Using zebrafish to model erythroid lineage toxicity and regeneration

The zebrafish is a vertebrate model organism widely used to study developmental and malignant hematopoiesis. All major mammalian blood cell lineages are present in zebrafish, and many genes and pathways involved in vertebrate hematopoiesis are conserved between fish and mammals.[1-3] The small size of zebrafish embryos, the ease of handling and culture, and the transparent nature of the embryonic and larval stages, make it an accessible, *in vivo* imaging-friendly vertebrate model. Additionally, early stage embryos are permeable to chemicals added to water, which makes them suitable for large-scale pharmaceutical testing.[4] Blood cell toxicity is one of the most common drug side effects;[5,6] current screening strategies rely on *in vitro* assays with limited readouts. Classical *in vivo* mammalian models, although very informative, are laborious and not suitable for high-throughput experiments.[7,8] Herein we report a novel zebrafish-based model for analyses of erythroid toxicity and regeneration.

Erythroid populations have been previously monitored using a *Tg(gata1:DsRed)* line driving expression from the gata1 promoter mostly expressed in progenitor cells[9] and the *Tg(globin:GFP)* line in which an α-LCR and proximal globin promoter activate expression specifically in erythroid cells.[10] Here we crossed homozygous strains of these lines to obtain double transgenic *Tg(gata1:DsRed;globin:GFP)* zebrafish (Figure 1A). Single and double fluorescent signals were readily detected by confocal microscopy in double transgenic embryos (*Online Supplementary Figure S1*). To quantitate fluorescent populations, embryos were dissociated to single cells, labeled with DRAQ5 to recognize the nucleated zebrafish erythrocytes and scored by flow cytometry from day 1 to 5 of development (Figure 1B and *Online Supplementary Figure S2A-D*).

Descriptions of procedures and image acquisition are available in the *Online Supplementary Material.*

In both assays, we observed two distinct populations:

