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E-cadherin and β-catenin across the branches of hematopoiesis in evolution

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Chapter 6

Anemia Links β-Catenin with FOXO3 Signaling during Erythroid Differentiation

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Abstract

Wnt/β-catenin signaling is well known to promote stem cell self-renewal and/or proliferation. At the cell membrane, β-catenin is stabilized by cadherin family members which association sustains adherence junction formation. We previously revealed β-catenin to bind to and be stabilized by E-cadherin within erythroid precursors (erythroblasts) of humans and rats but not in mice. E-cadherin/β-catenin protein expression is upregulated in erythroblasts of anemic rats, and activation of β-catenin signaling stimulated terminal erythroid differentiation. The underlying molecular mechanism by which β-catenin can control erythroid differentiation remains, however, to be defined. Here we report that activation of β-catenin/TCF7/LEF signaling is restricted to early-stage erythropoiesis. Upon erythroblast development, TCF7/LEF expression disappeared which coincided with a strong elevation of FOXO3 expression. Accordingly, a part of β-catenin bound FOXO3 in unstimulated cultured human erythroblasts. Loss of FOXO3 delayed terminal erythroid differentiation of cultured human erythroblasts, indicating FOXO3 to control terminal erythroid differentiation in humans. In response to anemia, FOXO3 protein levels strongly increased in rat erythroblasts, similarly as we previously found for β-catenin. RNA-seq analysis, accordingly, revealed erythroblasts to display a FOXO3 gene signature in response to anemia, that is linked to heme metabolism. Since β-catenin binding of FOXO3 has been described to stimulate FOXO3-driven transcription, we propose β-catenin binding of FOXO3 signaling to promote erythroid differentiation, particularly in response to anemia. Further investigations are however needed to dissect the spatial temporal molecular interplay between β-catenin and FOXO3 signaling, and the underlying molecular mechanisms which regulate terminal erythroid differentiation.

Introduction

Erythropoiesis ensures the lifelong formation of erythrocytes, with an estimated production of more than 2.5×10^6 red blood cells (RBCs) per second (1). A reduction of circulating RBCs will increase their production to secure healthy oxygen levels, through a process that is known as stress erythropoiesis. The mouse displays a valuable model to elucidate the molecular pathways underlying stress erythropoiesis (2–5). Yet, stress erythropoiesis in mice may not fully represent the molecular mechanisms that control stress erythropoiesis in humans, as stress erythropoiesis in mice mostly occurs in the spleen whereas in humans this predominantly occurs in bone marrow (BM) (6). Stress erythropoiesis in humans, therefore, may be regulated by different or additional environmental niche factors as compared to mice. In line with this notion, our group identified E-cadherin, a cell adhesion protein, to mark the erythroid lineage in humans but not in mice (7). Moreover, E-cadherin protein expression is conserved in the erythroid lineage of rats, which similar to humans predominantly execute stress erythropoiesis in their BM (6).

In rat and human erythroblasts, E-cadherin binds and stabilizes β-catenin (7). Aside from controlling cell-cell adhesion, β-catenin signaling is well-known to mediate canonical Wnt signaling (8).Wnt/β-catenin signaling is activated upon Wnt stimulation, which inactivates the β-catenin destruction complex. Stabilization of β-catenin protein levels promotes its accumulation in the nucleus, leading to β-catenin/TCF/LEF transcriptional signaling (8). Aside from canonical activation of TCF/LEF signaling, FOXO transcription family members also bind β-catenin, particularly FOXO3 (9). FOXO3 competes with TCF/LEF binding for β-catenin, which binding enhances FOXO3 transcriptional activity (10,11).

In erythropoiesis, FOXO3 has been indicated to promote RBC formation by stimulating terminal erythroid differentiation (12). Moreover, FOXO3 seems to underlie stress erythropoiesis in mice, as FOXO3 KO mice were unable to recover from phenylhydrazine (PHZ) induced hemolytic anemia (13). Our group revealed that in response to anemia, E-cadherin/β-catenin protein levels increase, both at the peripheral cell membrane as well as in the nuclear/cytoplasmic fraction of rat erythroblasts (7). Moreover, we found that the expression of a stabilized active β-catenin variant accelerated terminal differentiation (7).

In this study, we investigate the molecular mechanisms by which β-catenin may stimulate erythropoiesis. We reveal that β-catenin does not control erythroid terminal differentiation by TCF/LEF signaling as both transcriptional factors are not expressed within the basophilic erythroblast differentiation stage. This was also evident by the lack of TCF/LEF-TOP reporter activity during terminal erythroid differentiation. Instead, we identify FOXO3 to be highly expressed and to partly associate with β-catenin in cultured human erythroblasts. Loss of FOXO3 expression, induced with CRISPR/Cas9, does not hamper the formation nor expansion of cultured human erythroblasts but

was found to delay their terminal differentiation. Aside we reveal that, like β-catenin, FOXO3 protein expression is increased in rat erythroblasts in response to anemia. In response to anemia, rat erythroblasts reflect a FOXO3 transcriptional signature representing genes that are linked to hemoglobin formation. Overall, our findings link β-catenin with FOXO3 signaling in erythroblasts, which association in response to anemia, may enhance erythroid differentiation.

Methods

Human BM, PBMC and CD34+ HSPC isolation

Human specimens were collected with informed consent and by the principles of the Declaration of Helsinki, as well as the ethical guidelines of the Dutch National and Sanquin Internal Ethic Boards. Peripheral blood mononuclear cells (PBMCs) were purified from human peripheral blood using density gradient separation with Ficoll (GEHealthcare, 17-1440-03), following the manufacturer's protocol. For the isolation of hematopoietic stem and progenitor cells (HSPCs), mobilized peripheral blood (MPB) was collected through leukapheresis. CD34+ HSPCs were isolated using MACS beads (Miltenyi, 130-046-703) targeting the CD34+ marker, following the manufacturer's instructions. The isolated cells were cryopreserved in Iscove's Modified Dulbecco's Medium (IMDM) with 20% fetal calf serum (FCS) and 5% dimethyl sulfoxide (DMSO), as previously described by Voermans et al. (1999)(14).

Cell culture

Primary cells were cultivated in a modified IMDM-based Cellquin medium (PAN Biotech, P04-20250) as outlined in Supplementary Table S1. Depending on the specific stage of cell differentiation, supplements were added according to the culturing protocol as described by Heshusius et al. (2019)(15). In short, CD34+ hematopoietic stem and progenitor cells (HSPCs), Cellquin medium was augmented with 50 ng/mL Nplate (Amgen, 70068673), 10 ng/mL interleukin-3 (IL-3, Stemcell Technologies, 78040), 10 ng/mL IL-6 (Stemcell Technologies, 78050), 1 U/mL EPO (Brocacef, EP069503), and 50 ng/mL of human recombinant stem cell factor (SCF, produced in-house). When specified, cells were treated with 10 μM of CHIR99021 (Sigma-Aldrich, Cat. No. SML1046)

Following four days of expansion of erythroblasts was triggered using Cellquin medium supplemented with 50 ng/ml human recombinant SCF, 1 U/mL EPO, and 1 μM dexamethasone (DEX, Sigma, D4902). After expansion, erythroblast differentiation was initiated with 10 U/mL EPO, 700 μg/mL holotransferrin (Sanquin, blood supply), 5% omniplasma (Sanquin, blood supply), and 5 U/mL heparin (LEO Pharma BV).

