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Definitive erythropoiesis in the trunk of zebrafish embryos

In a recent paper in *Development*, Jin et al. (Jin et al., 2009) reported that, in zebrafish embryos, the definitive haematopoietic cells of the trunk and tail have different differentiation outputs; that is, whereas cells in the tail can give rise to erythrocytes, those remaining in the trunk cannot. This finding that definitive erythropoiesis is absent in the trunk depends entirely on the absence of a whole-mount in situ hybridisation (WISH) signal for a single erythroid marker (α*e1-globin*) in these cells. They then describe a series of elegant experiments to examine and to attempt to explain this differing fate. If correct, these findings would be interesting, although surprising.

The finding that no definitive erythropoiesis occurs in the trunk is puzzling, because it contradicts our previously published results (Zhang and Rodaway, 2007). In this paper, we clearly showed expression of the erythroid markers *gata1* and β*e1-globin*, as well as histochemical staining for haemoglobin, in haematopoietic clusters in the trunk of embryos at 4 days post-fertilisation (dpf). In light of the Jin et al. (Jin et al., 2009) study, we repeated our experiments and confirmed that β*e1-globin* is expressed in both trunk and tail haematopoietic cells (see Fig. S1A-C in the supplementary material), implying that both regions are erythropoietic. Detection of β*e1-globin* expression in the trunk required a longer incubation in chromogenic substrate than that in the tail, but was clearly specific. It is well known in the field that it is more difficult to detect gene expression in the trunk of older zebrafish embryos than in other tissues.

To avoid reliance on the expression of a single globin mRNA, we re-examined the emergence of definitive erythropoiesis by histochemistry for haemoglobin (see Fig. S1D-P in the supplementary material). At 2 dpf, staining is strong in circulating primitive erythrocytes and clear, but weaker, staining is seen in cells lying ventral to the dorsal aorta (DA) in the tail (see Fig. S1E in the supplementary material). These might be erythroblasts derived from the transient tail erythro-myeloid progenitors, as described by Bertrand et al. (Bertrand et al., 2007). In the trunk at this stage, no staining above background can be detected in the mesenchyme between the DA and the posterior cardinal vein (PCV). By 3 dpf, however, scattered haemoglobin-expressing cells are located in the mesenchyme between the major vessels in both trunk and tail. By 4 dpf, this expression has coalesced into clusters in the trunk (typically adjacent to somite boundaries) and into a more continuous strip of cells in the tail. In both trunk and tail, the erythroblasts are on the dorsal aspect of the major vein [PCV in trunk, caudal vein (CV) in tail; see Fig. S1J,K in the supplementary material]. This expression pattern is maintained at least as late as 7 dpf. At this age, erythropoiesis is also clearly detectable in the pronephros. Both trunk and tail haematopoietic tissues are, therefore, erythropoietic.

The presence of histochemically detectable haemoglobin in the trunk, along with the expression of β*e1-globin* mRNA, implied that at least one α-globin was likely to be expressed in this region. Although Jin et al. (Jin et al., 2009) reported that α*e1-globin* was not expressed in the trunk, we re-tested the expression of this gene by WISH. We detected expression in mesenchyme overlying the cardinal vein both in the tail and (after longer staining) in the trunk (see Fig. S1Q-T in the supplementary material). There are two possible reasons for the discrepancy between our data regarding α*e1-globin* expression and that of Jin et al. (Jin et al., 2009). Firstly, WISH detection of gene expression in the trunk of older embryos requires careful optimisation of proteinase K treatment. However, the fact that Jin et al. (Jin et al., 2009) were able to detect other mRNAs [for example, *L-plastin* (*lcp1* – Zebrafish Information Network) and *cmyb*] in this region makes this explanation less likely. The second possibility is that, when probing for α*e1-globin*, Jin et al. (Jin et al., 2009) stopped the chromogenic reaction once staining had appeared in the tail, and did not continue the reaction until trunk expression was detected.

The fact that it is difficult to detect mRNA in the trunk vessels and the haematopoietic clusters of older embryos by WISH is well known in the field. The difference in WISH sensitivity is likely to result from the fact that most of the posterior blood island (PBI) is covered only by a thin layer of epidermis, whereas the trunk clusters are surrounded by other tissues that might impede access of fixative and that, once fixed, can present a barrier to probe and/or antibody penetration. Our findings also show that although globin mRNA is much harder to detect by WISH in the trunk than in the tail, the amount of haemoglobin (assayed by rate of staining by peroxidase histochemistry) is similar in the two sites. Therefore, we believe that the difficulty of detecting mRNA in trunk haematopoietic clusters in older embryos is a limitation of the WISH technique, rather than a result of genuinely lower expression levels.

