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► **To cite this version:**

David Sibon, Tereza Coman, Julien Rossignol, Mathilde Lamarque, Olivier Kosmider, et al.. Enhanced Renewal of Erythroid Progenitors in Myelodysplastic Anemia by Peripheral Serotonin. Cell Reports, 2019, 26 (12), pp.3246-3256.e4. 10.1016/j.celrep.2019.02.071 . hal-02086600

HAL Id: hal-02086600

<https://hal.sorbonne-universite.fr/hal-02086600>

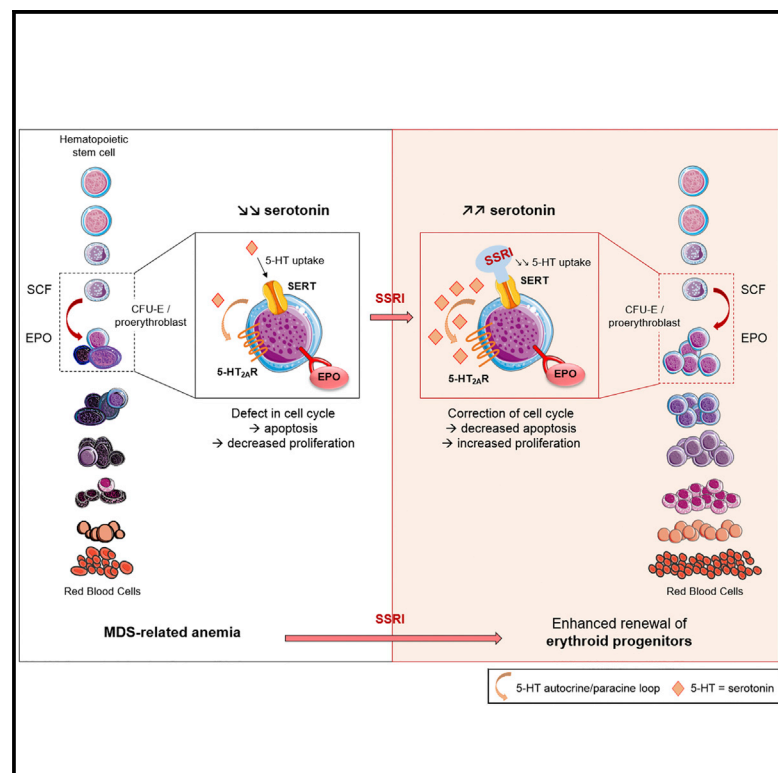
Submitted on 1 Apr 2019

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Enhanced Renewal of Erythroid Progenitors in Myelodysplastic Anemia by Peripheral Serotonin

Graphical Abstract



Authors

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In Brief

Sibon et al. identify a cell-autonomous serotonergic network in human and mouse erythroid progenitors. Reduced levels of serotonin lead to decreased proliferation and survival of erythroid progenitors. Increasing serotonin's concentration through fluoxetine, commonly used to treat depression, could be a valuable therapeutic intervention to correct myelodysplastic-syndrome-related anemia.

Highlights

- In mouse, bone-marrow-derived serotonin (5-HT) contributes to normal erythropoiesis
- Serotonin-deficient mice exhibit anemia and an MDS-like phenotype
- Lack of 5-HT decreases proliferation and survival of human erythroid progenitors
- Pharmacologic restoration of 5-HT can rescue anemia, whether MDS related or not



Enhanced Renewal of Erythroid Progenitors in Myelodysplastic Anemia by Peripheral Serotonin

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<https://doi.org/10.1016/j.celrep.2019.02.071>

SUMMARY

Tryptophan as the precursor of several active compounds, including kynurenine and serotonin, is critical for numerous important metabolic functions. Enhanced tryptophan metabolism toward the kynurenine pathway has been associated with myelodysplastic syndromes (MDSs), which are preleukemic clonal diseases characterized by dysplastic bone marrow and cytopenias. Here, we reveal a fundamental role for tryptophan metabolized along the serotonin pathway in normal erythropoiesis and in the physiopathology of MDS-related anemia. We identify, both in human and murine erythroid progenitors, a functional cell-autonomous serotonergic network with pro-survival and proliferative functions. *In vivo* studies demonstrate that pharmacological increase of serotonin levels using fluoxetine, a common antidepressant, has the potential to become an important therapeutic strategy in low-risk MDS anemia refractory to erythropoietin.

INTRODUCTION

Myelodysplastic syndromes (MDSs) are a heterogeneous group of myeloid malignancies highly prevalent among older individuals. Bone marrow cellular morphology showing dysplasia of any of the three hematopoietic lineages and the percentage of blasts leads to the diagnosis and classification of MDSs (Mufti

et al., 2008; Arber et al., 2016). For instance, low-risk MDSs, which include refractory anemia with ringed sideroblasts (RARS), have been associated with impaired erythroid-proliferating capacities of multifactorial causes, including molecular abnormalities as well as microenvironmental factors (Malcovati and Cazzola, 2016). Despite the fact that erythropoietin (EPO) has demonstrated some clinical benefit, many patients, especially those with RARS, are EPO refractory, thereby remaining transfusion dependent and at higher risk for transfusion-related comorbidities (Malcovati et al., 2005; Cazzola and Malcovati, 2005). Hence, identifying mechanisms of anemia and drugs to improve ineffective erythropoiesis at initial stages of MDS will contribute to increase the quality of life of patients, reduce comorbidities, and eventually increase overall survival.

Tryptophan is an essential amino acid that can be metabolized to several active compounds, including kynurenine, kynurenic acid, and quinolinic acid. The synthesis of these metabolites is regulated by an enzymatic cascade, known as the kynurenine pathway. Key enzymes of the pathway are indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO) (Chen and Guillemin, 2009). IDO is ubiquitously expressed and present in most extrahepatic tissues, and TDO present in the liver is responsible for regulating systemic tryptophan levels. Tryptophan is also the precursor of serotonin (5-hydroxytryptamine or 5-HT), a monoamine highly conserved throughout evolution. 5-HT synthesis is initiated by the rate-limiting enzyme, tryptophan hydroxylase (Tph), with two isoforms: Tph1 in the periphery and Tph2 in the central nervous system (CNS) (Walther et al., 2003; Côté et al., 2003). 5-HT synthesized by Tph2 has a well-recognized role as neurotransmitter, acting on 5-HT receptors in various brain regions. 5-HT receptors have also been shown



to exist in several nonneuronal tissues (Hannon and Hoyer 2008). To explore *in vivo* the functions involving extra-cerebral 5-HT, we and others generated a Tph1 knockout mouse model (Tph1^{-/-}) and revealed unexpected roles for the monoamine in normal and pathological conditions (reviewed in Amireault et al., 2013 and Spohn and Mawe, 2017).

A tight control of tryptophan metabolism is critical for maintaining healthy homeostasis, as changes in tryptophan concentrations have been associated with physiological and behavioral processes (Platten et al., 2012; Oxenkrug, 2010). Interestingly, MDSs were reported to be associated with greater tryptophan breakdown and higher kynurenine levels, implying that enhanced tryptophan metabolism and elevated levels of kynurenine metabolites in the patients' sera could inhibit hematopoietic progenitor amplification (Berthon et al., 2013).

Yet, a fundamental issue has not been carefully investigated in MDS patients. Due to enhanced tryptophan metabolism, the amino acid may no longer be available for 5-HT production? Hence, the lack of 5-HT itself may lead to the emergence of myelodysplastic-related anemia?

The pharmacological and genetic evidences presented here demonstrate that tryptophan metabolism along the kynurenine pathway and the accumulation of tryptophan metabolites do not appear as a primary event in the development of low-risk MDS. Our results reveal that a functional autocrine serotonergic network exists in both murine and human erythroid lineages and that lack of 5-HT participates to the physiopathology of MDS-related anemia. We identified Tph1 as an erythroid gene and uncovered a fundamental role for 5-HT in regulating hematopoietic stem cell fate along the erythroid pathway. EPO induces *TPH1* expression and 5-HT synthesis necessary for erythroid progenitor's survival and proliferation. Importantly, as one of the hallmarks of MDS is ineffective hematopoiesis with anemia, our findings argue that the use of selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine—a common antidepressant, in the earliest stages of MDS, where anemia is a major sign, may represent an innovative therapeutic strategy.