CRISPR-Cas9 genome editing

Two of the crRNAs were sourced from Celestini et al. (2018)(16). The remaining CRISPR RNAs (sgRNAs) were generated using CRISPOR (Version 5.01). The sequences of all crRNAs can be found in Table 1. To generate a single guide RNA (sgRNA), crRNA (300 µM, Alt-R, IDT) was combined with Tracer RNA (100 µM, IDT), heated to 95ºC for 5 minutes, and allowed to cool down to room temperature over 20 minutes. Subsequently, spCas9-3xNLS (produced in-house, Verhagen et al., 2022) (17) was added to the sgRNA mixture and incubated at room temperature for 10 minutes and allowed form ribonucleoprotein complexes (RNPs). For the nucleofection process, 1 million cells per reaction were suspended in homemade M1 buffer for primary cells or nucleofection buffer for cell lines (17), and nucleofected using the 4D-Nucleofector system from Lonza. Program EO100 was applied for HSPCs $CD34^+$, while program CA137 was employed for the KU812 cell line. Following nucleofection, cells were immediately supplemented with Cellquin medium. Finally, to eliminate nucleofection buffer, cells were centrifuged (5 minutes, 400 rcf) and then cultured. $CD34^+$ CRISPRed HSPCs were cultured according to the procedure described above.

Table 1. sgRNA sequences against FOXO3

sgRNA name	Sequence
FOXO3 exon 2_1	GTCTTCATCGTCCTCCTCCT (16)
FOXO3 exon 2_2	AGGAGGAGGACGATGAAGAC (16)
FOXO3 exon 3_1	CCTGCCATATCAGTCAGCCG
FOXO3 exon 3_2	CTGCCTTCTTCTTGGCTGCG
FOXO3 exon 3_3	CCACGGCTGACTGATATGGC
FOXO3 exon 3_4	CGTGCACGGTGGAACTGCCA

Flow cytometry

Cells were rinsed with PBS, suspended in PBS solution containing antibodies (table 2), and incubated at room temperature for 30 minutes. Dead cells were identified using Near-IR live-dead marker, (APC-Cy7, Thermo Fisher L34975). When specified, DRAQ5 was used to differentiate nucleated cells from enucleated reticulocytes and pyrenocytes (APC, Abcam 108410). Subsequently, the cells were washed with PBS and analyzed on a BD Symphony flow cytometer. FlowJo software (version 10.8.1) was used for the analysis.

Western blot

1 million cells used to make protein lysates with lysis buffer containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM EDTA (pH 8.0), 100 mM NaF, 10% Glycerol, 1% NP-40, 0.1 nM okadaic acid, 1 U of benzonase nuclease (Sigma, E1014-25KU), 1 mM sodium orthovanadate (New England BioLabs, NEB P0758L), 100 µM PMSF, and cOmplete™ proteinase inhibitor Cocktail. The samples

were further supplemented with NuPAGE™ LDS sample buffer (Invitrogen™, NP0008) and 1% β-mercaptoethanol. Afterward, the proteins were sonicated for 15 minutes and then denatured by heating in a 95ºC heat block for 5 minutes. Protein lysates were loaded onto precast NuPAGE Bis-Tris gels (Thermo Fisher, NP0321PK2) and run for 1.5 hours at 0.04A using an XCell SureLock Electrophoresis Cell (Thermo Fisher, EI0001) and Powerpac 200 (Bio-Rad). Subsequently, the proteins were transferred from the gel onto iBlot 2NC Mini Stacks (Thermo Fisher, IB23002) for 7 minutes at 20V using the iBlot 2 Gel Transfer Device (Thermo Fisher, IB21001). To prevent nonspecific binding, the membrane was blocked with 5% BSA (Sigma, A7030). Next, the membrane was incubated overnight at 4ºC with primary antibodies diluted in 5% BSA in TBST. Subsequently, it was incubated with secondary antibodies in 5% BSA in TBST for 1 hour at room temperature. The details of the antibodies used can be found in Supplementary Table S5. β-actin served as the loading control. Finally, the membrane was developed using Pierce ECL Western Blotting Substrate (Thermo Fisher, 32106) following the manufacturer's protocol, and imaging was conducted with the ChemiDoc XRS+ imaging system (BioRad). Processing of Western blotting images was performed using Adobe Illustrator software (version 27.0).

Immunoprecipitation

1 million cells were rinsed with cold PBS, lysed in ice-cold lysis buffer (described above) . The lysates were incubated on ice for 5 minutes and centrifuged at max speed (20,817 RCF) for 5 minutes at 4ºC to remove insoluble debris. Protein G Sepharose beads (Cytiva, 17-0618-01) was pre-treated with 5% BSA in lysis buffer and placed on a vertical rotator for 1 hour at 4ºC. The total lysates were mixed with antibodies against epitopes of interest and rotated for 1 hour at 4ºC. Subsequently, the blocked beads were introduced into the total lysate-antibody mixture and allowed to incubate on the vertical rotator overnight at 4ºC. Subsequently, the beads were washed three times with lysis buffer. Input samples were collected before immunoprecipitation, and flow-through samples were collected before bead washing. All samples were supplemented with NuPAGE LDS Sample buffer (Invitrogen, NP0008), and proteins were denatured in a 95ºC heat block for 15 minutes. Finally, the samples were analyzed using the Western blotting procedure described in the section above.

Immunohistochemistry

Rat bone marrow (BM) and spleen samples were fixated in 10% neutral buffered formalin for 48 hours, after which bones were decalcified in 6,5% formic acid. Subsequently, the samples were embedded in paraffin, sectioned, and subjected to hematoxylin and eosin (H&E) staining. Immunohistochemistry (IHC) procedures were conducted following previously established protocols (18,19). All prepared slides were digitally processed utilizing the Aperio ScanScope (Aperio) and captured with ImageScope software version 12.0.0 (Aperio). Used antibodies are listed in table 2.

Table 2. Antibodies used in this study

Technique	Species	Epitope/ target	Clone	Dye	Host species	Conjugate	cat. no.	manufacturer
FC	Human	CD71	AC102		mouse	vioblue	30-128-947	Miltenyi
FC	Human	CD235a	GA-R2		mouse	BUV395	563810	BD Biosciences
FC	Rat	CD71	OX-26		Mouse	FITC	554890	BD Biosciences
FC	Rat	His49a	His49		Mouse	APC	562348	BD Biosciences
FC	Human	Dead cells	-	LIVE/DEAD™ Fixable Near-IR Dead Cell Stain	-	Excitation 637 nm	Invitrogen	L-34975
FC	Human	DNA	-	DRAQ5	-	Excitation 637 nm	Ab108410	Abcam
WB	Human, rat	FOXO3	75D8		Rabbit	-	2497S	Cell Signaling
WB	Human	FOXO3	D19A7		Rabbit	-	12829	Cell Signaling
WB	Human	β-catenin	14/β-catenin		Mouse	-	b16051	Abcam
WB	Human	β-catenin	polyclonal		Rabbit	-	610153	BD Biosciences
WB	Human	E-cadherin	36/E-cadherin		Mouse	-	610181	BD Biosciences
WB	Human	E-cadherin	HECD		Mouse	-	-	Sahai laboratory, gift
WB	Human, rat	β-actin	polyclonal		Rabbit	-	4967S	Cell Signaling
WB	Human	Anti Rabbit IgG	polyclonal		Goat	HRP	P0448	DAKO
WB	Human	Anti Mouse IgG	polyclonal		Goat	HRP	P0447	DAKO
IHC	Rat	Foxo3	D19A7		Rabbit	-	12829	Cell Signaling

Rat PHZ experiment

Lewis rats (Janvier) were housed in the animal facility at the Netherlands Cancer Institute (NKI). Animal experiments were conducted in accordance with both institutional and national guidelines, following the approved protocol with permit ID: 9.4.9961. PHZ was freshly dissolved in PBS on the same day it was intraperitoneally injected with a dose of 40 mg/kg of body weight for three consecutive days. Blood samples were obtained either by tail vein puncture or heart puncture on the day of sacrifice. The blood parameters were measured on the Scil Vet abc Plus machine (Horiba Medical), utilizing the pre-installed Rat program. On specified days, rats were euthanized using CO_2. Spleen, femur, lung, intestine, and tibia tissues were collected and either preserved in formalin for immunohistochemistry or kept in PBS for flow cytometry and subsequent procedures.

