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Abstract: The hematopoietic stem cell (HSC) compartment consists of a small pool of cells capable of replenishing all blood cells. Although it is established that the hematopoietic system is assembled as a hierarchical organization under steady-state conditions, emerging evidence suggests that distinct differentiation pathways may exist in response to acute stress. However, it remains unclear how different hematopoietic stem and progenitor cell subpopulations behave under sustained chronic stress. Here, by using adult transgenic mice overexpressing erythropoietin (EPO; Tg6) and a combination of in vivo, in vitro, and deep-sequencing approaches, we found that HSCs respond differentially to chronic erythroid stress compared with their closely related multipotent progenitors (MPPs). Specifically, HSCs exhibit a vastly committed erythroid progenitor profile with enhanced cell division, while MPPs display erythroid and myeloid cell signatures and an accumulation of uncommitted cells. Thus, our results identify HSCs as master regulators of chronic stress erythropoiesis, potentially circumventing the hierarchical differentiation-detour.

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Hematopoietic stem cells but not multipotent progenitors drive erythropoiesis during chronic erythroid stress in EPO transgenic mice

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Running title: EPO induces transcriptional rewiring in HSCs.
SUMMARY

The hematopoietic stem cell (HSC) compartment consists of a small pool of cells capable of replenishing all blood cells. Although it is established that the hematopoietic system is assembled as a hierarchical organization under steady-state conditions, emerging evidence suggests that distinct differentiation pathways may exist in response to acute stress. However, it remains unclear how different hematopoietic stem and progenitor cell subpopulations behave under sustained chronic stress. Here, by using adult transgenic mice over-expressing erythropoietin (EPO; Tg6) and a combination of in vivo, in vitro, and deep sequencing approaches, we found that HSCs respond differentially to chronic erythroid stress than their closely related multipotent progenitors (MPPs). Specifically, HSCs exhibit a vastly committed erythroid progenitor profile with enhanced cell division, while MPPs display erythroid and myeloid cell signatures and an accumulation of uncommitted cells. Thus, our results identify HSCs as master regulators of chronic stress erythropoiesis, potentially circumventing the hierarchical differentiation-detour.
INTRODUCTION

All lineages of hematopoietic cells, including those of the erythroid lineage, arise from a unique population of hematopoietic stem cells (HSCs) (Clapes et al., 2016). However, whether all mature blood cells are derived from these HSCs or from downstream multipotent progenitors (MMP) during steady state hematopoiesis remains contentious (Busch et al., 2015; Sawai et al., 2016; Sun et al., 2014). Previous work in the field of hematopoietic stem cell biology has mainly focused on acute stress, including bone marrow injury or infection (Haas et al., 2015), which typically triggers an increase of HSCs that enter the cell cycle; HSCs return to quiescence when homeostasis is re-established (Baldridge et al., 2010; Trumpp et al., 2010). Interestingly, a number of studies have also suggested that HSCs and MPPs can circumvent these distinct intermediate stages (Haas et al., 2015; Pietras et al., 2015; Sanjuan-Pla et al., 2013; Yamamoto et al., 2013).

Recently, attention is being drawn to the chronic effects of emergency signals (such as IL-1), their impact on the immune system, and their long-term consequences on the HSC compartment (Pietras et al., 2016). However, much remains to be understood regarding how chronic over-expression of lineage-specific proteins such as erythropoietin (EPO) may influence HSC/MPP fate. The latter hormone is the principal stimulator of erythropoiesis, a complex multistep process during which erythroid progenitors develop into mature red blood cells. EPO binding to its receptor (EPO-R) activates multiple pathways, including the JAK/STAT and the MAPK-ERK1/2 pathways, which reduce apoptosis and promote expansion and differentiation of erythroid progenitors (Dunlop et al., 2006; Franke et al., 2013a; Gassmann and Muckenthaler, 2015). Increased EPO production resulting from mutations in specific members of the hypoxia inducible factor (HIF) pathway (e.g. VHL, HIF2α, or PHD2) may lead to secondary erythrocytosis, representing the aberrant increase in red blood cell numbers (Franke et al., 2013b). In this report, using the well-defined EPO transgenic mouse line (Tg6) (Ruschitzka et al., 2000), we establish that HSCs and MPPs respond differently to chronic and sustained erythroid stress. Specifically, HSCs display a substantial
erythroid progenitor profile in a quest to differentiate into erythrocytes potentially bypassing numerous downstream progenitors, while MPPs limit their internal commitment towards myeloid progenitors.

RESULTS
To understand the role of chronic and sustained erythroid stress on the entire hematopoietic compartment, we used the well-defined EPO transgenic mouse line (Tg6). Independent of the oxygen conditions, these mice constitutively show high serum levels of human EPO, resulting in excessive production of red blood cells (RBCs) (Figure S1A and (Ruschitzka et al., 2000)). The bipotent pre-megakaryocyte erythocyte progenitor (Pre-MgE), which is upstream of the more committed erythroid-restricted progenitor (Pre-CFUe), the CFUe (colony forming unit erythroid) and erythroblasts (EB), have been suggested as the first cells in the hematopoietic tree to robustly express EPO-R during hematopoiesis in humans (Seita et al., 2012). Although Tg6 mice did not display any alteration in Pre-MgE as compared to WT mice, the relative proportions of Pre-CFUe, but not CFUe, were significantly higher in Tg6 mice. Conversely, megakaryocyte progenitors (MkP) and pre-granulocyte-monocyte lineage cells (Pre-GM) were significantly reduced (Figure 1A, 1B and S1B; gating according to (Pronk et al., 2007)). Furthermore, compared to pre-CFUe, Tg6 CFUe’s were progressing significantly more through the cell cycle compared to WT CFUe’s (Figure 1C and 1D), strongly indicating a prominent proliferation/differentiation impulse towards downstream EBs ((Figure 1E and S1C); gating according to (Dumitriu et al., 2010)) and RBCs (Figure S1A). Upstream of Pre-GM, common myeloid progenitors (CMP) were also reduced in Tg6 BM (Figure 1F and S1D; gating according to (Akashi et al., 2000)). We next analyzed the HSC/MPP compartment immediately upstream of the CMPs, which consists of a heterogeneous population. Interestingly, although the HSC fraction (CD48+/CD150+ Lineage- SCA-1+ Kit+ (LSK) (= LT-HSC and MPP1 (Wilson et al., 2008)) displayed a significant increase in absolute numbers
but not in its relative abundance, the MPP population (CD48+/CD150- LSK cells (= MPP3 and MPP4 (Wilson et al., 2008)) was both relatively and absolutely expanded more than 2-fold, compared to WT littermates (Figure 2A and 2B). Together, these observations suggest a long-term surge in erythroid progenitor populations at the expense of myeloid progenitors, starting with CMP (Figure 2C).

