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TO THE EDITOR:

Lack of *Gdf11* does not improve anemia or prevent the activity of RAP-536 in a mouse model of β-thalassemia

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β-thalassemia (BT) is a global health problem affecting millions of patients, and current therapies to treat BT present major clinical challenges.[1,2] Luspatercept (ACE-536), an erythroid maturation agent that functions independently of the erythropoietin (EPO) pathway,[3] has been shown to improve anemia in BT.[4,5] In clinical trials, treatment with ACE-536 increased hemoglobin (Hb) levels and significantly reduced red blood cell (RBC) transfusions in adults with BT, with little to no adverse effects.[4,5] ACE-536 is a fusion protein composed of a modified extracellular domain of activin receptor IIB (ACVR2B) and the Fc part of human immunoglobulin G1. This fusion protein competes with ACVR2B to bind members of the transforming growth factor-β (TGF-β) superfamily.[6] Studies in murine models of BT using the murine analog of ACE-536 (RAP-536) show that RAP-536 targets the protein growth differentiation factor 11 (GDF11).[3,7,8] These studies have proposed that overexpression of GDF11 blocks terminal erythroid differentiation by increasing oxidative stress and that treatment with the ligand trap ACE/RAP-536 sequesters GDF11, unblocking terminal erythroid differentiation and, thereby, ameliorating ineffective erythropoiesis. However, ACE-536 and RAP-536 have been shown to stimulate RBC synthesis in healthy humans and mice, where GDF11 overexpression has not been reported.[5,9] Because of the incongruency of the proposed model, our study resorted to genetic tools to reduce GDF11. We investigated whether deletion of the *Gdf11* gene improved anemia in a BT mouse model ($Hbb^{th3/+}$).[10] We also examined whether RAP-536 was efficacious in the absence of *Gdf11*.

Gdf11 was functionally inactivated in the entire hematopoietic compartment of mice ($Gdf11^{\Delta 2\text{-}3/\Delta 2\text{-}3}$) by crossing Vav^{Cre}-transgenic animals[11] with $Hbb^{+/+}$ and $Hbb^{th3/+}$ LoxP flanked *Gdf11* ($Gdf11^{flox/flox}$) mice[12] to produce $Hbb^{+/+}Vav^{Cre}Gdf11^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ and $Hbb^{th3/+}Vav^{Cre}Gdf11^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ mice (supplemental Figure 1, available on the *Blood* Web site). The resulting progeny were viable, and complete blood count (CBC) results did not show changes in RBC, Hb, hematocrit (Hct), or reticulocyte levels (Figure 1A-H). *Gdf11* recombination in the spleens of $Hbb^{th3/+}Vav^{Cre}Gdf11^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ mice was confirmed by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) (Figure 1Q). Similarly, crossing $EpoR^{Cre}$-transgenic animals[13] with $Gdf11^{flox/flox}$ mice, in which *Gdf11* was reduced in the early erythroid progenitor, did not result in changes to the hematological profile (supplemental Figure 2).

We then tested whether GDF11 produced by nonhematopoietic tissues indirectly influenced erythropoiesis. Because *Gdf11*-null mice ($Gdf11^{-/-}$) are embryonically lethal,[12] we generated mice with a pancellular deletion of *Gdf11* using the tamoxifen (TAM)-inducible $Rosa^{Cre}$ strain[14] and generating $Hbb^{+/+}Rosa^{Cre}Gdf11^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ and $Hbb^{th3/+}Rosa^{Cre}Gdf11^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ mice (supplemental Figure 3). Neither $Hbb^{+/+}Rosa^{Cre}Gdf11^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ mice nor $Hbb^{th3/+}Rosa^{Cre}Gdf11^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ mice showed any alterations in RBC, Hb, Hct, or reticulocyte levels 2 weeks (Figure 1I-P), 5 weeks (supplemental Figure 5A-H), or 5 to 6 months (supplemental Figure 5I-P) post-TAM treatment. We confirmed *Gdf11* recombination by qRT-PCR in the spleens of $Hbb^{th3/+}Rosa^{Cre}Gdf11^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ mice (Figure 1Q) and by polymerase chain reaction in the spleen, liver, heart, duodenum, kidney, and bone marrow of $Hbb^{+/+}Rosa^{Cre}Gdf11^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ and $Hbb^{th3/+}Rosa^{Cre}Gdf11^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ animals (supplemental Figure 4). These findings were consistent in females (supplemental Figure 6) and males (supplemental Figure 7). No differences were detected in the spleen-to-body weight ratios of $Vav^{Cre}Gdf11^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ or $Rosa^{Cre}Gdf11^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ mice compared with controls (supplemental Figure 8). GDF11 has also been proposed to exert a negative effect on late-stage erythropoiesis in myelodysplastic syndromes, a heterogeneous group of clonal hematopoietic disorders.[3,15] To test this, we crossed *NUP98-HOXD13*[16] (*NHD13*), a mouse model that recapitulates all key features of myelodysplastic syndromes, with $Vav^{Cre}Gdf11^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ mice. Results show that genetic removal of *Gdf11* from all hematopoietic lineages in *NHD13* mice did not confer a therapeutic benefit (supplemental Figure 9).

