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Stem Cells, Tissue Engineering, and Hematopoetic Elements

Protein Phosphatase 2A Catalytic Subunit α (PP2Acα) Maintains Survival of Committed Erythroid Cells in Fetal Liver Erythropoiesis through the STAT5 Pathway

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Suppression of programmed cell death is critical for the final maturation of red blood cells and depends largely on the anti-apoptotic effects of EpoR–STAT5–Bcl-x_L signaling. As the major eukaryotic serine/threonine phosphatase, protein phosphatase 2A (PP2A) regulates multiple cellular processes, including apoptosis. However, whether PP2A plays a role in preventing erythroid cells from undergoing apoptosis remains to be elucidated. We conditionally inactivated the catalytic subunit α of PP2A (PP2Acα), which is the predominant form of PP2Ac, during early embryonic hematopoiesis. Loss of PP2Acα in hematopoietic cells perturbed definitive erythropoiesis characterized by fetal liver atrophy, reduced $Ter119^+$ cell number, abnormal expression patterns of molecular markers, less colony formation, and a reduction in definitive globin expression. Levels of erythropoiesis-promoting cytokines and initial seeding with hematopoietic progenitors remained unchanged in $PP2Ac\alpha^{TKO}$ fetal livers. We noted impaired expansion of the fetal erythroid compartment, which was associated with increased apoptosis of committed erythroid cells. Mechanistically, PP2Acα depletion markedly reduced Tyr^{694} phosphorylation of STAT5 and expression of Bcl-x_L. Unexpectedly, PP2Acα-deficient embryos did not manifest any early embryonic vascular defects. Collectively, these data provide direct loss-of-function evidence demonstrating the importance of PP2Acα for the survival of committed erythroid cells during fetal liver erythropoiesis. ***(Am J Pathol 2011, 178:2333–2343; DOI: 10.1016/j.ajpath.2011.01.041)***

The production of red blood cells (RBCs) is normally maintained at a constant level by well-tuned regulation of erythropoiesis. During terminal maturation, mammalian erythroblasts accumulate hemoglobin, assemble the RBC cytoskeleton, extrude their nuclei, and give rise to RBCs.[1] Suppression of programmed cell death is considered to be critical for the final maturation of RBCs and depends strongly on anti-apoptotic effect of erythropoietin (EPO) stimulation and the intracellular EpoR-STAT5-Bcl-x_L signaling axis.[2] $EPO^{-/-}$ and $EpoR^{-/-}$ mice die at embryonic day (E) 13.5 owing to a failure in definitive erythropoiesis.[3] $STAT5^{\triangle N}$ mice are embryonic anemic due to decreased survival of RBC progenitors.[4,5] *Bcl-x* is a member of the BclII gene family, the members of which share homology in four conserved regions (BH1-4 domains), which control their dimerization and function. The expression of Bcl-x is increased in terminally differentiated erythroblasts,[6] during which stage it positively regulates the survival of these cells.[7] Indeed, Bcl-x–deficient mice are embryonic lethal owing to massive apoptosis of immature erythroid cells in the fetal liver.[8] Factors that regulate the survival of maturing erythroblasts are also relevant to clinical anemia induced by chemotherapy and chronic diseases, such as renal disorders, myeloma, and myelodysplasic syndromes.[9–11]

In eukaryotic cells, at least 30% of proteins can be modulated by reversible phosphorylation. Controlled protein phosphorylation, mediated by protein kinases and phosphatases, regulates multiple cellular processes, including apoptosis.[12,13] Deregulation of apoptosis can lead to many human diseases, including cancer, Alzheimer's disease, cardiac dysfunction, and inflammation,[14,15] most of which have been reported to involve deregulation of protein phosphatase 2A (PP2A),[13,16,17] the major eukaryotic serine/threonine phosphatase. However, whether PP2A also plays a role in preventing erythroid cells from undergoing programmed cell death is

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still largely unknown. The only previously reported function of PP2A in erythropoiesis is associated with activation of K-Cl cotransport.[18]

Heterotrimeric PP2A is composed of a scaffold subunit (A subunit), a catalytic subunit (PP2Ac), and a regulatory subunit (B subunit).[19] The scaffold subunit is flexible and structurally links PP2Ac with various regulatory subunits to form different holoenzymes. Molecular cloning has revealed the existence of two mammalian PP2Ac isoforms: PP2Acα (encoded by the *Ppp2ca* gene) and PP2Acβ (encoded by the *Ppp2cb* gene). These two isoforms share 97% amino acid identity, and seven of the eight residues that differ between them are located within the first 30 amino acids (encoded by exon I).[13,20] Both PP2Ac isoforms are ubiquitously expressed, and PP2Acα transcripts are generally 10-fold more abundant than are PP2Acβ transcripts owing to transcriptional regulation.[21,22] However, detailed interpretation of the specific contribution of these two isoforms has long been hampered owing to the lack of reliable antibodies or specific chemical inhibitors that can distinguish between them. We sought to address a role for PP2Acα in erythropoiesis using a genetic approach.

Conventional deletion of the *Ppp2ca* allele in mice results in embryonic lethality after E6 due to absent mesoderm formation,[23] making it impossible to determine the functional importance of *Ppp2ca* in embryonic erythropoiesis. Herein, we conditionally inactivated the *Ppp2ca* allele during early embryonic hematopoiesis by using *Tie2Cre* transgenic mice, which are ideal models to induce early gene recombination in hematopoietic and endothelial cells.[24] We discovered that loss of the *Ppp2ca* allele perturbed fetal liver erythropoiesis. PP2Acα was not required to establish the fetal liver hematopoietic stem and progenitor cells (HSCs/Ps) pool; however, it enhanced the cell survival function of EPO. It acted, at least in part, by promoting the STAT5–Bcl-x_L axis and, thereby, inhibiting apoptosis of committed erythroid cells.

Materials and Methods

Mice and Genotyping

Mice carrying the conditional *Ppp2ca* allele (*Ppp2ca*$^{fl/fl}$) were bred with *Tie2Cre* mice,[25] which were purchased from The Jackson Laboratory (Bar Harbor, ME). To visualize cells with recombined alleles, mice bearing the *Tie2Cre* transgene were crossed with *ROSA26* reporter mice.[26] All mice used in this study were of a mixed 129/B6 background. Mice were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care International–credited specific pathogen–free animal facility, and animal welfare and experimental procedures were approved by the Animal Care and Use Committee of the Model Animal Research Center, the host for the National Resource Center for Mutant Mice in China, Nanjing University. Genotyping was performed by PCR analyses of genomic DNA isolated from mouse tails or yolk sacs. Genotyping primer sets and PCR reaction programs are listed in Table 1 and in Supplemental Table S1 (available at *http://ajp.amjpathol.org*), respectively.

Table 1. Genotyping Primers

Primer name	Primer sequence
First loxP	F: 5′-AAGTTACTGAGTGCAGTGTGCCTTG-3′ R: 5′-TTATACCCTTCCTCATTCGCTCTGC-3′
Second loxP	F: 5′-TAGCCCATGCCTTTAATCTCAGAGC-3′ R: 5′-CACTCGTCGTAGAACCCATAAACC-3′
Cre	F: 5′-GCCTGCATTACCGGTCGATGC-3′ R: 5′-CAGGGTGTTATAAGCAATCCC-3′
DNA-EXdel	F: 5′-AACCCACCCTTGAGAAACAGC-3′ R: 5′-TACCCTTCCTCATTCGCTCTG-3′
RNA-EXdel	F: 5′-ATTACCTGTTTATGGGAGACTATGT-3′ R: 5′-AAGTGGTCACGGCTGTTGATG-3′

F, forward; R, reverse.