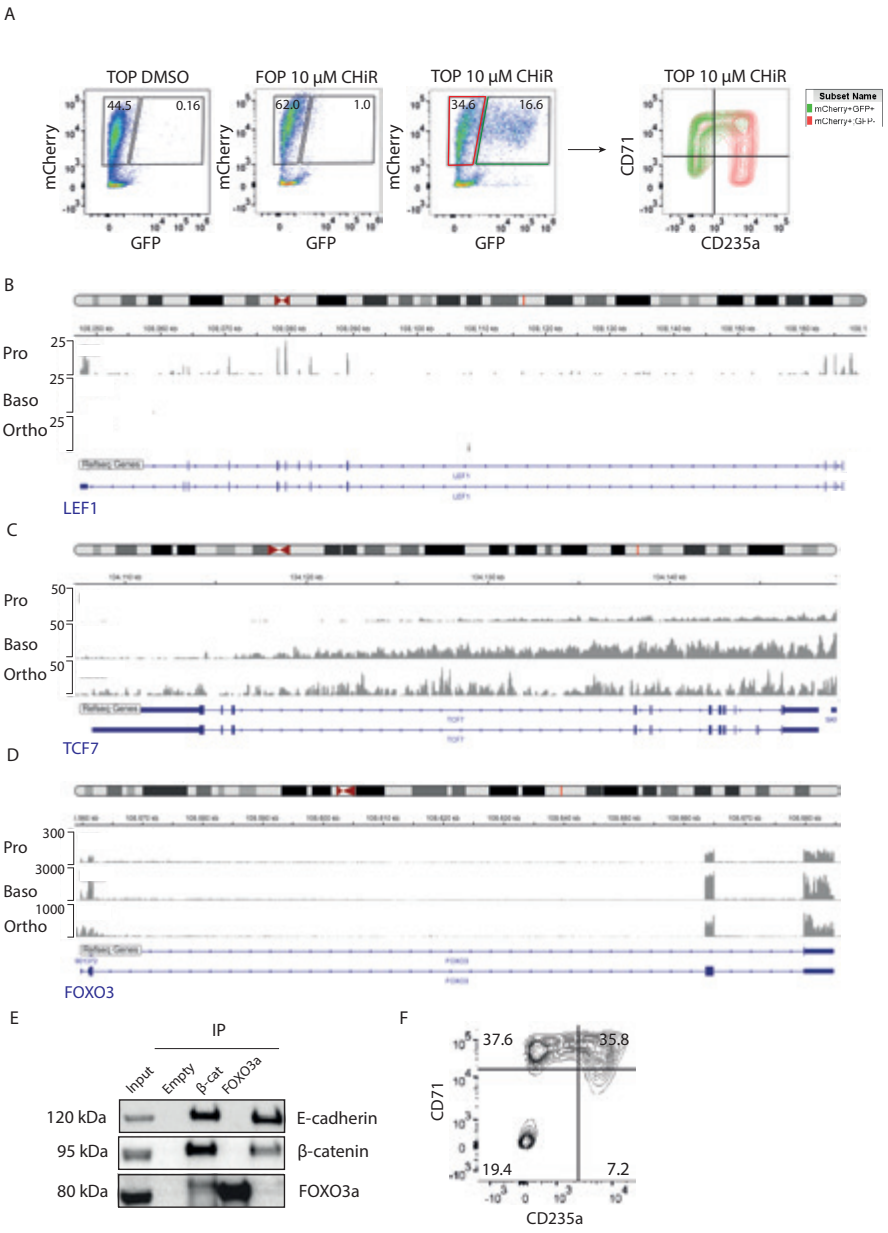


Figure 1. β-catenin signaling and FOXO3 expression during terminal differentiation. *(A) Representative flow cytometry plots of GFP (marking TCF7/LEF activity) and/or mCherry (marking lentiviral expression) fluorescence in TOP-GFP.mC or control FOP-GFP.mC transduced human erythroid cells, 48 h post DMSO or 10 µM ChiR treatment. GFP-positive cells in the 10 µM ChiR treated, TOP-GFP.mC transduced cells were analyzed based on CD71 and CD235a expression. (n=3) (B-D) RNA expression of LEF1 (B) TCF7 (C) and FOXO3 (D) in synchronized pro-erythroblasts (pro), basophilic erythroblasts (baso) and orthochromatic erythroblasts, as analyzed using Altanalyze software (4 donors) (E) Western blot for β-catenin, FOXO3 and E-cadherin protein expression in immunoprecipitates for β-catenin, FOXO3, E-cadherin or without antibody (empty, negative control) obtained from (F) human erythroblasts as defined by flow cytometry analysis of CD71 and CD235a cell surface expression.*

Results

Terminal erythroid differentiation links β-catenin with FOXO3 signaling

To investigate β-catenin signaling in erythropoiesis, we used a canonical Wnt reporter construct with constitutive mCherry expression (a TOP reporter (20)). The reporter contains seven binding sites for the canonical β-catenin binding partners TCF7/LEF in front of a GFP reporter gene. Erythroid progenitors/pro-erythroblast (CD71dim/high CD235a−) generated from lentiviral transduced CD34+ HSPCs, comprised ~40%–60% transduced mCherry-positive cells (Figure 1A).

The generated TOP-expressing erythroblasts were treated with CHIR99021, a selective inhibitor of GSK3β activity, to activate Wnt/β-catenin signaling. We used 10μM CHIR99021, which concentration we previously defined to elevate β-catenin protein levels by ~twofold in cultured human erythroblasts (7). CHIR99021 treatment triggered GFP expression in ~30% of mCherry-marked transduced cells 48 hours after treatment as defined by flow cytometry (Figure 1A). Intriguingly, CHIR99021 treatment predominantly activated TCF7/LEF signaling in CD71dim/highCD235a− erythroid progenitors and pro-erythroblasts, as evident by GFP expression (Figure 1A). In contrast, basophilic erythroblasts (mCherry+CD71+CD235a+) did not express GFP, as also found for control (DMSO) treated TOP transduced erythroblasts (Figure 1A). GFP expression was also not detected in CHIR99201 treated erythroblasts that were transduced with a GFP/mCherry FOP control construct, that served as a negative control (Figure 1A) (20). Finally, treatment of TOP transduced cells with 1μM CHIR99021, which does not elevate β-catenin protein levels (as previously defined (7)), also did not induce GFP expression (data not shown).

Next, we examined RNA-sequencing data that was previously generated in our department from cultured synchronized pro-erythroblasts, and subsequent derived basophilic and orthochromatic erythroid differentiation stages (15). We detected LEF expression in pro-erythroblasts, albeit at very low detection levels (<25 reads/exon) (Figure 1B). LEF expression was subsequently lost within basophilic/orthochromatic erythroblast differentiation stages. Additionally, specific TCF7 expression was not detected throughout these erythroblast maturation stages (Figure 1C). These findings are in line with the results that we obtained with the TOP-reporter, which together indicate that canonical β-catenin/TCF7/LEF signaling is mostly restricted to early erythroid/pro-erythroblasts.