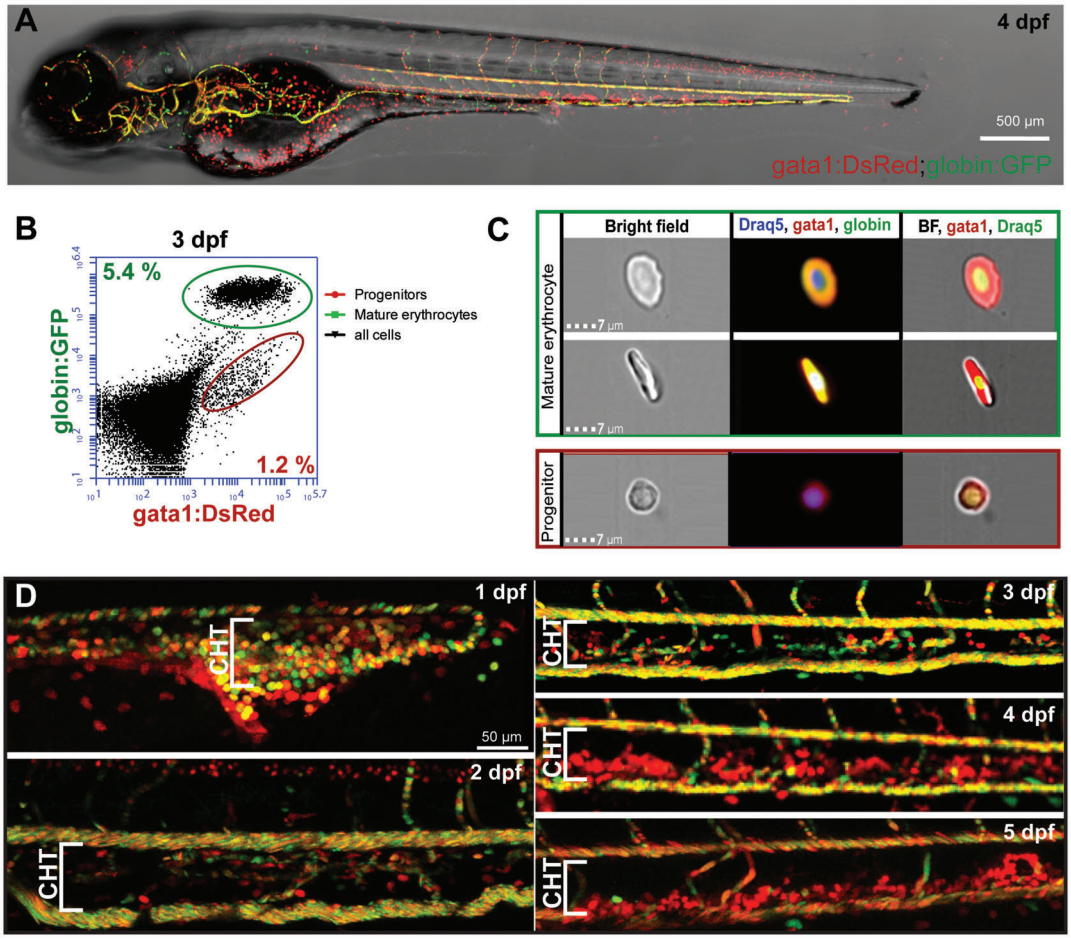

Figure 1. (Figure 1. Analyses of double transgenic zebrafish embryos *Tg(gata1:DsRed;globin:GFP)*. (A) A confocal image representing the 4 dpf double transgenic zebrafish embryo with the bright field channel. (B) A flow cytometry plot representing cells from homogenized whole zebrafish embryos at 3 dpf. The GFP versus DsRed plot demonstrates the distribution of fluorescent cells; two populations are marked: double positive gata1+/globin+ mature erythrocyte population (green frame) and gata1+/globin- erythroid progenitor population (red frame). (C) Representative images from ImageStream imaging flow cytometer of a mature erythrocyte cell from the double positive gata1+/globin+ gate, with en face and sidewise view (green frame) and an erythroid progenitor from the gata1+/globin- gate (red frame). DRAQ5 marks the nucleus. (D) Close-up confocal images of the caudal hematopoietic tissue region (CHT) in the double transgenic zebrafish embryos over the first 5 days of development. At 1 dpf the CHT is full of newly formed erythrocytes. At 2-3 dpf the double fluorescent gata1+/globin+ mature erythrocytes are circulating within the embryonic blood vessels. At 3-5 dpf the gata1+/globin- erythroid progenitors are accumulating in the CHT.

a double-fluorescent population of mature erythrocytes (gata1+/globin+) and a red fluorescent-only population (gata1+/globin-) of erythroid progenitors (Figure 1B and *Online Supplementary Figure S2D*). Notably, the red signal of double positive cells was partly very faint and therefore more reliably detected by flow cytometry than in overlay maximal projections of confocal images. We found that within this time frame, the number of mature erythrocytes per embryo increased along with the number of all fish cells, resulting in fairly steady relative erythrocyte numbers (as % of all fish cells). Instead, erythroid progenitors linearly increased from ~250 at 1 day post fertilization (dpf) to ~1500 cells at 5 dpf (*Online Supplementary Figure S2E,F*). Double positive gata1+/globin+ and red fluorescent-only gata1+/globin- cells were further analyzed using ImageStream imaging flow cytometer (Amnis) evaluating cell morphology in conjunction with classical flow cytometry (Figure 1C). Indeed, gata1+/globin+ cells had a shape typical for mature erythrocytes (flat-elliptical ~10 μm long and ~5 μm wide, with a small nucleus) while gata1+/globin- cells showed progenitor-like morphology (spherical, smaller, ~5 μm in diameter, and with a relatively large nucleus). Confocal images showed double positive cells circulating within the embryonic blood vessels (Figure 1A and *Online Supplementary Figure S1*), while gata1 positive progenitors accumulated in the caudal hematopoietic tissue (CHT) of the embryo's tail region starting at ~48 hpf, as reported before[11] (Figure 1D). Consistent with our flow cytometry results, progenitor numbers increased noticeably in the CHT between 2 and 4 dpf (Figure 1D). Around 5 dpf, the stem and progenitor cells migrate from the CHT to the kidney marrow where the adult hematopoiesis takes place.[12] In line, gata1-positive progenitors slightly decreased in the CHT at 5 dpf (Figure 1D), whereas overall numbers increased (*Online Supplementary Figure S2E,F*).

Next, we used this model to study erythroid lineage drug-induced toxicity and regeneration. The topoisomerase I inhibitors irinotecan (IRI) and topotecan (TOP) (see *Online Supplementary Material*) were used as antimitotic reference compounds. Various drug exposure schemes were developed to analyze the effects of compounds on dividing erythroid progenitors and mature cell populations, while ensuring survival of the embryos.