Timed Mating and Tissue Harvesting

Female *Ppp2ca*$^{fl/fl}$ mice were mated with male *Tie2Cre*$^{+}$/*Ppp2ca*$^{fl/+}$ mice for embryo collection. Fetal livers were dissociated mechanically by pipetting for fluorescence-activated cell sorting (FACS) analyses. Single-cell suspensions were prepared by drawing medium and cells up and down through a 1-mL syringe and 27-gauge needle.

Cell Cultures and EPO Stimulation

Fetal liver cells from E12.5 embryos were cultured in Iscove's modified Dulbecco's medium with 2% fetal bovine serum. Cells were maintained at 37°C and 5% CO_2 in the presence or absence of 10 U/mL recombinant human EPO. For the annexin V binding assay, stimulation lasted 18 hours.[27] For the Western blot assay, stimulation lasted 15 minutes.

Immunostaining, Flow Cytometric Analyses, and Cell Sorting

Freshly isolated fetal liver cells were stained with different combinations of Ter119-PE, CD45-FITC, Gr-1–FITC, CD41-FITC, c-Kit–APC, CD71-biotin, and streptavidin-PECy5. Megakaryocyte progenitors and megakaryocytes were sorted as Lin^{-}c-Kit^{+}CD41^{+} and Lin^{-}c-Kit^{-}CD41^{+} cells, respectively.[28] For analysis of Lin^{-}Sca-1^{+}c-Kit^{+} (LSK) cells, fetal liver cells were stained with c-Kit–APC, Sca-1–FITC, and a lineage marker cocktail containing CD3-PE, CD5-PE, B220-PE, Gr-1–PE, and Ter119-PE. Apoptotic cells were verified using annexin V–FITC and Ter119-PE double staining. Endothelial cells were selected as CD31^{+}CD45^{-}.[29] Stained cells were analyzed using a FACSCalibur flow cytometer equipped with CellQuest software or were sorted using LSR II and four-laser FACSAria II sorters (all from Becton Dickinson, San Jose, CA). Sorted cells were collected in buffer containing RNase inhibitor and were stored at −70°C. The calculated absolute fetal liver cell numbers and the percentages of Ter119^{+}, CD45^{+}, Gr-1^{+}, Lin^{-}c-Kit^{+}CD41^{+}, Lin^{-}c-Kit^{-}CD41^{+}, LSK, and CD31^{+}CD45^{-} cells allowed for the determination of absolute cell numbers of these particular cell lineages in whole fetal liver samples.

Table 2. Primers Used for Quantitative PCR

Gene	Forward primer	Reverse primer
Ppp2cb	5′-GAGGGTACTACTCTGTGGAGAC-3′	5′-CCGGCTTTCGTGATTTCCT-3′
β$^{maj/min}$	5′-ATGGCCTGAATCACTTGGAC-3′	5′-ACGATCATATTGCCCAGGAG-3′
βH1	5′-TCCTTGGGCTTGGGGGTTA-3′	5′-TGTGGGACAGAGCATTGGC-3′
εy	5′-TGGCCTGTGGAGTAAGGTCAA-3′	5′-GAAGCAGAGGACAAGTTCCCA-3′
EPO	5′-CAGGCCCTGCTAGCCAATT-3′	5′-ACGTAGACCACTGATGGCTTTGT-3′
SCF	5′-CCCTGAAGACTCGGGCCTA-3′	5′-CAATTACAAGCGAAATGAGAGCC-3′
Bclx	5′-ACTGTGCGTGGAAAGCGTAGA-3′	5′-TGCTGCATTGTTCCCGTAGAG-3′
Pim1	5′-TTCTGGACTGGTTCGAGAGG-3′	5′-GCTCCTCGTTCGGTGATAAA-3′
Cis1	5′-CCACTGGCTTTGTCAAGAAGG-3′	5′-AGGCCACATAGTGCTGCACAA-3′
Socs3	5′-CCGCTTCGACTGTGTACTCAAG-3′	5′-TCTTCTCGCCCCCAGAATAGAT-3′
Osm	5′-AACTGAGCAAGCCTCACTTCC-3′	5′-ATGCCGAGGATATTGTGCCG-3′
GAPDH	5′-TGCCCAGAACATCATCCCT-3′	5′-GGTCCTCAGTGTAGCCCAAG-3′

In Vitro *Colony Formation Assays*

Fetal liver cells from E12.5 embryos were harvested in Iscove's modified Dulbecco's medium with 2% fetal bovine serum. For the erythroid colony-forming unit (CFU-E) assay, 2×10^4 cells were plated in 1 mL of methylcellulose medium supplemented with EPO (MethoCult M3334; STEMCELL Technologies Inc., Vancouver, BC, Canada) and were cultured for 2 days. For the erythroid blast-forming unit (BFU-E) assays, 1×10^5 cells were plated in 1 mL of methylcellulose medium and were cultured for 7 days. Erythroid colonies were stained for hemoglobin using benzidine. CFU-E contained 8 to 32 benzidine-positive cells and BFU-E contained 3 or more clusters of CFU-E. The reported values are mean ± SEM as determined for three or four embryos from each genotype.

Phosphatase Activity Determination

Tissue protein was extracted in a phosphatase extraction buffer containing 20 mmol/L imidazole-HCl, 2 mmol/L EDTA, 2 mmol/L EGTA (pH 7.0), 1 mmol/L benzamidine, 1 mmol/L phenylmethylsulfonyl fluoride, and protein inhibitor cocktails. Phosphatase activity was quantified using a malachite green–based PP2A Assay Kit (Upstate Biotechnology, Waltham, MA). Briefly, total proteins were immunoprecipitated with anti-PP2Ac, and PP2Ac-bound beads were incubated with synthetic phosphopeptide for the dephosphorylation reaction. The reaction supernatant was then mixed with malachite green reagent for color development. Changes in absorbance were measured at 650 nm.

RNA Isolation, Reverse Transcription, and Quantitative PCR

RNA from sorted cells was extracted using RNAspin Mini Kit (GE Healthcare Bio-Sciences Corp, Piscataway, NJ), whereas RNA from fetal livers was isolated using RNAiso (Takara Bio Inc., Shiga, Japan). Reverse transcription was performed using a first-strand cDNA synthesis kit. Quantitative PCR–based measurements of RNA abundance were performed using SYBR green reagents in an ABI 7300 sequence detector (Applied Biosystems, Foster City, CA). Amplification of the *GAPDH* gene served as an input control of cDNA templates. Primers used in this study are listed in Table 2. Efficiency of amplification for all primers was validated by determining the slope of CT versus dilution series.

Western Blotting

Tissue or cell proteins were extracted using 1% Nonidet P-40 (Caledon Laboratories Ltd., Georgetown, ON, Canada), 20 mmol/L Tris · HCl (pH 8.0), 5 mmol/L EDTA, 0.5 mmol/L EGTA, 150 mmol/L NaCl, 10% glycerol, 20 mmol/L β-glycerophosphate, protein inhibitors, and phosphatase inhibitors. Primary antibodies used in this study included anti-PP2Ac (Upstate Biotechnology), anti-PP2A-Aα/β (Santa Cruz Biotechnology, Santa Cruz, CA), anti-pTyr694–STAT5 (Cell Signaling Technology Inc., Beverly, MA), anti-STAT5 (Cell Signaling Technology Inc.), and anti–Bcl-x_L (eBioscience Inc., San Diego, CA).

Whole-Mount LacZ Staining

Embryos were fixed on ice in fixation buffer containing 0.2% glutaraldehyde, 5 mmol/L EGTA (pH 8.0), and 2 mmol/L $MgCl_2$ in PBS. Samples were washed with rinse buffer containing 0.01% sodium deoxycholate, 0.02% Nonidet P-40, 5 mmol/L EGTA (pH 8.0), and 2 mmol/L $MgCl_2$ in PBS. Samples were then stained with lacZ staining buffer containing 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 10 mmol/L $K_3Fe(CN)_6$, 10 mmol/L $K_4Fe(CN)_6$, and 20 mmol/L Tris · HCl (pH 7.3) in rinse buffer. Stained tissues were paraffin embedded, sectioned, and counterstained with nuclear fast red.

