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Purification of zebrafish erythrocytes as a means of identifying a novel regulator of haematopoiesis

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Summary

Zebrafish embryos are useful to study haematopoietic gene function in vertebrates, although lack of antibodies to zebrafish proteins has limited the purification of specific cell populations. Here, we purified primitive zebrafish erythrocytes using 1, 5-bis{[2-(di-methylamino)ethyl]amino}-4, 8-dihydroxyanthracene-9, 10-dione (DRAQ5™), a DNA-staining fluorescent dye. At 48-h post-fertilization, we sorted small-sized cells from embryos using forward scatter and found that they consisted of DRAQ5^{high} and DRAQ5^{low} populations. DRAQ5^{high} cells contained haemoglobin, lacked myeloperoxidase activity and expressed high levels of embryonic globin (*hbae3* and *hbbe1.1*) mRNA, all characteristics of primitive erythrocytes. Following DRAQ5™ analysis of

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Contribution: K.K., Tom.I. and Tor.I. performed experiments, analysed the data and wrote the manuscript; Y.N. and L.I.Z. designed the study and interpreted the data; and D.S. designed the study, interpreted the data and wrote the manuscript.

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The authors declare no competing financial interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

gata1:dsRed transgenic embryos, we purified primitive DRAQ5^{high} dsRed+ erythrocytes from haematopoietic progenitor cells. Using this method, we identified docking protein 2 (Dok2) as functioning in differentiation of primitive erythrocytes. We conclude that DRAQ5™-based flow cytometry enables purification of primitive zebrafish erythrocytes.

Keywords

zebrafish; primitive erythrocyte; DRAQ5™; flow cytometry

Zebrafish (*Danio rerio*) is a useful model to study vertebrate haematopoiesis, owing to highly conserved functions of haematopoiesis-related genes. Several zebrafish mutant strains relevant to erythropoiesis-related human diseases, such as sherocytosis (Liao *et al*, 2000), sideroblastic anaemia (Brownlie *et al*, 1998) and thalassemia-like disorder (Brownlie *et al*, 2003), have been generated. Adult transgenic zebrafish in which blood cells express particular oncogenes also serve as models of leukaemogenesis (Langenau *et al*, 2003) and leukaemia progression (Feng *et al*, 2007; Feng *et al*, 2010).

Zebrafish embryos and larvae are also useful models to study vertebrate haematopoiesis. Their small size and large number plus their extra-utero development allow high-throughput genetic screens that have identified numerous genes that function in erythropoiesis (Jing & Zon, 2011), some of which were later confirmed as underlying pathophysiology of human anaemia. Moreover, in zebrafish embryos, genome editing techniques, including TALEN (Transcription activator-like effector nucleases) and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) (Xiao *et al*, 2013), are currently being applied to genetic screens of erythropoiesis (Chung *et al*, 2015) and macrophage formation (Shiau *et al*, 2015) as well as the establishment of human blood disease models (Lin *et al*, 2016).

Flow cytometry allows quantitative analysis and isolation of human and mouse erythroid cells due to availability of fluorescence-labelled, erythrocyte-specific antibodies. However, there is a limited number of antibodies available that specifically bind membrane proteins in zebrafish erythrocytes. A previous report shows that flow cytometry can separate erythrocytes from the kidney tissue of adult zebrafish based on light scatter characteristics (Traver *et al*, 2003). However, its application in zebrafish embryos has not been investigated. Besides antibodies, several DNA-binding fluorochromes are available for flow cytometry. One such fluorochrome, the red fluorescing agent, 1, 5-bis{[2-(di-methylamino) ethyl]amino}-4, 8-dihydroxyanthracene-9, 10-dione (DRAQ5™), is a synthetic anthraquinone (Smith *et al*, 2000) that rapidly enters living cells and specifically binds DNA with high affinity. DRAQ5™ fluorescence intensity depends on DNA content and chromatin complexity, which affects accessibility to DRAQ5™ (Smith *et al*, 1999). To expand application of flow cytometry for study of zebrafish erythropoiesis, we have developed an antibody-free DNA staining-based approach to purify zebrafish primitive erythrocytes. Here, we applied it to the identification of docking protein 2 (Dok2) as a novel regulator of zebrafish erythropoiesis.

Methods

Fish maintenance

Adult zebrafish (*Danio rerio*, India strain) were housed in tanks of recirculated dechlorinated tap water at 26·5°C (water temperature). The transgenic line *Tg*(*gata1:dsRed*) (Traver *et al*, 2003) and *Tg*(*myb:GFP*) (North *et al*, 2007) was obtained from the Aquatic Research Program, Boston Children's Hospital, MA, USA. Animal care was performed in accordance with institutional and national guidelines and regulations. The study protocol was approved by the institutional review board of the Medical Institute of Bioregulation, Kyushu University.

Cell preparation

Figure 1A shows a diagram of cell preparation from 48-h post-fertilization (hpf) embryos. Embryos were washed three times with 0·9 × phosphate-buffered saline (PBS) and allowed to settle by gravity after each wash. After removing 0·9 × PBS, the embryos were immersed in 0·9 × PBS containing 2% fetal bovine serum (FBS), crushed with the plunger of 1-ml syringe and passed through a 30-μm nylon filter (Falcon® Cell Strainer, Corning, Durham, NC, USA). The resulting cells were washed three times with 0·9 × PBS containing 2% FBS. Cells were pelleted by centrifugation at 200 × g for 5 min at room temperature and then re-suspended in 0·9 × PBS containing 2% FBS. Cells were stained with trypan blue, and living cells were counted using a haematocytometer.

DRAQ5™ staining and flow cytometric analysis

DRAQ5™ staining was undertaken using the manufacturer's instruction (BioStatus Ltd., Loughborough, UK). Briefly, 1×10^5 cells were suspended in 1 ml of 5 μmol/l DRAQ5™ in 0·9 × PBS containing 2% FBS and kept in the dark for 15 min at room temperature. Cells were pelleted by centrifugation at 200 × g for 5 min at room temperature and analysed by flow cytometry without washing. Dead cells were excluded by incubation with TO-PRO®-1 iodide (Invitrogen, Eugene, OR, USA) in the case of both wild-type and *Tg*(*gata1:dsRed*) lines or SYTOX® Blue (Invitrogen) in the case of the *Tg*(*myb:GFP*) line. A filter of Per-Cp-Cy5 was set to detect the emitted fluorescent signal of DRAQ5™. Cells were sorted into RNAlater® (Life Technologies, Carlsbad, CA, USA) for gene expression analysis and into 0·9 × PBS containing 2% FBS for morphological analysis. Flow cytometry and cell sorting were performed using a BD FACSAria (BD Bioscience, San Jose, CA, USA).

May-Grünwald Giemsa staining

Sorted cells were attached onto glass slides (SUPERFROST®, Matsunami Glass Ind., Ltd., Osaka, Japan) using a CytoSpin™4 Cytocentrifuge (Thermo Fisher Scientific, Waltham, MA, USA) at 23 × g for 7 min and then rapidly air-dried. Cells were fixed and stained using May-Grünwald reagent (Muto Pure Chemicals, Tokyo, Japan) for 5 min at room temperature. After a brief wash in running tap water, cells were incubated with phosphate buffer (pH = 6·4) for 2 min and stained with 1:18 diluted Giemsa solution (Muto Pure Chemicals) at room temperature for 30 min. After one wash with running tap water, slides were air-dried and covered with glass coverslips (Matsunami Glass Ind., Ltd., Osaka, Japan)

with a drop of MGK-S mounting solution (Matsunami Glass Ind., Ltd.). Cell morphology was assessed using an Olympus CKX41 microscope (Olympus, Tokyo, Japan).

