Mice over-expressing human erythropoietin indicate that *erythropoietin* enhances expression of its receptor via up-regulated *Gata1* and *Tal1*

The development of medullary hematopoiesis is characterized by a specific expression profile of hematopoietic transcription factors, including GATA transcription factors. At mid-gestation, when hematopoiesis is newly established in the bone marrow of human fetuses, initially high *GATA2* expression becomes subsequently down-regulated, while *GATA1* expression increases in parallel.[1] Both transcription factors bind to overlapping sets of hematopoietic downstream target genes, often at distinct sites, to regulate the balance between proliferation and differentiation. Chromatin occupancy by GATA1 and GATA2 can change in the course of hematopoietic differentiation, leading to the so-called GATA switch.[2] Thus, a spatio-temporal regulation of GATA1 or GATA2 activities is required within lineage-specific differentiation. During erythroid differentiation GATA1 expression peaks at the level of colony-forming units (CFU-E),[3] where erythropoietin (Epo) exerts most specifically its effects, but blocks terminal maturation if constitutively over-expressed.[4] In CFU-E progenitors, the Epo receptor (*EpoR*) gene is a prerequisite downstream target of GATA1 that activates *EpoR* expression in concert with several co-factors.[5] Notably, EpoR-mediated signals in turn strongly enhance *GATA1* gene expression in erythroid progenitor cells *in vitro*.[5,6] There is also *in vitro* evidence that Epo induces *EpoR* expression by activating the GATA1-mediated *EpoR* transcription.[5]

An alternate link between Epo and excessive erythropoiesis, which includes GATA1 activity, is given by the basic helix-loop-helix protein TAL1 (T-cell Acute Leukemia-1 transcription factor).[7] *In vitro*, Epo stimulates expression of TAL1 and phosphorylation of its protein products.[8] TAL1 directly up-regulates EpoR transcription and increases by nucleosome shifting the association of a transcription factor complex that includes GATA1, TAL1, LMO2 (Lim-Only Protein-2) and LDB2 (Lim-Domain Binding Protein 2), to a regulatory domain in the 5' untranslated region of the *EpoR* gene.[7] In this way, TAL1 causes hypersensitivity to Epo and promotes excessive erythrocytosis.

Transgenic mice, constitutively over-expressing the human EPO (*hEPO*) gene (*tg6* mice), represent a valuable model to further elucidate the *in vivo* implication of Epo in fine-tuning transcriptional activities that may modulate *EpoR* expression and explain the gradual increase in erythropoiesis from normal hematocrit levels at birth to maximum hematocrit levels of up to 90% after several weeks.[9]

Our analysis indicates that the two erythroid master regulators Gata1 and Tal1 co-operatively act as developmental-stage specific enhancers of *EpoR* expression in response to constitutive EPO overexpression.

While *Gata1* mRNA expression in the newly established bone marrow of wild-type mice declined with increasing postnatal age (Figure 1A), its expression remained on a constant and significantly higher level in *hEPO* over-expressing *tg6* mice. However, Gata2 mRNA expression remained similar in *hEPO* over-expressing *tg6* mice compared to controls throughout all ages (Figure 1B). In *hEpo* over-expressing *tg6* mice, *EpoR* mRNA expression significantly increased with age, but declined in control animals (Figure 1C).

The significant upregulation of *Gata1* and *EpoR* mRNA expression in *hEPO* over-expressing *tg6* mice was confirmed by analyzing the spleen as a major source of hematopoiesis (Figure 2A and B). To further dissect the complexity of changes in the transcriptional network, the analysis of *Myb* mRNA expression served as marker for adult definitive erythroblasts,[10] showing significantly