Statistical Analysis

Data were analyzed using two-tailed *t*-tests and are presented as mean ± SEM.

Results

In Vivo *Inactivation of PP2Acα in Tie2-Expressing Cell Lineages (PP2Acα^{TKO} Mice)*

For this study, we generated the *Ppp2ca$^{fl/fl}$* mice, in which two loxP sites were introduced into the 3′ and 5′ ends of

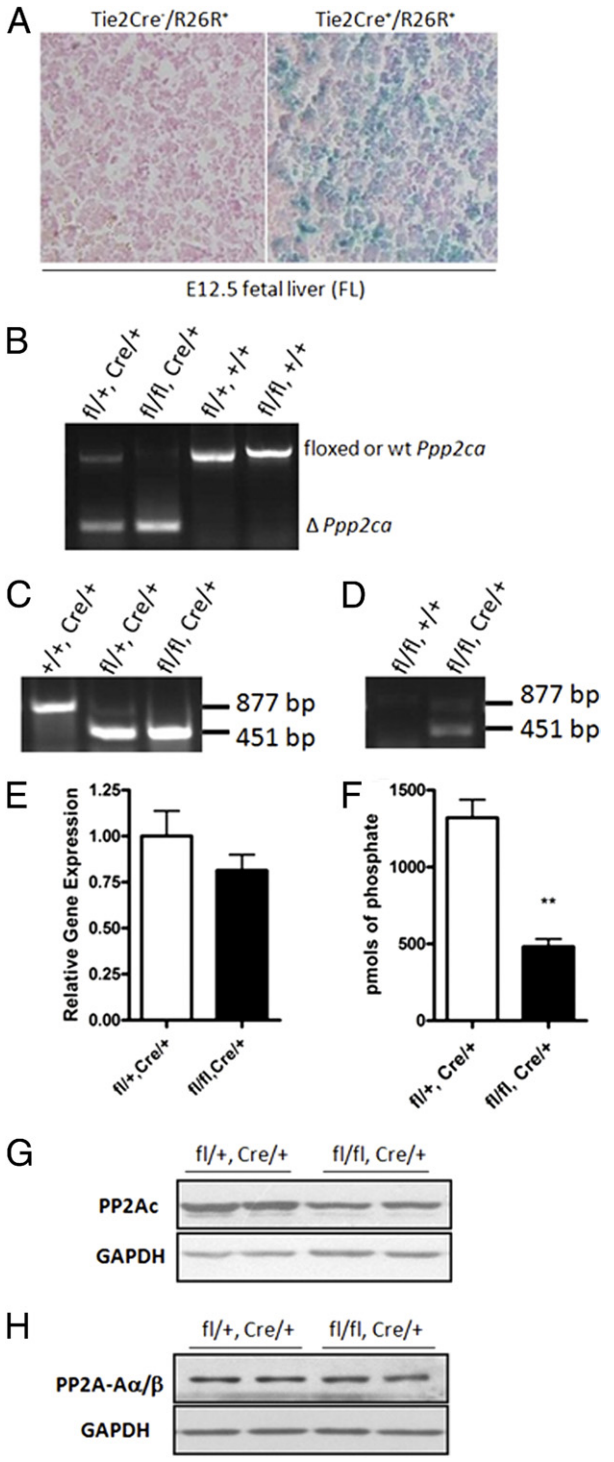

Figure 1. Deletion efficiency of PP2Acα in E12.5 PP2Acα^{TKO} fetal livers. **A:** LacZ-stained sections of Tie2^{+}Cre fetal liver carrying a *ROSA26* allele show the relatively sporadic distribution of blue cells. **B:** Deletion efficiency of the *Ppp2ca* allele was analyzed using genomic DNA from the fetal liver. A 339-bp fragment should appear with "DNA-EXdel" primers if Cre-mediated recombination occurs. **C:** RT-PCR was performed to verify PP2Acα mRNA. The cDNA containing exons 2 to 7 was amplified with "RNA-EXdel" primers. The 877-bp and 451-bp products reflect wild-type and mutant PP2Acα, respectively. **D:** RT-PCR was performed to verify PP2Acα mRNA in sorted HSCs in fetal livers. **E:** Quantitative PCR assay for PP2Acβ mRNA in E12.5 fetal livers ($n = 6$; average ± SEM). **F:** PP2A phosphate activity in the fetal liver was tested ($n = 5$; $^{**}P < 0.05$; average ± SEM). **G:** Total PP2Ac protein level was determined using an antibody detecting PP2Acα and PP2Acβ. **H:** Expression of the A subunit of PP2A was determined with an antibody specific for PP2A-Aα and PP2A-Aβ.

the region spanning exons 3 to 5 of the *Ppp2ca* gene (data not shown). To conditionally inactivate the *Ppp2ca* allele during embryonic hematopoiesis, we used two mouse lines, *Ppp2ca$^{fl/fl}$* mice and *Tie2Cre* mice, in which the *Cre* transgene is directed under the receptor tyrosine kinase Tek promoter/enhancer. Because *Tie2Cre* mice also exhibit Cre expression in the female germline, the *Cre* allele was maintained only in male mice in this study. To generate PP2Acα^{TKO} (*Tie2Cre^{+}/Ppp2ca$^{fl/fl}$*) mice, we first set up mating between male *Tie2Cre^{+}* and female *Ppp2ca$^{fl/+}$* mice. Male *Tie2Cre^{+}/Ppp2ca$^{fl/+}$* mice were subsequently mated with female *Ppp2ca$^{fl/fl}$* mice to generate PP2Acα^{TKO} mice (see Supplemental Figure S1A at http://ajp.amjpathol.org). PCR analyses of genomic DNA from mouse tails were performed to verify the expected genotypes (see Supplemental Figure S1, B and C, at http://ajp.amjpathol.org).

PP2Acα Recombination Efficiency in the Fetal Liver

In addition to endothelial cells, HSCs/Ps in mouse embryos have also been reported to express Tie2.[30–32] On initiation of the hematopoietic program, *Tie2* regulatory elements are transiently active in hematopoietic progenitors. To determine the extent of Cre expression in the fetal liver, we bred Tie2Cre mice with *ROSA26*-LacZ reporter mice. The relatively sporadic blue staining in fetal livers of mice carrying the *Cre* and the floxed *Ppp2ca* alleles indicated the distribution pattern of hematopoietic cells having undergone Tie2Cre-mediated gene recombination (Figure 1A). To verify the excision efficiency of the *Ppp2ca* allele, we harvested genomic DNA, cDNA, and protein from E12.5 fetal livers. Figure 1B shows data confirming that recombination occurred in the floxed *Ppp2ca* genomic locus in *Tie2Cre^{+}/Ppp2ca$^{fl/+}$* and *Tie2Cre^{+}/Ppp2ca$^{fl/fl}$* embryos. To confirm that deletion of the targeted exons indeed altered *Ppp2ca* expression, we measured mRNA and protein levels of PP2Acα and PP2Acβ in fetal livers. RT-PCR analyses showed that transcription of the *Ppp2ca* gene was truncated in total fetal liver cells (Figure 1C) and sorted HSCs (LSK) of PP2Acα^{TKO} embryos (Figure 1D), although intact transcripts were still observed. Transcription of *Ppp2cb* was not altered by excision of the *Ppp2ca* allele (Figure 1E). Owing to the high sequence similarity between PP2Acα and PP2Acβ, we did not have a reliable antibody to distinguish between these two isoforms. However, total quantity of catalytic subunit was still substantially reduced in PP2Acα^{TKO} fetal livers (Figure 1G). PP2A phosphatase activity in PP2Acα^{TKO} samples was approximately 36.4% of the activity observed in *Tie2Cre^{+}/Ppp2ca$^{fl/+}$* fetal livers (Figure 1F). Expression of the scaffolding subunit of PP2A remained unchanged despite loss of the *Ppp2ca* allele (Figure 1H). PP2Ac protein quantity and PP2A phosphatase activity were similar between *Tie2Cre^{+}/Ppp2ca$^{+/+}$* and *Tie2Cre^{+}/Ppp2ca$^{fl/+}$* fetal livers (see Supplemental Figure S2 at http://ajp.amjpathol.org). In subsequent experiments, we used *Tie2Cre^{+}/Ppp2ca$^{fl/+}$* mice as controls (CTRs).