Haemoglobin staining with o-dianisidine

Sorted cells were attached to glass slides as described above. Cells were incubated with o-dianisidine solution (0·62 mg/ml of o-dianisidine in 0·65% H_2O_2, 43% ethanol in 10 mmol/l sodium acetate) for 10 min at room temperature in a moisture chamber. After 3 washes in tap water, cells were counterstained in Mayer's Haematoxylin solution (Muto Pure Chemicals) at room temperature for 10 min. After washing slides with running tap water, cells were air-dried and covered with glass coverslips with a drop of MGK-S mounting solution (Matsunami Glass Ind., Ltd.). Cell morphology was assessed as described above.

Analysis of myeloperoxidase activity

Myeloperoxidase activity was assessed following the manufacturer's instructions (Sigma-Aldrich, St Louis, MO, USA). Briefly, sorted cells were attached onto glass slides (Matsunami Glass Ind., Ltd.) using a CytoSpin™4 Cytocentrifuge (Thermo Fisher Scientific) at 23 × g for 7 min and then rapidly air-dried. Cells attached to glass slides were fixed at room temperature for 30 s in 3·7% formaldehyde in 85·5% ethanol. After washing in running tap water and air-drying, glass slides were placed in freshly prepared peroxidase indicator reagent (Sigma-Aldrich) containing 0·01% H_2O_2 at 37°C for 30 min in the dark. After washing with running tap water and air-drying, cells were counterstained in acid haematoxylin solution at room temperature for 10 min. Cells on slides were then washed with running tap water, air-dried and covered with glass coverslips with a drop of MGK-S mounting solution (Matsunami Glass Ind., Ltd.). Cell morphology was assessed as described above.

RNA extraction and real-time polymerase chain reaction (PCR)

Total RNA was extracted from sorted cells using an RNA-queous®4PCR Kit (Ambion, Austin, TX), and cDNA was prepared using a High-Capacity RNA-to-cDNA Kit (Life Technologies). Expression of *runx1, myb, gata2a, ptprc* (*cd45*), *spi1b* (*pu1*), *gata1, klf1* (*klfd*), *hbbe3 hbae1, hbbe1.1, hbaa1* and *ba1* was assessed by StepOnePlus™ real-time PCR (Life Technologies) with a SYBR Green Gene Expression System (Life Technologies). Primer sets were designed using Primer3 and validated for specific amplification using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). mRNA levels were normalized to *eukaryotic translation elongation factor 1 alpha 1, like 1* (*eef1a1l1*) mRNA (Tang *et al*, 2007), and relative transcript levels were calculated using a relative standard curve method.

PCR-amplification of zebrafish *dok2* cDNA and RNA preparation

dok2 cDNA was prepared from haematopoietic cells isolated from adult zebrafish kidney marrow using PCR. Primers were designed based on a cDNA reference sequence (GenBank accession number XM_001341478.7) and using software Primer Express® version 3.0 (Applied Biosystems, Life Technologies). Primer sequences used for *dok2* amplification were: 5′-tttgaattcatggaggaagacattc-3′ and 5′-tttctcgagttcttttgcattacttttac-3′. The amplicon was cloned into the pCS2P+ vector (kindly provided by Dr. Ishitani Tohru, Kyushu

University), and the sequence of cloned cDNA was verified by DNA sequencing. The *dok2*-pCS2P+ vector was then linearized with XhoI endonuclease and subjected to RNA synthesis using an mMESSAGE mMACHINE kit (Ambion, Life Technologies, Vilnius, Lithuania). Synthesized RNA was purified using an RNeasy® Plus Micro kit (Qiagen, Hilden, Germany) prior to injection.

Morpholino and RNA injection

Morpholino antisense oligonucleotides (MO) against *dok2* were designed and obtained from Gene Tools (Philomath, OR, USA). The MO sequence was 5′-gcatgttcgccttaccttcccgaac-3′, which binds to the exon 1/intron 1 junction. Oligonucleotides (2·5 ng) were injected into embryos at the one- or two-cell stage using a GD-1 glass capillary needle (Narishige, Tokyo, Japan) and a nitrogen gas-pressurized injection system (Narishige). A random control oligo 25-N obtained from Gene Tools served as a negative control. For rescue experiments, a mixture of the *dok2* MO plus *dok2* RNA was injected at a ratio (MO:RNA) of 2·5 ng:15 pg. After injection, total RNA was extracted from MO-injected embryos at 24 hpf and subjected to cDNA synthesis. *dok2* expression was assessed by real-time PCR as described above. Primers used for real-time PCR were 5′-at ggaggaagacattcgaaagaag-3′ and 5′-tgcggtttgctttctccagcgt-3′, which bind *dok2* at exons 1 and 2, respectively.

In vitro differentiation assay

Kidney stromal cells were prepared following a previous report (Bertrand *et al*, 2007). Briefly, kidney was dissected out from adult zebrafish and bleached in 0·000525% sodium hypochlorite. After washing with 0·9 × PBS, kidney was gently crushed with the plunger of 1-ml syringe. To remove haematopoietic cells, the dissociated cells were passed through a 40-μm nylon filter (Falcon® Cell Strainer, Corning). The remaining cells were cultured in medium [50% L-15, 35% Dulbecco's modified Eagle medium (DMEM), 15% Ham's F-12 media, 10% FBS, 2% penicillin/streptomycin (Wako Pure Chemical Industries, Osaka, Japan), 1% L-glutamine, 150 mg/l sodium bicarbonate, and 1·5% HEPES] at 32°C, 5% CO_2. To prepare immature primitive erythroid cells (defined as Gata1+Myb+), adult *gata1:dsRed* Tg zebrafish were crossed with *myb:GFP* Tg zebrafish. Embryos having dsRed+ and GFP+ were selected. At 30 hpf, embryos were dissociated as described in the cell preparation section. *gata1*:dsRed+ *myb*:GFP+ cells were sorted out using flow cytometry and co-cultured with kidney stromal cells for 2 days. The cultured floating cells were analysed for expression of *gata1*:dsRed and *myb*:GFP using flow cytometry.

Results

Flow cytometric profile of cells prepared from 48-hpf zebrafish embryos

In zebrafish embryos, blood circulation is established by 24 hpf, and the number of erythrocytes increases after 48 hpf. Thus, we performed flow cytometric analysis of cells prepared from 48-hpf zebrafish embryos and separated cells into four fractions based on forward scatter (FSC) using a blue laser. The designations F1, F2, F3 and F4 were assigned to cells having the lowest, low, intermediate and highest FSC (Figure S1A). The F1 fraction contained cell debris and melanin-containing granules, while F2 contained erythrocyte-like cells, which were round and exhibited a small, condensed nucleus. In F2 we also observed

round cells having high nuclear/cytoplasmic ratio, which is characteristic of blast cells. F3 and F4 fractions contained mixtures of cells size larger than cells in F2. Some erythrocyte-like cells were observed in F4, but they were larger than erythrocyte-like cells in F2 (Figure S1B).

To confirm the presence of erythroid cells in the 4 fractions, we conducted the same analysis using *gata1*:dsRed transgenic zebrafish, in which erythroid cells are marked by dsRed (Figure S1C). We observed dsRed+ erythroid cells in the F2 (7·6 ± 1·8%, $n = 4$) and F3 (2·3 ± 1·4%, $n = 4$) fractions (Figure S1D). The number of dsRed+ erythroid cells in the F2 fraction was 3·3-fold higher than that seen in the F3 fraction. Therefore, the rest of our analysis focuses on cells of the F2 fraction.