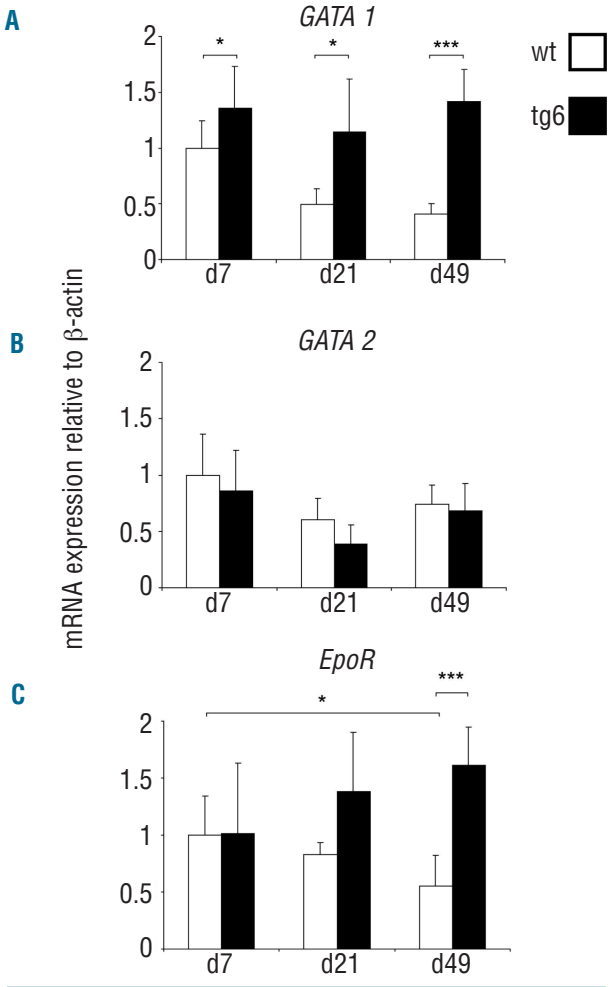

Figure 1. Longitudinal expression of *Gata1*, *Gata2* and *EpoR* mRNA transcripts in the bone marrow of *tg6* and wild-type mice (at postnatal Day 7 (d7), d21, d49). The transgenic mouse line *tg6*, in which the *hEPO* transgene is transmitted in autosomal dominant manner, was bred at the Institute of Veterinary Physiology, University of Zurich. The protocol was approved by the local Institutional Review Board and all procedures were performed according to the Swiss Animal Protection Law. To obtain hematopoietic cells from the bone marrow, femurs and tibias of 7, 21 and 49 day-old mice were prepared and flushed with ice-cold PBS. Bone marrow specimens of each single animal were pooled. Total mRNA was purified following the TRIzol protocol (Invitrogen, Carlsbad, CA, USA), and cDNA was generated by a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). 5'-FAM-labeled probes were used for detection of murine *Gata1* (Assay ID: Mm00484678_m1), *Gata2* (Mm00492301_m1) or *EpoR* mRNA (Mm00438760_m1) transcripts and β-actin (ACTB 4352664-0602004; all from Applied Biosystems) by real-time PCR technology with TaqMan 2× Universal PCR Mastermix Mix as recommended in the manufacturer's protocol (Applied Biosystems). Data (mean and standard deviation) are presented as ratio between *Gata1*, *Gata2* or *EpoR* mRNA transcript and β-*actin* mRNA transcript levels (n=4-6 sets). All reactions were performed in duplicates, and all four transcripts were analyzed in parallel. Statistical significance was examined by one-way ANOVA *post hoc* comparisons. $P<0.05$ was considered significant. *$P<0.05$; ***$P<0.001$.