We then evaluated whether the effect of RAP-536 is mediated by the synthesis of *Gdf11* from erythroid or nonerythroid cells. We administered 12 doses of RAP-536 to $Hbb^{+/+}$ and $Hbb^{th3/+}$ mice of the $Vav^{Cre}Gdf11^{\Delta 2\text{-}3/\Delta 2\text{-}3}$- and $Rosa^{Cre}Gdf11^{\Delta 2\text{-}3/\Delta 2\text{-}3}$-derived lines. In all mice treated, RAP-536 significantly increased RBC, Hb, and Hct parameters; additionally, reticulocyte counts were normalized in $Hbb^{th3/+}$ mice (Figure 2A-P). Thus, lack of *Gdf11* did not prevent responsiveness to RAP-536. Because activin receptor ligand traps have been shown to stimulate RBC synthesis and Hb increases in healthy humans,[5,17] we investigated whether CD34$^+$ cells respond to RAP-536 treatment in vitro. CD34$^+$ were differentiated and expanded in a 3-phase liquid erythroid-specific differentiation medium containing RAP-536.[18] Cell counts were conducted at the end of an 8-day differentiation assay to determine whether cells cultured in RAP-536 produced more mature cells. No differences were observed in the number of hemoglobinized cells after treatment with 5 μg/mL or 150 μg/mL RAP-536 (Figure 2Q).