The DRAQ5^{high} sub-fraction exhibits erythroid, but not myeloid, characteristics

Because DRAQ5™ fluorochrome intensity depends on the accessibility of DRAQ5™ to genomic DNA (Smith *et al*, 1999), we employed DRAQ5™ to sub-fractionate F2 based on intensity and observed two sub-fractions: (i) low intensity DRAQ5 cells (DRAQ5^{low}, 11·8 ± 6·1%, $n = 3$) and (ii) high intensity DRAQ5 cells (DRAQ5^{high}, 4·6 ± 2·0%, $n = 3$) (Fig 1B, C). DRAQ5-negative cells were seen in debris and excluded from analysis. May-Giemsa staining revealed that the DRAQ5^{low} sub-fraction contained round cells with a high nuclear/cytoplasmic ratio, a characteristic of immature blood cells, whereas the DRAQ5^{high} sub-fraction contained round cells with a small and condensed nucleus (Fig 1D).

In zebrafish embryos, primitive erythroid cells, erythroid-myeloid progenitors (EMPs) and primitive myeloid cells (macrophage and neutrophils) are present by 48 hpf (Bennett *et al*, 2001; Burns *et al*, 2002; Kalev-Zylinska *et al*, 2002; Berman *et al*, 2005; Bertrand *et al*, 2007; Chen & Zon, 2009). Moreover, staining for haemoglobin as well as assessment of myeloperoxidase activity as a marker of myeloid cells can distinguish erythroid cells from other cell types. Only the DRAQ5^{high} sub-fraction contained haemoglobin-expressing cells, and no cell in either the DRAQ5^{low} or DRAQ5^{high} sub-fraction exhibited myeloperoxidase activity compared to myelomonocytic cells prepared from kidney of adult zebrafish (Fig 1D). We conclude that the DRAQ5^{high} sub-fraction of F2 possesses erythroid, but not myeloid, characteristics.

DRAQ5^{high} cells express high levels of erythroid-specific transcripts

After 24 hpf, zebrafish embryonic erythroid cells express the erythroid transcription factors *gata1* and *klf1*, which are orthologues of human *GATA1* and *KLF1*, respectively. Embryonic α-globin (*hbae3*) and β-globin (*hbbe1.1*) mRNAs are reportedly detected at 48 hpf (Ganis *et al*, 2012). PCR analysis of the DRAQ5^{low} sub-fraction revealed expression of haematopoietic progenitor-regulated transcription factors *myb* and *gata2a* (Fig 2A) and myeloid progenitor-specific *spi1b* and *ptprc* genes (Fig 2B) at levels higher than in the DRAQ5^{high} sub-fraction. By contrast, the DRAQ5^{high} fraction showed *gata1* and *klf1* expression at levels higher than the DRAQ5^{low} sub-fraction (Fig 2C). Respective levels of *hbae3* and *hbbe1.1* mRNAs in the DRAQ5^{high} sub-fraction were approximately 20 000- and 10 000-fold higher than those in the DRAQ5^{low} sub-fraction. These data confirm that DRAQ5^{high} cells are primarily comprised of primitive erythroid cells.

Purification of erythroid cells using DRAQ5™ and *gata1:dsRed* transgenic zebrafish

Human GATA1 is an erythroid-specific transcription factor regulating erythropoiesis and haemoglobin synthesis. We employed transgenic zebrafish, in which dsRed is expressed under control of the promoter of *gata1a*, an orthologue of human *GATA1*. The DRAQ5^{high} sub-fraction contained dsRed+ erythroid cells (67·7 ± 19·3%) at levels 75-times higher than the DRAQ5^{low} sub-fraction (0·9 ± 0·4%) (Fig 3A). Morphological analysis showed that DRAQ5^{high} *gata1*:dsRed+ cells were round with small and condensed nuclei, while DRAQ5^{high} *gata1*:dsRed− cells exhibited larger nuclei with apparently more loosely compacted content and scant cytoplasm. By contrast, DRAQ5^{low} *gata1*:dsRed+ cells were heterogeneous: some showed a high nucleus/cytoplasm ratio with dark blue cytoplasm, which is indicative of early haematopoietic progenitors (Bertrand *et al*, 2007), and others exhibited a small, kidney-shaped nucleus with pink-orange cytoplasm (Fig 3B). Moreover, o-dianisidine staining revealed haemoglobin in DRAQ5^{high} *gata1*:dsRed+ but not in DRAQ5^{low} *gata1*:dsRed+ cells, indicating that the latter were relatively more mature than DRAQ5^{low} *gata1*:dsRed+ erythroid cells (Fig 3C). In accord with morphological observations and haemoglobin staining, DRAQ5^{high} *gata1*:dsRed+ cells expressed *hbae3* and *hbbe1.1* mRNAs at respective levels 10 000- and 4000-times higher than DRAQ5^{low} *gata1*:DsRed+ cells (Fig 3D).

By contrast, DRAQ5^{low} *gata1*:dsRed+ cells expressed higher levels of mRNAs encoding the myeloid-specific transcription factor Spi1b than did DRAQ5^{high} *gata1*:dsRed+ cells. Co-expression of *gata1*:dsRed and *spi1b* mRNA suggests that DRAQ5^{low} *gata1*:dsRed+ cells contain EMPs and/or haematopoietic progenitor cells. To confirm this, we first assessed expression level of *lmo2* mRNA, which was reportedly expressed in EMPs (Bertrand *et al*, 2007). DRAQ5^{high} *gata1*:dsRed+ cells expressed *lmo2* mRNAs at respective levels 3·3-times higher than DRAQ5^{low} *gata1*:dsRed+ cells (Fig 3d). Secondly, we utilized *myb*:GFP transgenic embryos, in which Myb-expressing haematopoietic progenitor cells are GFP+ (Thompson *et al*, 1998; Bertrand *et al*, 2008). The DRAQ5^{low} fraction contained 14·7 ± 0±92% GFP+ cells, whereas the DRAQ5^{high} fraction contained 2±3 ± 0±28% GFP+ cells. The presence of *myb*:GFP+ progenitors in the DRAQ5^{high} fraction was consistent with our morphological observation of round cells with a high nuclear/cytoplasmic ratio (Fig 3B).

Reportedly, primitive erythroid cells (Thompson *et al*, 1998), EMPs (Bertrand *et al*, 2008) and non-EMP haematopoietic progenitor cells (Bertrand *et al*, 2008) also express Myb. To further characterize the DRAQ5^{high} *myb*:GFP+ cells, we prepared single cell suspension of 48-hpf *gata1:dsRed;myb:GFP* Tg embryos by crossing adult *gata1:dsRed* Tg with *myb:GFP* Tg zebrafish and analysed the cells by flow cytometry. The DRAQ5^{high} *myb*:GFP+ fraction contained 89·9 ± 8·6% of DsRed+ cells, suggesting that this fraction mainly contained primitive erythroid cells and EMP, which is consistent with high expression levels of embryonic globin and *lmo2* genes (Fig 3D). The DRAQ5^{low} *myb*:GFP+ fraction contained 0·14 ± 0·12% of DsRed+ cells (n = 3), also implying that *myb*:GFP+ cells in the DRAQ5^{low} fraction contain non-EMP haematopoietic progenitor cells. Overall, analysis of *gata1*:dsRed transgenic zebrafish with DRAQ5™ enabled us to distinguish primitive erythroid cells and EMPs from non-EMP haematopoietic progenitor cells by flow cytometry.