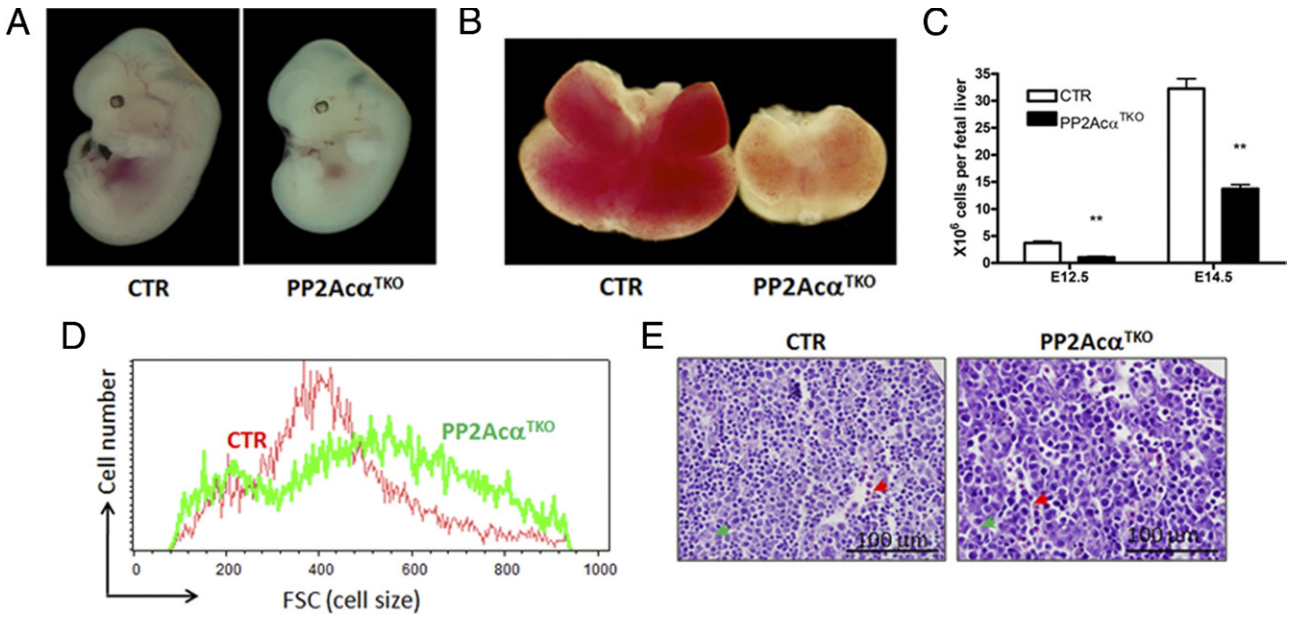

Figure 2. Impaired hematopoiesis in PP2Acα^{TKO} embryos. Gross morphologic features of E12.5 embryos (**A**) and isolated E12.5 fetal livers (**B**). **C:** Cell number per fetal liver was calculated (n = 5 to 8; $^{**}P < 0.05$; average ± SEM). **D:** Cell size of CTR and PP2Acα^{TKO} fetal liver cells derived from E12.5 embryos was determined by forward scatter (FSC) measurement by FACS. **E:** H&E staining of E12.5 fetal liver sections showing hepatocytes (**green arrows**) and hematopoietic cells (**red arrows**).

PP2Acα^{TKO} Embryos Manifest Fetal Anemia

PP2Acα^{TKO} embryos were pale, indicating defective hematopoiesis (Figure 2A). The CTR fetal liver showed a bright red appearance compared with the pale fetal liver of PP2Acα^{TKO} embryos (Figure 2B). The total cellularity of PP2Acα^{TKO} fetal liver was dramatically reduced at E12.5 and E14.5 (Figure 2C). PP2Acα^{TKO} fetal liver cells were also larger (macrocytic) than CTR cells (Figure 2D). As determined by histologic analyses, CTR livers contained numerous hematopoietic elements. In contrast, PP2Acα^{TKO} fetal livers consisted primarily of hepatic cells and, infrequently, hematopoietic progenitors or nucleated primitive RBCs (Figure 2E).

Erythropoiesis Is Impaired in PP2Acα^{TKO} Fetal Livers

To confirm and clarify hematopoietic defects in PP2Acα-deficient embryos, we analyzed the expression of several hematopoietic lineage markers in E14.5 fetal livers, such as Ter119$^+$ for erythrocytes, CD45$^+$ for leukocytes,[33] Gr-1$^+$ for granulocytes, Lin$^-$c-Kit$^+$CD41$^+$ for megakaryocyte progenitors, and Lin$^-$c-Kit$^-$CD41$^+$ for megakaryocytes[28] (Figure 3A). The absolute number of Ter119$^+$ cells in PP2Acα^{TKO} fetal liver was dramatically reduced to approximately 11.8% of that in CTRs (CTR versus PP2Acα^{TKO}: $3.39 \times 10^7 \pm 5.79 \times 10^6$ versus $4.02 \times 10^6 \pm 1.86 \times 10^6$ cells per fetal liver), whereas no statistically significant differences were observed in the mean ± SEM absolute number of CD45$^+$ cells (CTR versus PP2Acα^{TKO}: $1.28 \times 10^6 \pm 1.51 \times 10^5$ versus $1.15 \times 10^6 \pm 1.91 \times 10^5$ cells per fetal liver), Gr-1$^+$ cells (CTR versus PP2Acα^{TKO}: $4.94 \times 10^5 \pm 1.52 \times 10^5$ versus $3.90 \times 10^5 \pm 1.34 \times 10^5$ cells per fetal liver), Lin$^-$c-Kit$^+$CD41$^+$ cells (CTR versus PP2Acα^{TKO}: $1.29 \times 10^4 \pm 0.26 \times 10^4$ versus $1.49 \times 10^4 \pm 0.34 \times 10^4$ cells per fetal liver), and Lin$^-$c-Kit$^-$CD41$^+$ cells (CTR versus PP2Acα^{TKO}: $1.66 \times 10^4 \pm 0.20 \times 10^4$ versus $1.75 \times 10^4 \pm 0.78 \times 10^4$ cells per fetal liver). These findings suggest that the severe anemia observed in PP2Acα^{TKO} embryos was mainly due to erythropoietic abnormalities, with no apparent impairment in other hematopoietic lineages. RT-PCR analyses of sorted Ter119$^+$, CD45$^+$, Gr-1$^+$, and CD41$^+$ cells revealed incomplete knockout of PP2Acα in these particular hematopoietic lineages (Figure 3B). To explain the erythroid impairment phenotype, we hypothesize that considering their similar deletion efficiency, the maturation of erythroid and other hematopoietic lineages are differentially regulated.