To understand this differential response between HSCs and MPPs, and the reduction in downstream myeloid progenitors, we studied cell cycle progression in the hematopoietic stem cell progenitor (HSPC) compartment. To this end, we found that a significantly higher number of Tg6 HSCs progressed through the cell cycle compared to WT HSCs. In contrast, Tg6 MPPs were more quiescent compared to their WT counterparts (Figure S2A and 2D). As the latter displays an apparent discrepancy with the increase in total MPP numbers, we decided to define the telomere lengths in MPPs from Tg6 and WT mice, as these are a measure of the number of times a (stem) cell has divided in time (Chiu et al., 1996). Our analyses revealed significantly shorter telomeres in Tg6 MPPs than in WT MPPs, which suggests longer overall survival and subdued differentiation (less downstream progenitors) of Tg6 MPPs, despite reduced instant proliferation. Furthermore, we also examined MPPs in 4-week-old mice and found a clear trend towards lower numbers of Tg6 MPPs compared with WT littermates (Figure S2B). Conversely, we found no increased cycling of Tg6 MPPs in these 4-week-old mice (Figure S2C), although MPP cell numbers in 6-week-old animals were not different anymore (Figure S2B) and further reverted in adult mice (Figure 2B). This further supports the notion that already at an early stage, Tg6 MPPs show more preference to self-renew than to differentiate in comparison to WT MPPs.

To unravel the differential response of HSCs and MPPs to chronic and sustained EPO exposure, we performed a global transcriptome analysis, which uncovered a strong divergence between hematopoietic progenitor populations from Tg6 and WT mice (Figure 3A and S3A). Specifically, HSCs from Tg6 and WT mice revealed more than 6600 significantly differentially expressed genes
(DEGs) while that for MPPs was about 3800 DEGs (Figure 3A and S3B). Ontology analyses of genes that were at least 2-fold up-regulated in the HSCs and MPPs of Tg6 mice (Huang da et al., 2009), indicated that HSCs from Tg6 mice were significantly enriched in genes directly related to RBC production (Figure 3B). These included transcripts involved in erythrocyte structure (Dmtn, Epb4.1 and 4.2), heme biosynthesis (e.g. all 8 core members, namely Als2, Alad, Cpox, Fech, Hmbs, Uros, Urod, and Ppox; (Nilsson et al., 2009)), as well as a set of transcription factors directly related to erythropoiesis, including Gata1, E2F4, Klf1 and Sox6 (Figure 3B, 3C; (Dumitriu et al., 2010; Gutierrez et al., 2008; Humbert et al., 2000; Shah et al., 2015)). Parallel to these findings and our initial observations in Tg6 HSCs, numerous gene ontology (GO)-terms related to the “Cell Cycle Process” were uncovered, which corresponded to more than 100 Tg6 HSCs genes (≥ 2-fold) significantly over-expressed in the diverse phases of the division process (Figure 3B and 3C).

To support these results, we performed immunofluorescent staining for GATA1 on sorted cells. We stained LT-HSC and MPP1 separately (CD34− and CD34+ HSCs respectively) as it was shown previously that the latter population can give rise to stress erythroid progenitors (Harandi et al., 2010). Interestingly, both Tg6 fractions showed a significant increase in GATA1 staining compared to their respective WT control cells (Figure 3D). Further, we found an increase in all target genes with ‘GATA1-prefered sites’ that contribute to erythroid differentiation (Figure S3C; (Suzuki et al., 2013)), and a significant downregulation of Gata2 (Figure S3D), an HSC-specific transcription factor that is directly and transcriptionally repressed, and physically replaced from chromatin, by GATA1 (Bresnick et al., 2010).

In stark contrast with RNAseq data from HSPCs after acute exposure to high EPO (Grover et al., 2014), the Tg6 MPPs displayed GO-terms that are positively associated with the innate immune system (Figure 4A). Moreover, a large set of myeloid lineage markers such as mst1r (macrophage stimulating protein 1 receptor), Tlr1 (Toll-like receptor 1), Cebpb (CCAAT/enhancer-binding protein beta), and Csf1r (Macrophage colony-stimulating factor 1 receptor) were highly
upregulated, with only a less pronounced link towards erythropoiesis, especially in comparison to the erythroid genes induced in Tg6 HSCs (Figure 4B and S4A). CSF1R is a receptor for the macrophage colony-stimulating factor (M-CSF) and is known to influence HSPC differentiation into monocytes/macrophages (Stanley and Chitu, 2014). Consistently, we also demonstrate that a significantly higher fraction of Tg6 MPPs display CSF1R on their surface (Figure 4C); this is however independent of a potential increase in the myeloid-biased MPP3 cell population, as their relative percentages remained comparable between Tg6 and WT mice (Figure S4B). Additionally, we found no difference in GATA1 staining between both genotypes in a majority of their MPPs (CSF1R-), although CSF1R+ Tg6 MPPs contained significantly less GATA1 than their control littermates (Figure 4D).