Use of DRAQ5™ to identify genes functioning in erythropoiesis

We previously employed global gene transcription analysis to show that treatment of mouse bone marrow-derived haematopoietic cells with Ninjin'yeito, a herbal medicine known as Kampo, accelerated myelopoiesis and suppressed erythropoiesis *in vitro*. In that study, expression of *Dok2* mRNA was suppressed by Ninjin'yeito treatment, suggesting that Dok2 positively regulates erythropoiesis (Inoue *et al*, 2014). Here, to confirm this possibility, we used morpholinos (MO) to knock down *dok2* mRNA to ~10% of levels detected in control MO-injected zebrafish at 24 hpf (Fig 4A). Staining of whole embryos for haemoglobin at 48 hpf showed decreased intensity of haemoglobin (red-orange in Fig 4B) in *dok2* MO-injected compared to control MO-injected embryos. When we applied DRAQ5™ plus flow cytometric analysis at 48 hpf, we observed a 2·7-fold decrease in the percentage of erythroid cells in the DRAQ5^{high} fraction ($P = 0·01$, Fig 4C, D). Although haemoglobin levels were unchanged (Fig 4E), embryonic β-*globin* (*hbbe1.1*) mRNA levels in *dok2* MO-injected embryos significantly decreased to 52% of that seen in control MO-injected embryos ($P = 0·0056$, Fig 4F). These phenotypes were rescued by co-injection of *dok2* RNA with the *dok2* MO (Fig 5A–E).

To investigate which stage of erythroid differentiation is altered by *dok2* knockdown, we injected *dok2* MO into *gata1:dsRed* transgenic embryos and analysed them by flow cytometry. We observed a 3-fold decrease in the percentage of *gata1*:dsRed+ erythroid cells in *dok2* MO-injected embryos relative to control MO-injected embryos (Fig 4G). When we analysed *gata1*:dsRed+ cells based on DRAQ5™ intensity, the percentage of haematopoietic progenitor cells (defined as DRAQ5^{low} *gata1*:dsRed+ cells) slightly increased (Fig 4H). By contrast, the percentage of primitive erythrocytes (defined as DRAQ5^{high} *gata1*:dsRed+ cells) decreased 3·3-fold (Fig 4H), suggesting inhibition of differentiation. These phenotypes were rescued by co-injection of *dok2* RNA with the *dok2* MO (Fig 5F–G).

To confirm that Dok2 impairs differentiation of primitive erythrocytes, we isolated *gata1*:dsRed+ *myb*:GFP+ cells and performed *in vitro* differentiation on adult kidney stromal cells (Fig 6A). We observed a 2-fold decrease in the percentage of *gata1*:dsRed+ *myb*:GFP− primitive erythrocyte ($P = 0·024$) and a 1·75-fold increase in the percentage of *gata1*:dsRed+ *myb*:GFP+ primitive erythroid cells and EMPs ($P = 0·024$) in *dok2* MO-injected relative to control MO-injected embryos (Fig 6B, C). There was no difference in cell morphology and haemoglobin levels (Fig 6D), whereas embryonic β-*globin* (*hbbe1.1*) mRNA level in *dok2* MO-injected embryos decreased to 53% of that seen in control MO-injected embryos ($P = 0·002$, Fig 6E). These phenotypes were rescued by co-injection of *dok2* RNA with the *dok2* MO (Fig 6B–D). These findings suggest that Dok2 is required for differentiation of primitive erythrocytes.

Discussion

Flow cytometry has been utilized to separate cells of different blood lineages in kidney marrow of adult zebrafish. Based on light-scatter characteristics, adult zebrafish erythrocytes can be distinguished from other blood lineages (Traver *et al*, 2003). Here, we developed a way to purify primitive mature erythrocytes from zebrafish embryos using flow cytometry. Although *gata1:dsRed* transgenic zebrafish enable isolation of Gata1-expressing erythroid

cells by flow cytometry, our morphological observations revealed that dsRed+ cells are heterogeneous and are comprised of EMP-like cells and primitive mature erythrocytes (Fig 3B). Combining DRAQ5™ analysis with *gata1:dsRed* transgenic zebrafish, we defined DRAQ5^{low} *gata1*:dsRed+ cells as EMP-like cells and DRAQ5^{high} *gata1*: dsRed+ cells as primitive mature erythrocytes.

By 48 hpf, we were able to observe primitive erythrocytes, EMPs and haematopoietic progenitor cells but not myeloid cells, findings compatible with several previous reports (Berman *et al*, 2005; Brownlie *et al*, 2003; Chen & Zon, 2009; Bennett *et al*, 2001; Bertrand *et al*, 2007; Burns *et al*, 2002; Kalev-Zylinska *et al*, 2002). At 12 hpf, *gata1*-expressing erythroid progenitors differentiate into primitive erythrocytes that enter the circulation around 24 hpf (Berman *et al*, 2005) and persist until 96 hpf (Chen & Zon, 2009). Primitive erythrocytes express embryonic globin genes (*hbae3* and *hbbe1.1*) (Brownlie *et al*, 2003). Around 24 hpf, bipotent EMPs are generated and differentiate into *gata1*$^{+}$ erythroid (Bennett *et al*, 2001) and *spi1*$^{+}$ myeloid cells (Bertrand *et al*, 2007). Haematopoietic progenitor cells are generated from dorsal aorta at 33 hpf (Burns *et al*, 2002; Kalev-Zylinska *et al*, 2002). However, definitive myelopoiesis starts around 72 hpf in the caudal haematopoietic tissue, as marked by *lcp1* expression (Berman *et al*, 2005). Overall, these findings strongly suggest that our approach is a reliable method to purify and analyse haematopoietic cells in embryonic zebrafish.

Analysis of erythropoiesis in zebrafish embryos was facilitated by use of the DNA-staining fluorochrome DRAQ5™. There are several advantages of DRAQ5™ compared to other DNA-staining fluorochromes. First, it specifically binds at high affinity to DNA, eliminating background signals from RNA and enabling co-staining with RNA-binding fluorochromes. Second, DRAQ5™ can rapidly enter living cells at ambient temperature (Smith *et al*, 1999) and its application does not require cell fixation or RNase treatment. Thus, damage to zebrafish cells exposed to 37°C temperature is minimized. Moreover, the DRAQ5™ fluorescence emission spectrum does not overlap with spectra of phycoerythrin, Texas Red, Cy3, or GFP. Thus, DRAQ5™ signals can be detected in transgenic zebrafish expressing other fluorescent markers with minimal overlap in spectrum. Importantly, DRAQ5™ undergoes minimal photobleaching (Martin *et al*, 2005) and is thus stable over long periods of analysis. Overall, we conclude that DRAQ5™ is probably preferable for flow cytometry over other DNA-staining fluorochromes, such as propidium iodide, TOTO™-1, TOTO™-3, Hoechst33258, ethidium bromide and 4′,6-diamidino-2-phenylindole (DAPI).

DRAQ5™ has reportedly been applied to analysis of human peripheral blood (Smith *et al*, 1999). Enhanced staining potential of DRAQ5™ in monocytes and lymphocytes is related to the accessibility of DRAQ5™ to DNA binding sites in chromatin (Smith *et al*, 1999). Thus, our observation of DRAQ5^{high} and DRAQ5^{low} populations probably reflects differential chromatin complexity in those groups, although further characterization of nuclei of zebrafish erythroid cells is required.

We also demonstrated the utility of this staining method to analyse gene function in erythropoiesis. Dok2 is an adaptor protein that binds to the intracellular domain of a transmembrane receptor protein tyrosine kinase and inhibits signalling. *Dok2* knockout mice

show increased incidence of lung adenocarcinoma (Berger *et al*, 2010) but normal haematopoiesis. *Dok1/Dok2* double knockout mice develop myeloproliferative disease but do not exhibit abnormalities in erythropoiesis (Niki *et al*, 2004). DOK1 and DOK2 reportedly function as inhibitors of signalling pathways in lymphopoiesis (Celis-Gutierrez *et al*, 2014; Guittard *et al*, 2009) and myelopoiesis (Mihrshahi *et al*, 2009; Yasuda *et al*, 2004). However, the function of DOK2 in erythropoiesis remains unknown. Our data suggest that in zebrafish embryos, Dok2 regulates differentiation of primitive erythrocytes.

