fewer CD8 single positive cells as compared to control (*Online Supplementary Figure S2G and H*). Many of these seem to be immature single positive CD8 cells since the majority of them do not co-express the mature T-cell marker CD3 (*data not shown*). In conclusion, SKI favors myeloid differentiation at the expense of lymphoid development.

SKI overexpression impairs erythropoiesis

SKI has been shown to negatively regulate erythroid development through direct interaction with GATA1.[28] Accordingly, SKI overexpression resulted in macrocytic anemia with a 25% decrease in red blood cell counts and reduced hematocrit (Figure 3A-C), as well as thrombocytosis (Figure 3D). PB morphology demonstrated that SKI over-expressing mice displayed elevated numbers of circulating polychromatic erythrocytes consistent with reticulocytosis (Figure 3E), and a greater than normal variation in size and shape of platelets (Figure 3E). Histological analysis of BM sections revealed increased numbers of megakaryocytes relative to control (Figure 3F and *Online Supplementary Figure S3A*), in keeping with the increased number of circulating platelets. We also noticed a decrease in adipocytes in the BM (*Online Supplementary Figure S3B*), potentially due to the increase in total number of hematopoietic cells. The erythroid maturation was impaired at an early erythroblast stage in the BM as determined by both morphology (Figure 3G and H) and FACS analysis (Figure 3I and J). Further fractionation of LKS- cells into distinct myeloerythroid progenitor populations (Figure 3K)[29] demonstrated an 83% increase in the frequency of pre-GM progenitors (Figure 3L) in SKI over-expressing mice, and a 67% decrease in the frequency of erythroid CFU-Es (Figure 3L) consistent with the observed anemia. Compensatory stress erythropoiesis with increased CD71$^+$/Ter110$^+$ erythroblasts was observed in the spleen (Figure 3M and N). Although mature erythroid cells can lose their GFP expression upon enucleation, complicating the analysis of the Ter119$^+$CD71^{low} cells, this would be the same for MIG and SKI and should not affect the comparative result. Collectively these analyses demonstrate that SKI overexpression resulted in the development of a myeloproliferative-like disease in mice affecting myeloid, erythoid/megakaryocyte and lymphoid lineages.

SKI-mediated myeloid expansion is modulated by genetic background

Although there was a substantial increase in the numbers of myeloid cells in both PB and BM, total cell counts in these organs were only mildly elevated (Figure 1C and *Online Supplementary Figure S1F*). Overexpression of SKI resulted in mildly disordered myeloid differentiation with occasional immature myeloid cells in the PB (*Online Supplementary Figure S1C*) and an expanded pool of intermediate myeloid cells in the BM (Figure 3H). However, no significant increase in blasts was seen, even though the number of BM blasts was slightly elevated in secondary recipients as compared to primary (*Online Supplementary Figure S3D and E*). Hence, there was no evidence of overt leukemia, and granulocyte production and turnover remained coupled consistent with skewing toward myeloid cell fate at the expense of lymphoid differentiation.

To further investigate the leukemia-inducing potential of SKI, we over-expressed it on Balb/c background, which is more susceptible to myeloid disease than C57BL/6 mice.[30] The phenotype was more pronounced with a rapidly developing myeloproliferative disease characterized by a 2-fold increase in PB leukocyte counts (*Online Supplementary Figure S4A-C*), an extensive increase in PB blasts (*Online Supplementary Figure S4D*), granulocytic hyperplasia in the BM with more than 90% of granulocytes derived from SKI-transduced cells (*Online Supplementary Figure S4E-G*), a 3.8-fold increase in splenic size (*Online Supplementary Figure S4H*), and a vast increase in myeloid cells in the spleen (*Online Supplementary Figure S4I*). However, no leukemia was observed.

SKI activates a stem cell signature and genes involved in myeloid differentiation and HGF signaling

To elucidate the mechanism of the effects of SKI, we performed gene expression analysis. Elevated levels of SKI resulted in a significant change in the expression of a large number of genes, a subset of which was con-

firmed by qPCR (Figure 4A and B). Gene set enrichment analysis (GSEA) was performed to determine if specific pathways were deregulated by SKI.[31] As anticipated, the TGF-β signaling pathway was repressed in cells over-expressing SKI (Figure 4C). Additional gene sets negatively regulated by SKI included a number of pathways associated with glucose metabolism and gluconeogenesis (Figure 4C).