To better define EPO-induced alterations in HSCs and MPPs, we used published profile data on committed progenitors (Li et al., 2014; Sanjuan-Pla et al., 2013); specifically, we evaluated the gene signatures of various lineages using gene set enrichment analyses (GSEA; (Mootha et al., 2003; Subramanian et al., 2005). GSEA revealed that both stem cells and progenitors from Tg6 mice displayed matching pre-CFUe signatures, which contrasted with the strong negative signature for the MkP gene set (Figure 5A). Downstream of the Pre-CFUe, HSCs and MPPs exhibited a positive signature for transcripts known to be upregulated in CFUe cells (CFUe ‘Up’). Remarkably, the Tg6 MPP fraction also contained a large set of significantly induced genes that, according to CFUe signature, are expected to be downregulated (NES: +3.03; CFUe ‘Down’) (Li et al., 2014), while Tg6 HSCs, showed an anticipated negative correlation for the same gene set (NES: -2.98). Interestingly, analysis of a set of 50-upregulated genes in MPPs (from 65 genes that were significantly changed and correlated with the ‘CFUe down’ signature), showed a highly significant association with monocytes, whilst the majority of the downregulated genes correlated with B-lymphocytes (Enrichr/Jensen TISSUES analysis; Figure 5B) (Kuleshov et al., 2016; Lachmann et al., 2010). Further, we compared significantly modulated Tg6 transcripts with a set of Pre-GM
genes but were able to confirm a highly significant and positive correlation with only Tg6 MPPs (Figure 5A). Together, these analyses clearly reveal that Tg6 HSCs display an exclusive erythroid signature, whereas the Tg6 MPP population exhibited erythroid as well as myeloid signatures.

As our RNAseq data revealed a significant 2-fold induction of Epo-R mRNA in Tg6 HSCs, we sought to assess the in vivo activation of the JAK/STAT and the MAPK-ERK1/2 pathways in both HSCs and MPPs using mass cytometry (Cytof). As expected, Tg6 CFUe cells showed significantly more phosphorylation of STAT5 compared to WT cells (Figure 6A); however, EPO/EPO-R pathways were not differentially activated in Tg6 HSCs, MPPs, or preMgE cells (Figure 6A, S5A and S5B). Subsequently, we performed a semi-solid colony-forming assay (no added EPO) using sorted HSCs and MPPs from both Tg6 and WT mice to understand the presence and extent of fate changes. In concurrence with our in vivo and deep sequencing data, Tg6 HSCs yielded significantly higher erythroid progenitors (BFUe), while Tg6 MPPs exhibited a lower differentiation potential towards granulocyte/monocyte precursors (GM-CFU) (Figure 6B). Additionally, we also defined the differentiation potential of MPP2 (CD48+/CD150+ LSK cells), positioned between HSC and MPP, with clear erythroid potential (Pietras et al., 2015). We reveal significantly more MPP2 in the BM of Tg6 mice, although without increase in cell cycle progression (Figure S5C and S5D). Moreover, and in clear contrast to HSC or MPP, MPP2 showed no change in their differentiation potential to any of the observed progenitors (Figure S5E). These findings primarily show that the enhanced differentiation of Tg6 HSC to BFUe is independent of direct EPO signaling and suggest that Tg6 HSCs do not necessarily have to differentiate into MPPs (e.g. MPP2 or 3) to become erythroid progenitors. Furthermore, we examined the in vivo repopulation capacity of Tg6 HSCs and MPPs (Figure S5F), and demonstrated that Tg6 HSCs were able to produce significantly higher fractions of RBCs compared to WT HSCs at 4 weeks after transplantation, and that chimerism in RBCs and PMNs was not significantly different between the two donor groups at 16 weeks (Figure 6C). Tg6 MPPs, on the other hand, lost their PMN differentiation potential more rapidly than WT.
MPPs (Figure 6D), confirming our initial findings (Figure 2C and 6B). Taken together, these results strongly suggest that chronic exposure to excessive EPO leads to differential fate changes in HSCs and MPPs.

**DISCUSSION**

In the current study, we have used the EPO transgenic mouse line Tg6 and a combination of *in vivo, in vitro,* and deep sequencing approaches to study the effects of chronic EPO stress on HSCs and their closest progenitors, the MPPs. We show that HSCs and MPPs display a clear differential response to such sustained stress. HSCs exhibit a committed erythroid progenitor profile with enhanced cell division and differentiation activity, while MPPs display opposing cell signatures and an accumulation of uncommitted cells.