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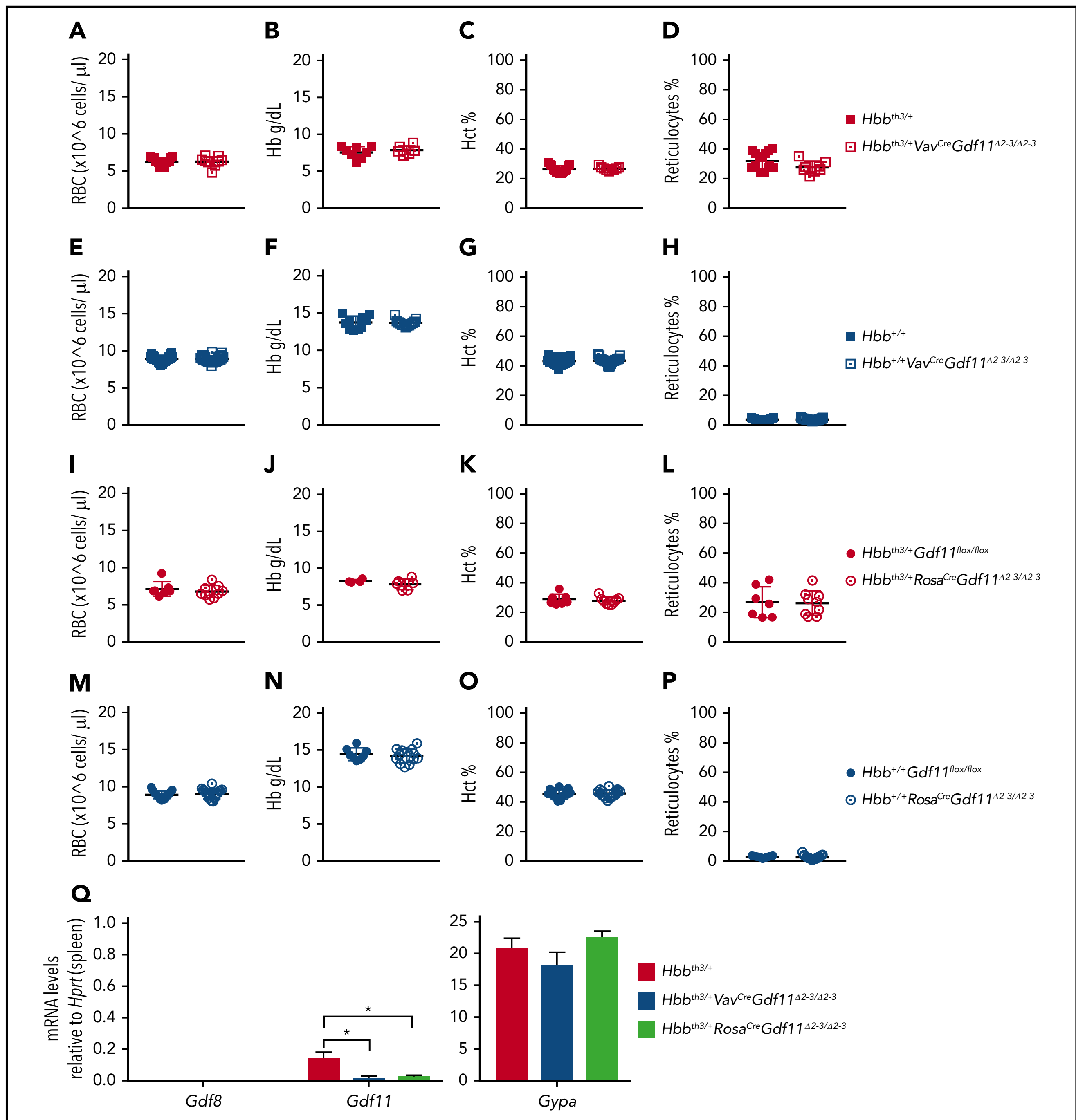

Figure 1. Conditional deletion of *Gdf11* in the entire hematopoietic compartment or pancellularly in thalassemic (*Hbb*$^{th3/+}$) mice does not improve hematological parameters. (A-D) Conditional deletion of *Gdf11* in the entire hematopoietic compartment of thalassemic mice (*Hbb*$^{th3/+}$ *Vav*^{Cre}*Gdf11*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$) (n = 9) does not result in any differences in RBC count, Hb, Hct, or reticulocytes compared with *Hbb*$^{th3/+}$ controls (n = 15). (E-H) Conditional deletion of *Gdf11* in erythroid cells of nonthalassemic wild-type animals (*Hbb*$^{+/+}$) does not result in altered hematopoietic parameters in *Hbb*$^{+/+}$ *Vav*^{Cre}*Gdf11*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ mice (n = 23) compared with *Hbb*$^{+/+}$ controls (n = 22). CBCs were analyzed at 2 months of age. Ubiquitous deletion of *Gdf11* in *Hbb*$^{th3/+}$*Rosa*^{Cre}*Gdf11*$^{flox/flox}$ mice treated with TAM (*Hbb*$^{th3/+}$*Rosa*^{Cre}*Gdf11*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$) does not improve hematological parameters. (I-L) *Hbb*$^{th3/+}$*Rosa*^{Cre}*Gdf11*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ mice (n = 9) did not show increases in RBC, Hb, or Hct or lower reticulocyte counts compared with *Hbb*$^{th3/+}$ *Gdf11*$^{flox/flox}$ control mice (n = 7). (M-P) No hematological differences were detectable in *Hbb*$^{+/+}$*Rosa*^{Cre} *Gdf11*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ mice (n = 17) compared with *Hbb*$^{+/+}$ *Gdf11*$^{flox/flox}$ controls (n = 15). *Rosa*^{Cre} *Gdf11*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ mice and *Gdf11*$^{flox/flox}$ controls were analyzed between 3 and 6 months of age. CBCs were analyzed 2 weeks post-TAM administration. Females and males were included in the analysis. (Q) Messenger RNA analysis of *Gdf11*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ mice confirms reduction of *Gdf11* in spleens. Messenger RNA from *Hbb*$^{th3/+}$ *Vav*^{Cre}*Gdf11*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ (n = 3) and *Hbb*$^{th3/+}$*Rosa*^{Cre}*Gdf11*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ (n = 3) age-matched males (5 months old) was assessed for *Gdf11* reduction in the spleen, by qRT-PCR, using a *Gdf11* probe specific for exon 2. Both show significant reductions normalized by *Hprt* (left panel). *Gdf8* was undetectable in all samples tested. No statistical differences were found in the *Gypa* positive control (right panel). Data are mean ± standard deviation. *$P \leq .05$, Student *t* test.