Flow cytometry was performed to further verify the maturation stage of fetal liver erythroid cells. Expression of Ter119 marks committed erythropoietic precursors beyond the CFU-E stage,[34,35] whereas c-Kit marks HSCs capable of long-term reconstitution.[36] Proerythroblasts, characterized as the c-Kit$^+$Ter119$^-$ cell fraction, differentiate into c-Kit$^-$Ter119$^+$ erythroblasts during maturation. However, this process was severely impaired in PP2Acα^{TKO} embryos (Figure 3C). CD71 antibodies bind to the transferring receptor, which is highly expressed in proerythroblasts and early erythroblasts and decays in late erythroblasts and reticulocytes. Cells from CTR livers contained at least four distinct cell populations, defined by the following characteristic staining patterns: CD71^{med}Ter119^{low}, CD71^{high}Ter119^{low}, CD71^{high}Ter119^{high}, and CD71^{med} Ter119^{high} (Figure 3D, left, regions R1-R4, respectively). However, cell populations from PP2Acα^{TKO} livers showed far fewer mature erythroblasts (Figure 3D, right, regions R1-R4, respectively). *Tie2Cre$^+$/Ppp2ca$^{+/+}$* and *Tie2Cre$^+$/Ppp2ca$^{fl/+}$* fetal livers had a similar, bright red appearance and c-Kit–Ter119 and CD71-Ter119 staining pat-

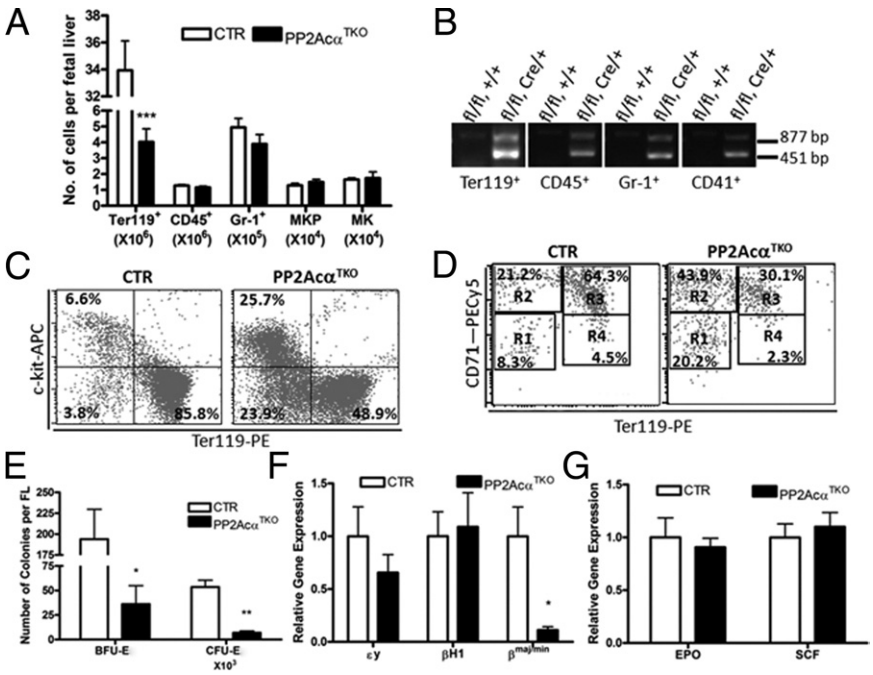

Figure 3. Perturbed erythropoiesis in PP2Acα^{TKO} fetal livers. Fetal livers from E14.5 embryos were harvested, and single-cell suspensions were prepared by passing livers through a 1-mL pipette and filtering them through a 100-μm cell strainer. Cell number was counted using a hemocytometer. Forty thousand cells were used for FACS analysis. **A:** The absolute numbers of Ter119$^+$, CD45$^+$, Gr-1$^+$, megakaryocyte (MK; Lin$^-$c-Kit$^-$CD41$^+$), and megakaryocyte progenitor (MKP; Lin$^-$c-Kit$^+$CD41$^+$) cells from CTR and PP2Acα^{TKO} fetal livers were calculated (n = 4 to 7; ***$P < 0.001$; mean ± SEM). **B:** RT-PCR was performed to verify PP2Acα mRNA in sorted Ter119$^+$, CD45$^+$, Gr-1$^+$, and CD41$^+$ fetal liver cells, as indicated. The cDNA containing exons 2 to 7 was amplified with "RNA-EXdel" primers. FACS analysis was performed using anti–c-Kit and anti-Ter119 (**C**) or anti-CD71 and anti-Ter119 (**D**) antibody combinations on E12.5 fetal liver cells. **E:** Absolute numbers of BFU-E and CFU-E per E12.5 fetal liver (FL) were calculated (n = 3 to 5; *$P < 0.05$; **$P < 0.01$; average ± SEM). Quantitative RT-PCR analysis of εy-, βH1-, and $\beta^{maj/min}$-globin transcripts (**F**) and EPO and stem cell factor (SCF) (**G**) transcripts in E12.5 fetal livers (n = 6; *$P < 0.05$; average ± SEM).

terns, confirming that *Tie2Cre*$^+$*/Ppp2ca*$^{fl/+}$ fetal livers had normal embryonic erythropoiesis (see Supplemental Figure S3 at *http://ajp.amjpathol.org*).

The generation of mature RBCs involves the commitment of pluripotent HSCs that progress through the BFU-E and CFU-E stages and the proerythroblast and erythroblast stages and, finally, differentiate into enucleated erythrocytes. Fetal liver erythroid progenitors are dependent on EPO for survival and growth during the final 48 hours of differentiation. To better define the observed impairment in definitive erythropoietic differentiation in PP2Acα^{TKO} embryos, we measured the ability of fetal liver cells to form CFU-E and the more immature BFU-E. These colony types reflect the presence of committed erythroid progenitors. As indicated in Figure 3E, the total numbers of BFU-E and CFU-E in PP2Acα^{TKO} fetal livers were dramatically lower than those in CTR littermates (BFU-E: decreased by 81.4%; CFU-E: decreased by 87.0%).

Further evidence for a defect in definitive erythropoiesis in PP2Acα^{TKO} embryos was provided by quantitative RT-PCR analyses of globin gene expression. The transcriptional levels of εy- and βH1-globin, which are expressed predominantly during primitive erythropoiesis,[37] were similar in CTR and PP2Acα^{TKO} fetal livers. However, expression of $\beta^{maj/min}$-globin, which first occurs in fetal livers during definitive erythropoiesis, was significantly reduced in PP2Acα^{TKO} embryos (Figure 3F). Collectively, these results indicate that primitive erythropoiesis proceeds normally in PP2Acα^{TKO} embryos but that definitive erythropoiesis is severely dampened.

Several cytokines, including EPO and stem cell factor, are required for erythropoiesis.[3,38–40] Quantitative RT-PCR analyses revealed no statistically significant differences in the relative expression levels of EPO and stem cell factor between CTR and PP2Acα^{TKO} samples, which precluded the possibility that PP2Acα^{TKO} embryos experience a deficiency in the production of erythropoiesis-promoting cytokines (Figure 3G).