Acute but short exposure of HSCs/MPPs to high EPO levels has been shown to result in the creation of an “erythroid superhighway” that generates erythroid progenitors via the classical intermediates (Grover et al., 2014); however, we show striking differences between HSC and MPP lineages in mice chronically exposed to high EPO. Essentially, while Tg6 HSCs showed enhanced proliferation with an induction of numerous cell cycle related genes, Tg6 MPPs remained more quiescent and displayed restrained differentiation. This highlights the differential response of both cell populations and strongly points to differentiation/proliferation of HSCs into erythroid progenitors in an environment of sustained exposure to high EPO. Further, although both Tg6 HSCs and MPPs showed a clear erythroid signature, only HSCs displayed a prominent increase in erythroid-related transcription factors and a 5-fold induction in *Klf1,* one of the genes with a GATA1-preferred site, which is regulated by it (Suzuki et al., 2013). Conversely, *Klf1* is even slightly downregulated in Tg6 MPPs (1.4-fold). Since this transcription factor was shown to stabilize GATA1’s occupancy in the β-globin locus (Kang et al., 2015), it is conceivable that the stability of the GATA1 protein, but not *Gata1* mRNA is reduced in Tg6 MPP, an effect that might
eventually also be reflected in the overall increased expression of erythroid genes. KLF1 is also an important and tight regulator of erythroid lineage commitment that stimulates the expression of the EPO-R in erythroid progenitors, whilst inhibiting the thrombopoietin receptor (TPO-R or MPL; (Shah et al., 2015). Tg6 HSCs indeed display almost 2-fold higher Epo-R and lower Mpl expression, which resembles an erythroid-commitment model similar to megakaryocyte erythrocyte progenitors (Pre-MgE). Moreover, mass cytometry experiments revealed no differential activation of EPO/EPO-R signaling pathways in the HSC/MPP/Pre-MgE fractions of adult Tg6 mice versus WT littermates. Although one study has demonstrated a functional EPO-R on HSCs in vitro (Shiozawa et al., 2010), another report demonstrated no detectable EPO-R expression on non-erythroid hematopoietic progenitors using in vivo lineage tracing studies (Singbrant et al., 2011). Therefore, it is possible that specific but divergent fate changes in Tg6 HSCs and MPPs are indirectly induced by EPO-triggered erythroid progenitors or through stromal cells in the bone marrow as suggested before (Lodish et al., 2010). In this respect, we recently showed an effect of chronic EPO exposure on the bone niche, as Tg6 mice revealed significant reduction of bone volume related to diminished osteoblast and increased osteoclast activity (Hiram-Bab et al., 2015; Rauner et al., 2016). Endothelial cells are also BM niche cells containing a functional EPO-R (Trincavelli et al., 2013). Previously, it was shown that AKT1-activated BM endothelial cells (BMEC) display a distinct transcription factor and cytokine profile that can support functional HSCs under severe stress conditions (Poulos et al., 2015). Furthermore, in the colony assays using methylcellulose-containing medium without additional EPO, Tg6 HSCs preferentially differentiate into BFUe progenitors. The same cells also produce more erythrocytes up to 4 weeks after transplantation into WT mice. These results show that even preceding chronic exposure to high EPO leads to erythroid fate decision in HSCs, suggesting for EPO-induced epigenetic changes. Further research is therefore required to unravel the (in)direct and/or temporal role of EPO/EPO-R on the bone/BM.
Although acute exposure to EPO decreases the Pre-GM signature in the HSC/MPP compartment (Grover et al., 2014), our comprehensive GSEA data analysis clearly linked Tg6 MPPs, but not HSCs, to the myeloid network, implying the existence of two clearly distinct signatures that apparently limited the contribution of MPPs to the response to the enhanced RBC demand. Despite the definitive innate immune signature, our observations from colony assays and Tg6 MPP transplantation experiments clearly show hampered differentiation into more mature myeloid progenitors. In addition, CSF1R\(^+\) Tg6 MPPs expressed significantly less GATA1, indicating the existence of distinct erythroid and myeloid Tg6 MPP fractions.

To conclude, we show that EPO can induce transcriptional rewiring, which leads to continuous and chronic over-production of erythroid progenitors largely driven by HSCs rather than MPPs. Moreover, our data show that a stepwise progression through specific differentiation stages is probably not essential for HSC lineage commitment, as also suggested previously (Yamamoto et al., 2013). Tg6 MPPs display two cell signatures with different functionalities that also result in hampered myeloid progenitor differentiation and the absence of erythroid progenitor overproduction. Our findings not only provide insights into the role of HSCs during erythroid stress, but may also have implications for other hematopoietic-related chronic disorders and long-term clinical therapies, including the application of EPO stimulating agents (ESAs).

**EXPERIMENTAL PROCEDURES**

**Mice**

All transgenic mice (Vogel and Gassmann, 2011) and their wild type littermates were bred and maintained in our animal facility with ad libitum water and food. C57BL/6 (B6), B6.SJL-PtprcaPep3b/BoyJ (B6.SJL), and Ubc:GFP mice were purchased from the Jackson Laboratory.
Experiments were performed with male and female mice (aged 3-5 months), and experimental procedures were approved by the local ethics committee.

**Flow-cytometry and sorting**

Flow cytometry, cell sorting, cell cycle analyses were performed as described previously (Singh et al., 2013).

**Transplantation**

HSCs or MPPs were isolated from Ubc:GFP-Tg6 and Ubc:GFP WT littermates. 5000 Lin- cKIT+ SCA-1+ CD48+CD150- (MPP) from WT-Ubc:GFP and littermate Tg6-Ubc:GFP or 300 cKIT+ SCA-1+ CD48- CD150+ CD34- (HSC) were competitively transplanted together with non-fractionated 5x10⁵ B6.SJL (CD45.1) BM cells into lethally irradiated (9Gy) congenic WT recipients. Peripheral blood was analyzed on BD FACS Canto or on a Sysmex.

**Telomere lengths**

Sorted 5x10⁴ MPPs were fixed with 70% Formamid, stained with PNA Cy3 probe and incubated for 10 min at 82°C. Consequent incubation overnight at RT after which samples were washed twice with 70% Formamid. DAPI solution was used to discriminate between G0/G1 and S/G2/M phases of the cell cycle. Samples were analyzed on LSRII and MFI for cells in G0/G1 phases was estimated.