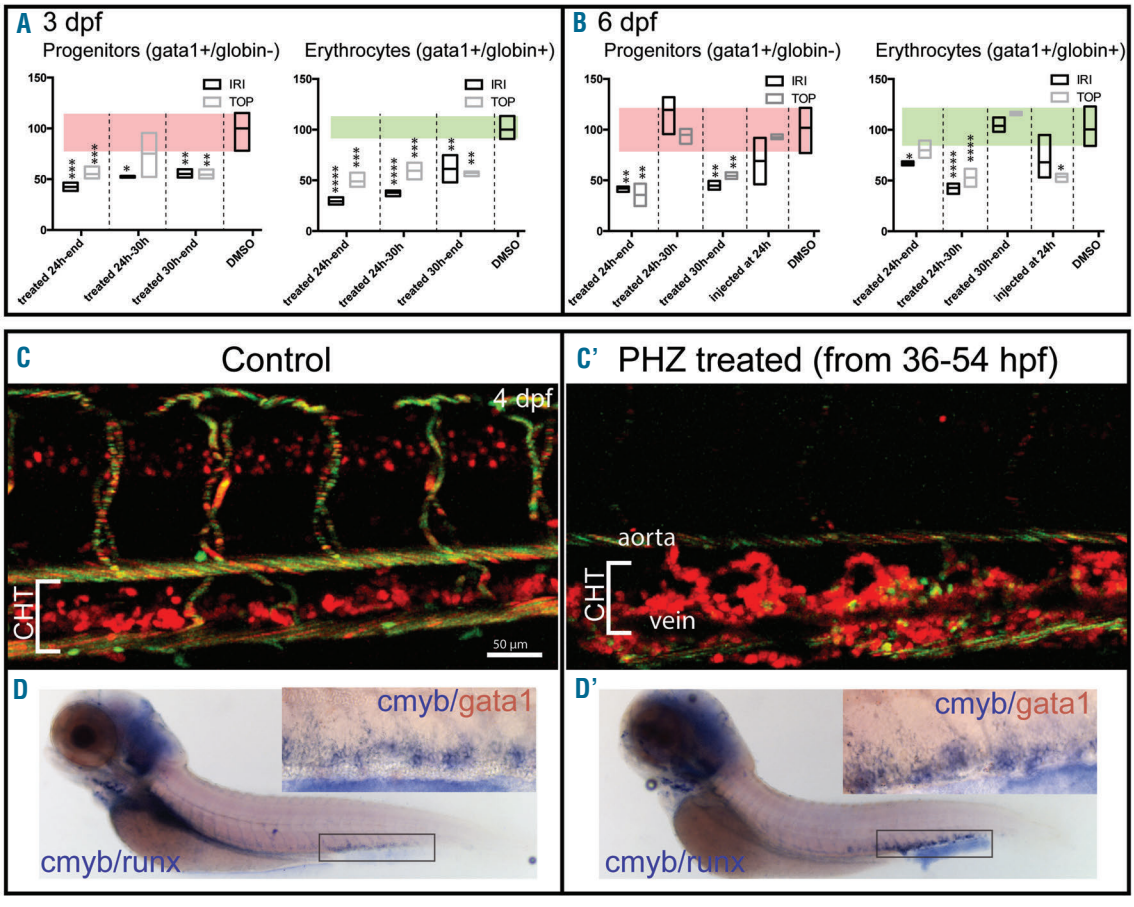

Figure 2. Differential compound toxicity to erythroid lineages. (A-B). Graphs representing flow cytometry results of irinotecan (IRI) and topotecan (TOP) exposure experiments on double transgenic zebrafish embryos *Tg(gata1:DsRed;globin:GFP)*, analyzed as percentage change in regards to the average control value. The range of control values is presented as a red or green rectangle for progenitors or erythrocytes, respectively. Each result is an average of 3 biological replicates with average and standard deviation. Irinotecan was used at a concentration of 4 μM in E3 medium and Topotecan was used at a concentration of 20 μM in E3. The different incubation schemes are indicated on the X-axis. Embryos were analyzed at 3 dpf (A) or at 6 dpf (B). (C-D) Confocal images of the CHT tail region of double transgenic zebrafish embryos *Tg(gata1:DsRed;globin:GFP)* after treatment with phenylhydrazine. At 4 dpf (C') very few circulating erythrocytes are visible in the blood vessels. The CHT is filled up with proliferating, red-only erythroid progenitors, a few of which are turning green, suggesting transformation into mature erythrocytes. (D) *In situ* hybridization analyses of stem cell markers (*cmyb* and *runx*) expression in zebrafish embryos at 3.5 dpf. Embryos treated with PHZ (D') show an increase of cmyb/runx staining in comparison to control (D).