Next, we investigated messenger RNA (mRNA) expression levels of *GDF11* and *ACVR2B* in early erythroid progenitors derived from healthy and BT donor CD34^{+} cells. Because GDF11 has been proposed to inhibit early erythroid progenitors from differentiating, cells were cultured as previously mentioned[18] and collected for mRNA early in the differentiation phases: 24 hours

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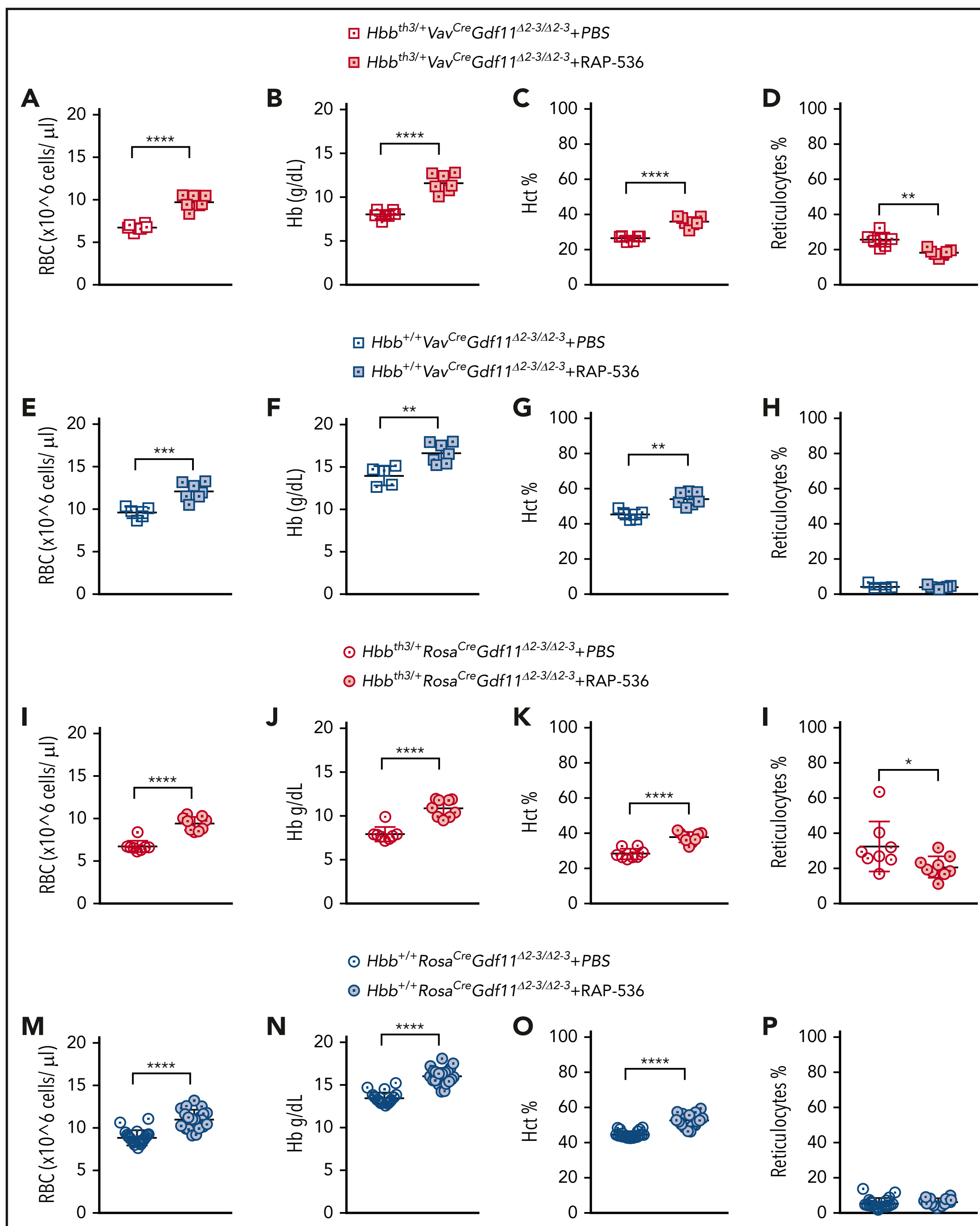