In addition, a number of gene sets up-regulated in response to SKI were highly consistent with the *in vivo* phenotype. There was an enrichment of pathways associated with HSCs from both mouse and human (Figure 4D).[32-34] Furthermore myeloid gene sets were activated within the LKS population, which may contribute to myeloid lineage priming and the myeloproliferative phenotype (Figure 4D).[35] Specific examples of myeloid genes up-regulated in response to SKI were bone marrow X-linked kinase (BMX), previously reported to be expressed and activated in neutrophils,[36] as well as Tribbles homolog 2 (Trib2), a myeloid oncoprotein recently described to induce AML in cooperation with HoxA9 (Figure 4B).[37] In addition, a significant number of gene sets associated with AML and MLL were enriched in SKI over-expressing LKS (*Online Supplementary Figure S5*).[38,39] Interestingly, the HGF signaling pathway was significantly up-regulated in response to SKI (Figure 4D), which was confirmed by qPCR (Figure 4B).

The effects of SKI are independent of TGF-β signaling

In accordance with the gene expression analysis, the ability of SKI to inhibit TGF-β signaling has been implicated as the main mechanism underlying its transforming capacity.[40] To determine whether the impact of SKI is dependent on its ability to inhibit TGF-β, we used the ARPG mutant form of SKI, which has lost its ability to interact with N-CoR and Smad4.[19,26,41] Hence, the ARPG mutant can still bind Smad2/3 and inhibit TGF-β and Activin signaling, but can no longer prevent BMP signaling (Figure 5A). Accordingly, enhanced expression of the ARPG mutant protected against TGF-β-mediated growth retardation to the same extent as SKI (Figure 5B). However, ARPG mutant over-expressing cells had no repopulating advantage compared to control (Figure 5C) and contributed normally to all lineages in both primary (Figure 5D) and secondary (*data not shown*) recipients. Mice transplanted with ARPG mutant over-expressing cells also displayed normal phenotypic stem and progenitor populations in the BM (Figure 5E), and the contribution of transduced cells to these primitive cell fractions was similar to control mice (Figure 5F). This was not due to lower levels of