We previously, however, revealed that activation of β-catenin signaling in basophilic erythroblast stimulates their terminal differentiation (7). Whereas canonical β-catenin/LEF signaling seems unlikely to be activated in basophilic erythroblasts, we next studied FOXO3 expression during terminal differentiation of cultured human erythroblasts. FOXO3 stimulates erythroid terminal differentiation in mice and has been defined to directly bind and control β-catenin transcriptional

signaling in tumor cells (13,21,22). RNA-expression analysis revealed pro-erythroblasts to express relatively low FOXO3 levels (~100 reads/exon), whereas expression strongly increased during the formation of basophilic erythroblasts (~3000 reads/exon) (Figure 1D). FOXO3 expression diminished within the orthochromatic erythroblast stage (~1000 reads/exon), which precedes enucleation and reticulocyte formation.

Since β-catenin has been reported to enhance FOXO3 transcriptional activity and/or to modify signal transduction that regulates FOXO3 activity, we next examined the potential interaction between β-catenin and FOXO3 in CD34+-derived cultured (pro)erythroblast with immunoprecipitation experiments. In suspension cultures of unstimulated erythroblast, a small fraction of β-catenin seemed to associate with FOXO3 (Figure 1E-F). Interestingly, β-catenin-bound FOXO3 ran slightly higher on the Western blot, which may display a posttranslational form (e.g. phosphorylation) of FOXO3. Reciprocal IP of FOXO3 however did not detect any β-catenin binding. The majority of β-catenin however was bound to E-cadherin, which is in agreement with our previous findings (7). Overall, these findings reveal that within erythropoiesis, β-catenin can stimulate TCF7/LEF signaling in early-stage/pro-erythroblasts formation and subsequently may crosstalk with FOXO3 signaling during terminal erythroid differentiation.

FOXO3 is dispensable for the formation and expansion of cultured human erythroblasts

To gain more insights into the role of FOXO3 in erythropoiesis during the differentiation of human erythroblasts, unique single guides were designed to target exons 2 and 3 of the FOXO3 gene with CRISPR/Cas9 to generate FOXO3 KO erythroblasts (Figure 2A). Guide 4 against exon 3 induced a nearly 100% KO efficiency in the basophilic leukemia cell line KU812 and was used to study the effect of FOXO3 loss on the expansion of cultured human erythroblasts (Figure 2B). HSPCs deficient of FOXO3 were cultured for 3 days in erythroid-defined progenitor medium, that was switched at culture day 4 (exp day 0) towards (pro)-erythroblast expansion medium (comprising EPO, SCF and Dexamethasone) and followed for 17 days (Figure 2C). Cas9-driven inactivation of FOXO3 protein expression was confirmed by Western-blot analysis in all CRISPR/Cas9 edited donor-derived CD34+ HSPCs (Figure 2D). Loss of FOXO3 did not perturb the expansion of pro-erythroblasts (CD71+CD235a−) nor the formation of basophilic erythroblasts (CD71+CD235a+) as compared to erythroblasts that were generated from control CRISPR/Cas9 edited HSPCs (Figure 2E-G). Overall these findings indicate that FOXO3 protein expression is not required to generate or expand CD34+derived human pro-erythroblast in vitro.

FOXO3 deficiency delays terminal differentiation of cultured human erythroblasts

We next investigated the differentiation of the generated FOXO3 KO erythroblasts in media favoring erythroid terminal differentiation and the formation of reticulocytes. After a seven-day expansion phase, FOXO3 KO and CRISPR/Cas9 control edited pro-erythroblast cell cultures were switched to media devoid of SCF and DEX, but supplemented with elevated levels of EPO (10 U/L), heparin, omni plasma, and holotransferrin (differentiation day 0). In Cas9 control-edited cells, FOXO3 protein expression was detected by Western blot up to day 4 of differentiation, which rapidly diminished at days 7 and 10 of differentiation (Figure 3A). These findings are also in line with the obtained RNAseq data for FOXO3 expression during differentiation. As a control, β-actin expression was assessed, although actin levels also decreased during terminal differentiation at differentiation days 7 and 10. At the start of differentiation, Cas9-edited control and FOXO3 KO pro-erythroblast cell cultures (n=3), harbored ~30% of pro- (CD71+CD235a−) and ~40% basophilic (CD71+CD235a+) erythroblasts, which did not significantly differ between the two experimental conditions (Figure 3B-C). Four days after differentiation, all pro-erythroblasts differentiated towards basophilic erythroblasts (Figure 3B-C). Interestingly, accumulation of basophilic erythroblasts was increased at day 4 within the FOXO3 KO cell culture as compared to Cas9 control edited cells (73,23% vs 62,73%, p=0.03) (Figure 3D). Subsequent orthochromatic (CD71−CD235a+) erythroblast formation (25.87% vs 35%, n=3 p=0.04) was decreased in FOXO3 KO erythroid cell cultures (Figure 3E). Loss of FOXO3 thereby seems to hamper erythroblast differentiation, as also seen for differentiation days 7 and 8 (Figure 3D-E). FOXO3 loss however did not alter cell proliferation, as the accumulation of cells during differentiation did not differ with that of Cas9 control-edited cells (Figure 3F).

By day 10 of differentiation, all FOXO3-deficient erythroblasts were differentiated into CD71−CD235a+reticulocytes, indicating that FOXO3-deficient cells are delayed in terminal differentiation rather than completely hampered in their differentiation.

Enucleation of FOXO3-deficient orthochromatic erythroblasts was compared to Cas9 control-edited cells after staining the cells with DRAQ5, a membrane-permeable nuclear dye. Over a ten-day differentiation period, FOXO3-deficient cultures exhibited delayed enucleation, with the largest delay visible on day seven showing ~25% less enucleated cells as compared to control cells ($p<0.005$, n=3) (Figure 3G-H). In sum, these data revealed FOXO3 to stimulate the differentiation of human basophilic erythroblasts.