RESULTS

Patients with Low-Risk Myelodysplastic Syndrome Exhibit an IDO/Tph1 Enzyme Activity Imbalance

Altered gene expression, associated with a disease in a cohort of patients, may be revealed by the identification of upregulated or downregulated genes. To further investigate a potential role for the kynurenine pathway in MDSs, we performed gene expression analysis of enzymes involved in tryptophan metabolism. Compared to the healthy control population (n = 5), in MDS patients (n = 27), we observed a deregulated expression between *TPH1* and *IDO*, and the expression of *TDO* was similar (Figure 1A). As previously proposed, these findings support the hypothesis that deregulation of tryptophan metabolism, involving an increase in *IDO* activity, may be a rate-limiting step leading to the emergence of MDSs (Berthon et al., 2013). Yet, in our cohort, a puzzling result is the decrease in *IDO* gene expression in MDS patients as compared to control individuals (Figure 1A). We hypothesized that the % of *IDO*

activity in MDS patients (as measured by the Kyn/Trp ratio) could result in diversion of some of the available tryptophan toward the kynurenine pathway and deprived cells of 5-HT. Along that line, when taking the Kyn/Trp ratio as a measure of tryptophan metabolism, we next performed analysis of *IDO* in a separate cohort of MDS patients (n = 71). Figure 1B provides evidence of an inverse correlation ($r^2 = 0.2943$; $p < 0.0001$) between the Kyn/Trp ratio and tryptophan plasma levels, indicating that an increase in *IDO* activity leads to greater tryptophan metabolism. In that cohort of MDS patients, *IDO* activity was inversely correlated with hemoglobin (Hb) levels ($r^2 = 0.2376$; $p < 0.0110$; Figure 1C). Considering all MDS patients of the cohort, no correlation was observed between Hb values and tryptophan levels ($r^2 = 0.045$; $p < 0.0732$; Figure 1D). But in patients with low MDS severity scores (n = 19; assigned in accordance with the International Prognosis Scoring System [IPSS]; Greenberg et al., 1997), a significant correlation ($r^2 = 0.3406$; $p < 0.0049$) between plasma tryptophan and whole-blood Hb levels was observed (Figure 1E). Likewise, analysis of those patients with the highest levels of *TPH1* gene expression (n = 11 in red in Figure 1A) revealed lower IPSS scores and a trend toward a positive correlation between *TPH1* expression and Hb levels (Figure 1F). These findings suggest that enhanced *IDO* activity may have resulted in diversion of the available tryptophan, depriving cells of 5-HT, leading to the emergence of MDS. Indeed, measurement of 5-HT levels from freshly drawn blood demonstrated a significant decrease in MDS patients (n = 15) as compared to control individuals (n = 14; Figure 1G). At last, a positive correlation between 5-HT and Hb levels was observed in low-risk MDS patients ($r^2 = 0.4119$; $p < 0.0454$; Figure 1H).

Together, the results indicate that disruption of tryptophan metabolism between the Tph1 and *IDO* pathways and diversion of the available tryptophan away from 5-HT synthesis can be an important event prior to the development of myelodysplastic anemia.

Identification of a Serotonergic System in Human Erythroid Progenitors to Increase Their Survival and Proliferation

Although it is well established that *TPH1*, the rate-limiting enzyme for peripheral 5-HT synthesis, is mainly expressed in gut enterochromaffin cells, a growing body of evidence has led to the discovery of complete serotonergic systems, including the specific membrane serotonin transporter (SERT) and the full repertoire of serotonergic receptors, in unexpected locations (reviewed in Amireault et al., 2013 and Spohn and Mawe, 2017; Hannon and Hoyer, 2008), albeit little is known regarding components of the 5-HT system in hematopoietic cells. The previous results, indicating that MDS-related anemia could be explained by reduced 5-HT levels, prompted us to explore whether components of the system were present in hematopoietic cells of the erythroid lineage. In purified human CD36⁺ cord blood cells, using qRT-PCR, we demonstrate that mRNA for *TPH1*, the 5-HT_{2A} receptor (*5-HT_{2A}R-HTR2a*), and the 5-HT-specific membrane transporter (*SERT-slc6a4*) were highly expressed at the specific pro-erythroblast stage of differentiation (day 3 of the culture after CD36⁺ isolation; Zermati et al., 2000, 2001; Figures 2A–2C). At

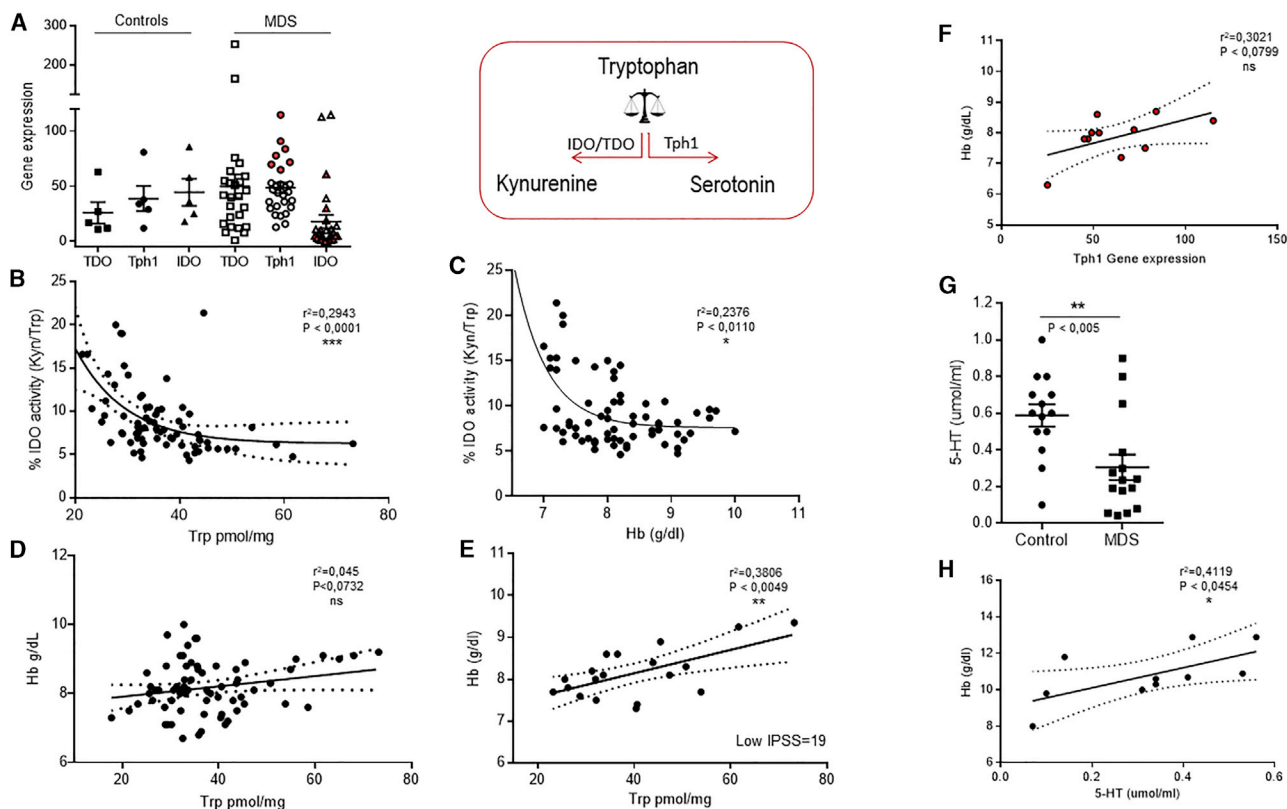


Figure 1. Patients with Low-Risk Myelodysplastic Syndrome Exhibit an IDO/Tph1 Enzyme Activity Imbalance

(A) Gene expression of TDO, TPH1, and IDO in MDS patients (n = 27) versus controls (n = 5). An inverse relationship between gene expression of *TPH1* and *IDO* is observed in MDS patients as compared to controls. No variation in the expression of TDO is shown.

(B) Inverse correlation between tryptophan levels and IDO activity as measured by kynurenine-to-tryptophan ratio (Kyn/Trp) in plasma of MDS patients (n = 71).

(C) Inverse correlation between Hb levels and IDO activity as measured by kynurenine-to-tryptophan ratio (Kyn/Trp) in MDS patients (n = 71).

(D and E) No significant correlation (D) between tryptophan and Hb levels in all MDS patients of the cohort (n = 71) but significant correlation (E) between tryptophan and Hb levels in MDS patients with lower IPSS (n = 19).

(F) Trend toward a positive correlation between high *TPH1* gene expression and Hb level in MDS patients with lower IPSS (n = 11 from Figure 1A).

(G) Significant decrease in 5-HT level in blood from MDS patients (n = 15) as compared to control individuals (n = 14).

(H) Significant correlation between 5-HT and Hb levels in low-risk MDS patients (n = 10).

Unpaired t test, Spearman correlation, Mann-Whitney test, and Pearson linear and non-linear regressions were used when appropriate. *p < 0.05; **p < 0.005; ***p < 0.0005.

the physiological dose of EPO (0.2 U/L), addition of 5-HT or the 5-HT_{2A}R agonist (PNU 22394) significantly enhanced the expansion of CD36⁺ cord blood cells (Figures 2D and 2E).

Because tryptophan can be metabolized through the IDO pathway, we also performed an analysis of IDO expression in CD36⁺ cells. Western blot analysis of the protein demonstrated that IDO is highly expressed from day 1 to day 3 of culture after CD36⁺ isolation, and the expression decreases rapidly thereafter. As elevated levels of kynurenine metabolites have been previously proposed to be responsible for inhibition of hematopoietic progenitor amplification, we next investigated whether inhibitors of the kynurenine pathway could function with 5-HT to enhance proliferation of human erythroid progenitors. Figures 2F and 2G demonstrate that, although a decrease in the expression of IDO is observed in the presence of the inhibitor 1-methyl-tryptophan (1-MT), there is no increase in the proliferation of CD36⁺ cells.

Together, the data show the existence of a functional serotonergic system within cells of the erythroid lineage and highlight the importance of the switch between IDO and TPH1 for tryptophan metabolism. At a specific point in time, tryptophan conversion into 5-HT appears to be required for efficient proerythroblast proliferation.