Initial Seeding of the Fetal Liver with Hematopoietic Progenitors Is Not Attenuated in PP2Acα^{TKO} Embryos

Hematopoietic progenitor cells are primitive cells capable of producing mature cells of one or more lineages. Fetal hematopoiesis consists of the following steps: i) formation of short-lived progenitors and immature HSC precursors in the yolk sac and aorta-gonad-mesonephros region, ii) maturation of the precursor cells into functional HSCs, iii) migration of HSCs to the fetal liver at E10 for proliferation and differentiation, and iv) shifting of hematopoiesis to the bone marrow after birth for the remainder of life.[41] To test for the possibility that the erythropoietic abnormality observed in PP2Acα^{TKO} mice may result from an insufficient HSCs/Ps pool, the number of hematopoietic progenitors was calculated by sorting LSK cells to obtain a precise enumeration in individual fetal livers (Figure 4A). The statistical result revealed that the mean ± SEM absolute quantity of LSK cells in PP2Acα^{TKO} fetal livers remained unchanged at E12.5 (CTR versus PP2Acα^{TKO}: $5.66 \times 10^3 \pm 2.10 \times 10^3$ versus $4.58 \times 10^3 \pm 2.74 \times 10^3$ cells per fetal liver) (Figure 4B). Mean ± SEM absolute LSK cell quantity of E14.5 PP2Acα^{TKO} embryos was even up-regulated, probably due to negative feedback modulation by ineffective erythropoiesis (CTR versus PP2Acα^{TKO}: $1.89 \times 10^4 \pm 5.36 \times 10^3$ versus $5.64 \times 10^4 \pm 1.09 \times 10^4$ cells per fetal liver) (Figure 4B). These results indicate that the initial colonization of the fetal liver with HSCs/Ps is not attenuated by loss of the *Ppp2ca* allele.

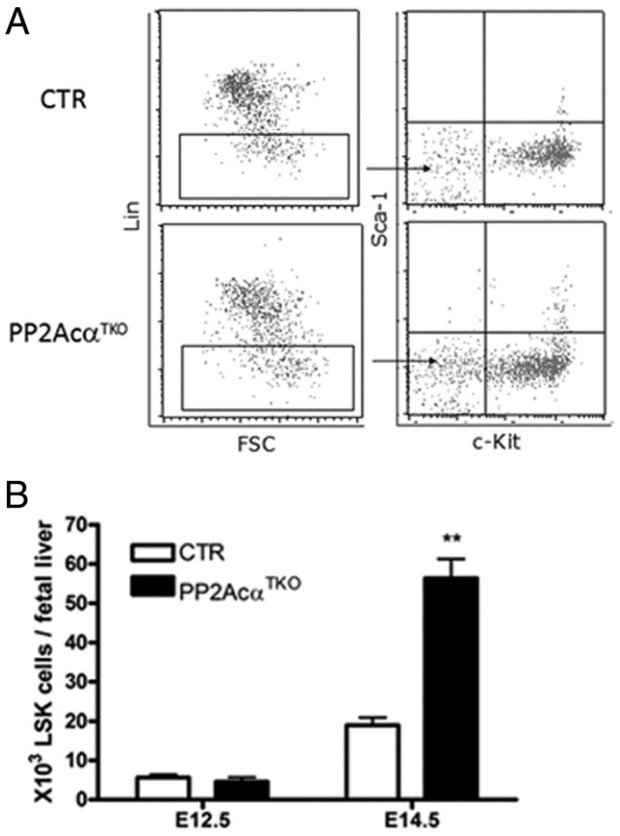

Figure 4. Analysis of LSK cell numbers in PP2Acα^{TKO} fetal livers. **A:** E12.5 fetal liver cells were sorted by representative lineage and sorting gates, as shown. **B:** Fetal liver cell numbers were counted using a hemocytometer and were analyzed using a flow cytometer. The cumulative counts of LSK cells obtained by FACS during the sorting procedure are shown ($n = 5$ to 8; $^{**}P < 0.01$; average ± SEM).

Increased Apoptosis in Cultured PP2Acα-Deficient Committed Erythroid Cells

Fetal liver erythropoiesis is considered to be mechanistically similar to stress erythropoiesis in adult mice, as both processes are EPO responsive, which is essential for the survival of CFU-E.[2,42] We, therefore, examined the ability of EPO to prevent apoptosis of PP2Acα^{TKO} erythroid cells. We cultured primary fetal liver cells and analyzed their sensitivity to apoptosis with or without EPO stimulation. After *in vitro* stimulation for 18 hours, cells were examined by FACS for the expression of Ter119 and annexin V.[27] As illustrated in Figure 5A, significant fractions of apoptotic cells were evident in committed erythroid cell cultures of PP2Acα^{TKO} fetal livers with or without EPO stimulation. This indicates that PP2Acα^{TKO} erythroid cells are much more sensitive to apoptotic stimulation than are CTR cells.

Defective STAT5–Bcl-x$_L$ Signaling Is Responsible for the Reduced Survival of PP2Acα^{TKO} Erythroid Cells

Stress erythropoiesis in the spleen depends sharply on the EpoR-STAT5 signaling axis. We, therefore, investigated whether decreased survival of committed erythroid cells in PP2Acα^{TKO} embryos could result from misregulation of this signaling pathway, thus indicating cross talk between PP2A and STAT5 signaling. We evaluated transcripts of the five known STAT5 downstream genes, including *Bcl-x*,[5] proviral integration site-1 (*Pim-1*),[43] *cis-1*,[44] *SOCS-3*,[45] and oncostatin-M (*OncoM*)[46] in E12.5 fetal livers. Each of these genes has been reported to be included in fetal liver or stress erythropoiesis. The present results indicate that loss of the *Ppp2ca* allele impaired transcription of *Bcl-x*, without overt influence on transcription of other genes (Figure 5B). *Bcl-x* is expressed predominantly in its long form, Bcl-x$_L$,[47] which can function as an anti-apoptotic factor. Down-regulation of basal Bcl-x$_L$ protein in PP2Acα^{TKO} fetal livers was confirmed by Western blot analysis (Figure 5C). Primary fetal liver cells were stimulated *in vitro* for 15 minutes with or without EPO. PP2Acα^{TKO} fetal liver cells exhibited an attenuated EPO response, as indicated by decreased tyrosine phosphorylation of STAT5 (pY694) and decreased Bcl-x$_L$ expression in basal and stimulated conditions (Figure 5D).

Loss of the Ppp2ca Allele in Tie2$^+$ Cells Results in Embryonic Lethality

To determine the precise survival rate of PP2Acα^{TKO} embryos, we examined embryos obtained from sched-

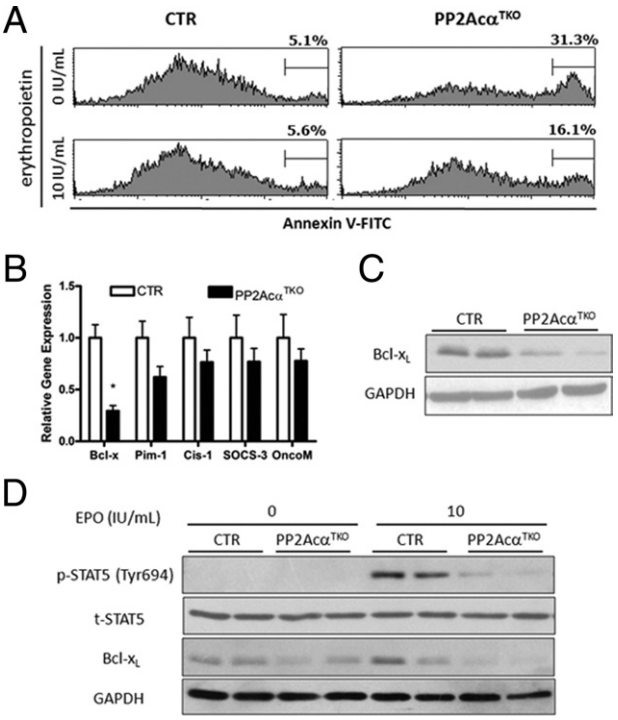

Figure 5. Increased apoptosis and impaired STAT5 signaling in PP2Acα-deficient fetal livers. **A:** Isolated E12.5 fetal liver cells were cultured in Iscove's modified Dulbecco's medium with 2% fetal bovine serum for 18 hours in the absence or presence of 10 U/mL recombinant human EPO, and combined Annexin V–Ter119 staining was performed and quantified by FACS analysis. **B:** Quantitative PCR analyses of downstream STAT5 targets, including *Bcl-x*, *Pim-1*, *cis-1*, *SOCS-3*, and *OncoM* ($n = 6$; $^{*}P < 0.05$; average ± SEM). **C:** Western blot analysis of basal Bcl-x$_L$ protein level in E12.5 fetal liver lysates. **D:** STAT5–Bcl-x$_L$ signaling after EPO stimulation. PP2Acα^{TKO} and CTR fetal liver cells were treated with or without human EPO for 15 minutes and were immunoblotted with the indicated antibodies.