**Differential gene expression analysis**

Complete cDNA (pre-amplified for all HSC samples) was synthesized with the SMARTer® Ultra HV Kit (Clontech). After ultrasonic shearing of the amplified cDNA (Covaris S2) samples were subjected to standard Illumina fragment library preparation using the NEBNext® chemistries (New England Biolabs). Libraries were equimolarly pooled and sequenced on an Illumina HiSeq 2500,
resulting in ca. 32 – 43 million single-end reads per library. After sequencing, FastQC (http://www.bioinformatics.babraham.ac.uk/) was used to perform a basic quality control on the sequencing data. As an additional control, library diversity was assessed by redundancy investigation in the reads. Next, the reads were aligned with BWA (v. 0.7.8) against the mouse reference (mm10) and an exon-exon junction library of 120 nucleotides, which was created according to the Ensembl annotation version 69. The uniquely aligned reads were counted with featureCounts (v1.4.4) and the same Ensembl annotation. Normalization of the raw read counts based on the library size and testing for differential expression between the two conditions was performed with the DESeq2 (v.1.4.0) R package. Genes with an adjusted p-value of less than 0.1 were considered as differentially expressed for comparison. Differential gene expression analysis was performed using the DEseq2 R package to compare gene expression levels between Tg6 and WT mice for both HSC and MPP cell types. DEseq gave 6629 significantly differentially expressed genes (DEGs) when comparing Tg and WT mice for HSC cells and 3801 DEGs for MPP cells (p-adjusted value <0.1). In order to compare DEGs between cell types we performed a simple overlap analysis (using Ensembl Gene IDs) to identify common and unique DEG, and performed a more fine-grained comparison by splitting DEGs into up-regulated and down regulated genes for each cell type. The RNAseq data are deposited in (GEO ID: GSE113053).

**Gene Ontology Enrichment analysis**

Significantly differentially up-regulated genes (p-adjusted value <0.1, fold-change ≥ 2) from both HSCs and MPPs (710 genes HSC, 248 genes MPP) were mapped to Entrez Gene and analyzed using the clusterProfiler R package for Gene Ontology term enrichment (enrichGO function, GO:BP ontology, p-value cut-off=0.1, BH adjusted p-value cut-off 0.00001; (Yu et al., 2012). A selection of the GO-terms represented in Figure 3B and 4A, include genes as shown in (Supplemental Table 1 and 2).
Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) was used to compare gene lists from other cell types, namely pre-MgE, MkP, Pre-GM, CLP (Sanjuan-Pla et al., 2013), CFUe Up and Down regulated genes from (Li et al., 2014). GSEA analysis was performed using GSEA software from the Broad Institute (Mootha et al., 2003; Subramanian et al., 2005). HSC and MPP were ranked by log2 fold-change, the two lists were imported, lists pre-ranked, and compared against gene lists from cell types stated above.

Overlap analysis

Comparisons between Pre-GM and DEGs from HSCs and MPPs were performed (Overlap diagram based on Gene Symbols) along with a similar comparison for CFUe Down, HSC, and MPP DEGs. Genes unique to Pre-GM and MPP gene lists were then input into the Cytoscape plugin CLUEGO for further characterization, and identical analysis was also performed on genes unique to CFUe Down and MPP sets. Plots to compare log2 fold-change for genes unique to Pre-GM & HSC, and Pre-GM and MPP were created, along with a similar analysis for CFUe Down genes.

Mass Cytometry

Bone marrow cells from two femurs and two tibia were used for mass cytometry obtained by crushing in PBS and immediately fixed with Maxpar Fix I Buffer (Fluidigm) for 30 min at room temperature. For surface staining, cells were incubated with antibodies labeled with Metal-tags (CD150-167Er (cat. nr. 3167004B), CD48-156Gd (cat. nr. 3156012B), CD117-173Yb (cat. nr. 3143001B), SCA-1 169Tm (cat. nr. 3169015B), CD41-143Nd (cat. nr. 3143009B) (Fluidigm) and CD105PE (cat. nr. 12-1051-82) (eBioscience), and for the secondary step anti-phycoerythrin (MaxPar Ready) labeled with 160Gd (cat. nr. 408105) (Biolegend). Next, cells were fixed and stained with specific metal tags antibodies against phosphorylated proteins (pAKT (S473)-152Sm (cat. nr. 3152005A), pSTAT5 (Y694) (cat. nr. 3150005A), pERK1/2(T202/Y204)-171Yb (cat. nr.
Cells were incubated overnight in MaxPar® Fix and Perm Buffer (Fluidigm) with 1:10000 dilution Cell-ID™ Intercalator-Ir (Fluidigm), and analyzed on CyTOF2.

**Immunocytochemistry**

Different cell populations were sorted directly on to slides, dried at RT, fixed, incubated with GATA1 Alexa Fluor 647 antibody (cat. nr. sc-265 AF647) (Santa Cruz Biotechnology), and analyzed on a Leica TCS SP5 confocal microscope.

**Semi solid colony forming assay**

100 HSCs and 500 MPPs were sorted and cultivated in duplicate in methylcellulose-containing medium (Methocult M3434). Colonies were counted microscopically at day 7.

**Statistics**

Data are presented as mean ± SEM. Significance was calculated using the Mann Whitney U test. All statistical analysis was performed using GraphPad Prism v 6.03 (GraphPad Inc., La Jolla, CA). Significance was set at p<0.05. “n” in the figure legends always denotes individual mice.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes six figures and two tables.

**AUTHOR CONTRIBUTIONS**

RPS and TG designed the study, performed the experiments, analyzed the data, and contributed towards writing the manuscript. BR, MM, and KF designed and performed experiments. KL and AD performed the deep sequencing analyses. IM, MG, and TC provided tools, contributed to the discussions, and in writing the manuscript. IH performed the bioinformatics analyses, provided
tools, contributed to the discussions, and in writing the manuscript. BW designed the study, supervised the overall project, analyzed the data, and wrote the manuscript.