Cell-Autonomous Action of Serotonin Contributes In Vivo to Normal Erythropoiesis

We next identified components of the 5-HT system in murine progenitor cells of the bone marrow and demonstrated the presence of mRNA for *Tph1* selectively and highly expressed at the colony-forming unit (CFU)-E-to-pro-erythroblast transition checkpoint (c-Kit⁺/CD71⁺/TER119⁻ to c-Kit⁻/CD71⁺/TER119⁻; Figure 3A). Correspondingly, *Tph1* enzymatic activity was detected in wild-type (WT) pro-erythroblasts as compared to pro-erythroblasts derived from *Tph1*^{-/-} animals (Figure 3B). High

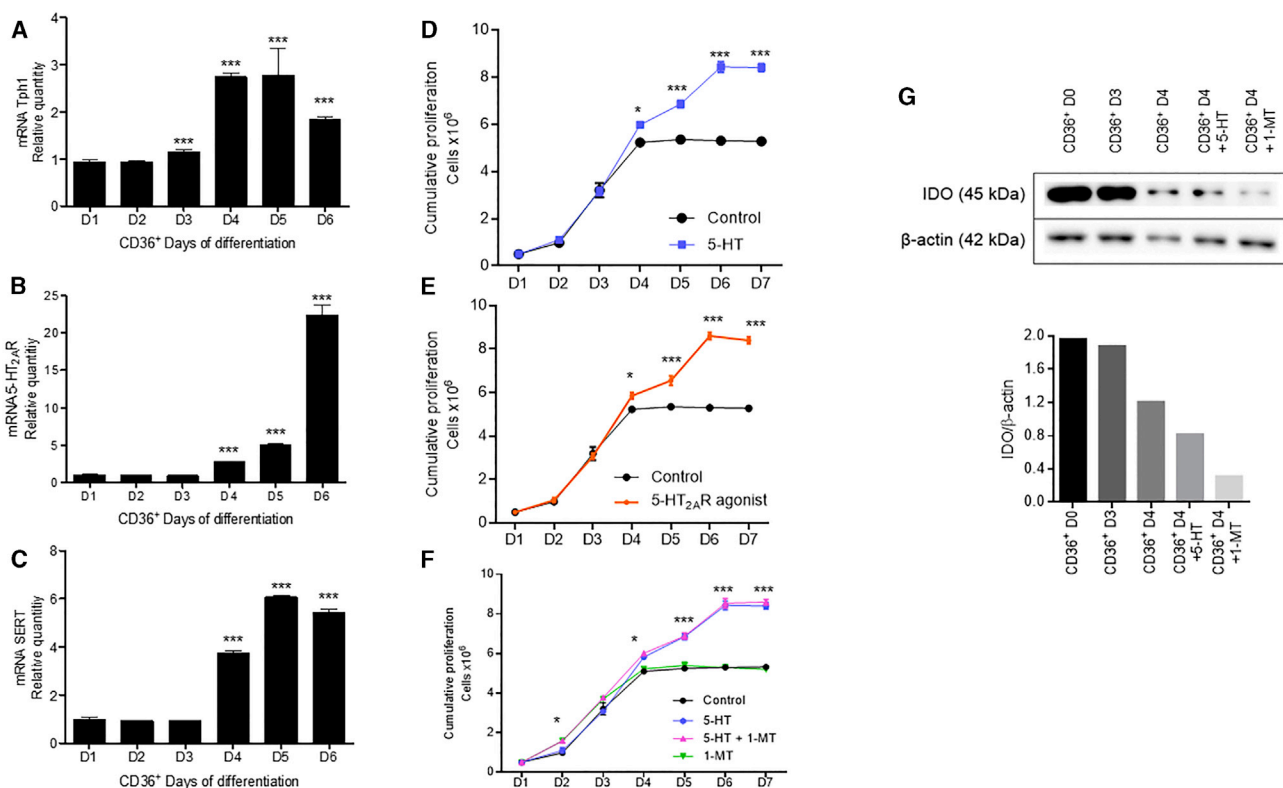


Figure 2. Identification of a Serotonergic System in Human Erythroid Progenitors to Increase Their Proliferation and Survival (A–C) *TPH1* (A), *5-HT_{2A}R* (B), and *SERT* (C) mRNA in cell cultures from human CD36⁺ cord bloods (data are from two independent experiments). (D and E) Use of 5-HT (D) or PNU 22394 (E), a 5-HT_{2A}R agonist, enhances erythroid proliferation (data are from 3 independent experiments). (F) No increase in proliferation of CD36⁺ cells in presence of the IDO inhibitor 1-methyl-tryptophan (1-MT) (data are from 3 independent experiments). (G) Western blot analysis of the protein IDO showed high expression from day 1 to day 3 of culture after CD36⁺ isolation (data are from one experiment). Throughout, data are mean ± SEM. Paired, unpaired t tests were used when appropriate. *p < 0.05; **p < 0.005; ***p < 0.0005.

levels of mRNA expression encoding for *SERT* and *5-HT_{2A}R* were also seen specifically in the pro-erythroblast population (*c-Kit^{+/+}/CD71⁺/TER119⁻*), derived from the bone marrow of adult WT mice (2 months old) (Figures 3C and 3D).

We further examined whether 5-HT was present in other hematopoietic organs involved in red blood cell (RBC) production and identified the fetal liver as an additional source of intrinsic nonneuronal or peripheral 5-HT. Analysis of primary cell cultures from WT murine fetal liver (as defined in Socolovsky et al., 2007) demonstrated high expression of *Tph1* mRNA in the pro-erythroblast population, precisely at E13.5, although we observed weak or no expression at other points of development (Figure 3E). We, as well, identified high levels of mRNA expression encoding for the *5-HT_{2A}R* and the 5-HT-specific transporter (*SERT*) in the same population in the fetal liver (Figures 3F and 3G), as seen in the adult bone marrow.

The presence of the enzyme responsible for 5-HT synthesis and key serotonergic components in both human and murine erythroid progenitors support the hypothesis that a local serotonergic network participates to the regulation of erythropoiesis. To better understand the mechanism of action, bone marrow transplant experiments (as illustrated in Figure 3) were performed. The results demonstrated that 5-HT plays a cell-auton-

omous role as the anemic phenotype was transplantable by *Tph1^{-/-}* bone marrow cells in WT mice. Specifically, WT mice that received a transplant from *Tph1^{-/-}* (KO/WT) bone marrow cells had lower bone marrow 5-HT levels associated with a decrease in RBCs and Hb levels, whereas *Tph1^{-/-}* mice that received a transplant from WT bone marrow cells (WT/KO) had normal bone marrow 5-HT levels and presented no anemic phenotype (Figures 3H–3J).

These results are in line with a general mechanism during development and in adult life involving a physiological circuit exerting a cell-autonomous action in hematopoietic organs. Fetal and bone-marrow-derived 5-HT appears to play an essential role at the transition checkpoint (CFU-E to pro-erythroblast) critical for pro-erythroblast expansion and proliferation.

***TPH1* Is an Erythroid-Specific Gene under the Control of Erythropoietin**

The previous findings identified 5-HT as an important autocrine regulator of erythropoiesis, a process highly dependent on EPO and stem cell factor (SCF) (Wu et al., 1995; Munugalavadi and Kapur, 2005; Kapur and Zhang, 2001). SCF, the ligand for the Kit receptor, plays a predominant role in the expansion of early progenitors, and EPO is crucial in regulating their survival

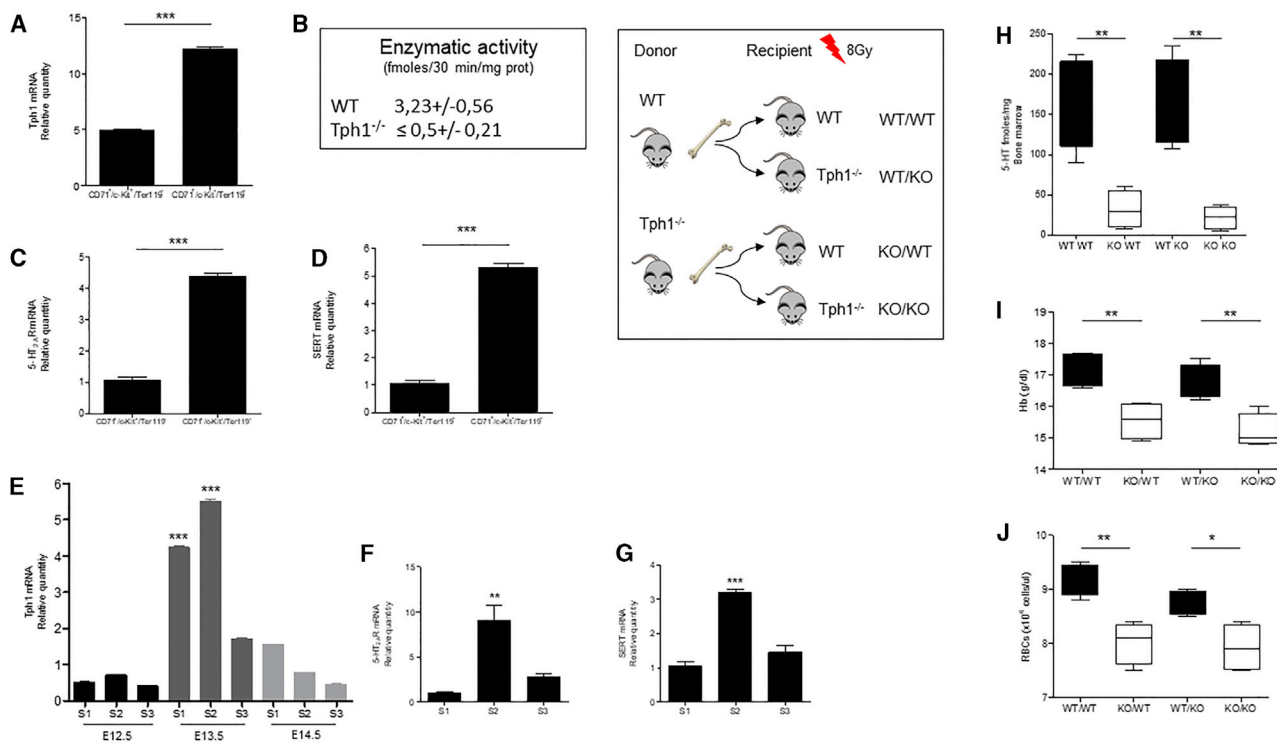


Figure 3. Cell-Autonomous Action of Serotonin Contributes *In Vivo* to Normal Erythropoiesis