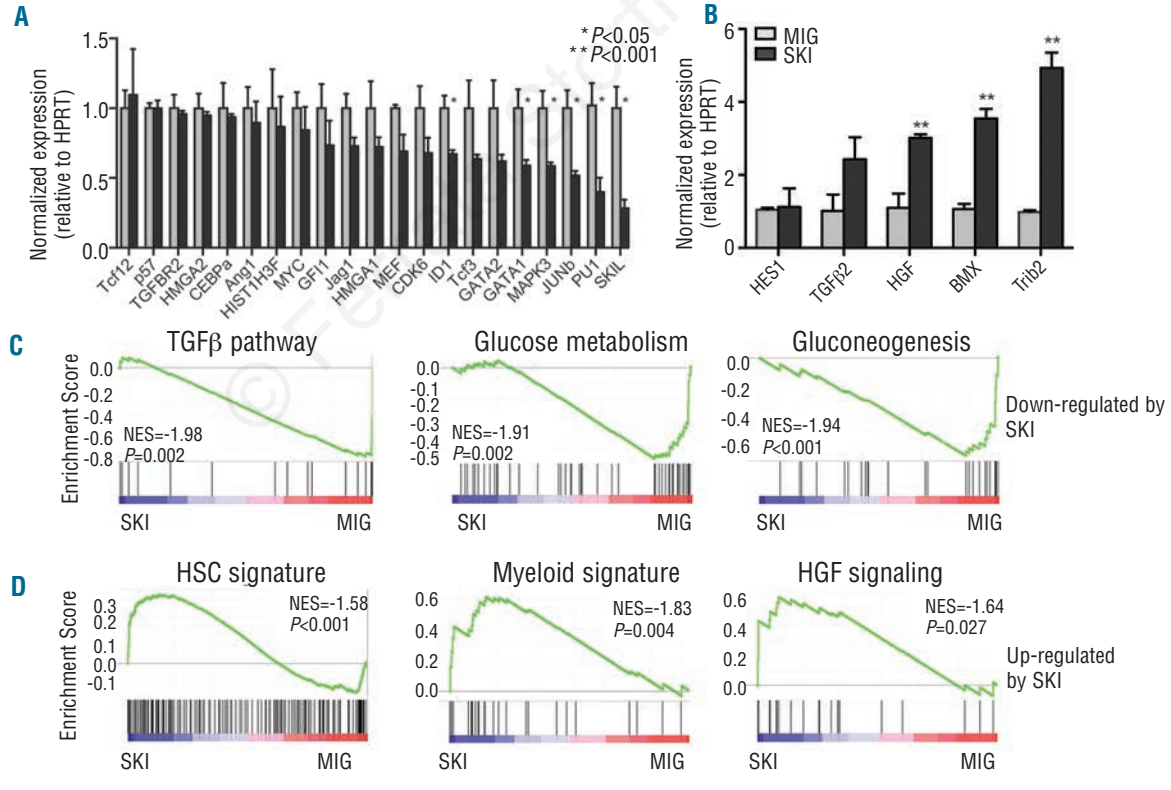

Figure 4. SKI induces a stem cell signature and activates gene expression involved in myeloid differentiation and HGF signaling. (A) Real-time PCR validation of genes that are down-regulated and (B) up-regulated by SKI (performed on LKS cells from 3 transductions separate from those used for the array). (C) Gene set enrichment analysis (GSEA) determining specific pathways that are negatively regulated by SKI including the TGF-β pathway and pathways associated with glucose metabolism and gluconeogenesis, and (D) up-regulated in response to SKI including gene sets enriched in HSCs, myeloid cells and hepatic growth factor (HGF) signaling. The data are presented as mean ± S.E.M (n=6 independent transductions), q-values<0.25 were considered significant for GSEA.

overexpression, since the ARPG mutant was even 2 and 3 times more highly expressed than SKI at the mRNA and protein level, respectively (*Online Supplementary Figure S6A-C*). This suggests that the effect of overexpression of SKI is independent of its ability to inhibit TGF-β and Activin signaling.

Since SKI inhibits BMP signaling whereas the ARPG mutant does not,[19] one hypothesis could be that inhibition of canonical BMP signaling might be the underlying mechanism for the SKI phenotype. However, in a previous study, we had generated a conditional Smad1/Smad5 double knockout mouse with complete disruption of canonical BMP signaling, demonstrating that Smad1/Smad5 deficient BM cells display unaffected self-renewal and differentiation capacity upon transplantation.[42,43] Canonical BMP signaling is hence dispensable for adult murine hematopoiesis, suggesting that inhibition of BMP signaling is not likely to be the primary cause of the phenotype observed when overexpressing SKI.

SKI-induced myeloproliferation is partially mediated by HGF signaling

SKI over-expressing cells had evidence of an activated HGF pathway by transcriptional profiling (Figure 4C and E). To determine whether autocrine HGF signaling played a role in SKI-induced myeloproliferation, we performed colony assays of sorted GFP+ LKS cells with increasing concentrations of MET inhibitor, inhibiting the receptor tyrosine kinase that mediates HGF signaling (Figure 5G). While the MET inhibitor had no effect on the colony forming ability of LKS cells expressing the control vector, SKI-induced colony formation was impaired in a dose-dependent manner. This confirms that SKI acts via mechanisms that include elevated HGF signaling (Figure 5H). In summary, overexpression of