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FIGURE LEGENDS

Figure 1. Sustained chronic exposure to high systemic EPO levels leads to dramatic changes in myeloid/erythroid progenitor. A. Gating strategy for the myeloid-erythroid-megakaryocyte progenitors gated on Lin−SCA-1−cKIT+CD41−CD16/32− according to Pronk and colleagues (Pronk et al., 2007). B. Bar graphs showing Pre-MgE (Lin−SCA-1−cKIT+CD41−CD16/32−CD105−CD150−), Pre-CFUE (Lin−SCA-1−cKIT+CD41−CD16/32−CD105−CD150−), CFUE (Lin−SCA-1−cKIT+CD41−CD16/32−CD105−CD150−), MkP (Lin−SCA-1−cKIT+CD41−CD16/32−CD150−) and Pre-GM (Lin−SCA-1−cKIT+CD41−CD16/32−CD105−CD150−) (n = 5-6), and one of three representative experiments is shown. C. Cell-cycle analysis of pre-CFUE and D. CFUE cells from WT and Tg6 mice. Bar graphs representing the fraction of progenitors in the quiescent G0 phase of the cell cycle (left) and progressing through the cell cycle (non G0, right) (n=4). Gating strategy for cell-cycle analysis of cells is as shown in Figure S2A for HSC and MPP. E. EB (CD71+Ter119+) (n = 4) F. CMP (Lin−SCA-1−cKIT+CD34+CD16/32−) (n = 5-6). C-F. Data are representative for 2-3 independent experiments. “n” denotes individual mice. Values are mean ± SEM. *, p < 0.05. See also Figure S1.

Figure 2. Tg6 mice display an increased HSC/MPP compartment. A. Gating strategy and representative FACS plots of hematopoietic stem cell progenitor populations. B. Proportion and total HSC and MPP numbers from WT and Tg6 mice (n = 5-7), one of more than five representative experiments is shown. C. The overall effect of chronic and continuous high EPO exposure on the classical representation of the hematopoietic tree taken from data presented in Figure 1, 2 and S1. D. Bar graphs representing the fraction of HSCs and MPPs in the quiescent G0 phase of the cell cycle (left) and progressing through the cell cycle (non G0, right), (n = 15-17), from 4 independent
experiments. Gating strategy as shown in Figure S2A. E. Relative telomere length of isolated MPPs from both genotypes (n= 5-6). Values are mean ± SEM. *, p < 0.05. See also Figure S2.

Figure 3. Sustained and chronic high EPO induces erythroid transcriptional reprogramming in HSCs. A. Schematic overview of the transcriptome sequencing setup to compare HSC/MPP from Tg6 and WT littermates (3 samples per genotype and each sample-contained cells from 3 individual mice). B. Adapted networks of the highest represented GO-terms of genes with ≥2-fold over-expression in Tg6 HSCs vs. WT littermate HSCs. C. Heat maps depicting all genes from the HSC GO-terms represented in B. D. Immunofluorescent staining of the GATA1 transcription factor in sorted LT-HSC and MPP1 isolated from adult WT and Tg6 mice. Scale bars represent 25μm. GATA1 expression in sorted single cells defined by Fluorescent intensity per cell. Data are representative for two independent experiments. Representation of GATA1-prefered sites are shown in Figure S3C. Values are mean ± SEM. *, p < 0.05. See also Figure S3, S4 and Table S1.

Figure 4. Sustained and chronic high EPO induces a dual signature in MPPs. A. Adapted networks of the highest represented GO-terms of genes with ≥2-fold over-expression in Tg6 MPPs vs. WT littermate MPPs. B. Heat maps depicting all genes from the MPP GO-terms represented in A. C. Proportion of CSF1R+ cells in Tg6 vs. WT MPP. D. GATA1 expression in sorted single cells defined by fluorescent intensity per cell. Data in C and D are representative for two independent experiments. Values are mean ± SEM. *, p < 0.05. See also Figure S4 and Table S2.

Figure 5. Tg6 HSCs has a clear erythroid-signature, while the MPP fraction displays two different signatures. A. GSEA of significantly upregulated genes from Tg6 HSCs and MPPs compared to sets of genes from different erythroid/myeloid/megakaryocyte progenitors, as shown in Figure 2C. Signs in the upper corner of the tables (+, -, =) designate correlation of the signature with respective progenitor (HSC→left, MPP→right) based on p-value. Both signs underneath the
CFUe tables reflect the potential subsequent effect of the ‘up’ or ‘down’ signatures (> <, opposing). p-value as indicated (if p = 0.000 then p < 0.0001). B. Heat map depicting all 65 significantly changed Tg6 MPP genes represented in the CFUe ‘down’ signature (GSEA).

**Figure 6. Differential Tg6 HSCs display a clear erythroid-signature, while the MPP fraction has two opposing signatures.** A. Mass cytometry to quantify the phosphorylated forms of STAT5 in freshly isolated CFUe’s, HSCs, and MPPs from WT and Tg6 mice (n = 3-5). B. Bar graphs representing the number of colonies in the semi-solid ex vivo assay using HSCs or MPPs from both genotypes (n = 8). C. Transplantation assay in irradiated WT mice as depicted in Figure S5F. All mice were bled at the time points shown and peripheral blood (PB) chimerism was defined by FACS (n = 4-5). Data are representative for 2-3 independent experiments. All values are mean ± SEM. *, p < 0.05. See also Figure S5.
Figure 1

A

Gated on Lin^−SCA1^−cKIT^+CD41^−CD16/32^−

CD105

33.9 7.16

34.7 24.3

19.5 17.3

PreGM

Pre-MgE

Pre-CFUe

CFUe

MkP

CD150

CD41

WT

Tg6

B

Pre-MgE

Pre-CFUe

CFUe

MkP

Pre-GM

Percent of total cells

WT  Tg6

WT  Tg6

WT  Tg6

WT  Tg6

WT  Tg6

C

pre-CFUe: G_0

pre-CFUe: non G_0

Percent of cells

WT  Tg6

WT  Tg6

E

EB

Percent of total cells

WT  Tg6

F

CMP

Percent of total cells

WT  Tg6
Figure 2

(A) Flow cytometry plots showing the distribution of Lin-CD48-CD150- (HSC) and Lin-cKit-SCA1+ (MPP) cells among WT and Tg6 mouse strains. The plots depict the percentage of cells in the G0 and non-G0 states.