(A–D) *Tph1* mRNA expression (A), *Tph1* enzymatic activity (B), *5-HT_{2A}R* (C), and *SERT* mRNA expression (D) in c-Kit^{+/+}/CD71⁺/TER119⁻ bone-marrow-derived cells from 8- to 10-week-old WT mice (data are representative of 4 independent experiments for A, C, and D and 2 independent experiments for B). (E–G) *Tph1* (E), *5-HT_{2A}R* (F), and *SERT* (*Slc6a*) (G) mRNA expression from S1, S2, and S3 (as described in Socolovsky et al., 2007) sorted cells from E12.5 to E14.5 WT fetal livers (data are representative of 5 independent experiments).

(H–J) 5-HT levels (H), Hb levels (I), and RBCs (J) number in WT or *Tph1*^{-/-} mice that received a transplant from WT or *Tph1*^{-/-} (data are from one bone marrow transplantation experiment; n = 4 WT, 4 *Tph1*^{-/-}).

Throughout, data are mean ± SEM. Paired and unpaired t tests were used when appropriate. *p < 0.05; **p < 0.005; ***p < 0.0005.

and proliferation, allowing differentiation of CFU-Es to pro-erythroblasts and further stages of maturation. To investigate whether 5-HT could be considered as an erythroid-stimulating agent in addition to EPO and SCF, murine bone-marrow-derived erythroid progenitors (c-Kit^{+/+}/CD71⁺/TER119⁻) from WT mice were expanded and submitted to various EPO and SCF concentrations. *Tph1* was positively regulated by EPO in a dose-dependent manner both at the mRNA and protein levels (Figures 4A and 4B), and 5-HT synthesis occurred as early as 3 h following EPO stimulation (Figure 4C). Increasing EPO concentration also upregulated *Htr2a* but downregulated *Slc6a4* (Figures 4D and 4E). Next, to identify the signaling pathway, cultured bone-marrow-derived erythroid progenitor cells (c-Kit^{+/+}/CD71⁺/TER119⁻) from WT mice were expanded and submitted to various signal transducer inhibitors. SCF significantly downregulated the expression of *Tph1* and, in the presence of EPO, inhibition of STAT5 downregulated *Tph1* expression, although inhibition of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) and mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) signaling pathways had no effect, indicating that the Janus kinase (JAK)/signal transducer and activator of transcription protein (STAT) pathway was the main one involved in *Tph1*-positive regulation (Figures 4F and 4G). More-

over, *in silico* analysis of the *Tph1* gene promoter led to the identification of the GATA1, STAT5, and ZBP89 binding sites (see Figure S1), which are key transcription factors involved in the regulation of erythroid-specific gene expression (Socolovsky et al., 2001; Ferreira et al., 2005; Woo et al., 2011). However, follow-up studies are needed to functionally validate that these transcription factors bind to the *Tph1* promoter and act as transcriptional regulator.

Overall, these data demonstrate that *Tph1* is a target gene of the EPO/erythropoietin receptor (EPOR) complex, which—through the orchestration of (1) increased 5-HT synthesis, (2) upregulation of 5-HT_{2A} receptor expression, and (3) downregulation of SERT—possibly raises the concentration of bone marrow 5-HT for local signaling, leading to renewal of erythroid progenitors.

Aged *Tph1*^{-/-} Mice Exhibit Anemia and MDS-like Phenotype

Low-risk MDSs are characterized by macrocytic anemia, dysplastic changes, possibly due to a discrepancy between proliferation and maturation as a result of a cell cycle anomaly of erythroid progenitors that induces multinuclearity, causing enhanced intramedullary death. Because MDS has an

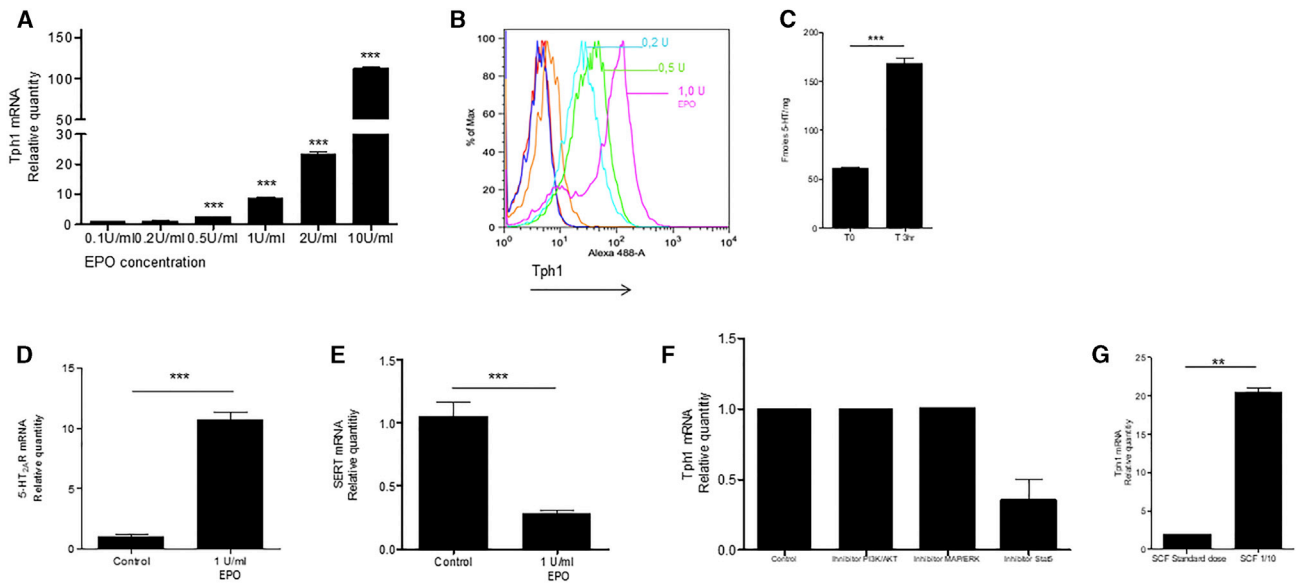


Figure 4. *TPH1* Is an Erythroid-Specific Gene under the Control of Erythropoietin

(A–C) *Tph1* mRNA (A), *TPH1* protein expression (B) following EPO stimulation (red line: no staining, blue line: staining with Tph Ab only; orange line: staining with 2nd Ab goat anti-rabbit immunoglobulin G [IgG] only), and turquoise, green, and pink lines staining with both Ab and (C) 5-HT synthesis following EPO stimulation (data are from 5 independent experiments for A and 2 independent experiments for B and C).

(D and E) *5-HT_{2A}R* (D) and *SERT* (E) mRNA following EPO stimulation (data are from 4 independent experiments).

(F) *Tph1* expression following inhibition of PI3K/Akt, MAP/ERK, and STAT5 signaling pathways (data are from 2 independent experiments).

(G) *Tph1* expression with different doses of SCF (data are from 3 independent experiments).

Throughout, data are mean ± SEM. Paired and unpaired t tests were used when appropriate. *p < 0.05; **p < 0.005; ***p < 0.0005.

age-related incidence, we specifically analyzed aged *Tph1*^{-/-} mice (>8 months old). Compared to WT mice, *Tph1*^{-/-} mice shared some features with MDS patients, including macrocytic anemia (Figures 5A–5C), peripheral blood cytological alterations with multinuclear, dysplastic cells (May-Grünwald-Giemsa stain; Figure 5D), and ring sideroblasts (Perl's staining) in bone marrow smears (Figure 5E).