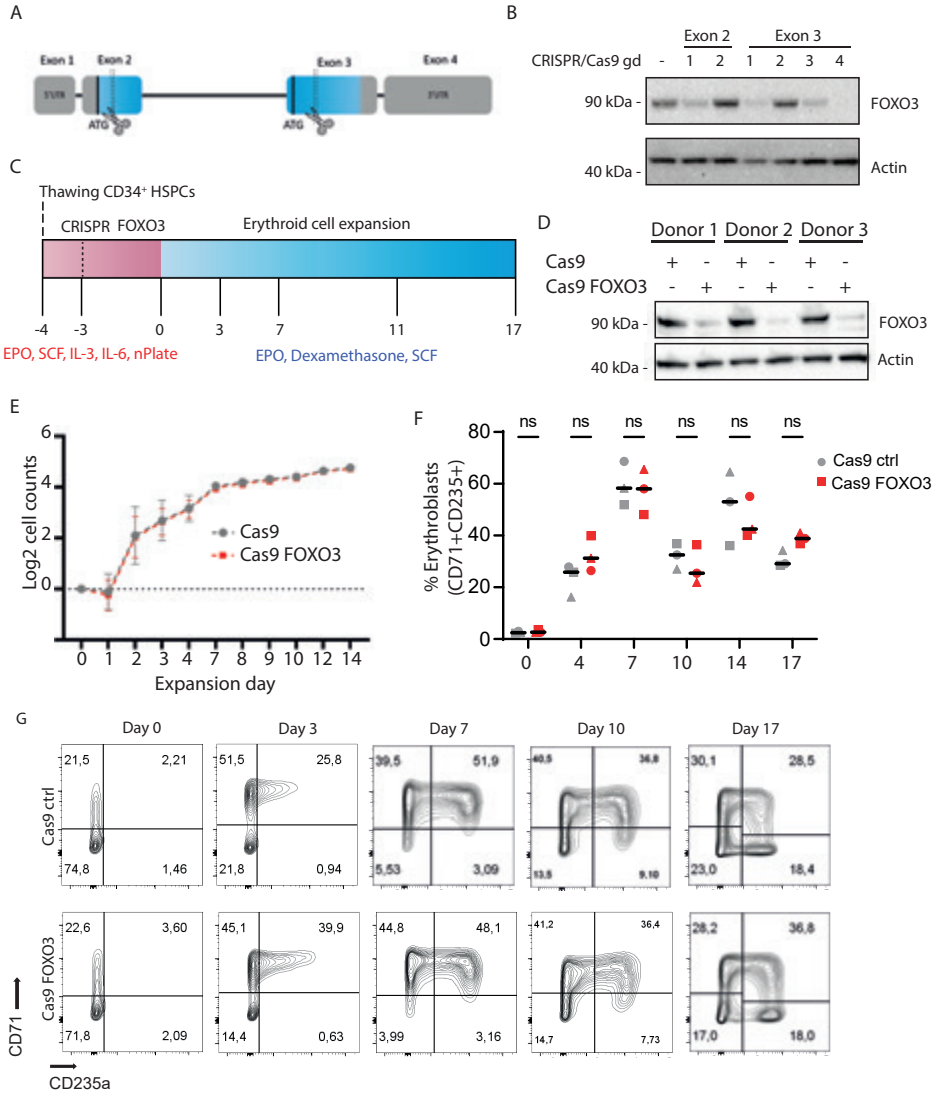


Figure 2. FOXO3 is dispensable for the formation and expansion of cultured human erythroblasts. *(A) Strategy to generate FOXO3 deficient human erythroblasts using CRISPR Cas9 technology. To knock out FOXO3 in human erythroblasts, sgRNAs were developed against exons 2 and 3 of FOXO3. (B) Western blot analysis showing knockout efficiency of 6 sgRNA targeting FOXO3 exon 2 or 3 respectively, in KU812 cells (n=1). (C) Experimental layout. MPB-derived CD34+ HSPCs were cultured for 4 days in a stem cells medium containing IL-3, IL-6, nPlate, EPO, and SCF, to skew HSPCs towards the erythroid lineage. On day 3 (day 1 of culturing), FOXO3 deficient cells were generated using CRISPR Cas9 technology. On day 0, cells were transferred to erythoid-defined expansion medium, containing EPO, Dexamethasone, and SCF. (D) Western blot analysis showing knockout efficiency of sgRNA guide #4 targeting exon 3 in FOXO3 in cultured erythroblasts derived from CD34+-derived HSPCs from 3 different donors. (E) Growth curves of CRISPR/Cas9 control and FOXO3 KO erythroblasts depicting cell expansion over 14 days of growing the cells in erythroblast expansion-defined medium (F) Quantification of the percentage of control and FOXO3 KO generated CD71+CD25a+ erythroblasts in erythroid-defined expansion medium over time. (G) Flow cytometry analysis, showing representative examples of CD71+CD235a− (pro-), CD71+CD235a+ (basophilic) and CD71−CD235a+ (orthochromatic) erythroblasts in CRISPR/Cas9 edited control and FOXO3 KO cells, in erythroid-defined expansion medium on culture days 3, 7 10 and 17.*