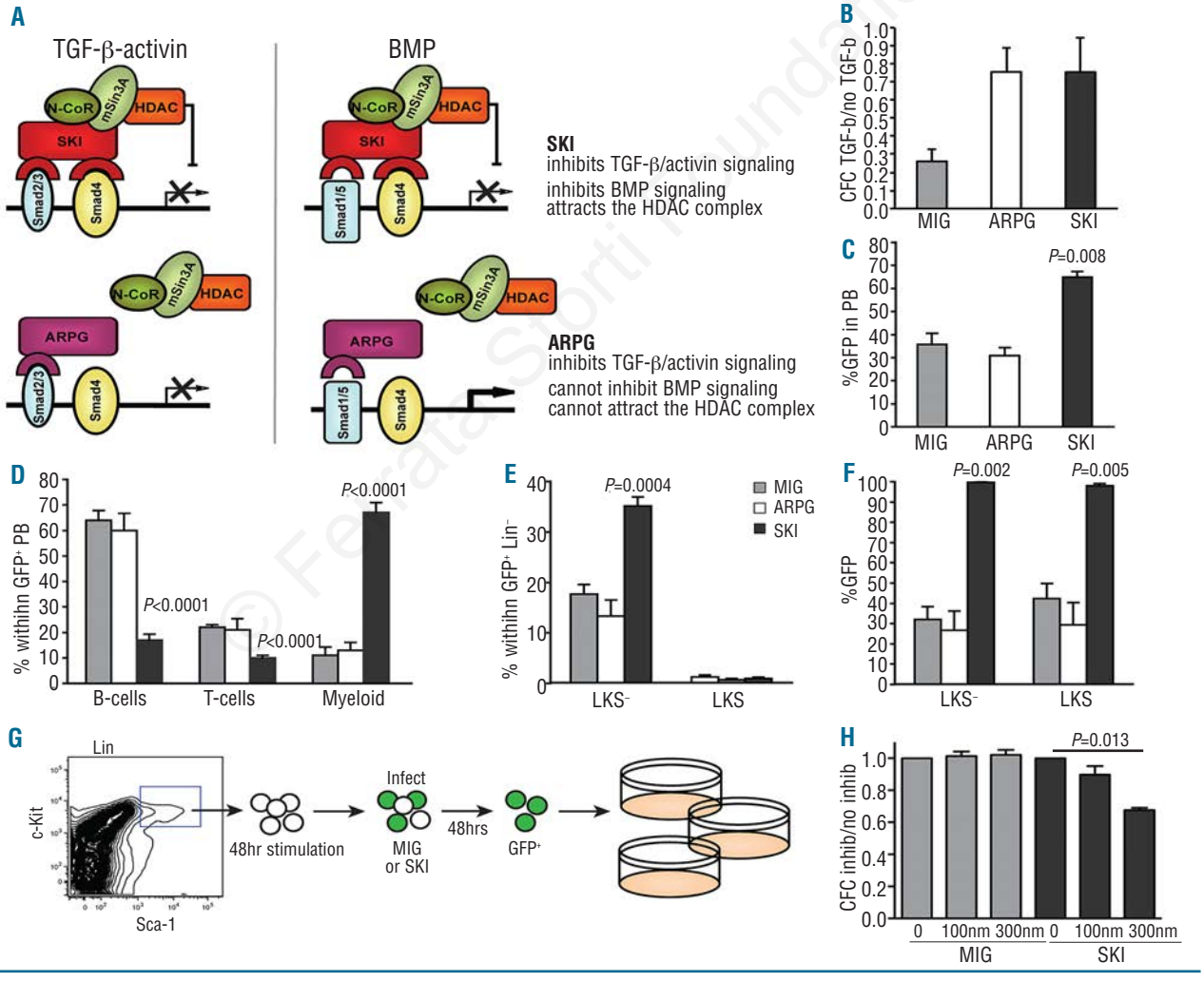

Figure 5. The effect of SKI on the hematopoietic system is partially dependent on HGF-signaling, but independent of its ability to inhibit TGF-β. (A) Schematic figure illustrating the inhibiting actions of SKI and the ARPG mutant (modified from Takeda *et al.*[19]). (B) Colony assay measuring TGF-β sensitivity plating GFP+ cells with or without 10 ng/mL TGF-β (n=2 independent transductions). FACS analysis of (C) donor reconstitution, (D) lineage distribution within GFP+ PB (n=8, 9 and 9 for MIG, ARPG and SKI, respectively, 2 separate transductions), (E) the percentage of stem and progenitor cells within GFP+ BM (LKS+/-; lineage- c-kit+ Sca1+/-), and (F) the contribution of transduced cells to each of these populations. Data were collected 16 weeks post-transplant and n=4, 4 and 5 for MIG, ARPG and SKI, respectively, if not stated otherwise. (G) Experimental outline of HGF signaling studies (n=3 independent transductions). (H) Colony forming assay plating GFP-sorted MIG or SKI transduced LKS cells with increasing concentrations of MET inhibitor.

SKI in early hematopoietic stem and progenitors resulted in an expansion of phenotypic HSC populations and a profound competitive advantage, accompanied by myeloproliferation at the expense of both lymphoid and erythroid differentiation. On a molecular level, enforced expression of SKI induced a gene signature associated with HSCs, myeloid differentiation and HGF signaling. Importantly, the significant impact of SKI on the hematopoietic system was independent of its ability to inhibit TGF-β signaling, while myeloid progenitors expressing SKI were dependent on functional HGF signaling.