Finally, we tested whether the caudal haematopoietic tissue (CHT; also known as the posterior blood island, PBI) is necessary for definitive erythropoiesis. Primitive and definitive erythrocytes can be distinguished by their morphology: primitive erythrocytes are disc shaped, whereas definitive erythrocytes are rugby ball shaped (Belair et al., 2001). Primitive cells enter circulation early in the second day of development and form the majority of circulating erythrocytes as late as 5 dpf. Thereafter, they are lost from circulation (Weinstein et al., 1996) and by 7 dpf almost all the circulating erythrocytes have definitive morphology (Belair et al., 2001). In order to determine the requirement for the CHT/PBI, we surgically removed the tails of embryos either before (1 dpf, 30 embryos) or after (3.5 dpf, 30 embryos) the colonisation of the CHT/PBI by trunk-derived cells described previously by Jin et al. (Jin et al., 2007). We examined these embryos at 7 dpf and, although we cannot completely exclude an effect on total number, there were ample circulating erythrocytes. Significantly, the proportion of erythrocytes with definitive morphology (~95%) was indistinguishable from unoperated controls (see Fig. S1U-Z and Appendix S1 in the supplementary material; 5 embryos per treatment). Thus, although erythropoiesis clearly does occur in the CHT/PBI, it is not the sole site of definitive erythropoiesis, nor is it indispensable.

In summary, our findings show that haematopoietic cells in the trunk of zebrafish embryos: (1) express both α*e1-* and β*e1-globin* mRNA; (2) contain histochemically detectable haemoglobin; and (3) can give rise to definitive erythrocytes in the absence of the PBI/CHT. Thus, in contrast to the findings of Jin et al. (Jin et al., 2009), definitive erythropoiesis does occur in the trunk of zebrafish embryos.

Supplementary material

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RESPONSE

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Erythrocytes in the trunk of zebrafish embryos

In our previous study (Jin et al., 2009), we presented evidence indicating that, in zebrafish embryos, hematopoietic stem cells (HSCs) in the ventral wall of the dorsal aorta (VDA), where HSCs originate, and in the caudal hematopoietic tissue (CHT)/posterior blood island (PBI), where HSCs later home, possess distinct differentiation outputs. Using expression analysis and fate mapping, we showed that HSCs only give rise to myeloid cells and not to erythroid cells in the VDA, whereas HSCs differentiate into erythroid cells upon migrating to the CHT/PBI (Jin et al., 2009). By analyzing the differentiation of HSCs in embryos in which HSCs were trapped in the VDA, we further showed that the erythropoietic ability of developing HSCs is dependent on the CHT/PBI (Jin et al., 2009).