Progenitors and mature erythrocytes were quantified by flow cytometry at different time-points (Figure 2A,B and *Online Supplementary Figure S3*). Since the major wave of erythroid cell production occurred between 24-48 hours post fertilization (hpf) (Figure 1D and *Online Supplementary Figure S2F*), we identified 24-32 hpf as a key time window for drug exposure analyses. We first exposed embryos to compounds from 24 hpf constantly until the time of analyses (Figure 2A,B) and observed a consistent decrease in erythroid mature and progenitor cells, along with overall reduced numbers of zebrafish cells (*Online Supplementary Figure S3A,B*). Next, we incubated embryos from 24 to 32 hpf and then washed the compounds away. At 3 dpf, these embryos showed decreased mature erythrocytes but only slightly decreased progenitors (Figure 2A), while at 6 dpf the embryos showed a significant decrease of mature erythrocytes but not of the progenitors, indicating that removal of the drug allowed progenitor recovery at this later time point (Figure 2B and *Online Supplementary Figure S3C,D*). This shows that the inhibition of progenitor proliferation in this key time window results in significant anemia at later stages, which is reversible if drug exposure is stopped. This corresponds to the delayed effects on mature erythrocytes observed in patients receiving such therapies. In contrast, in embryos constantly exposed to higher doses of the compound from 32 hpf to 3 dpf, all populations were still affected (*Online Supplementary Figure S3E-G*).

Since we observed differences in required compound dosages, we investigated whether drugs differed with respect to their bioavailability following water uptake. We analyzed concentrations of irinotecan and topotecan in embryos treated from 32 hpf to 3 dpf and in the corresponding incubation media. The concentrations in the embryo increased proportionally with higher medium concentrations but remained in the ng/ml range despite very high concentrations in the medium (μg/ml range) (*Online Supplementary Figure S4*). Interestingly, irinotecan showed higher accumulation in the embryo than topotecan (*Online Supplementary Figure S4B*). This result corresponds with our phenotypic analyses, indicating that higher concentrations of topotecan are needed for similar effects on erythroid cells. Interestingly, when administered via injection into the yolk at 24 hpf, topotecan was at least as equally potent as irinotecan at the same concentration (Figure 2A,B and *Online Supplementary Figure S3H*). Therefore, bioavailability of different compounds during water exposure varies dependent on their different physical, chemical and biological properties, and should also be routinely assessed when screening larger sets of compounds.