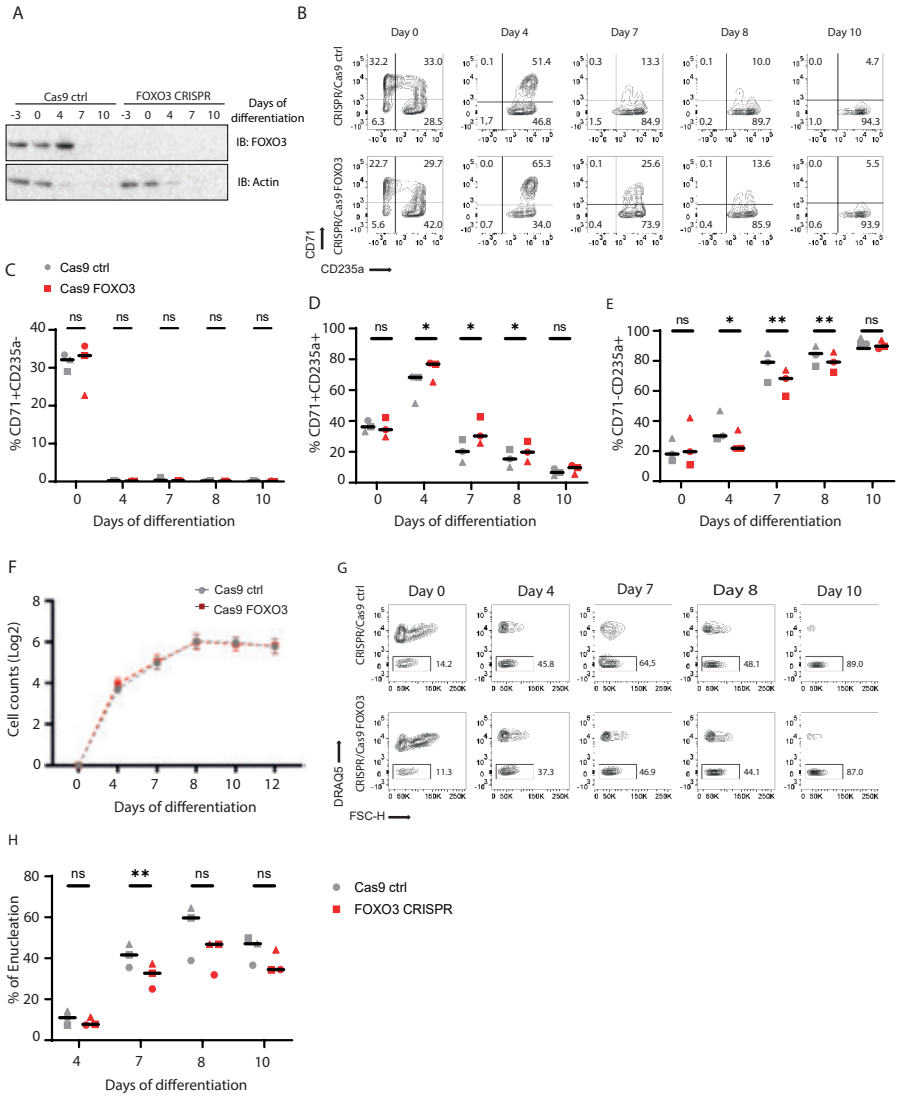


Figure 3. FOXO3 stimulates terminal the differentiation of cultured human erythroblasts *(A) Western blot analysis for FOXO3 protein expression on day -3 (comprising erythroid-defined expansion medium) and on culture days 0, 4, 7, and 10 in media favoring erythroid terminal differentiation. β-actin was used as loading control which during erythropoiesis declines and therefore used to compare protein expression at culture days -3, 0 and 4 (n=3) (B) A representative example of the accumulation of CD71+CD235a− pro-erythroblast, CD71+CD235a+ basophilic and CD71-CD235a+ orthochromatic erythroblasts in CRISPR/Cas9 edited control and FOXO3 KO cells, in erythroid-defined differentiation medium on culture days 3, 7 10 and 17, defined with flow cytometry. (C) Subsequent quantification of the percentages of CD71−CD235a+, (D) CD71+CD235a+, (E) CD71−CD235a+ CRISPR/Cas9 edited control and FOXO3 KO erythroblasts on culture days 0, 4, 7, 8, and 10 during terminal erythroid differentiation (F) Growth curves of FOXO3 deficient or control erythroblasts, in terminal differentiation medium (n=3). (G) Examination of reticulocyte formation with flow cytometry. Reticulocytes are marked as DRAQ5- with an FCS-H of ~50K, pericytes as DRAQ5+ with an FSC-H of ~ 40K and orthochromatic erythroblasts as DRAQ5+ with an FSC-H of ~ 100K. (H) Quantification of the formed reticulocytes depicted as percentages, in cultured control and FOXO3-deficient erythroblasts on experimental days 0, 4, 7, 8, and 10 during terminal erythroid differentiation (n=3).*

FOXO3 protein expression is upregulated in rat erythroblasts in response to anemia

We previously defined anemia to upregulate β-catenin protein levels in erythroblasts in rat BM (7). Because FOXO3, similar to β-catenin, can stimulate erythroid differentiation, we next examined FOXO3 protein expression in erythroblasts of anemic rats. We induced anemia in 8-week-old Wistar rats with 3 consecutive IP injections of Phenylhydrazine (PHZ, 40mg/kg) as previously described (6,23).

PHZ treatment induces a synchronized anemic response in Lewis rats, resulting in a maximal anemic response between 48 to 96 hours after PHZ treatment (23). We, therefore examined BM composition and FOXO3 protein expression in femurs and tibia with IHC 48 hours after PHZ treatment. Anemia strongly increased the number of FOXO3-positive cells (Figure 4A). IHC staining indicated that FOXO3 protein expression was also elevated. We analyzed whether the observed increase of FOXO3 occurred within the erythroid lineage by sorting CD71+HIS49+-erythroid-defined cells from BM as previously described (23). Western blot analysis revealed low expression levels of FOXO3 protein in rat erythroblasts in steady-state conditions (Figure 4B). However, 48 hours after treatment with PHZ, FOXO3 protein expression strongly increased in BM-derived rat erythroblasts (n=4).

In severe cases of anemia, humans and rats can regulate erythroid turnover in the spleen. We therefore also examined FOXO3 expression by IHC in the spleen of control and anemic rats. In steady-state conditions, FOXO3 is expressed in the rat spleen, in both the white and red pulp (Figure 4C). The red pulp of the anemic spleen harbored large numbers of erythroblasts in correspondence to previous results (6,23). The accumulation of erythroblasts in the anemic spleen was characterized by the clustered cellular and dense nuclear staining that was not seen in control conditions (Figure 4C). Moreover, generated erythroblasts could be distinguished by a strong staining for FOXO3 as defined with IHC (Figure 4C). Overall, these data reveal that hemolytic anemia, as induced with PHZ, strongly increases FOXO3 protein expression in erythroblasts in both BM and spleen.

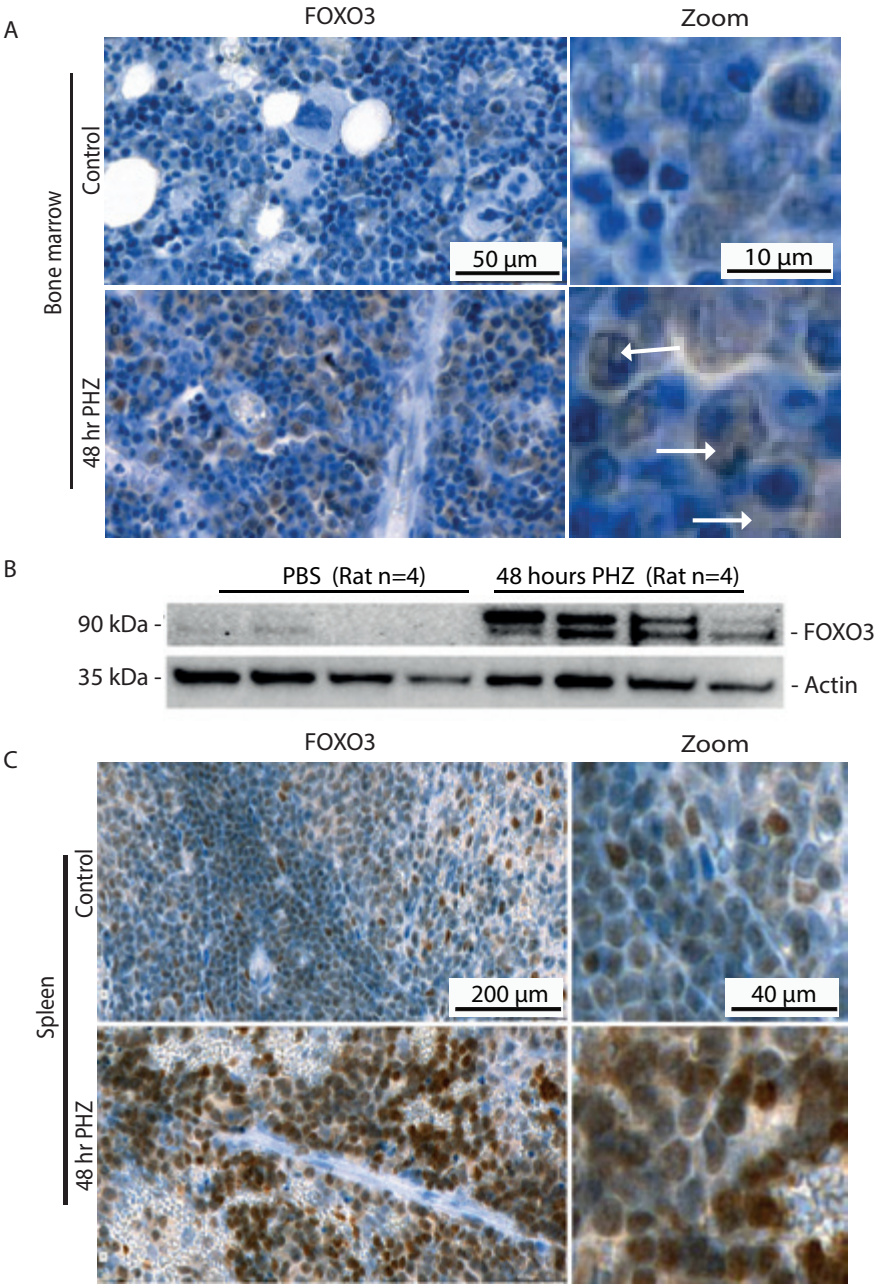
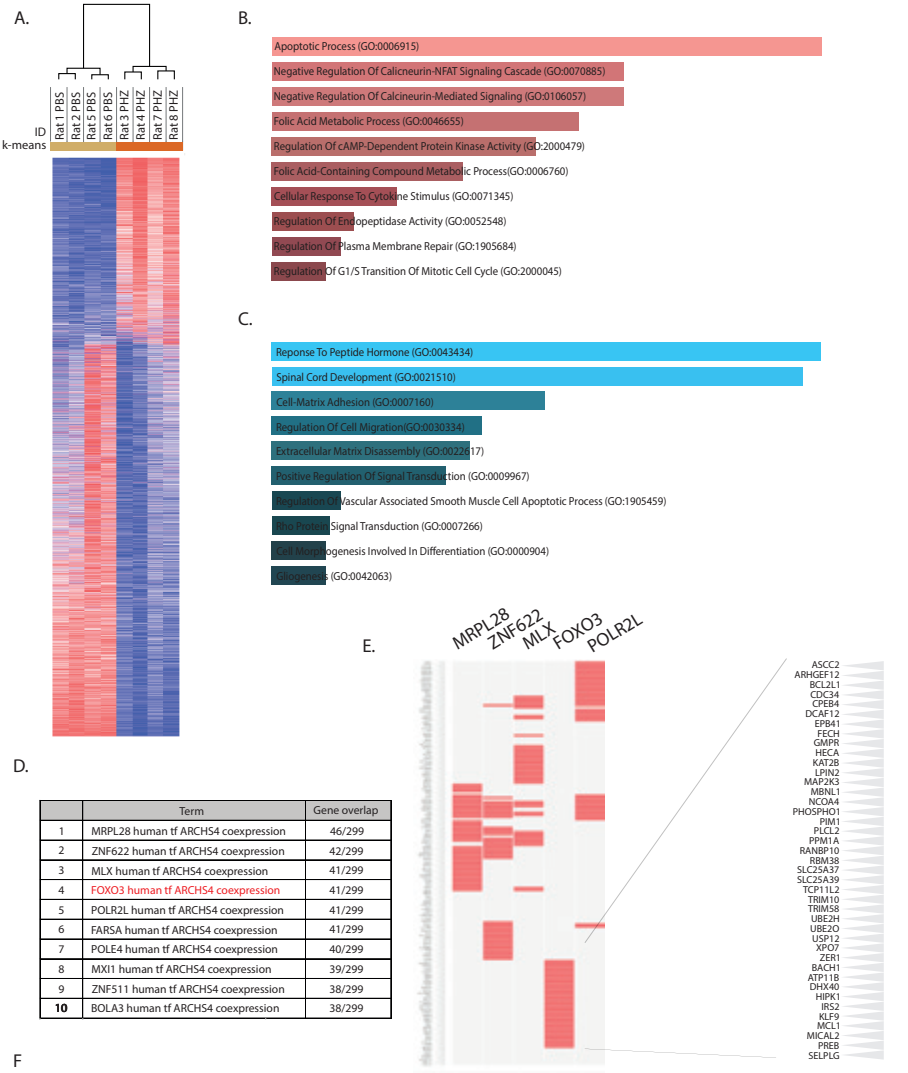