In their Correspondence (Falenta and Rodaway, 2011), Falenta and Rodaway have provided additional evidence to support their earlier finding (Zhang and Rodaway, 2007) and questioned the non-erythropoietic nature of the VDA because they have detected α*e1*- and β*e1-globin*-expressing cells (using α*e1*- and β*e1-globin* probes) and cells positive for hemoglobin peroxidase activity [using diaminofluorene (DAF) staining] in the trunk of zebrafish embryos. As suggested by Falenta and Rodaway, we stained 4 days post-fertilization (dpf) embryos for a longer time period of 24 hours (see Appendix S1 in the supplementary material), rather than 2 hours as in the original paper, by whole-mount in situ hybridization (WISH) to detect α*e1*- and β*e1-globin*. Whereas staining for 2 hours in our original study failed to detect α*e1*- and β*e1-globin* expression in the trunk, staining for 24 hours allowed the detection of α*e1*- and β*e1-globin* expression in the trunk of some 4 dpf embryos. However, these α*e1*- or β*e1-globin*-positive cells in the trunk were rare in comparison with the numbers of α*e1*- and β*e1-globin*-positive cells that were present in the CHT/PBI and a significant portion of 4 dpf embryos that were stained for 24 hours with α*e1*- or β*e1-globin* probes lacked staining in the trunk (see Table S1 in the supplementary material). Furthermore, 'per cell' staining intensity for α*e1*- and β*e1-globin* was much weaker in the trunk compared with that in CHT/PBI (see Fig. S1A,B in the supplementary material). By contrast, all the embryos stained with *lyz* or *mpx*, two myeloid markers, for a short period of 2 hours had detectable signals in the trunk (see Appendix S1 and Table S1 in the supplementary material) and 'per cell' staining intensity was comparable in the trunk and CHT/PBI (see Fig. S1C,D in the supplementary material). Because of the finding that staining intensity for α*e1*- and β*e1-globin* in the trunk was weaker than that observed in the CHT/PBI, whereas staining intensity for *lyz* and *mpx* was comparable in these two sites, we think that cells in the trunk might express genuinely low levels of α*e1*- and β*e1-globin*. Additionally, in these embryos stained for 24 hours, primitive erythrocytes were also weakly stained by α*e1-globin* and β*e1-globin* probes (see Fig. S1A,B in the supplementary material), which raises the possibility that the erythroid cells reported by Falenta and Rodaway to be present in the trunk are primitive erythrocytes. It is also possible that these rare erythrocytes come from the CHT/PBI and stop randomly in the blood vessel during the fixation step of WISH. Therefore, the nature and origin of these cells weakly positive for α*e1*- and β*e1-globin* in the trunk are not clearly addressed. Specifically, it is unknown whether these cells are derived from the differentiation of HSCs in the VDA, from primitive erythrocytes that carry residual globin transcripts, or from other sources. A lineage-tracing study would help to resolve the origin of these trunk erythrocytes in the future. Irrespective of the origin of trunk erythrocytes, embryos at 4 dpf, on average, contained many fewer α*e1*- and β*e1-globin*$^{+}$ clusters in the trunk than *lyz*$^{+}$ or *mpx*$^{+}$ clusters (see Table S1 in the supplementary material). Moreover, Falenta and Rodaway mentioned that there was no detectable hemoglobin expression between the DA and the posterior cardinal vein (PCV) at 2 dpf, which is consistent with our previous finding that myeloid cells but not erythroid cells are generated in situ at this time point in the VDA in our previous report (Jin et al., 2009). Altogether, the contention that HSCs in the VDA produce erythroid cells in situ is not well supported.

Falenta and Rodaway also raised concerns over the requirement of CHT/PBI for definitive erythropoiesis. They suggested that definitive erythrocytes could arise in the absence of CHT/PBI because the proportion of rugby ball-shaped 'definitive' erythrocytes was normal in the circulation of 7 dpf tail-transected embryos. Although the tail transection experiment is elegant, it suffers from two potential drawbacks. First, in embryos in which the CHT/PBI has been removed, other alternative definitive hematopoietic compartments might still function, such as the pronephros, which could compensate for the loss of the CHT/PBI. Second, the identification of rugby ball-shaped circulating cells as definitive erythrocytes is still questionable and is not generally accepted within the field, as these cells have not been shown to be lost from embryos that lack definitive hematopoiesis. Indeed, the high percentage (~94%) of definitive-like cells among the circulating erythrocytes of 7 dpf

wild-type embryos reported by Falenta and Rodaway conflicts with findings reported by Weinstein et al. that 75% of circulating cells are primitive erythrocytes at 7 dpf, and that at 10 dpf, 50% of circulating cells remain as primitive cells (Weinstein et al., 1996). Given such ambiguity, we revisited the identity of these rugby ball-shaped 'definitive' cells in this study. If these cells are of definitive origin, we anticipate that they would be absent or greatly reduced in *runx1* mutant embryos (*runx1*^{w84x}), in which definitive hematopoiesis is reported to be blocked at an early stage (Jin et al., 2009; Sood et al., 2010). All *runx1*^{w84x} mutants lack HSC- and lineage-specific markers in the AGM, CHT and thymus during early definitive hematopoiesis and, consequently, all *runx1*^{w84x} mutants become bloodless after 11-12 dpf, although 20% of *runx1*^{w84x} mutants regain circulating blood cells after 15 dpf (Jin et al., 2009; Sood et al., 2010). Thus, the *runx1*^{w84x} mutant is an appropriate system to evaluate whether rugby ball-shaped cells at 7 dpf are definitive hematopoietic cells. Largely consistent with the finding reported by Falenta and Rodaway, we found that 82.9% of circulating blood cells isolated from 7 dpf wild-type embryos were of rugby ball shape (see Appendix S1 and Fig. S1E,F in the supplementary material). However, the percentage of rugby ball-shaped cells did not decrease, but was instead maintained, in 7 dpf *runx1*^{w84x} mutants (in which 95.5% of circulating blood cells were of rugby ball shape), which would not be predicted if they are definitive in origin (see Appendix S1 and Fig. S1E,F in the supplementary material). Thus, it appears more likely that the rugby ball-shaped cells observed at 7 dpf reflect a more matured state of primitive erythrocytes rather than a definitive erythroid population. Our data also suggest that rugby ball shape is not a reliable criterion for scoring definitive erythrocytes in related experiments.