Discussion

The oncogene SKI, best known as a transcriptional co-repressor downstream of TGF-β signaling, is highly expressed in myeloid leukemias. To define its role in hematopoietic malignancies we over-expressed SKI, which resulted in a profound competitive advantage and myeloproliferation at the expense of both erythroid and lymphoid development. This myeloid-biased phenotype was apparent already at the early progenitor stage. Importantly, we were able to demonstrate that the SKI phenotype is independent of TGF-β signaling using the ARPG mutant form of SKI.

SKI over-expressing cells gave rise to the majority of all stem and progenitor cells and gene expression analysis indicated that HSC associated gene programs were activated in SKI expressing cells. Furthermore, SKI overexpression led to increased numbers of phenotypic long-term HSCs and an extensive repopulating advantage after serial transplantation. Together this indicated that SKI is important for regulating hematopoiesis at the stem cell level in a cell-intrinsic manner, in addition to its previously demonstrated non-cell-autonomous effect on HSCs.[9,10]

SKI expression is up-regulated in both CML and AML,[2,3] and analysis of gene expression changes in LKS cells at an early time point after SKI overexpression revealed enrichment of several gene sets associated with leukemia. Furthermore, enhanced expression of SKI resulted in altered expression of genes involved in glucose metabolism and gluconeogenesis, suggesting that expression of SKI in HSCs may play a role in the metabolic reprogramming associated with oncogenesis (the Warburg effect).[44,45] Interestingly, enforced SKI expression also correlated with increased HGF mRNA levels, and inhibition of HGF signaling via the receptor MET selectively impaired the proliferation of SKI overexpressing colony forming cells. It was recently demonstrated that AML cells are dependent on autocrine MET signaling achieved in part by upregulation of HGF production.[46] HGF signaling is also implicated in mediating stromal resistance to tyrosine kinase inhibitors in solid tumors.[47] Elevated HGF following SKI overexpression significantly contributed to the proliferative effects we observed and may play an important role in elevated SKI expression in human cancer. However, further investigation is warranted to decipher the connection between SKI and HGF, and its potential role in cancer development and maintenance. In the light of the increased expression of HSC-associated signatures following SKI overexpression, it is intriguing to speculate that this represents an important aspect of the role of SKI in leukemia.

The phenotype obtained when over-expressing SKI in mice is highly consistent with a myeloproliferative neoplasm[48] which most closely resembles human CNL and shows significant overlap with other closely related chronic myeloid neoplasms such as CML, atypical CML and CMML (*Online Supplementary Table S1*). CNL is a rare form of atypical CML (negative for the Philadelphia chromosome), which is characterized by sustained, mature neutrophilic leukocytosis with few or no circulating blasts.[49] These patients usually display splenomegaly and may transform to AML. In contrast to other myeloproliferative neoplasms, little is known about the molecular alterations involved in CNL. Intriguingly, SETBP1 was recently shown to be recurrently mutated in atypical CMLs, CNL and CMML, and a striking 92% of the mutations were located within a SKI homologous region present in the SETBP1 protein.[4] Considering the upregulation of SKI in myeloid leukemia, and the striking similarity in phenotype, it is possible to speculate that myeloid proliferation and enhanced self-renewal in human myeloid neoplasms such as CNL, aCML and CMML could be due at least in part to aberrant activity of SKI. Although primary recipients were monitored for up to 18 months, and BM from one cohort of mice was transplanted into tertiary recipients (*data not shown*), none of the mice in our study developed acute leukemia. This demonstrates that SKI itself is sufficient for the development of a myeloproliferative syndrome, but requires additional co-operative mutations to progress from chronic phase to blast phase.

In conclusion, we show that SKI is an important regulator of HSC activity and that its overexpression leads to myeloproliferative disease most similar to CNL, in keeping with the involvement of SETBP1 mutations in the SKI homologous region in human CNL and other closely related myeloid neoplasms. Importantly, in contrast to what has generally been assumed, the significant impact of SKI on hematopoiesis is independent of its ability to inhibit TGF-β signaling. Instead, we demonstrate that myeloid progenitor cells expressing SKI are dependent on functional HGF signaling.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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