Conclusions

Zebrafish primitive erythroid cells can be isolated using DRAQ5™ staining of *gata1:dsRed* transgenic zebrafish and flow cytometry. Using this method, we identified Dok2 as functioning in the differentiation of primitive erythrocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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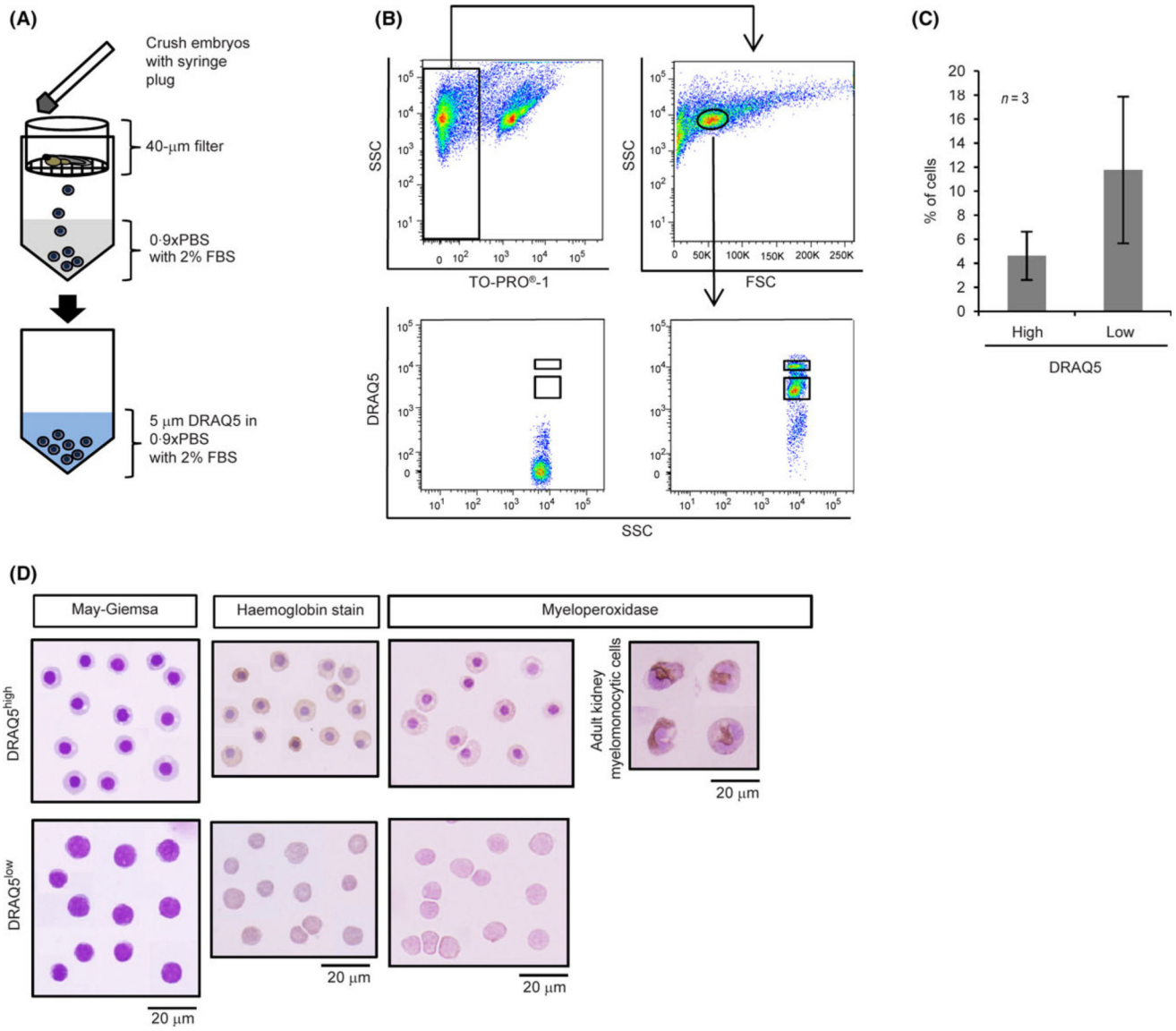

Fig 1.

DRAQ5^{high} cells are erythroid-like cells containing haemoglobin. (A) Schematic diagram of preparation and staining of 48-h post-fertilization zebrafish cells. (B) Flow cytometric analysis of zebrafish embryonic cells. Dead cells were excluded using TOPRO-1. TOPRO-1 negative cells were then gated according to SSC and FSC. Cells were analysed for DRAQ5™ intensity in FSC^{low} and SSC^{low} fractions, and detected via PerCp-Cy5. DRAQ5^{high} and DRAQ5^{low} cells were gated. (C) Percentage of DRAQ5^{high} and DRAQ5^{low} obtained from three independent flow cytometric analyses and shown as mean±SD. (D) May-Giemsa staining, haemoglobin staining and myeloperoxidase activity of DRAQ5^{high} and DRAQ5^{low} cells. Scale bars: 20 μm for all panels. Orange-red in cytoplasm indicates haemoglobin. Nuclei are stained by haematoxylin and appear as grey. DRAQ5, 1, 5-bis{[2-(di-methylamino)ethyl]amino}-4, 8-dihydroxyanthracene-9, 10-dione; FBS, fetal bovine serum; FSC, forward scatter; PBS, phosphate-buffered saline; SSD, side scatter. [Colour figure can be viewed at wileyonlinelibrary.com]

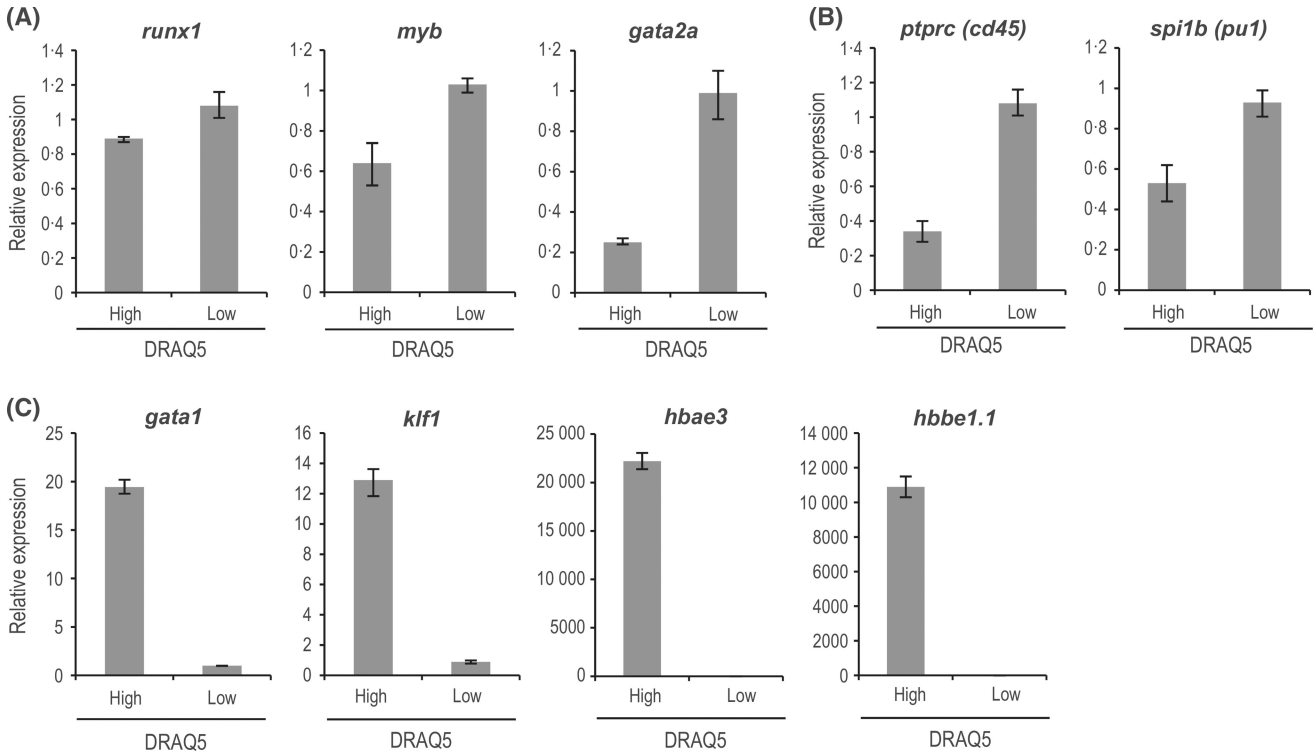

Fig 2.