(B) Bar graphs illustrating the percentage of total cells and cell number for HSC and MPP in WT and Tg6 strains. The bars are marked with asterisks (*), indicating statistical significance.

(C) A flow chart depicting the differentiation pathways from HSC to erythroblasts (RBC) with differentiation events indicated by arrows.

(D) Bar graphs showing the percentage of HSC and MPP in G0 and non-G0 states for WT and Tg6 strains.

(E) Bar graph depicting the mean fluorescence intensity of MPP telomeres in WT and Tg6 strains.
Figure 3

A. Schematic diagram showing RNA seq. analysis of HSC and MPP cells.

B. Heatmap displaying the -log(Adj. P value) for various biological processes.

C. Heatmap showing gene expression changes in HSC and MPP cells.

D. Fluorescence intensity per cell analysis for GATA1 expression in LT-HSC and MPP1 cells.

Erythropoiesis and Heme biosynthesis pathways are highlighted in the heatmaps.
Figure 4

A

MPP

innate immune response
erythrocyte development
erythrocyte homeostasis
erythrocyte differentiation
immune system process

-log(Adj. P value)

0 2 4 6 8

B

WT Tg6

Erythropoiesis

WT Tg6

C

CSF1R

Frequency of CSF1R⁺ cells in MPP [%]

WT Tg6

WT Tg6

D

GATA1

Fluorescence intensity per cell

CSF1R⁻ CSF1R⁺

WT Tg6
Figure 6

A

pSTAT5 (CFUe)  
Mean  
WT | Tg6  
0 | 25 | 50  
*  

pSTAT5 (HSC)  
WT | Tg6  
0 | 25 | 50  
*  

pSTAT5 (MPP)  
WT | Tg6  
0 | 25 | 50  
*  

B

HSC  
# colonies per 100 HSC  
GM-CFU | GME-CFU | BFUe  
WT | Tg6  
*  

MPP  
# colonies per 500 MPP  
GM-CFU | GME-CFU | BFUe  
WT | Tg6  
*  

C

RBC(HSC)  
Chimerism [%]  
Time (weeks)  
WT | Tg6  
*  

PMN(HSC)  
Chimerism [%]  
Time (weeks)  
WT | Tg6  
*  

D

RBC(MPP)  
Chimerism (%)  
Time (days)  
WT | Tg6  
*  

PMN(MPP)  
Chimerism (%)  
Time (days)  
WT | Tg6  
*
Inventory of Supplemental Information:

Supplemental Figures:

**Figure S1. Identification of the hematopoietic compartment in Tg6 mice.**
All graphs in this supplemental figure are directly linked to Figure 1. The majority displays the total amount of hematopoietic progenitor cells in Tg6 versus WT BM.

**Figure S2. Cell-cycle analysis of cells from WT and Tg6 mice.**
These panels are mainly linked to Figure 2. S2A represents the gating strategy to define cell cycle progress in the hematopoietic progenitor cells (Figure 1C – D, 2D and S2C) – S2B is related to 2A and 2B.

**Figure S3. Analysis of HSC and MPP RNAseq from WT and Tg6 mice.**
All figures are directly related to Figure 3. Panels A and B represent the overall analyses of the RNAseq experiment, while C and D are additional analyses linked to Figure 3B and C.

**Figure S4: Comparative analysis of erythropoiesis signatures in HSC and MPP.**
These panels display additional analyses to data shown in Figure 3 and 4. S4A shows a comparison between the erythropoiesis-genes induced in Tg6 HSC versus Tg6 MPP. S4B represents the impact of MPP3 versus MPP4.

**Figure S5. Epo/EpoR signaling in hematopoietic progenitors and the MPP2 population.**
All panels are related to Figure 6. S6A and B are additional results complementing Figure 6A. S6C-E represent an extensive MPP2 analysis connected to Figure 6B. S6F is a schematic representation of the transplantation assays (Figure 6C and D).

**Supplemental Tables:**
Table S1 and S2 contain gene ontology terms based on the significantly and ≥2-fold overexpressed genes in Tg6 HSC (S1) and Tg6 MPP (S2), both versus their WT littermates.
Figure S1. Identification of the hematopoietic compartment in Tg6 mice. A. Percentage of red blood cell volume per blood volume (hematocrit-hct) and amounts of RBCs per blood volume in Tg6 versus WT littermates (n = 6-9). B-D. Total cell numbers of all cell populations as shown in Figure 1B, 1E and 1F (n = 4-7). Data from one of 2-5 representative experiments is shown. Values are mean ± SEM; *, p < 0.05.
Figure S2. Cell-cycle analysis of cells from WT and Tg6 mice. (A) Cell cycle gating strategy: the lower gate (Ki67-ve) contains cells in the G0 phase of cell cycle, top-left represents cells in the G1 phase, and top-right in the S/G2/M phases – both together is non-G0. (B) Bar graphs representing total MPP cell numbers in the BM of WT versus Tg6 mice (4 and 6 week old mice). (C) Bar graphs representing the fraction of progenitors in the quiescent G0 phase of the cell cycle (left) and progressing through the cell cycle (non G0, right) in 4 week old WT versus Tg6 mice. Data represent a total of 2 independent experiments. Values are mean ± SEM; (n = 3-6).
**Figure S3. Analysis of HSC and MPP RNAseq from WT and Tg6 mice.**