To investigate whether, as seen in MDS patients, a disruption of tryptophan metabolism is occurring in *Tph1*^{-/-} mice, we analyzed metabolites of the kynurenine pathway in cells derived from the bone marrow. As illustrated in Figures 5H–5J, measurements of tryptophan and kynurenine levels are fairly similar to those reported in MDS patients. Specifically, in cells derived from the bone marrow of *Tph1*^{-/-} mice, there was significantly less tryptophan, a slight increase in kynurenine, and a significantly higher Kyn/Trp ratio, suggesting an increase in IDO activity. However, following western blot analysis of IDO in pro-erythroblasts derived from bone marrow of WT and *Tph1*^{-/-} mice, no variation in the expression of IDO was observed between the two genotypes (Figure 5F). In agreement with the previous results, metabolites of the kynurenine pathway (3-hydroxykynurenine [3-HK], xanthurinic acid [XA], and nicotinamide adenine [Nad]) were not increased in cells derived from bone marrow of *Tph1*^{-/-} animals as compared to WT (Figures 5K–5M). More importantly, *in vitro* addition of the IDO inhibitor (1-MT) on culture of pro-erythroblasts derived from bone marrow of *Tph1*^{-/-} mice indicated that, in absence of 5-HT, the inhibition of the kynurenine pathway did not result in an increase in proliferation of pro-erythroblasts (red line in Figure 5G). Whereas, as

previously shown (Amireault et al., 2011), addition of 5-HT to pro-erythroblasts derived from bone marrow of *Tph1*^{-/-} mice increased their proliferative capacity (blue line in Figure 5G).

Together, the findings imply that the anemia and MDS-like phenotype observed in 5-HT-deficient mice may not be due to toxicity following an increase of kynurenine metabolites as observed in MDS patients, where the metabolites have been proposed to inhibit hematopoietic progenitor amplification (Berthon et al., 2013). Rather, the findings demonstrate that decreased level of 5-HT available to erythroid progenitors can lead to the emergence of myelodysplastic-related anemia.

Pharmacologic Restoration of 5-HT Is Efficient to Rescue the Anemic Phenotype

We further studied the contribution of the identified source of 5-HT derived from pro-erythroblast cells of the bone marrow and asked whether the cytological features observed in *Tph1*^{-/-} pro-erythroblasts were associated with a defect in proliferation, differentiation, apoptosis, or some other processes (Zhang et al., 2003). We performed flow cytometry analysis (fluorescence-activated cell sorting [FACS]) by studying surface expression of the erythroid cell marker (TER119) and transferrin receptor (CD71; as described in Dussiot et al., 2014). Analysis of erythroid compartments in the bone marrow of aged *Tph1*^{-/-} mice (and of fetal livers; see Figure S2) indicated an overexpansion of pro-erythroblasts and early precursors (i.e., EryA and EryB). This was associated with decreased maturation to late-stage precursors in the bone marrow, leading to reduction of RBC production (EryC; Figure 6A). The results show that the apparent increase in

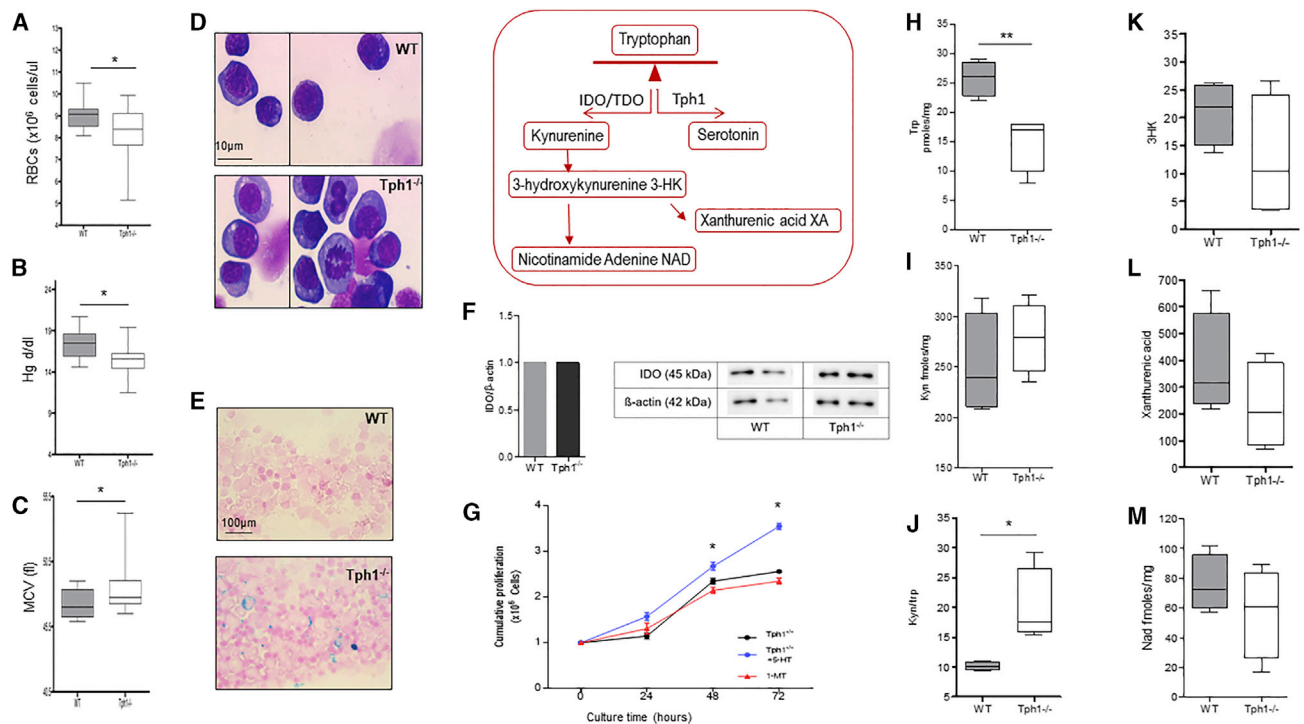


Figure 5. Aged *Tph1*^{-/-} Mice Exhibit Anemia and MDS-like Phenotype

30% of aged *Tph1*^{-/-} mice present with macrocytic anemia.

(A–C) Red blood cell counts (RBCs) (A), hemoglobin (Hb) (B), and mean corpuscular volume (MCV) (C; n = 32 WT; n = 45 *Tph1*^{-/-}).

(D and E) Representative image of bone marrow smears and pro-erythroblasts isolated from WT and *Tph1*^{-/-} mice (D) stained with MGG and Perle's Prussian blue (E).

(F) Western blot analysis showing no increase in IDO expression in pro-erythroblasts derived from *Tph1*^{-/-} mice in relation with actin loading control (data are from one experiment; n = 2 WT; n = 2 *Tph1*^{-/-}).

(G) Inhibition of the IDO enzyme did not increase proliferation of pro-erythroblasts derived from *Tph1*^{-/-} mice (data are from 2 independent experiments).

(H–J) Lower tryptophan level (H) with comparable level of kynurenine (I) and a significant increase in the ratio kynurenine to tryptophan (Kyn/Trp) (J) in cells isolated from the bone marrow of WT and *Tph1*^{-/-} mice (n = 4 WT; n = 4 *Tph1*^{-/-}).

(K–M) Measurements of metabolites of the kynurenine pathway showed no increase in cells derived from bone marrow of *Tph1*^{-/-} mice (n = 4 WT; n = 4 *Tph1*^{-/-}). 3-hydroxykynurenine (3-HK) levels (K), xanthurenic acid (XA) levels (L), and nicotinamide adenine (NAD) levels (M) are shown.

Throughout, data are mean ± SEM. Paired and unpaired t tests were used when appropriate. *p < 0.05; **p < 0.005; ***p < 0.0005.

the population of erythroid progenitors did not translate to more RBCs—due to heightened apoptosis, as revealed by FACS analysis of annexin V from *Tph1*^{-/-} mouse pro-erythroblasts (1.7-fold increase in the pro-erythroblast populations compared with WT animals; Figure 6B). In comparison to our previous results, where no difference was observed in annexin V binding in more mature cells of the erythroid lineage (c-Kit⁻/CD71⁺/Ter119⁺), the use of an improved *in vitro* method revealed an increase in annexin V binding in less mature erythroid progenitors derived from *Tph1*^{-/-} mice (c-Kit⁺/CD71⁺/Ter119⁻) (Dolznig et al., 2006). The findings suggest that the previously observed reduction in the total cellularity of the bone marrow in 5-HT-deficient mice resulted in a decrease in erythroid progenitors due to increased cell apoptosis. The cytological features and the defect in cells proliferation observed in pro-erythroblasts from *Tph1*^{-/-} mice were associated with an accumulation of the cells in S/4n phase of the cell cycle (Figure 6C). The presence of the 5-HT_{2A}R on pro-erythroblasts suggested that the effect on proliferation, cell cycle, and apoptosis might directly be mediated through activation of this receptor. Accordingly, *in vitro* addition

of a 5-HT_{2A}R agonist (PNU 22394) or of 5-HT rescued the defects in pro-erythroblasts from *Tph1*^{-/-} mice (Figures 6A–6C right, in pink).

To further understand the *in vivo* mechanism of action of 5-HT signaling on bone marrow cells, we use a well-described physiological model of anemia (Chen, 2005). As illustrated on the left part of Figure 6, WT mice were sub-lethally irradiated and, prior to treatment, were administered the well-known SSRI fluoxetine—a common antidepressant—or a placebo. We hypothesize that treatments with fluoxetine by blocking the action of SERT would lead to increased 5-HT extracellular concentrations and signaling (Olsson and Marcus, 2009). In agreement with our proposal, in WT mice treated with placebo, a prolonged anemia without normalization of Hb until day 22 was observed, whereas, in WT mice treated with fluoxetine, a rapid and significant increase in the number of reticulocytes was observed as early as day 3 and a close to normal Hb level was seen starting on day 11 (Figures 6D and 6E).