Figure 2. *Gdf11* deletion from the hematopoietic compartment or pancellularly from all tissues did not suspend RAP-536 action. *Hbb*$^{th3/+}$*Vav*^{Cre}*Gdf11*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ mice treated with RAP-536 (n = 7) showed increased RBCs (A), Hb (B), and Hct (C), as well as reduced reticulocytes (D), compared with phosphate-buffered saline (PBS)-treated controls (n = 6). *Hbb*$^{+/+}$*Vav*^{Cre}*Gdf11*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ mice also exhibited increased RBCs (E), Hb (F), and Hct (G). No statistically significant difference was found in reticulocyte number (H). *Vav*^{Cre}*Gdf11*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ mice were treated with RAP-536 between 3 and 4 months. Similarly, *Hbb*$^{th3/+}$*Rosa*^{Cre}*Gdf11*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ mice (n = 9) and *Hbb*$^{+/+}$*Rosa*^{Cre} *Gdf11*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ mice (n = 20) treated with RAP-536 exhibited increased RBC (I,M), Hb (J,N), and Hct (K,O) levels compared with *Hbb*$^{th3/+}$*Rosa*^{Cre}*Gdf11*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ (n = 8) and *Hbb*$^{+/+}$*Rosa*^{Cre} *Gdf11*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ (n = 20) PBS-treated animals. (L) *Hbb*$^{th3/+}$*Rosa*^{Cre}*Gdf11*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ mice showed a significant reduction in reticulocytes. (P) No statistical differences were observed in reticulocytes from *Hbb*$^{+/+}$*Rosa*^{Cre} *Gdf11*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ animals. *Rosa*^{Cre} *Gdf11*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ animals were treated with RAP-536 between 4 and 7 months. CBCs were assessed 2 days after last dose of RAP-536 treatment. Females and males were included in the analysis of all groups. CD34^{+} cells did not respond to RAP-536 in vitro. (Q) Treatment of CD34^{+}-derived cells isolated from healthy donors with 5 µg/mL or 150 µg/mL RAP-536 (n = 3) did not result in increased cell number at the end of erythroid differentiation assay day 8 (DD8). mRNA levels of *GDF11* and *ACVR2B* were low in human thalassemia and healthy erythroid progenitor cells. Quantification of *GDF11* and *ACVR2B* mRNA in thalassemia (R) and healthy (S) donor-derived erythroblasts showed low *GDF11* and *ACVR2B* expression relative to *HPRT* after day 1 (DD1) and day 4 (DD4) of differentiation in an erythroid differentiation assay, as determined by qRT-PCR (n = 3). BT CD34^{+} cells showed higher relative levels of *GDF11* compared with healthy CD34^{+} cells. *RHO* was used as a negative control (data not shown), and *TFRC* and *GYPA* were used as positive controls. *Gdf11* and *Acvr2b* mRNA was expressed at low levels in splenic Ter119^{+} cells isolated from PBS- and RAP-536–treated *Hbb*$^{th3/+}$ mice. (T) Erythroid cells, isolated from the spleens of PBS-treated *Hbb*$^{th3/+}$ mice and RAP-536–treated mice, were analyzed for Cd71 and Ter119 marker expression before mRNA extraction. (U) mRNA from Ter119^{+} cells isolated from the spleens of *Hbb*$^{th3/+}$ PBS- and RAP-536–treated mice was analyzed for *Gdf11* and *Acvr2b* by qRT-PCR; results show low expression relative to *Hprt* compared with *Tfrc* and *Gypa* in either group. *Rho* was used as a negative control (data not shown). Data are mean ± standard deviation. $^{*}P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .001$, $^{****}P \leq .0001$, Student *t* test.