A. Heat map and dendrogram showing genotype to genotype separation for HSC and MPP. B. Scheme representing the amounts of unique/overlapping, significantly up- or downregulated genes in Tg6 HSC versus MPP. C-D. Normalized expression level of genes from GATA1-preferred sites and Gata2 (HSCs) (n = 3). Values are mean ± SEM. *, p < 0.05.
Figure S4: Comparative analysis of erythropoiesis signatures in HSC and MPP. A. Comparison of the erythropoiesis-related gene induction of Tg6 HSCs genes (Figure 3C) versus Tg6 MPPs, displayed as ratio (in %). Bars in red show erythropoiesis-related genes that are more induced in Tg6 HSCs than in Tg6 MPPs. Bars in green show the erythropoiesis-related genes that are more induced in Tg6 MPP versus Tg6 HSC. D. Total cell numbers of MPP3 and MPP4. Different fractions of MPP3 and 4 in total MPP of WT and Tg6 mice (n = 5-7). Data represent one of 3 independent experiments. Values are mean ± SEM. *, p < 0.05.
Figure S5. Epo/EpoR signaling in hematopoietic progenitors and the MPP2 population. A-B. Mass cytometry using isolated BM cells from WT and Tg6 mice, using metal labeled antibodies against surface markers and phosphorylated proteins representing intracellular pathways activation though direct EPO/EPO-R interaction. C-E. Classification of the MPP2 population in Tg6 versus WT mice (total cell number, cell cycle phases and ex vivo colony assay). Data are representative of 2-3 independent experiments, (n = 3-7). F. Schematic overview of the competitive HSC/MPP transplantation model in lethally irradiated WT recipient mice. All mice were injected with WT or Tg6 HSC/MPP cells from Ubc:GFP or Ubc:GFP-Tg6 mice respectively and supplemented with 5.10^5 BM competitor cells (C57BL/6). Values are mean ± SEM. *, p < 0.05.
Table S1. Gene ontology terms based on significantly and ≥2-fold overexpressed genes in Tg6 HSC versus WT littermate cells, related to Figure 3B and 3C. (Blue: cell cycle, purple: heme biosynthesis and red: erythropoiesis).
| ID         | Description                           | GeneRatio | ByRatio | p.value  | p.adjust | q.value  | geneID | Count |
|------------|---------------------------------------|-----------|---------|----------|----------|----------|--------|-------|
| GO:0002376 | immune system process                 | 59/220    | 1677/16502 | 1.65E-12 | 4.75E-09 | 4.14E-09 | Isg15/Pld4/Hba-a2/Add2/Addora3/Alas2/Ank1/Bpgm/C1qa/C1qb/C1qc/Cd14/Cd36/Celbp/Cfh/Ccr2/Csf1r/Ednrb/Epb4.2/AF251705/Fcgr1/Gata1/Gpd1/Gzmb/Hba-a1/Hbb-b1/Hbb-b2/Mafb/Lgals3/Tlr8/Slc11a1/Ptcb1/Rhag/Trim10/Mst1r/Ccl2/Snca/Spta1/Cd300e/Tlr1/Tfcc/Dyrk3/Fcgr4/Podxl/Pia2g7/Hist1h4d/CSar2/Fcrl5/Cd300lg/Mefv/Cadm1/Isg20/Rasd2/Pmaip1/Ifi30/Crip2/Trim15/Fam213a/Susd2 | 59 |
| GO:0030218 | erythrocyte differentiation            | 15/220    | 104/16502 | 7.75E-12 | 1.12E-08 | 9.74E-09 | Isg15/Hba-a2/Alas2/Ank1/Bpgm/Epb4.2/Gata1/Hba-a1/Hbb-b1/Hbb-b2/Mafb/Ptcb1/Rhag/Trim10/Dyrk3 | 15 |
| GO:0034101 | erythrocyte homeostasis                | 15/220    | 114/16502 | 3.01E-11 | 2.90E-08 | 2.52E-08 | Isg15/Hba-a2/Alas2/Ank1/Bpgm/Epb4.2/Gata1/Hba-a1/Hbb-b1/Hbb-b2/Mafb/Ptcb1/Rhag/Trim10/Dyrk3 | 15 |
| GO:0048821 | erythrocyte development                | 9/220     | 31/16502  | 1.77E-10 | 1.02E-07 | 8.88E-08 | Hba-a2/Ank1/Bpgm/Epb4.2/Gata1/Hba-a1/Hbb-b1/Hbb-b2/Rhag | 9 |
| GO:0048872 | homeostasis of number of cells         | 18/220    | 243/16502 | 4.49E-09 | 1.85102E-06 | 1.61167E-06 | Isg15/Hba-a2/Alas2/Ank1/Bpgm/Epb4.2/Gata1/Hba-a1/Hbb-b1/Hbb-b2/Mafb/Ptcb1/Rhag/Trim10/Ccl2/Snca/Spta1/Dyrk3/Pmaip1 | 18 |
| GO:0042592 | homeostatic process                   | 41/220    | 1202/16502 | 2.07E-08 | 7.48432E-06 | 6.51653E-06 | Isg15/Hba-a2/Alas2/Ank1/Fabp4/Apopc2/Atp4a/Bpgm/Cln3/Ccr2/Csf1r/Ednrb/Epb4.2/Gata1/Gpd1/Hba-a1/Hbb-b1/Hbb-b2/Mafb/Lgals3/Tlr8/Slc11a1/Ptcb1/Rhag/Trim10/Ccl2/Sfrp4/Sltc4a1/Snca/Spta1/Tfcc/Dyrk3/Fam132b/Hemw2/Fcrl5/Pmaip1/Slc4a8/Ccdd109b/Clec4b1 | 41 |
| GO:0045087 | innate immune response                 | 22/220    | 407/16502 | 2.79E-08 | 8.96459E-06 | 7.80538E-06 | Isg15/C1qa/C1qb/C1qc/Cd14/Cd36/Cfh/Csf1r/Lgals3/Tlr8/Slc11a1/Trim10/Mst1r/Ccl2/Snca/Tlr1/Mefv/Cadm1/Isg20/Rasd2/Trim15 | 22 |