We next performed a second set of experiments with heterozygous (*Tph1*^{+/-}) animals. Of importance, *Tph1*^{+/-} mice have

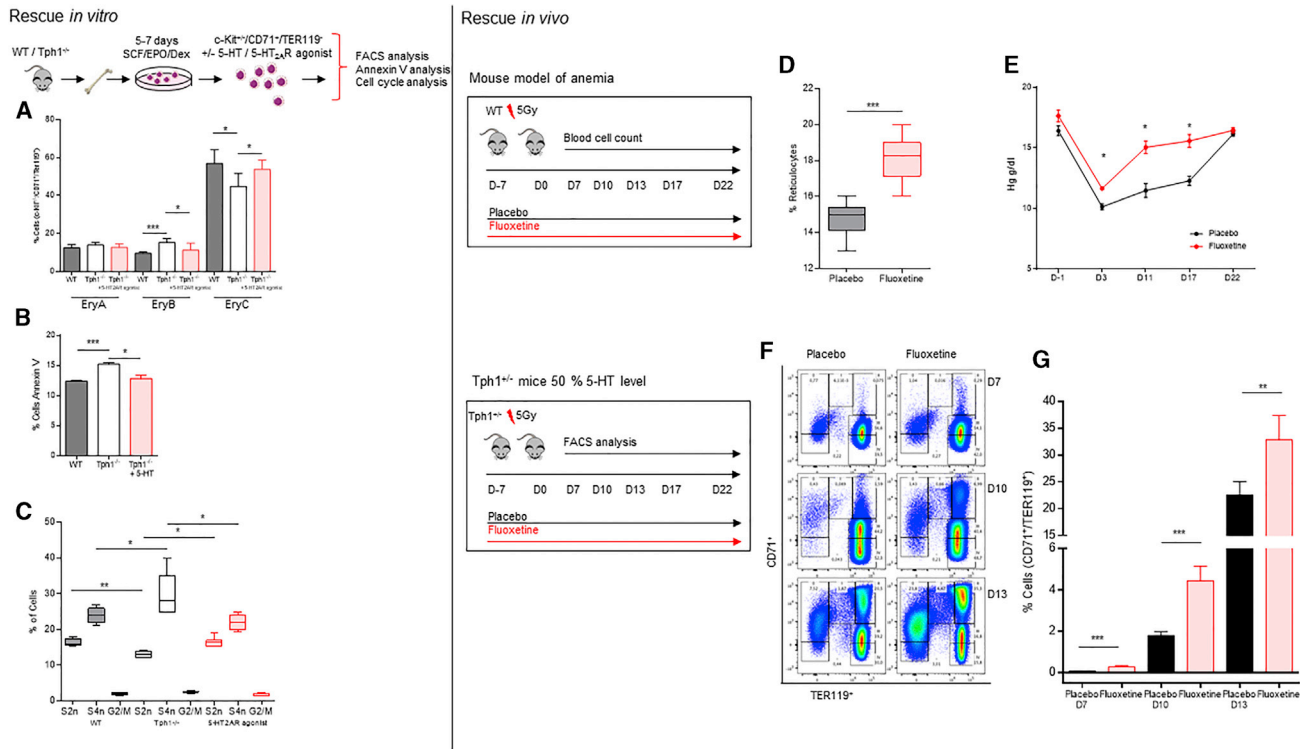


Figure 6. Pharmacologic Restoration of 5-HT Is Efficient to Rescue the Anemic Phenotype

(A–C) *In vitro* rescue experiment.

(A) Graph of the percentage of different erythroblast populations shown in WT (gray), $Tph1^{-/-}$ (white), and $Tph1^{-/-}$ +5-HT_{2A}R agonist (pink).

(B) Graphs showing the percentage of apoptotic pro-erythroblasts in WT (gray), $Tph1^{-/-}$ (white), and $Tph1^{-/-}$ +5-HT (pink; EryB population from Figure 6A).

(C) Cell cycle analysis of cultured pro-erythroblasts from WT (gray), $Tph1^{-/-}$ (white), and $Tph1^{-/-}$ +5-HT_{2A}R agonist (pink). Data are from 3 independent experiments.

(D–G) *In vivo* rescue experiment.

(D and E) Schematic representation of the two experimental models of anemia, percent of reticulocytes (D), and Hb level in WT mice (n = 7) (E) treated with placebo or fluoxetine for 7 days followed by sub-lethal irradiation (data are from 2 independent experiments).

(F and G) Representative image of a FACS profile of erythroid progenitors from freshly isolated bone marrow cell derived from $Tph1^{+/-}$ mice treated with placebo (n = 4–6 mice at each time point) or fluoxetine (n = 6–8 mice at each time point) for 7 days followed by sub-lethal irradiation (data are from one experiment).

(F) Representative images of FACS profiles of erythroid progenitors for mice treated with placebo or fluoxetine at day 7, day 10 and day 13 after irradiation.

(G) Corresponding percentages of CD71⁺/TER119⁺ cells for the same groups and time points.

Throughout, data are mean ± SEM. Paired and unpaired t tests were used when appropriate. *p < 0.05; **p < 0.005; ***p < 0.0005.

5-HT levels that are 50% of the WT control level, and under steady-state condition, there is a slight, but not significant, decrease in Hb level. Alongside, there is a proliferation defect in pro-erythroblasts cells derived from $Tph1^{+/-}$ mice, yet again the differences are not statistically significant (data not shown). As results from Figure 1H revealed a significant correlation between 5-HT and Hb levels in low-risk MDS patients, we hypothesize that individuals with lower 5-HT level may be more at risk to develop MDSs. We performed FACS analysis to identify proliferation and differentiation-stage-specific erythroid progenitors from freshly isolated bone marrow cells derived from $Tph1^{+/-}$ mice following sub-lethal irradiation and treated with fluoxetine or placebo. Starting on day 7, a significant increase is observed in the CD71⁺/TER119⁺ population in cells derived from the bone marrow of $Tph1^{+/-}$ mice treated with fluoxetine as compared to $Tph1^{+/-}$ mice treated with placebo (Figures 6F and 6G). Combined with the increase in erythroid progenitors and precisely on day 7 of treatment, we measured a significant increase in

5-HT levels (nM) in cells derived from the bone marrow of $Tph1^{+/-}$ mice treated with fluoxetine as compared to placebo (0.654 ± 0.023 versus $<0.2 \pm 0.019$).

Altogether, the *in vitro* and *in vivo* results indicate a mechanistic role for 5-HT to ensure proper RBC production. 5-HT protects the erythroid progenitors from apoptosis, specifically at the CFU-E-to-pro-erythroblast transition checkpoint, which involves intense cell division. In the absence of peripheral 5-HT, $Tph1^{-/-}$ animals exhibit impaired erythroid-proliferating capacities, morphological abnormalities, increased apoptosis, intramedullary death, and anemia similar to what is observed in patients with low-risk MDS. *In vitro* addition of the 5-HT_{2A}R agonist enhances cellular division of erythroid progenitors to rescue the proliferation defect. *In vivo* pharmacological increase of 5-HT levels through SSRI treatment prolongs and increases 5-HT_{2A}R stimulation, which in turn enhances cellular division of erythroid progenitors also to rescue the proliferation defect and the anemic phenotype.

DISCUSSION

Through the TPH and IDO pathways, the essential amino acid tryptophan is the precursor of important metabolites, including 5-HT and kynurenine. In this study, we confirmed, in cohorts of MDS patients, an increase in IDO activity and enhanced tryptophan breakdown. We further investigated whether, in MDS patients, enhanced IDO activity could result in diversion of the available tryptophan toward the Kyn pathway and deprived cells of 5-HT. As such, impaired erythroid proliferating capacities seen in MDS-related anemia could be explained by low 5-HT levels. Our work revealed lower 5-HT levels in blood of MDS patients, thereby providing evidence that lack of 5-HT can lead to the emergence of myelodysplastic-related anemia. However, the MDS-like phenotype observed in 5-HT-deficient mice does not appear to be due to toxicity following an increase of kynurenine metabolites as previously proposed in MDS patients. Rather the decreased level of 5-HT leads to cell cycle anomaly of erythroid progenitors, causing enhanced intramedullary death. The *in vivo* rescue experiments demonstrating the use of SSRIs to increase extracellular source of 5-HT in the earliest stages of MDS, where ineffective erythropoiesis and anemia are major signs, represent an innovative therapeutic strategy.

Our work as well demonstrated that EPO, the main cytokine for RBCs production, upregulated 5-HT synthesis at a critical transition checkpoint during erythroid progenitors' proliferation (CFU-E to pro-erythroblast). Moreover, the developmental defect and the unsolved significant reduction (15%) in the number of *Tph1*^{-/-} live pups at E13.5 (Amireault et al., 2013) is reminiscent of the phenotype of the EPO knockout, which is lethal for E13.5 embryos (Wu et al., 1995). A combined action between EPO and 5-HT is thus likely for efficient proliferation of erythroid progenitors and consistent with our previous result showing higher levels of EPO in the serum of adult *Tph1*^{-/-} mice yet without any erythropoietic response (Amireault et al., 2011).