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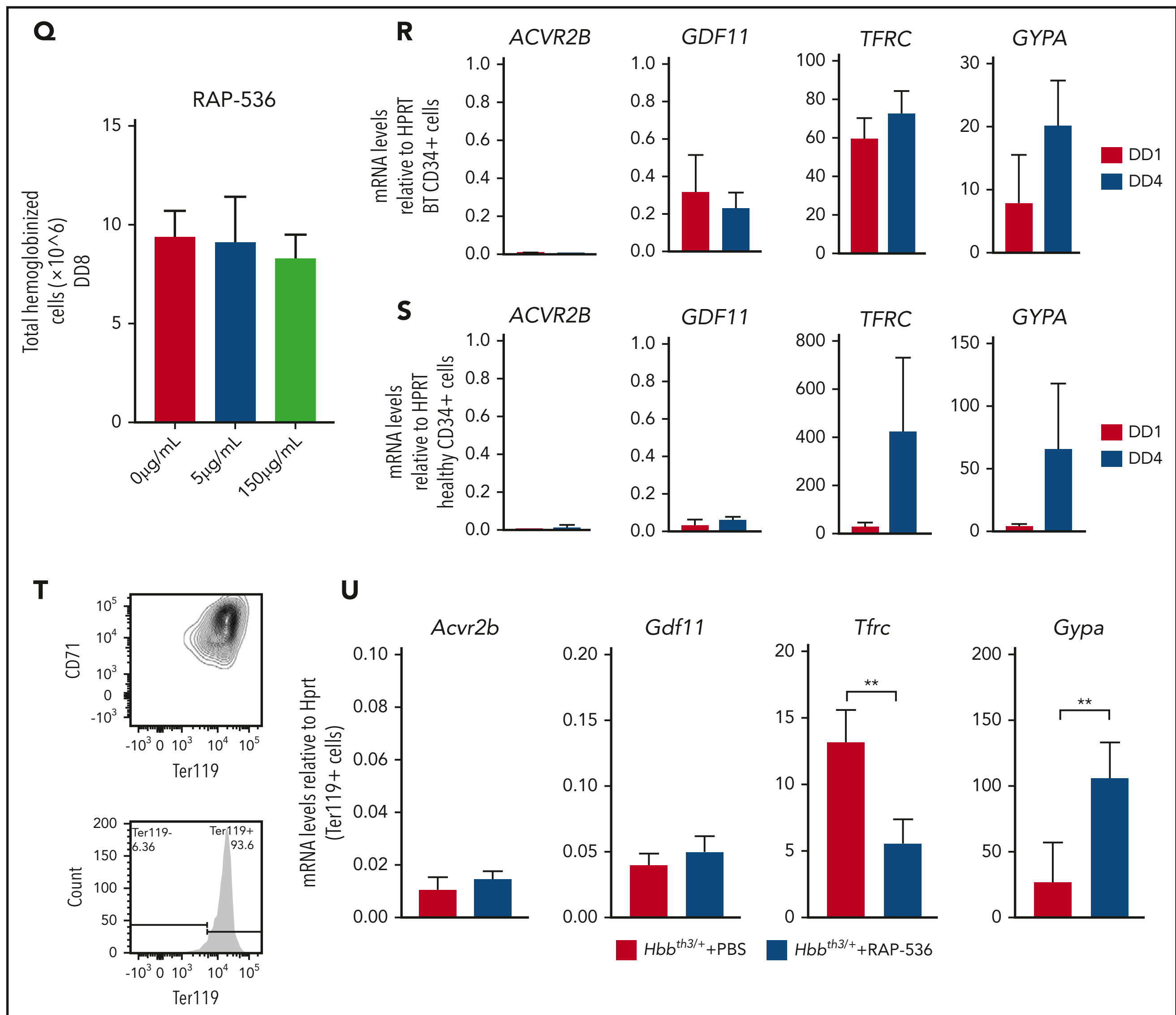