DRAQ5^{high} cells express high levels of erythropoiesis-related transcripts. (A) Gene expression analysis of DRAQ5^{high} and DRAQ5^{low} cells obtained from 48-h post-fertilization embryos. Expression levels of haematopoietic stem and progenitor-related mRNAs. (B) Expression levels of myeloid-related mRNAs in DRAQ5^{high} and DRAQ5^{low} cells obtained from 48-hpf embryos. (C) Expression levels of erythroid-related mRNAs. DRAQ5, 1, 5-bis{[2-(di-methylamino)ethyl]amino}-4, 8-dihydroxyanthracene-9, 10-dione.

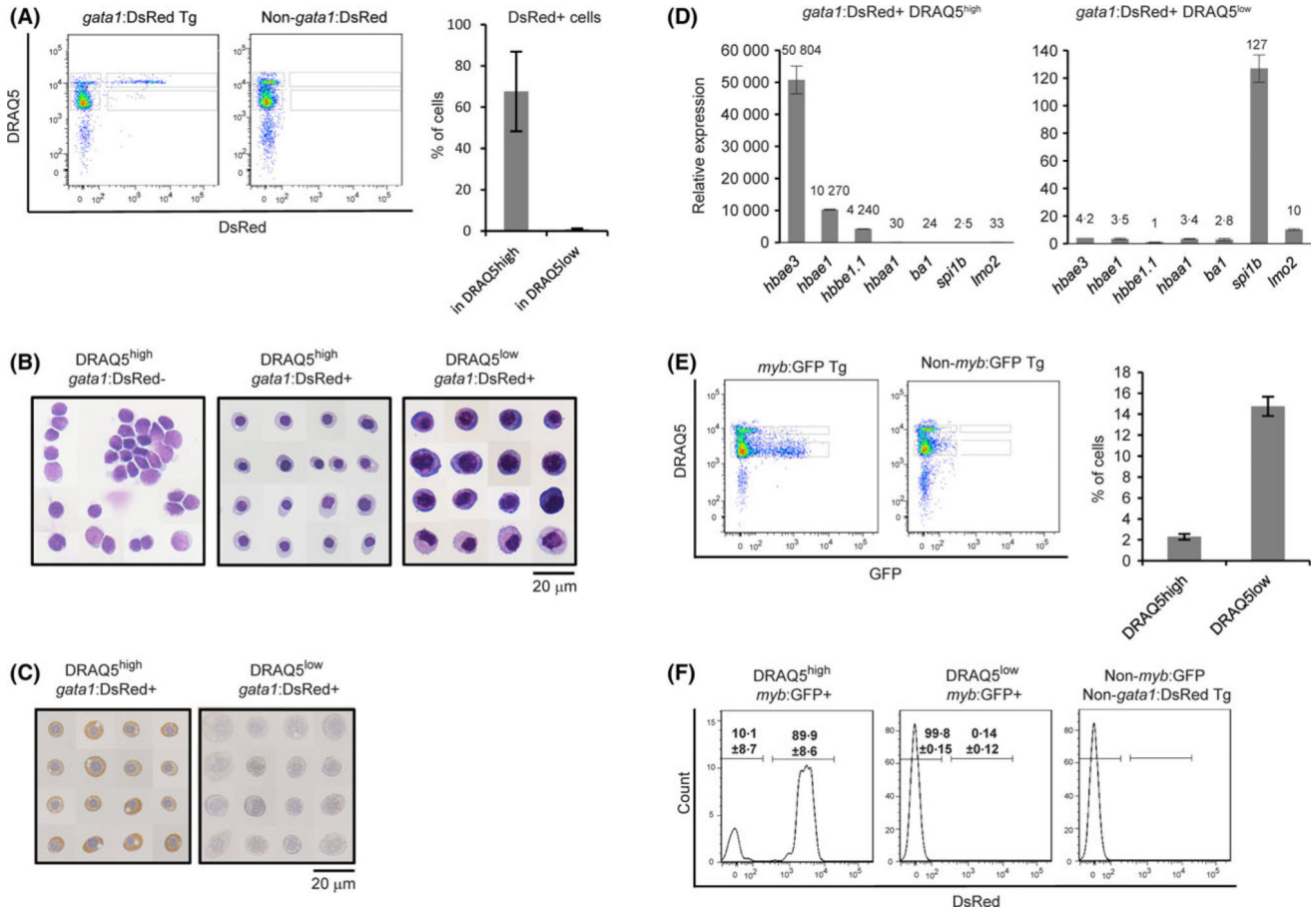

Fig 3.

DRAQ5^{high} *gata1*:dsRed+ cells are mature primitive erythroid cells. (A) The presence of *gata1*:dsRed+ erythroid cells in DRAQ5^{high} cells. Percentages of DRAQ5^{high} *gata1*:dsRed+ and DRAQ5^{low} *gata1*:dsRed+ erythroid cells obtained from three independent experiments. Data is shown as mean±SD. (B) May-Giemsa staining of DRAQ5^{high} *gata1*:dsRed−, DRAQ5^{high} *gata*1:dsRed+ and DRAQ5^{low} *gata1*:dsRed+ cells. (C) Haemoglobin staining of DRAQ5^{high} *gata*1:dsRed+ and DRAQ5^{low} *gata1*:dsRed cells. (D) Expression levels of erythroid and myeloid-related mRNAs in DRAQ5^{high} *gata*1:dsRed+ and DRAQ5^{low} *gata1*:dsRed cells. (E) Percentages of *myb*:GFP+ haematopoietic progenitor cells in DRAQ5^{high} and DRAQ5^{low} fractions were calculated from three independent experiments. (F) The presence of erythroid-myeloid progenitor cells was confirmed by analysing *gata1:dsRed; myb:GFP* transgenic zebrafish embryos. Data is shown as mean ± SD. DRAQ5, 1, 5-bis{[2-(di-methylamino)ethyl] amino}-4, 8-dihydroxyanthracene-9, 10-dione. [Colour figure can be viewed at wileyonlinelibrary.com]