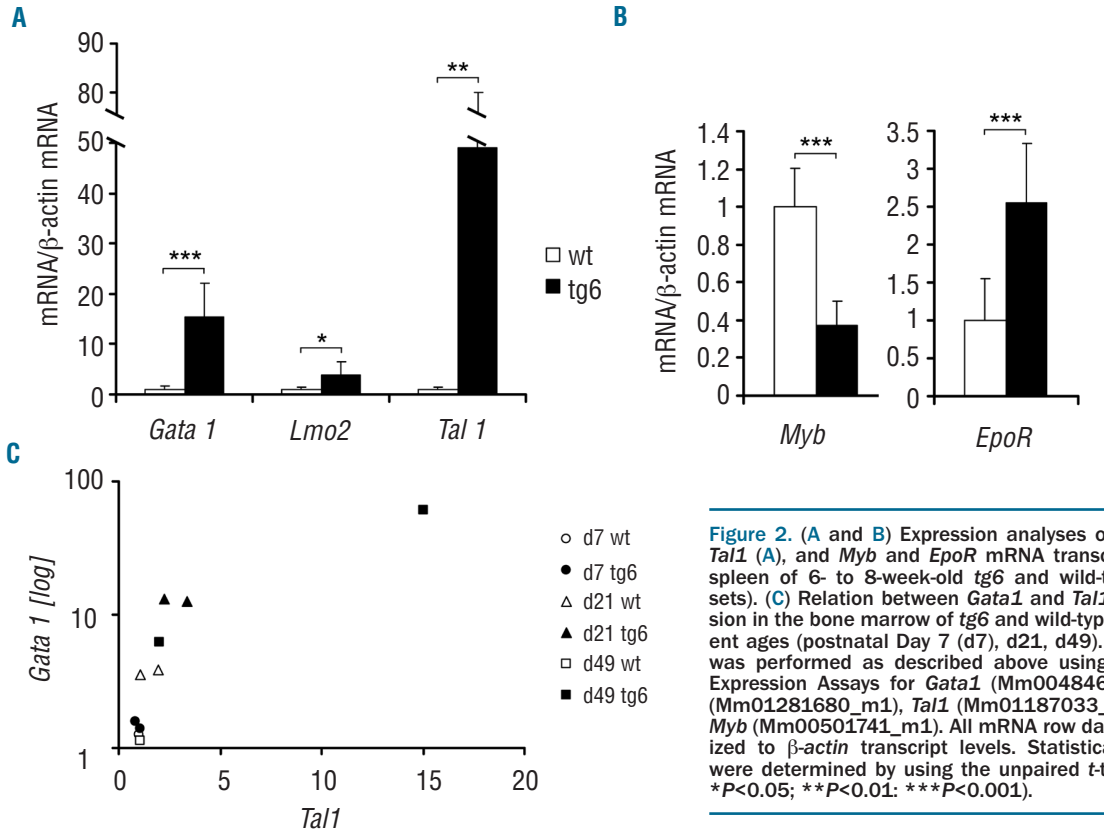

Figure 2. (A and B) Expression analyses of *Gata1*, *Lmo2*, *Tal1* (A), and *Myb* and *EpoR* mRNA transcripts (B) in the spleen of 6- to 8-week-old *tg6* and wild-type mice (n=4 sets). (C) Relation between *Gata1* and *Tal1* mRNA expression in the bone marrow of *tg6* and wild-type mice at different ages (postnatal Day 7 (d7), d21, d49). Q-PCR analysis was performed as described above using TaqMan Gene Expression Assays for *Gata1* (Mm00484678_m1), *Lmo2* (Mm01281680_m1), *Tal1* (Mm01187033_m1) as well as *Myb* (Mm00501741_m1). All mRNA row data were normalized to β-*actin* transcript levels. Statistical significances were determined by using the unpaired *t*-test. (n=2 pairs; **P*<0.05; ***P*<0.01: ****P*<0.001).

reduced expression in *hEPO* over-expressing *tg6* mice (Figure 2B). In contrast, expression levels of both *Tal1* and *Lmo2* were significantly up-regulated in *hEPO* over-expressing *tg6* mice (Figure 2A).

The longitudinal analysis in the developing bone marrow also indicated increasing *Tal1* mRNA levels, which were tightly correlated with *Gata1* mRNA expression (Figure 2C).

The combined data confirm that Epo recruits the erythroid transcriptional network to enhance its erythropoietic effect by mechanisms that directly induce *EpoR* upregulation and hypersensitivity to Epo *in vivo*. While such effects, predominately mediated by Gata1 and Tal1, are attributed to the stage of BFU-E (burst-forming unit erythoid) and CFU-E, the reduced expression of *Myb* in the *hEPO* over-expressing *tg6* mice may reflect its prerequisite role for Epo-induced differentiation commitment.[11] However, evidence is given that Gata1 protein induces *EpoR* expression by activating the 5' *EpoR* promoter.[5] In response to acute anemic stress, the Epo-induced increase in the CFU-E population is concomitant with upregulation of *EpoR*, *Gata1* and *Bcl-X*$_L$ expression in the murine bone marrow.[12] Thus, Epo stimulates erythropoiesis not only by activation of 'classical' downstream signaling, but also further enhances its effect by the Gata1-induced upregulation of *EpoR* expression. As recently reported, Epo regulates GATA1 through protein kinase D activation, promoting histone deacetylase 5 dissociation from GATA1, and subsequent GATA1 acetylation.[13] This post-translational modification resulted in increased DNA-binding activity of GATA1,[14] which may contribute to enhanced *EpoR* expression. Such GATA1 function is highly specific for erythroid precursor cells, because on activated CD4-positive lymphocytes EpoR expression depends only on the transcription factor Sp1, but not on GATA1.[15]

The lack of any differences in *Gata2* expression indicates that Epo does not directly interfere with the spatio-temporal regulation of Gata1 or Gata2 activities within the erythroid differentiation. The observation that *Gata1* expression in the developing bone marrow of control mice declined with increasing age, while its expression in *hEPO* over-expressing *tg6* mice remained constantly high, raises the question as to whether a critical threshold of the Gata1 induction has been reached during the observation period, and whether additional regulators are involved in the process of developing excessive erythropoiesis under constitutive *hEPO* overexpression. Indeed, an approximately 50-fold increase of *Tal1* mRNA expression in adult *hEPO* over-expressing tg6 mice as well the tight correlation between gradually increasing Gata1 and *Tal1* mRNA expression levels in their developing bone marrow direct to another mechanism of *EpoR* regulation. This mechanism has been previously explored in erythroid progenitor cells from a patient with excessive erythrocytosis.[7] While Tal1 can directly induce *EpoR* expression by binding to E-box motifs in the 5'-untranslated *EpoR* locus, Tal1 may synergize with Gata1 activity by increasing the association of the GATA1-TAL1-LMO2-LDB2 transcription factor complex to 5'-GATA and 3'-E-box motifs flanking the *EpoR* transcription start site.[7]

In summary, longitudinal analysis of *hEPO* over-expressing *tg6* mice provides novel *in vivo* information that Epo directly tunes activation of the erythroid transcriptional master regulators Gata1 and Tal1 to enhance *EpoR* gene expression. When a selective GATA1 inhibitor

eventually becomes available, these data may be important for developing novel therapeutic concepts for chronic polycythemia.

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