Figure 4. FOXO3 is upregulated in rat erythroblasts in response to anemia. *(A) Representative IHC staining for FOXO3 protein expression (brown) in bone marrow sections from rats that were treated for 48 h with PHZ (inducing anemia) or PBS (served as control) (n=4) (B) Western blot analysis of FOXO3 protein expression in FACS-sorted CD71+His49+ rat erythroblasts derived from anemic (48 h post-PHZ treatment) and control (48 h post-PBS treatment) rat BM (n=4). (C) Representative IHC staining for FOXO3 protein expression (brown) in spleen sections derived from anemic (48 h post-PHZ treatment) and healthy control (48 h post-PBS treatment) rats (n=4).*



D.

	Term	Gene overlap
1	MRPL28 human tf ARCHS4 coexpression	46/299
2	ZNF622 human tf ARCHS4 coexpression	42/299
3	MLX human tf ARCHS4 coexpression	41/299
4	FOXO3 human tf ARCHS4 coexpression	41/299
5	POLR2L human tf ARCHS4 coexpression	41/299
6	FARSA human tf ARCHS4 coexpression	41/299
7	POLE4 human tf ARCHS4 coexpression	40/299
8	MXI1 human tf ARCHS4 coexpression	39/299
9	ZNF511 human tf ARCHS4 coexpression	38/299
10	BOLA3 human tf ARCHS4 coexpression	38/299

F

Pathway	Overlap	P-value	Genes
Heme metabolism	14/200	$1.8*10^{-18}$	MAP2K3;RBM38;ARHGEF12;FECH;EPB41,NCOA4;XPO7;BACH1;KAT2B;SLC25A37;RANBP10,TRIM58;LPIN2;TRIM10
TNF-α signaling via NF-κB	4/200	$0.8*10^{-3}$	MAP3K3;KLF9;IRS2;MCL1
Apoptosis	2/161	0.04	MCL1;BCL2L1
Mitotic spindle	2/199	0.06	ARHGEF12;EPB41
IL-2/STAT5 signaling	2/199	0.06	PIM1;BCL2L1
Hypoxia	2/200	0.06	PIM1;IRS2

Figure 5. A FOXO3 signaling signature marks rat erythroblasts in anemic BM. *(A) Heatmap reflecting 1163 genes that were statistically significantly upregulated by 1.5 fold or more and 2472 genes that were downregulated by 1.5 fold or lower (Padj score < 10-5) in sorted CD71+His49+ rat erythroblasts derived 48hr post PHZ (anemic) vs PBS (healthy control) treatment from rat BM (n=4). (B) String pathway analysis depicting GO-defined processes linked to the 917 significantly upregulated genes and (C) 1060 downregulated genes (D) Top 10 transcriptional associated networks enriched in anemic (48 h post-PHZ treatment) as compared to healthy (48 post-PBS treatment) rat CD71+His49+ erythroblasts, defined with ENRICHR. (E) A reflection of the gene signatures that corresponded with the top 5 transcriptional networks that were enhanced in anemic (48 post-PHZ) rat CD71+His49+ erythroblasts as compared to healthy conditions, as defined with ENRICHR. (F) Pathways analysis of the FOXO3-linked enriched genes in anemic (48 post-PHZ) rat CD71+His49+ erythroblasts.*

A FOXO3 gene signature marks rat erythroblasts in response to anemia.

To identify pathways that may be linked or controlled by E-cadherin/β-catenin signaling in erythroblasts during stress erythropoiesis, we performed RNA-seq on sorted CD71+HIS49+ erythroblasts, isolated from control (PBS) and anemic BM (PHZ 48hrs) rats (n=4). Gene expression analysis defined 1163 genes that were statistically significantly upregulated by 1.5 fold or more and 2472 genes that were downregulated by 1.5 fold or lower (Padj score < 10-5) as displayed in a heatmap (Figure 5A). We examined the significantly (r>0.8 among replicates), upregulated (917) genes, and downregulated (1060) genes with String pathway analysis. Upregulated genes comprised pathways involved in metabolism, protein translation pathways, and sterol biosynthesis (Figure 5B). Genes that were downregulated in response to anemia reflected GO terms including cell adhesion and may be linked with the strong expansion of erythroblasts in anemic BM (Figure 5C).

To improve our understanding of the underlying active transcriptional networks, we next used ENRICHR pathway analysis (24–26). Within the upregulated gene set we retrieved active transcriptional networks, using 'ARCHS4 co-expression with transcription factors' including; MRPL28, ZNF622, MLX, FOXO3, POLR2L, FARSA, POLE4, MXL1, ZNF511, and BOLA3 as the top 10 gene signatures (Figure 5D). Among these, we identified FOXO3 signaling to mark erythroblasts in response to anemia. We identified 'heme metabolism' to reflect the most prominent pathway within the retrieved FOXO3 gene signature (14 out of 41 genes) (Figure 5E-F). Overall, these data reveal that BM-derived erythroblasts upregulate processes that control heme metabolism, protein/lipid metabolism and downregulate pathways that are linked to cell adhesion in anemic rats. Moreover, we identified a FOXO3 signature, that is linked to heme metabolism, to mark erythroblasts in anemic rat BM, which may underlie the control of FOXO3 on terminal erythroid differentiation.