The identification of a serotonergic system in erythroid progenitors is an additional indication for the concept of “microserotonergic systems,” where locally restricted 5-HT synthesis plays a predominant role in comparison to the traditional view of 5-HT as a pleiotropic hormone synthesized in the gut, taken up by platelets and distributed throughout the entire organism (reviewed in Amireault et al., 2013 and Spohn and Mawe, 2017). The data are consistent with those of Kirouac and colleagues (2010), who reported that hematopoietic stem cells expansion correlated with G-protein-coupled receptors neurotransmitter signaling (especially members of the 5-HT_{2R} family) in regulating stem cell fate. The authors proposed that the signal of nonneuronal origin was activated endogenously by hematopoietic progenitor cells rather than by infiltrating distant cells (Kirouac et al., 2010). Although we do not exclude that neuronal 5-HT might exert its activity on hematopoietic stem and progenitor cells proliferation, our results established the direct effect of locally derived 5-HT on erythroid progenitors that does not require any downstream effectors signal, such as the hypothalamic, pituitary, adrenal axis observed in zebrafish embryos (Kwan et al., 2016). Also, even though we have identified proerythroblasts as a source of 5-HT in the bone marrow, we cannot exclude that additional cell types of the hematopoietic lineage

are capable of synthesizing 5-HT. Nevertheless, in contrast to a suggested role played by neuronal 5-HT on hematopoietic stem and progenitor cells during murine early development (Lv et al., 2017), we have identified two sources of nonneuronal or peripheral 5-HT and provide pharmacological and genetic evidences for 5-HT synthesis in a specific erythroid progenitor cell type at a critical transition checkpoint.

With reference to 5-HT and its well-known effect on mood—low levels being associated with the onset of depression—a recent publication by Vulser et al. (2016) showed a robust association between depression and anemia, offering further support to the emerging link between decreased levels of 5-HT and anemia. Along that line, it is tempting to speculate that, in patients with anemia, SSRI could be used and particularly for the group of MDS patients not responding to EPO treatment. For instance, in RARS patients, a subgroup of low-risk MDS, EPO treatment is usually inefficient to improve anemia. Likewise, a retrospective cohort analysis suggested that low-risk MDS patients may benefit from SSRI treatment, as improvement in overall survival was observed among patients diagnosed with low-risk MDS treated with SSRIs, including fluoxetine (Li et al., 2017). If our findings are further validated by the ongoing clinical trial (NCT02452983), the proposal to target the serotonergic network in the earliest stages of MDS, where anemia is a major sign, may indeed represent a therapeutic intervention.

In summary, our study led to significant advances in understanding of the molecular mechanisms that could be targeted in MDSs. The identification of drugs to improve ineffective erythropoiesis at initial stages of MDS will contribute to reduce the need for transfusions and the transfusion-related risks and importantly improve the quality of life of patients suffering from erythroid disorders, including MDS.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found with this article online at <https://doi.org/10.1016/j.celrep.2019.02.071>.

ACKNOWLEDGMENTS

The authors thank Aurelia Dujardin (LEAT Institut *Imagine*) and Corinne Cordier, Jérôme Mégret, and Olivier Pellé for their help with cell sorting (SFR Necker). This work was supported in part by grants from Laboratory of Excellence GR-Ex, reference ANR-11-LABX-0051. The labex GR-Ex is funded by the program “Investissements d’avenir” of the French National Research Agency, reference ANR-11-IDEX-0005-02. T.C. was supported by a PhD fellowship from Institut Imagine, D.S. was supported by a post-doctoral fellowship from INSERM, and J.R. was supported by a PhD fellowship from the Ministère Supérieur de l’Enseignement et de la Recherche. L.M. received support from the Centre National de la Recherche Scientifique; the INSERM; the Sorbonne Université Sciences; and by grants from the Fondation pour la Recherche sur le Cerveau, the Fondation de France, the Fondation pour la Recherche Médicale “Equipe FRM DEQ2014039529,” ANR-17-CE16-0008, and the Investissements d’Avenir programme ANR-11-IDEX-0004-02) as part of the Bio-Psy Labex. Funding from Programme Hospitalier de Recherche Clinique (PHRC) MDS-04, INCa-DGOS_5480 (to M.F.) was also received. The authors would also like to thank Jason Miller for editing of the manuscript.

AUTHOR CONTRIBUTIONS

Conceptualization, D.S., T.C., J.R., O.H., and F.C.; Methodology, D.S., T.C., J.R., M.L., E.B., G.F., R.R., S.T., P.B., F.B., M.D., K.D., L.L., J.C., J.-M.L., L.M., C.V., G.C., and F.C.; Resources, O.K. and M.F.; Writing—Review & Editing, T.C., O.H., and F.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: April 18, 2018

Revised: December 30, 2018

Accepted: February 20, 2019

Published: March 19, 2019

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD71 FITC	BD PharMingen	Cat# 553266; RRID:AB_394743
TER119 PE	BD PharMingen	Cat# 553673; RRID:AB_394986
c-Kit APC	BD Biolegend	Cat# 105811; RRID:AB_313220
Antibody Tph1	Abcam	Cat# ab52954; RRID:AB_2207555
IDO	BioLegend	Cat# 122402; RRID:AB_2280140
DyLight 488 goat anti-rabbit IgG	Abcam	Cat# ab96899; RRID:AB_10679361
Chemicals, Peptides, and Recombinant Proteins		
Fluoxetine	Prozac ® 20mg/5mL soln	N/A (Necker Hospital)
Erythropoietin		N/A (Necker Hospital)
1-methyl-tryptophan	Sigma-Aldrich	Cat# 860646
Pimozide		Cat# P1793
StemPro-34 serum-free stem cell expansion medium	Thermo Fischer Scientific	Cat# 10639011
Stem Cell factor	Immunotools	Cat# 12343323
Dexamethasone	Sigma	Cat# D1756
Annexin V	BD Biosciences	Cat# 556419; RRID:AB_2665412
PNU 22394 HCl	Tocris Bio-Science	Cat# 2201-10
5-HT HCl	Sigma	Cat# H9623
Critical Commercial Assays		
BrdU flow kit	BD Biosciences	5 Cat# 59619
Experimental Models: Cell Lines		
Human CD34 cord blood cells	This paper	N/A
Mouse Pro-erythroblasts	This paper	N/A
Experimental Models: Organisms/Strains		
Tph1 KO mice	Côté et al., 2003	N/A
Oligonucleotides		
Primers Tph1 mouse	Life technologies	Cat# Mm01202614_m1
Primers Tph1 human	Life technologies	Cat# Hs00188220_m1
Primers SERT human	Life technologies	Cat# Hs00984349_m1
Primers SERT mouse	Life technologies	Cat# Mm00439391_m1
Primers GAPDH mouse	Life technologies	Cat# Mm99999915_g1
Primers 18S transcript mouse	Life technologies	Cat# Mm03928990_g1
Primers GAPDH human	Life technologies	Cat# Hs03929097_g1
Primers 18S transcript human	Life technologies	Cat# Hs99999901_s1
Primer 5-HT2AR human	Life technologies	Cat# Hs01033524_m1
Primers 5-HT2AR mouse	Life technologies	Cat# Hs01566408_m1
RNA seq: data will be made available upon request		N/A

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Francine Côté, francine.cote@parisdescartes.fr.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse models

For all experiments animals were housed 5 mice/cage. Tph1^{-/-} mice were generated as described (Côté et al., 2003). Tph1^{-/-}, Tph1^{+/-} and WT animals (males and females) were derived from pure C57BL/6J genetic backgrounds. For some experiments, C57/bl6 mice (males and females) were purchased from Janvier Labs (CS 4105 LE GENEST ST ISLE, SAINT BERTHEVIN Cedex France). For all experiments, littermates of the same sex were randomly assigned to experimental groups. Mice were given *ad libitum* access to food and water with a 12 hours light/ 12 hours dark cycle.

For experiments involving mouse embryos, Tph1 wild-type, heterozygous, and homozygous males (10-12 weeks old) bred to Tph1 wild-type, heterozygous, and homozygous females (10-12 weeks old).

For *in vivo* experiments involving fluoxetine and sub-lethal irradiation, WT mice and Tph1^{+/-} were used (males and females 8-10 weeks old and >8 months old). For bone marrow transplantation experiments, WT females and Tph1^{-/-} 8-10 week's old female mice were used. For murine erythroid *in vitro* cell culture and immunophenotyping of murine erythroid precursors, cells were obtained from fetal livers or flushed from femur/tibia (WT and Tph1^{-/-} males and females 8-10 weeks old and >8 months old).

Animal procedures

Tph1^{-/-} mice were generated as described (Côté et al., 2003). Tph1^{-/-}, Tph1^{+/-} and WT animals (males and females) were derived from pure C57BL/6J genetic backgrounds. For some experiments, C57/bl6 mice (males and females) were purchased from Janvier Labs (CS 4105 LE GENEST ST ISLE, SAINT BERTHEVIN Cedex France). Animal experiments were performed according to the recommendations of the French Institutional Committee (A75-15-34).

Blood counts

To analyze blood counts, peripheral blood from the tail vein was collected in EDTA tubes and analyzed using an electronic hematology particle counter (type MS9-5; Melet Schloesing Laboratories).