uled matings (see Supplemental Table S2 at *http://ajp.amjpathol.org*). Mice with all four genotypes existed at the expected Mendelian ratio at E10.5. At E12.5, however, only 54 of the expected 69 PP2Acα^{TKO} embryos were harvested, with 47 PP2Acα^{TKO} embryos moribund and pale looking. At E14.5, the survival ratio of PP2Acα^{TKO} embryos became even smaller. However, 8 of the expected 69 PP2Acα^{TKO} pups survived until postnatal day 10. Adult PP2Acα^{TKO} mice did not show any visible abnormalities. They even exhibited a normal steady-state hematocrit (see Supplemental Figure S4 at *http://ajp.amjpathol.org*).

PP2Acα^{TKO} Embryos Exhibit No Obvious Early Embryonic Vasculature Defects

Because Tie2Cre mediates the excision of floxed DNA in hematopoietic and endothelial cells, we analyzed the vascular potential of blood vessels in PP2Acα^{TKO} embryos. Platelet endothelial cell adhesion molecule-1 and endomucin[48] staining of whole-mount E10.5 embryos indicated that PP2Acα^{TKO} embryos displayed no obvious abnormalities in early embryonic vasculature. Whole embryo views and close examination of the trunk and head regions confirmed these findings (Figure 6, A-E). LacZ whole-mount staining of embryos carrying a *ROSA26* allele also revealed normal blood vessel development in PP2Acα^{TKO} embryos at E10.5 (Figure 6F). Although the mean ± SEM absolute number of nonhematopoietic cells ($CD45^-$) was dramatically reduced (CTR versus PP2Acα^{TKO}: $2.17 \times 10^6 \pm 0.66 \times 10^6$ versus $7.60 \times 10^5 \pm 2.15 \times 10^5$ cells per fetal liver) (Figure 6G), the endothelial population ($CD31^+CD45^-$)[29] remained unchanged in E12.5 PP2Acα^{TKO} fetal livers (CTR versus PP2Acα^{TKO}: $2.93 \times 10^4 \pm 0.52 \times 10^4$ versus $3.15 \times 10^4 \pm 0.91 \times 10^4$ cells per fetal liver) (Figure 6G). RT-PCR analyses of sorted $CD31^+CD45^-$ cells revealed the complete absence of PP2Acα mRNA, which precluded the possibility that the observed normal embryonic vasculature was due to inefficient knockout of PP2Acα mRNA in endothelial cells of PP2Acα^{TKO} fetal liver (Figure 6H).

Discussion

In this study, we delineate that sustained activity of PP2Acα is crucial for fetal liver erythropoiesis. Considering that the colonization of PP2Acα^{TKO} fetal livers with HSCs/Ps is not attenuated, we believe that the observed decrease in the survival of erythroid cells, itself the result of defective STAT5–Bcl-x_L signaling, is responsible for the impaired erythropoiesis phenotype in PP2Acα^{TKO} fetal livers. These results also indicate that signaling through PP2Acα is not essential for early embryonic vasculature development.

PP2Acα Is Essential for the Survival of Committed Erythroid Cells

Several lines of evidence suggest that PP2A activation is linked to apoptosis. PP2A plays pivotal dual roles in the induction of neutrophil apoptosis through dephosphorylation of p38 MAPK and its substrate, caspase 3.[49] PP2A is a Bcl-2 phosphatase and controls its major functional phosphorylation site (Ser70).[50] PP2A has also been shown to colocalize with Bcl-2 at the mitochondrial membrane and may be activated by ceramide to dephosphorylate Bcl-2.[51] Activation of caspase-3 causes cleavage of the A subunit of PP2A, which, in turn, increases PP2A activity.[52] Herein, we reported that conditional genetic deletion of PP2Acα inhibited the survival of erythroid cells through regulation of Bcl-x_L expression. *Bclx* is expressed predominantly in its long form, Bcl-x_L; however, other alternative splice variants, such as Bcl-x_S, have also been detected.[53,54] Although Bcl-x_S can function as a pro-apoptotic factor *in vitro*, its expression has not been detected in erythroid cells.[6] Bcl-x_L is a direct target of STAT5, as STAT5 binds to its consensus element within intron 1 of the *Bclx* gene and induces immediate early expression of Bcl-x_L in HCD-57 cells.[5]

It is not clear why some PP2Acα^{TKO} embryos showed such a severe anemia phenotype and a few PP2Acα^{TKO} embryos survived to adulthood and demonstrated a normal steady-state hematocrit (see Supplemental Figure S4 at *http://ajp.amjpathol.org*). Possible explanations include i) the mixed 129/B6 genetic background; ii) the efficiency and/or timing of the recombination of the *Ppp2ca* floxed allele in the PP2Acα^{TKO} population; and iii) the heterogeneity in the recombined cells' ability to compensate for deficient STAT5–Bcl-x_L signaling. The regulation of Bcl-x_L in erythroblasts is multifactorial and involves synergistic interaction of the EPO and GATA signaling pathways.[55] In a manner similar to STAT5$^{\triangle N}$ mice,[4,5] the extent to which candidate pathways can compensate for impaired STAT5 activity and maintain sufficient Bcl-x_L expression seems, at least in part, to determine the extent of anemia.

Adult PP2Acα^{TKO} mice have normal steady-state hematocrit. We speculate that this is because of the different characteristics of bone marrow and fetal liver erythropoiesis. Fetal liver erythropoiesis is similar to stress erythropoiesis in that embryos have to maintain high rates of RBC generation. The embryos must produce their entire RBC mass in only a few days (E9 to E15). This leaves little erythropoietic reserve. In contrast, the adult has a remarkable erythropoietic reserve. In steady-state erythropoiesis, the rate of RBC production needs only match the rate of senescent RBC loss. From a molecular perspective, unlike steady-state erythropoiesis, stress and fetal liver erythropoiesis depend dramatically on the anti-apoptotic effects of the EpoR-STAT5 signaling axis. Dominant-negative STAT5 increases apoptosis and inhibits growth of cultured fetal liver erythroid progenitors.[56] The five STAT5-downstream genes mentioned in this study (Figure 5B) have all been implicated in fetal liver or stress erythropoiesis. Bcl-x functions as an erythroblast survival factor, as conditional gene disruption results in failed reticulocyte formation.[57] *Pim-1*$^{-/-}$ mice exhibit decreased CFU-E number and compound *Pim-1*$^{-/-}$*;Pim-2*$^{-/-}$ mice have microcytic anemia.[58] *SOCS-3* is associated with receptor kinase inhibition and degradation. *OncoM*–deficient mice exhibit reduced circulating erythrocytes. Finally, *cis-1* provides negative feedback, par-