Discussion

In this study, we investigated the role of β-catenin and FOXO3 in erythropoiesis. Previously, we revealed that expression of a constitutively active β-catenin construct in cultured human erythroblasts accelerated terminal differentiation (7). However, whereas β-catenin stabilization is typically associated with activation of the canonical transcription factors TCF/LEF, we here demonstrate that this does not occur after the pro-erythroblast stage. This suggests that β-catenin may drive erythroid terminal differentiation through alternative pathways. Our preliminary data suggest β-catenin to associate with FOXO3 in cultured erythroid progenitors. In mice, FOXO3 signaling has been linked to erythroid differentiation and enucleation (12,13). This is in line with our data, which show that FOXO3 deficiency delays the differentiation and enucleation of human erythroblasts. Moreover, we identified FOXO3 protein expression to strongly increase in erythroblasts in the BM and spleen of anemic rats. Analysis of the transcriptome of rat

erythroblasts identified FOXO3 as a key transcriptional regulator upregulated in erythroblasts in response to anemia.

Both β-catenin and FOXO3-associated signaling have been reported to control erythroid recovery in response to anemia in mice (13,23,27). Additionally, an association of β-catenin potentiates FOXO3-mediated transcription during oxidative stress and thereby stimulates cell survival (10,11). Of note, FOXO3 has been demonstrated to inhibit canonical TCF/LEF transcriptional activation by sequestering β-catenin (28).

While β-catenin-FOXO3 interaction has been described within epithelial tumor cell lines (29–31), immunoprecipitation of β-catenin and FOXO3 is a notorious technical challenge (10). This could be the consequence of the ratio between FOXO3 and β-catenin expressed within the cell. Alternatively, interference from other proteins such as E-cadherin or the antibodies used in the assay potentially prohibit the interaction. Previously, the interaction is more clearly visualized by the molecular tools HA-tags and Flags. In our erythroblast cultures in unchallenged conditions, we observed a faint endogenous FOXO3-β-catenin one-sided interaction. Besides technical tools, the detection of β-catenin-FOXO3 binding perhaps requires additional challenges or stimulation. For instance, the elimination of SCF from our erythroblast expansion cultures before immunoprecipitation might aid this interaction. SCF-induced activation of Akt is known to inhibit FOXO3 signaling, as p-Akt-mediated phosphorylation of FOXO3 results in its nuclear export and sequestering to the cytoplasmic 14-3-3 protein. We hypothesize that this in turn may hamper β-catenin-FOXO3 binding. In future studies, we will investigate the effect of SCF/AKT signaling on the interaction between β-catenin and FOXO3.

In our previous study on active β-catenin signaling, we observed β-catenin to accelerate terminal differentiation as seen in this study for FOXO3 in comparison to FOXO3 KO cells. This assay therefore provides a candid strategy to further investigate the putative interaction and interplay between β-catenin and FOXO3, by combining overexpression with CRISPR/Cas9-driven KO studies of either FOXO3 and/or β-catenin. If the acceleration of erythroid terminal differentiation is indeed driven by a β-catenin-FOXO3 interaction and/or interplay, FOXO3 KO erythroblasts would perturb constitutionally active β-catenin to enhance erythroblast differentiation.

Our immunoprecipitation studies however revealed that the majority of β-catenin protein is associated with E-cadherin in human erythroblasts. Moreover, we found β-catenin protein stability to rely on E-cadherin expression in human erythroblasts (7). It is therefore likely that the majority of β-catenin is sequestered by E-cadherin on the cytoplasmic membrane. This thereby prevents its binding by FOXO3, considering the overlapping binding domains for β-catenin of both E-cadherin and FOXO3 (10,32), E-cadherin binding thus may prevent β-catenin from binding with FOXO3. Dissociation of β-catenin from E-cadherin therefore may be required to stimulate the

association and nuclear accumulation of FOXO3 with β-catenin in the nucleus. Notably, through various mechanisms, β-catenin has been reported to be able to translocate to the nucleus in response to mechanical tension occurring at epithelial cell junctions (33–35). Mechanical tension among adherent erythroid cells therefore may induce the release of β-catenin from E-cadherin and potentially facilitate its interaction with FOXO3.

Alternatively, β-catenin-FOXO3 interaction may become more evident upon challenging culture conditions such as oxidative stress as also reported by Essers and colleagues (10). FOXO3 typically functions in defense against oxidative stress by enhancing the expression of antioxidant enzymes (36). In FOXO3-deficient mice, RBCs have decreased levels of ROS scavenging enzymes, resulting in higher levels of ROS and a shortened lifespan (13). The regulation of oxidative stress during hematopoiesis is critical because hematopoietic cells are sensitive to abnormal ROS levels. Further validation of ROS levels and the expression of antioxidant genes in our FOXO3-deficient erythroid cells would be necessary to confirm these findings.

Strikingly, in our erythroblast cultures, we find a delay in terminal differentiation in FOXO3 KO cells by an accumulation in the immature CD71+CD235+ stage and a delayed maturation towards CD71−CD235+ reticulocytes, corresponding with the data of the Ghaffari lab retrieved with FOXO3 KO mice (13). Results by Marinkovic and colleagues additionally (13) suggest that the delayed differentiation observed in FOXO3-deficient mouse erythroblasts may result from a cell cycle arrest, induced by elevated p53 and P21CIP/WAF/Sdi1, likely a response to elevated oxidative stress. FOXO3 deficient mice have increased ROS in their BM and blood during steady state that, upon PHZ treatment, accumulate even further (13). In this work, we however revealed that loss of FOXO3 protein expression did not result in cell cycle arrest as its loss did not alter the expansion of human erythroblasts but rather delayed their differentiation. We also reveal FOXO3 to increase in rat erythroblasts in response to anemia. Moreover, we identified erythroblasts to display upregulation of the FOXO3 target gene signature involved in heme production upon PHZ-induced hemolytic anemia. This is likely an essential response for survival, as FOXO3-deficient mice have been reported to succumb upon PHZ treatment, in contrast to WT controls, which upon perturbation may delay erythroblast differentiation (13).

Aside from FOXO3 accumulation we previously observed accumulation of β-catenin and E-cadherin upon PHZ-induced anemia in rat BM ((7) and figure 4). We and others observe in rat BM, but not mouse BM, a major architectural reorganization during anemia as it also occurs in human BM (6). Adipocytes decrease and concomitantly, erythropoiesis increases. Possibly, this provides the mechanical tension that we speculate to be beneficial for β-catenin translocation into the nucleus. However, these questions remain to be answered in future studies, as rat models for erythropoiesis are still in their infancy.

In conclusion, rather than canonical TCF/LEF factors, we reveal that β-catenin stabilization in erythropoiesis may be linked to FOXO3 signaling. We report here that FOXO3 deficiency in cultured primary CD34+ HSPCs-derived human erythroblasts, similarly as observed in mice, leads to delayed erythroblast differentiation. During PHZ-induced hemolytic anemia in rats, FOXO3 expression increases, and a FOXO3 transcription signature is upregulated comprising genes related to heme metabolism, further emphasizing FOXO3's role in regulating erythropoiesis in response to anemia.

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