As a result of these findings, we stand by our original conclusions.

Supplementary material

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Appendix S1

Supplementary methods and results

Except where stated otherwise, embryos were raised at 28.5°C in 0.1× Hanks Balanced Salt Solution (HBSS) without Phenol Red (Sigma). At 24 hours post-fertilisation (hpf), PTU (1-phenyl-2-thiourea) was added to a final concentration of 0.003% w/v to prevent pigment formation.

Following overnight fixation of zebrafish embryos in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), whole-mount in situ hybridisation (WISH) was performed by standard methods, exactly as described in (Broadbent and Read, 1999). Proteinase K digestion prior to hybridisation was optimised for each new batch of enzyme. For 4 days post-fertilisation (dpf) embryos, digestion was continued until the tail-fin had just begun to disintegrate. For our current batch of enzyme, this required a 1 hour incubation in 20 μg/ml Proteinase K at 37°C. The probes used were full-length β*e1-globin* (GenBank accession AF082662), which was a gift from Andre Quinkertz (Quinkertz and Campos-Ortega, 1999), and full-length α*e1-globin* (IMAGE clone 6906170, GenBank accession BC071550). Clone identities were verified by sequencing.

For photography, embryos were mounted in 80% glycerol:20% PBS (with 0.1% Tween20). For sectioning, embryos were embedded in 1.5% agarose and soaked in 30% sucrose overnight. After freezing in liquid nitrogen, the blocks were equilibrated to –30°C and 15 μm sections were cut on a cryostat.

The peroxidase activity of haemoglobin was detected using 2,7-diaminofluorene (Fluka). This was performed as described in Weinstein et al. (Weinstein et al., 1996) except that the pre- and post-incubation fixation was in 4% paraformaldehyde in PBSA rather than in BT-fix. Staining times were extended to 2 hours at room temperature to allow staining of the more weakly expressing erythroblasts in addition to the strongly expressing primitive erythrocytes.

For tail amputations, embryos were anaesthetised with 0.016% w/v MS222 (Sigma) in 50:50 mixture of Liebovitz L-15 (Sigma) and 0.1× HBSS (supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin) for 15 minutes prior to operation. The tail (all tissue posterior to the opening of the cloaca) was then amputated. Amputations were performed using a sapphire blade (World Precision Instruments) in order to minimise tissue damage to the embryo. Anaesthesia was maintained for 4 hours after operation, followed by transfer to fresh 0.1× HBSS with antibiotics for further development at 28.5°C. Most embryos rapidly re-established circulation to the trunk via development of arteriovenous shunts near the amputation site and mortality was minimal (greater than 95% survival to 7 dpf). Other than the (obvious) lack of tail, the development and behaviour of the embryos appeared normal, with the exception of delayed inflation of the swim bladder.

To obtain blood, anaesthetised embryos were exsanguinated then killed by over-anaesthesia. To prevent the rapid blood clotting that occurs in older embryos, this was performed in a 4:1 mixture of 0.9× SSC, 20 mM EDTA, 10 U/ml heparin in 1× Trypsin/EDTA (0.5 mg/ml Trypsin, 6 mM EDTA in PBS). The trunk was severed with a sapphire knife just anterior to the cloaca, and the blood cells were collected with a 2 μl pipette tip. For scoring of morphology, the cells were transferred to a haemocytometer slide. For each treatment, a minimum of 200 blood cells from each of five 7 dpf embryos were immediately scored as either definitive or primitive in morphology. For unoperated control embryos, an average of 94% of erythrocytes (range 92-97%) had definitive morphology, which is in agreement with Belair et al. (Belair et al., 2001). This proportion was

not reduced by removal of the tail at 1 dpf (average 96%, range 94-97%) or 3.5 dpf (average 96%, range 94-98%). Although we would not exclude a reduction in total numbers of erythrocytes following removal of the tail, it is clear that this tissue is not required for the switch from embryonic to definitive erythropoiesis. For photography, blood samples were placed on poly-L-lysine coated slides. After fixation with 4% PFA in PBSA (1 hour) and methanol (5 minutes) the slides were washed with water then mounted in 80% glycerol.

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