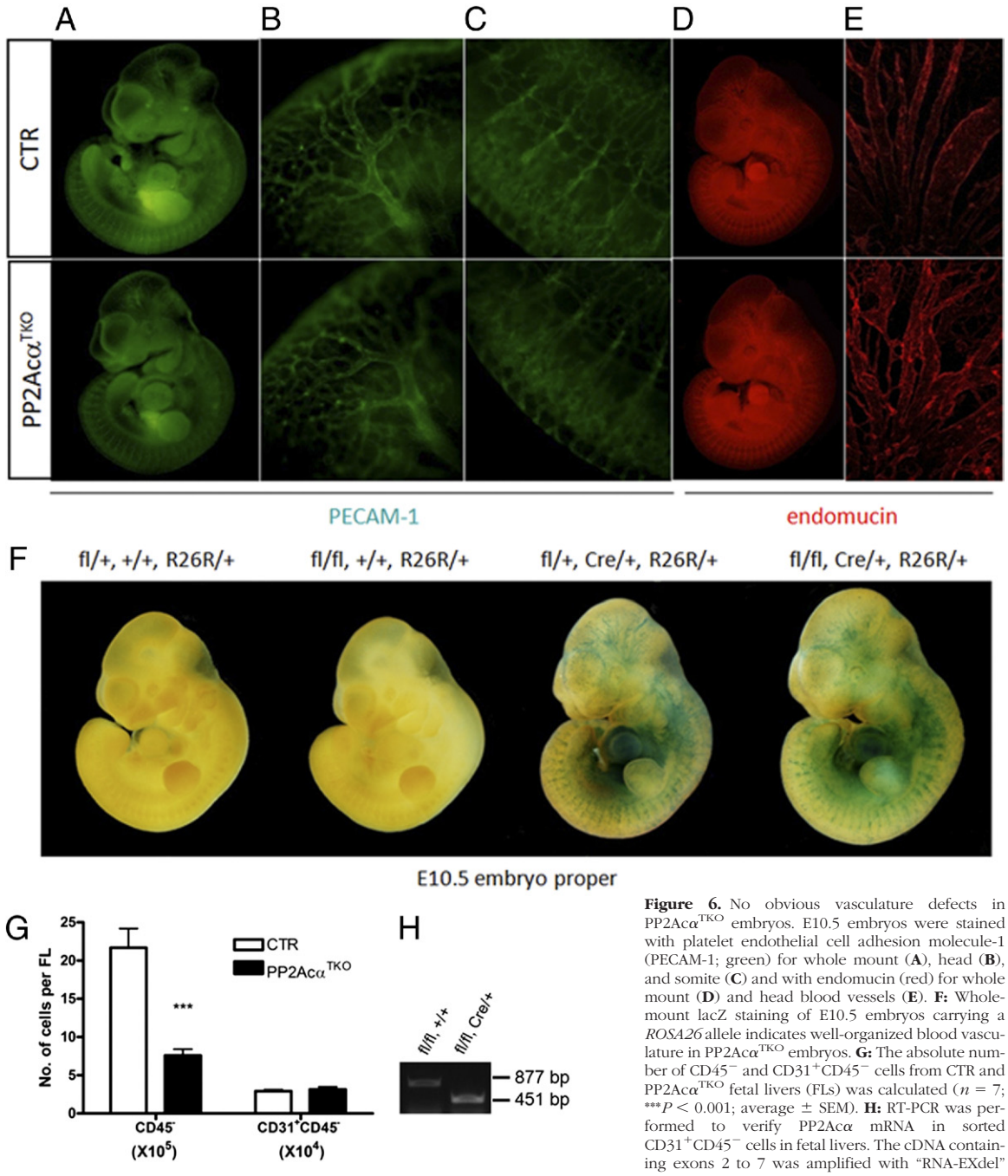

Figure 6. No obvious vasculature defects in PP2Acα^{TKO} embryos. E10.5 embryos were stained with platelet endothelial cell adhesion molecule-1 (PECAM-1; green) for whole mount (**A**), head (**B**), and somite (**C**) and with endomucin (red) for whole mount (**D**) and head blood vessels (**E**). **F:** Whole-mount lacZ staining of E10.5 embryos carrying a *ROSA26* allele indicates well-organized blood vasculature in PP2Acα^{TKO} embryos. **G:** The absolute number of CD45$^-$ and CD31$^+$CD45$^-$ cells from CTR and PP2Acα^{TKO} fetal livers (FLs) was calculated ($n = 7$; $^{***}P < 0.001$; average ± SEM). **H:** RT-PCR was performed to verify PP2Acα mRNA in sorted CD31$^+$CD45$^-$ cells in fetal livers. The cDNA containing exons 2 to 7 was amplified with "RNA-EXdel" primers.

tially via inhibition of JAK2 at activated EpoR complexes.[44,45] However, in the present study, only Bcl-x$_L$ was found to be down-regulated as a result of decreased tyrosine phosphorylation of STAT5 (pY694) (Figure 5B).

PP2A Functions as a Multitarget Phosphatase

The phenotype of PP2Acα^{TKO} mice is more severe than several STAT5 or Bcl-x knockout models. Although STAT5$^{\triangle N}$ mice, which still encode an N-terminally truncated and partially functional STAT5 protein, are embryonic anemic, they can be born and recover from anemia. Adult STAT5$^{\triangle N}$ mice have a normal basal hematocrit but are impaired in recovery from anemia-promoting chemical stress.[4,5] STAT5a/b-null mice, which are completely null of STAT5a/b, are more than 99% perinatal lethal due to severe anemia combined with other unidentified physiologic defects. However, the survival ratio of STAT5a/b-null mice at E18.5 is 100%.[59,60] Bcl-x–deficient mice die at approximately E13, showing extensive apoptosis in postmitotic, immature neurons of the developing brain, spinal cord, and dorsal root ganglia and in immature erythroid cells in the fetal liver.[57] To explain the discrepancy between the present model and others, it should be

borne in mind that for those key and multitargeting proteins, such as PP2A holoenzyme, one cannot attribute their effects to just one, or even several, downstream molecules. The phenotype we observe is likely the combined result of broad signaling pattern changes of multiple PP2A substrates. Genetic modification of PP2A itself should yield more severe phenotypes than modification of any single downstream gene.

PP2Acα Is Nonessential for Primitive Erythropoiesis and Early Embryonic Vasculature

There are two waves of embryonic erythropoiesis, each with distinctive features. Primitive erythropoiesis occurs in the yolk sac at approximately E7.5. Primitive RBCs are predominantly nucleated and express embryonic hemoglobins (ε and βH1). Definitive erythropoiesis takes place in the fetal liver at approximately E10, generating smaller, adult-type, enucleated RBCs that express adult hemoglobins ($\beta^{maj/min}$ and α). PP2Acα^{TKO} embryos showed impaired fetal liver, but normal yolk sac erythropoiesis, as indicated by comparable expression of primitive globins in both samples (Figure 3F). Consistent with the present data, embryos deficient for STAT5 or Bcl-x have not been reported to have primitive erythropoiesis defects, indicating that STAT5–Bcl-x_L signaling is not crucial for primitive erythropoiesis. However, *Xenopus* STAT5 has been shown to act as a repressor of primitive erythropoiesis and to function epistatically to fibroblast growth factor signaling.[61]

The finding that PP2Acα^{TKO} embryos show no obvious early embryonic vasculature defects was unexpected. Consistent with the present observations, however, basal PP2A activity in skeletal microvascular endothelial cells is not high, despite the abundance of the protein in this tissue.[62] This reported low PP2A activity correlates with the tyrosine-phosphorylated state of PP2Ac. In this case, the partially diminished PP2A activity may not be sufficient to induce embryonic vascular defects in PP2Acα^{TKO} embryos.

Another explanation for normal primitive erythropoiesis and early embryonic vascular development in PP2Acα^{TKO} embryos is the redundancy by PP2Acβ, which shares 97% primary sequence similarity with PP2Acα.[13,20] Nevertheless, without reliable antibodies to distinguish between these two isoforms, it is difficult to determine to what extent PP2Acβ can compensate for PP2Acα in these two processes.

Acknowledgments

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