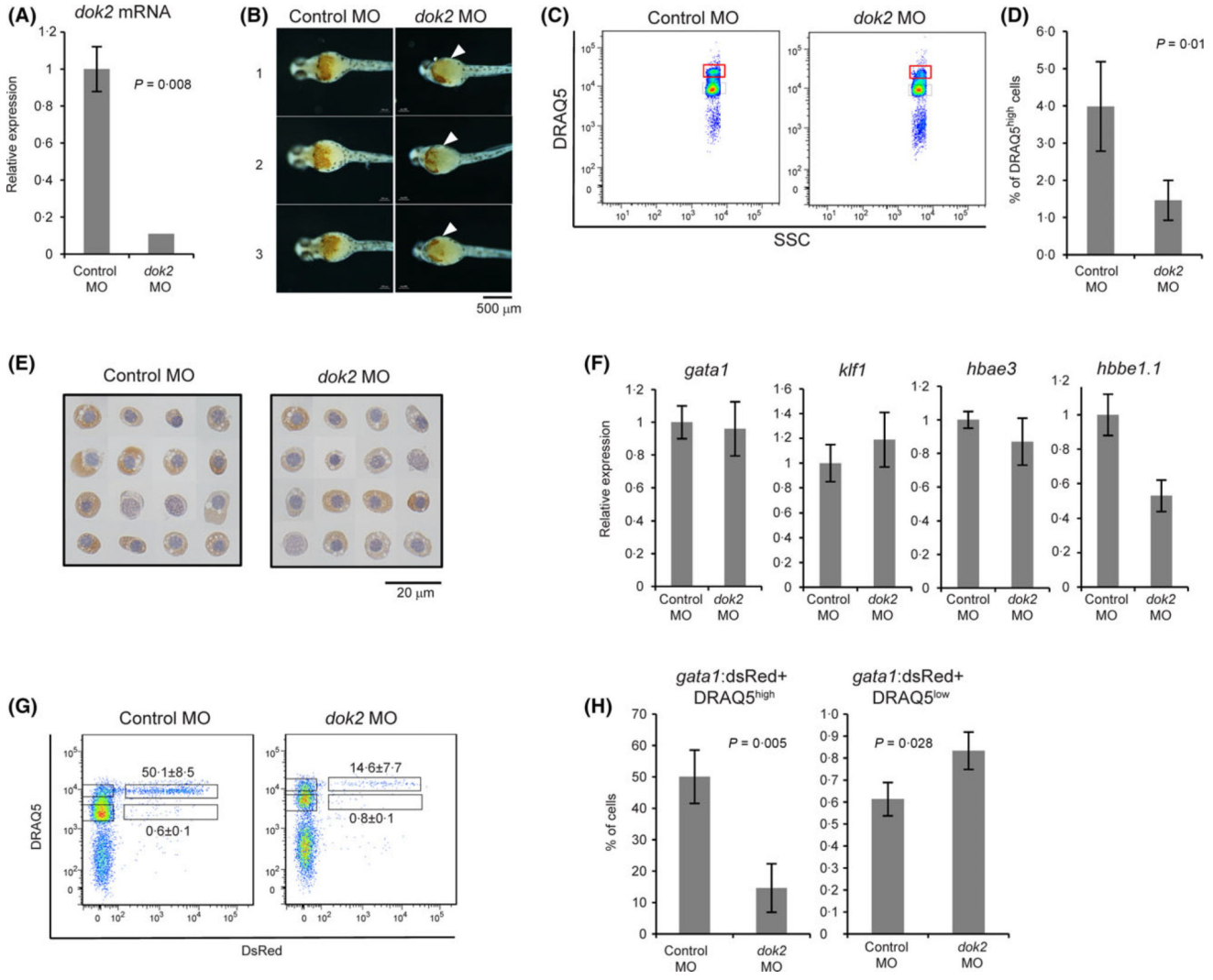

Fig 4.

Application of DRAQ5™-based flow cytometry to dok2 functional analysis. (A) Knockdown efficiency of *dok2* MO in 24-h post-fertilization (hpf) embryos. (B) Haemoglobin staining of *dok2* knockdown and control 48-hpf embryos. Haemoglobin-containing cells appear as orange-red. Arrowheads indicate areas where the number of haemoglobin-containing cells decreased after *dok2* knockdown. Representative images are obtained from three independent injections of MO and are shown in a ventral view. Scale bar: 500 μm. (C) Flow cytometric analysis of cells prepared from 48-hpf embryos. (D) Percentages of DRAQ5^{high} primitive erythroid-like cells per total cells prepared from 48-hpf embryos were calculated from three independent experiments. Data is shown as means ± SD. (E) Haemoglobin staining of DRAQ5^{high} erythroid-like cells prepared from 48-hpf embryos. Orange-red in cytoplasm indicates haemoglobin. Nuclei are stained by haematoxylin and appear as grey. Scale bar: 20 μm. (F) Expression of erythropoiesis-related genes in DRAQ5^{high} cells sorted from control MO and *dok2* MO-injected embryos. (G) Flow cytometric analysis of cells prepared from 48-hpf *gata1*:dsRed embryos. (H) Shown are percentages of *gata1*:dsRed+ DRAQ5^{low} hematopoietic progenitors in total DRAQ5^{low}

cells and *gata1*:dsRed+ DRAQ5^{high} primitive erythrocytes in DRAQ5^{high} cells. DRAQ5, 1, 5-bis{[2-(di-methylamino)ethyl]amino}-4, 8-dihydroxyanthracene-9, 10-dione; MO, morpholino antisense oligonucleotide; SSC, side scatter. [Colour figure can be viewed at wileyonlinelibrary.com]