Figure 2. Continued.

later and 4 days later. qRT-PCR results showed low levels of *GDF11* and *ACVR2B* in CD34$^+$ cells from BT (Figure 2R) and healthy (Figure 2S) donors compared with *TFRC* and *GYPA* controls (supplemental Figure 10B for probes used). Similarly, Ter119$^+$ erythroid progenitors isolated from *Hbb*$^{th3/+}$ spleens (Figure 2T; supplemental Figure 10A) showed low expression of *Gdf11* and *Acvr2b* in phosphate-buffered saline– and RAP-536–treated mice (Figure 2U). However, in RAP-536–treated mice, *Tfrc* and *Gypa* levels were significantly different compared with phosphate-buffered saline controls, which is consistent with a reduction in the erythroid progenitor pool and an increase in RBC differentiation.

The EPO-independent ability of RAP-536 to increase RBC number and Hb content offers the potential for understanding an undiscovered pathway in erythropoiesis. Studies have identified GDF11 as the primary target of RAP-536.[3,7,8] However, evidence for overexpression of GDF11 was obtained using nonspecific antibodies for GDF11[19] in BT erythroid cells[7] and does not explain how RAP-536 is also effective in *Hbb*$^{+/+}$ mice, in which GDF11 overexpression in erythroid cells has not been observed. RAP-536 has also been reported to bind other members of the TGF-β family, including GDF8 and activin B.[3,20] Based on these observations, analyses were performed in *Hbb*$^{+/+}$ mice using antibodies against activin B, GDF8, or GDF8/11.[20] These studies indicate that the antibodies had modest effects on RBC synthesis, and none could recapitulate the effect of RAP-536.[20] Here, we show that lack of *Gdf11* does not improve anemia in *Hbb*$^{th3/+}$ mice or increase hemoglobin in *Hbb*$^{+/+}$ mice. In accordance with these findings, *Hbb*$^{th3/+}$ and *Hbb*$^{+/+}$ animals, with a deletion in the hematopoietic compartments and a pancellular deletion of *Gdf11*, respond to RAP-536. In addition, we show that *Gdf11* and *Acvr2b* expression is low in a BT mouse model and in human erythroid cells. Altogether, our studies strongly indicate that decreasing GDF11 in erythroid cells has no effect on anemia. Lastly, GDF11 has been reported to have protective properties on cardiovascular health; patients with decreased levels of GDF11 had increased incidences of heart failure, stroke, and death.[21] Animal studies have also implicated GDF11 as important in neuronal vascularization

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and skeletal muscle development.[22-24] Targeting GDF11 as an agent to increase hematological parameters could compromise cardiovascular protection or affect other important pathways in development. Future work will focus on the role of the TGF-β superfamily in erythropoiesis and on identifying the targets responsible for the therapeutic effects produced by RAP-536, because the mechanism for RAP-536 remains to be elucidated.

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Authorship

Contribution: A.G., P.R.O., L.B., and S.R. designed experiments, analyzed and interpreted data, and wrote the manuscript; A.G., P.R.O., C.R.H., S.S., J.Z., V.L.P., C.C., P.L., and A.C.M. performed experiments and collected data; and S.S., A.C.M., L.B., A.K.S., and M.F. edited the manuscript.

Conflict-of-interest disclosure: S.R. is a member of the scientific advisory board for Ionis Pharmaceuticals and Meira GTx, has acted as a consultant for Disc Medicine, Protagonist, and La Jolla Pharmaceutical Company, and is a coinventor for patents US8058061 B2 C12N 20111115 and US7541179 B2 C12N 20090602. The consulting work and intellectual property of S.R. did not in any way affect the design, conduct, or reporting of this research. The remaining authors declare no competing financial interests.

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Footnotes

The online version of this article contains a data supplement.

There is a *Blood* Commentary on this article in this issue.

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