Embryos

Embryos were obtained from crosses between Tph1 wild-type, heterozygous, and homozygous males bred to Tph1 wild-type, heterozygous, and homozygous females (8-10 weeks old). The day at which the vaginal mating plug was seen was taken as E0.5. Mice were sacrificed by cervical dislocation; the fetal livers were removed from E10.5 to E18.5 and stained with appropriate antibodies. For BrdU incorporation, pregnant females were injected i.p. a dose of BrdU (1 mg) dissolved in PBS 2 hours before sacrifice. For cell cycle analysis, BrdU incorporation level in CD71⁺/c-Kit^{+/-}/TER119⁻ cells was assessed using a BrdU flow kit according to the manufacturer's instructions (BD PharMingen). For RT-qPCR analysis, CD71⁺/c-Kit^{+/-}/TER119⁻ cells were isolated from fetal livers using a FACS-Ariall cell sorter (BD Biosciences).

Fluoxetine treatment

Fluoxetine was administrated orally (40 mM in water bottle (Prozac ® 20mg/5mL solution) to WT mice and Tph1^{+/-} (males and females 8-10 weeks old C57/bl6 purchased from Janvier labs) for 7 days before inducing anemia and continue throughout the experiment.

Sub-lethal irradiation

Sub-lethal irradiation was induced by submitting WT or Tph1^{+/-} mice to 1.09 Gy during 4 minutes (WT C57/bl6 Males 8-10 weeks old purchased from Janvier labs for Figures 6D and 6E). For Figures 6F and 6G Tph1^{+/-} mice were males and females > 8 months old.

Bone marrow transplantation experiment

Bone marrow transplantation was performed as described in D'Aveni et al., 2015.

Histological analysis

For histology, tissues were fixed in 10% formalin overnight, embedded in paraffin blocks, and sectioned (5 μM). Sections were stained with Prussian blue. Smears and culture of pure pro-erythroblasts cells (c-Kit^{+/-}/CD71⁺/TER119⁻) derived from bone marrow of WT and Tph1^{+/-} aged mice (males and females > 8 months old) were stained with May-Gruenwald Giemsa for analysis.

Patients

Low and int-1 MDS patients (n = 71) were enrolled after they gave their informed consent according to the recommendations of the institutional ethics committee (CPP Ile-de-France X). Peripheral blood plasma (n = 71) and bone marrow RNA (n = 27) were obtained at diagnosis for tryptophan and kynurenine dosages, and for RNA-sequencing, respectively. Plasmas and bone marrows from age-matched subjects were used as controls. For 5-HT measurements, whole blood was collected from MDS patients (n = 15; 9 men, 6 women) and aged-matched controls (n = 14) on sodium-citrate tubes. For the RNA seq experiment (Figure 1A), n = 27; 22 men, 5 women. For the IDO, Hb, Trp measurement (Figures 1B-1E) n = 71; 48 men, 23 women.

Human cell studies

Cord blood samples were collected from umbilical veins following normal full-term vaginal deliveries and processed within 24 hours. Erythroid cells were generated from CD34⁺ cord blood progenitor cells.

METHOD DETAILS

Cell culture

Murine Erythroid in Vitro Cell Culture

Cells from fetal livers or flushed from femur/tibia were grown in StemPro-34 serum-free stem cell expansion medium supplemented with human recombinant EPO (1 U/mL), murine recombinant stem cell factor (100 ng/mL) (Immunotools), and dexamethasone (1 μ M) (Sigma). After 5–7 days in StemPro-34, a culture of pure pro-erythroblasts was obtained. In some conditions, 5-HT HCl (1 μ M; Sigma), PNU 22394 HCl (1 μ M) (Tocris Bio-Science) and 1-methyl-tryptophan (1-MT) (Sigma) was added to the proliferation/differentiation medium. Proliferation was monitored by cell counts. Treatment of CD71⁺/c-Kit^{+/-}/TER119⁻ mouse bone marrow cells for 2–4 hours with STAT5 inhibitors (10 μ M pimozide, Sigma), followed by RT-qPCR analysis for *Tph1* mRNA. Apoptosis was measured using APC-labeled annexin V (BD PharMingen) following staining with cell surface markers. For TPH1 protein expression, CD71⁺/c-Kit^{+/-}/TER119⁻ cells derived from the bone marrow were stained with the tryptophan hydroxylase antibody (ab52954; Abcam, Cambridge, England) according to the manufacturer's protocol. Briefly, CD71⁺/c-Kit^{+/-}/TER119⁻ were fixed with 80% methanol (5 minutes) and then permeabilized with 0.1% PBS-Tween for 20 minutes. Cells were then incubated in 1X PBS/10% normal goat serum followed by the antibody (ab52954, 1:100 dilution) for 30 minutes at room temperature. The secondary antibody used was DyLight 488 goat anti-rabbit IgG (ab96899) at 1:500 dilution for 30 minutes at room temperature. Isotype control antibody was rabbit IgG (monoclonal) used under the same conditions. Cells were analyzed on a FACS Cantoll coupled with FlowJo software version X.0.7 (Tree Star, Ashland, OR).

Cell cycle analysis

For cell cycle analysis, BrdU incorporation level in CD71⁺/c-Kit^{+/-}/TER119⁻ cells was assessed using a BrdU flow kit according to the manufacturer's instructions (BD PharMingen). For RT-qPCR analysis, CD71⁺/c-Kit^{+/-}/TER119⁻ cells were isolated from bone marrow using a FACS-Ariall cell sorter (BD Biosciences).

Human cord blood Erythroid in Vitro Cell Culture

Erythroid cells were generated from CD34⁺ cord blood progenitor cells in serum-free medium in the presence of EPO (2 mU/ml) + IL-3 (10 ng/ml) + stem cell factor (SCF; 50 ng/ml) as previously described in [Zermati et al., \(2000, 2001\)](#). The study was performed according to the Helsinki Declaration with the approval from the ethics committee of our institution (Comité de Protection des personnes (CPP) "Ile de France II"). All patients gave written informed consent. Cord blood samples were collected from umbilical veins following normal full-term vaginal deliveries and processed within 24 hours.

Immunophenotyping of Murine Erythroid Precursors

Cells from fetal livers or BM cells flushed from femur and tibia were resuspended in Hank's buffered saline before being passed through a 100- μ M strainer. Cells were washed, counted, and immunostained at room T° in PBS with PE-conjugated anti-TER119, FITC-conjugated anti-CD71 and APC-conjugated anti-CD117 (c-Kit) (BD PharMingen and BioLegend) antibodies for 20 min and analyzed on a FACS Canto II coupled with FlowJo software version X.0.7 (Tree Star, Ashland, OR)

Measurements of 5-HT, tryptophan, kynurenine Levels by HPLC

Tph1^{-/-} and WT littermates were sacrificed by cervical dislocation and the femur/tibia were removed, weighed, and sonicated for 5 s in 10 volumes (vol/wt) of 0.1 N perchloric acid 0.05% disodium EDTA 0.05% sodium metabisulfite. 5-HT was extracted, and 10-l samples were injected onto a Beckman Ultrasphere 5-m IP column (Beckman). Eluted 5-HT, tryptophan, kynurenine, hydroxykynurenine, xanthurenic acid and nicotinamide adenine were quantified electrochemically (at 0.65 V) and concentrations were calculated in nanograms per gram of tissue or in μ mol/ml plasma/serum 5-HT).

RT qPCR

Total RNA was extracted from fetal liver cells, bone marrow cells and CD34⁺/CD36⁺ cord blood cells using the RNeasy Kit (QIAGEN). Reverse transcription was performed using iScript Reverse Transcription Supermix for RT-qPCR (BioRad). Real time PCR was performed on a STEPONE cycling machine (Applied Biosystems) using oligos from Taqman (Life technologies). Two biological replicates were used for each condition. Data were analyzed by StepOne Plus RT PCR software v2.1 and Microsoft excel. β -actin, GAPDH and 18S transcript levels were used for normalization of each target (= Δ CT). Real-time PCR CT values were analyzed using the 2⁻($\Delta\Delta$ CT) method to calculate the fold expression ($\Delta\Delta$ CT method).

RNA-sequencing

Libraries were constructed using the TruSeq Stranded mRNA Sample Preparation Kit (Illumina, San Diego, CA) and sequenced on an Illumina HiSeq 2500 platform using a 100-bp paired-end sequencing strategy. An average depth of global sequence coverage of 114 million and a median coverage of 112 million was attained. For analysis, TopHat (v2.0.6) was used to align the reads against the human reference genome Hg19 RefSeq (RNA sequences, GRCh37) downloaded from the UCSC Genome Browser (<http://genome.ucsc.edu>). The read counts were transformed by voom from the Limma R package which converts the counts to log-counts per million with associated precision weights and flags were computed using a custom algorithm within R as follows. Assuming that a maximum of 80% of genes are expressed, we select the 20% lowest counts for each sample as background. A threshold is fixed at two standard deviations over the mean of the background. All transcripts for which normalized counts were lower than the

computed threshold were designated as background for each array. When comparing normalized counts between two groups, a transcript was included in the analysis if its counts exceeded the background. Normalized data were used to estimate the expression of selected genes.

Data will be made available upon request.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Throughout data are mean \pm SEM. Unpaired t test, Spearman correlation, Mann-Whitney test and Pearson linear and non-linear regressions were used when appropriate. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.