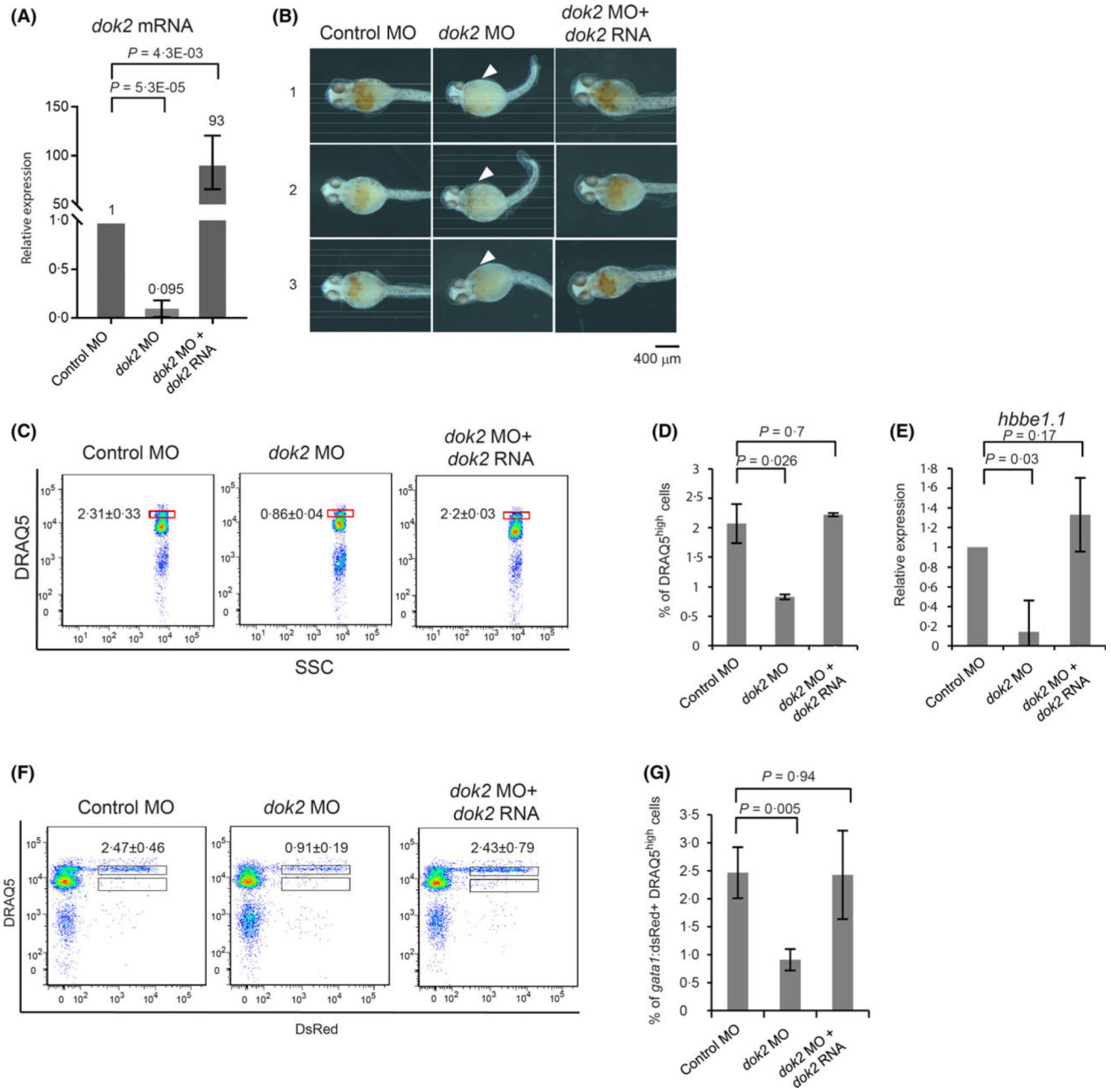

Fig 5.

Rescue of *dok2* knockdown phenotypes. (A) Levels of *dok2* in zebrafish embryos at 24 h post-fertilization (hpf) injected with both *dok2* MO and *dok2* RNA (rescue). Data is shown as means±SD of three independent experiments. (B) Haemoglobin staining of control, *dok2* knockdown and rescue embryos at 48 hpf. Haemoglobin-containing cells appear as orange-red. Arrowheads indicate areas where the number of haemoglobin-containing cells decreased after *dok2* knockdown. Shown are representative images of a ventral view. Scale bar: 400 μm. (C) Flow cytometric analysis of cells prepared from indicated 48-hpf zebrafish embryos. (D) Percentages of DRAQ5^{high} primitive erythroid-like cells per total cells prepared from 48-hpf embryos were calculated from three independent experiments. (E)

Expression of *hbbe1.1* in DRAQ5^{high} cells sorted from control MO, *dok2* MO and dok2 MO plus dok2 RNA (rescue)-injected embryos. (F) Flow cytometric analysis of cells prepared from 48-hpf *gata1*:dsRed embryos. (G) Shown are percentages of *gata1*:dsRed+ DRAQ5^{high} primitive erythrocytes in total cells prepared from 48-hpf embryos. Data were calculated from three independent experiments. DRAQ5, 1, 5-bis{[2-(di-methylamino)ethyl]amino}-4, 8-dihydroxyanthracene-9, 10-dione; MO, morpholino antisense oligonucleotide; SSC, side scatter. [Colour figure can be viewed at wileyonlinelibrary.com]

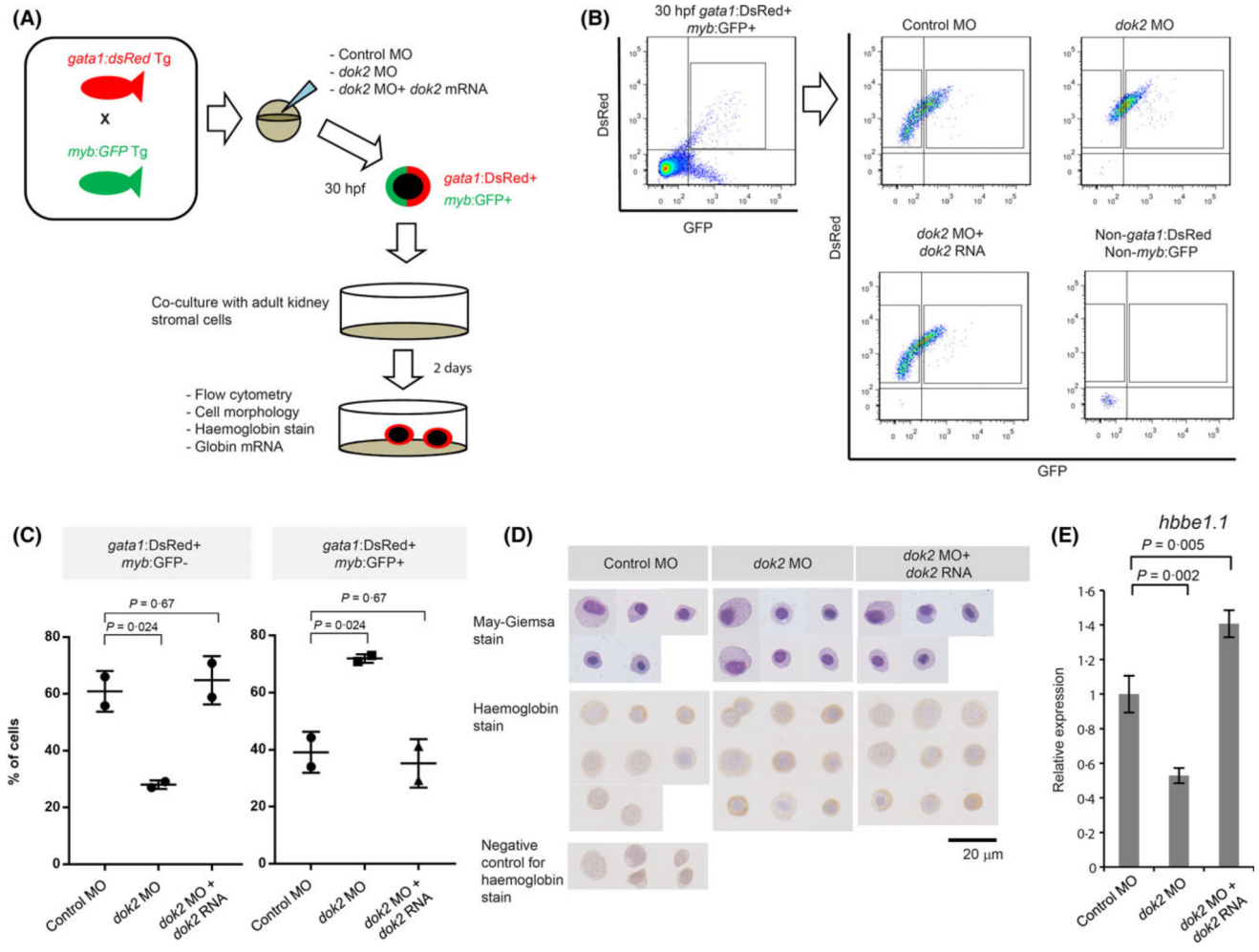

Fig 6.

In vitro functional analysis of dok2. (A) Diagram shows experimental design. Adult *gata1:dsRed* Tg fish were crossed with *myb:GFP* Tg fish. After injecting *dok2* MO, only DsRed+ GFP+ embryos were selected. Primitive erythroid cells and erythroid-myeloid progenitors (EMP, defined as *gata1*:dsRed+ *myb*:GFP+) were prepared from 30-hpf embryos and cultured on kidney stromal cells for 2 days. (B) Flow cytometric analysis of cultured floating cells. Shown are representative images of two independent experiments. (C) Shown are percentages of *gata1*:dsRed+ *myb*:GFP− cells and *gata1*:dsRed+ *myb*:GFP+ cells. Data is shown as mean ± SD calculated from two idenpedent experiments. (D) May-Giemsa and Haemoglobin staining of all cultured cells. Scale bar: 20 μm. (E) Expression of embryonic *hbbe1.1* globin gene in *gata1*:dsRed+ *myb*:GFP− cells sorted from 2-day cultured cells. GFP, green fluorescence protein; MO, morpholino antisense oligonucleotide. [Colour figure can be viewed at wileyonlinelibrary.com]