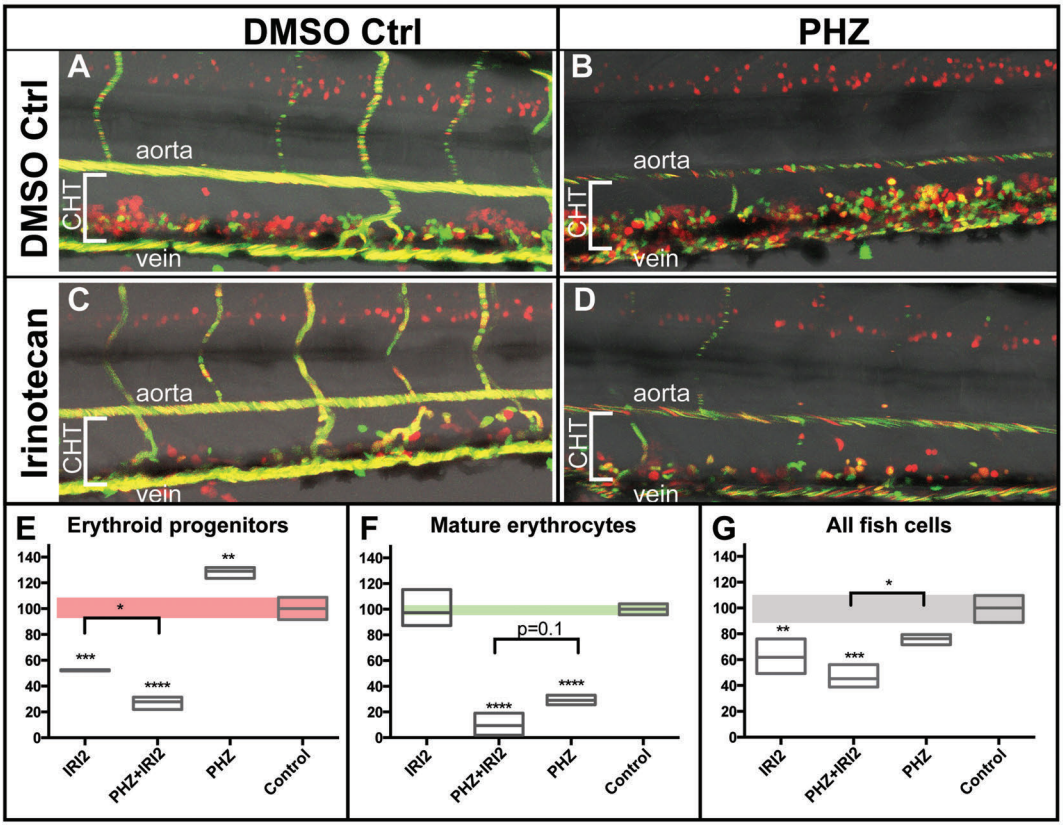

Figure 3. Irinotecan inhibits erythrocyte regeneration after hemolytic anemia. (A-D) Confocal images representing transgenic embryos *Tg(gata1:DsRed;globin:GFP)* treated with: DMSO (A, control), 2 μM irinotecan (B, IRI2), 1 μg/ml phenylhydrazine (C, PHZ) and show a combination of irinotecan and phenylhydrazine (D, PHZ+IRI2). Images were taken at 4 dpf. (E-F) Flow cytometry analyses of erythrocyte populations in double transgenic embryos *Tg(gata1:DsRed;globin:GFP)* for the treatments shown in A-D, presented as relative change in regards to the control average. Colored rectangles mark the control range. Erythroid progenitors (E) are diminished for irinotecan and irinotecan+PHZ treatment, whereas they increase in number after PHZ-only treatment. Mature erythrocytes (F) are not affected by irinotecan alone, but are diminished after PHZ and PHZ+irinotecan treatment. Irinotecan also affects the total cell number (G).

Phenylhydrazine is a chemical known to cause hemolytic anemia through the oxidization of hemoglobin and has been previously used in zebrafish.[13,14] We incubated our double transgenic zebrafish embryos from 36-54 hpf in 24-well plates with 1 µg/ml phenylhydrazine (PHZ, see *Online Supplementary Material*) and then washed them 4 times with fresh E3 medium to remove the compound. Depletion of mature erythrocytes was clearly visible by 2.5-3 dpf (Figure 2C, *Online Supplementary Video S1*). Moreover, the CHT region accumulated clumps of erythrocytes of irregular morphology, which were probably dying cells affected by the compound since time-lapse imaging showed they were phagocytized by large cells of macrophage morphology (*Online Supplementary Video S2,S3, Online Supplementary Figure S5*).

Interestingly, together with the depletion of mature, circulating erythrocytes, gata1-positive progenitors increased in the CHT at 3-4 dpf (Figure 2C,D) preceding the regeneration of mature erythrocytes (*Online Supplementary Figure S6*). Therefore, erythroid progenitors from the CHT appear responsible for the erythroid lineage regeneration after PHZ-induced hemolytic anemia in zebrafish. Indeed, time-lapse experiments revealed that gata1-positive cells divided and transformed into globin positive cells (*Online Supplementary Video S4 and S5 and Online Supplementary Figure S7*). Additionally, immunofluorescence experiments with anti-PH3 antibodies on double transgenic embryos *Tg(gata1:DsRed;globin:GFP)* showed increased numbers of proliferating cells in the CHT of PHZ treated embryos (*Online Supplementary Figure S6E*). Interestingly, the staining was strongest in cells that did not yet express gata1 or globin, suggesting that the regenerative response induces not yet specified progenitors or even stem cells to proliferate. In line, *in situ* hybridization experiments, performed as described,[15] showed a pronounced elevation of cmyb/runx expression in the CHT, suggesting the expansion of hematopoietic stem and early progenitor cells in response to hemolytic anemia (Figure 2D,D' and *Online Supplementary Figure S8B*). Double *in situ* with *gata1* and *cmyb* showed that some of the cmyb+ cells were also gata1 positive, suggesting their transformation into erythroid progenitors upon anemia induction (Figure 2D and *Online Supplementary Figure S8C*). Supporting the notion that erythroid regeneration arises from gata1-positive progenitors, imaging flow cytometry performed at 4 dpf showed that in the PHZ treated embryos, almost 40% of the gata+/globin+ cells had a progenitor type morphology, *versus* only 20% in the control (*Online Supplementary Figure S9F,G*). Finally, co-treatment of PHZ with the previously described anti-mitotic compounds affecting stem/progenitor cells (IRI, TOP) clearly impaired erythrocyte recovery. While treatment with PHZ alone resulted in ~40% increase in progenitor numbers, this was suppressed by co-supplementation with IRI (Figure 3). Quantification by flow cytometry further confirmed these results. Together these data show that irinotecan prevents the erythroid population regeneration after hemolytic anemia through its anti-mitotic activity on dividing early and erythroid progenitor cells.

In conclusion, we present a new zebrafish based tool for *in vivo* assessment of erythroid toxicity and regeneration that captures differences between effects on mature erythrocytes *versus* progenitor cells. This new multiplexed *in vivo* model may expand predictive and mechanistic preclinical erythroid safety testing.

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