Generating parabiotic zebrafish embryos for cell migration and homing studies

Doris Lou Demy1,2,4, Zachary Ranta1,2,4, Jean-Michel Giorgi3, Magali Gonzalez3, Philippe Herbomel1,2 & Karima Kissa1–3

Parabiosis, the surgical generation of conjoined organisms sharing a common bloodstream, has been a powerful tool for studying hematopoietic cell migration and interaction with stromal niches in rodent and avian systems. We describe a technique to generate parabiotic zebrafish embryos based on blastula fusion. This procedure permits the in vivo visualization of hematopoietic cell migration and homing to niches and peripheral tissues in zebrafish parabiotics of different genetic backgrounds.

In zebrafish, as in mammals, hematopoiesis occurs in two waves. The ‘primitive’ wave produces erythrocytes and myeloid cells; the latter differentiate in the yolk sac and then invade the embryonic tissues to become tissue-resident leukocytes1–2. The ‘definitive’ wave involves long-term hematopoietic stem cells (HSCs) that will generate all blood cell lineages. In mammals, HSCs originate from an intraembryonic region called the aorta-gonad-mesonephros and enter the bloodstream to colonize the fetal liver, where they expand and differentiate and subsequently seed the definitive hematopoietic organs, the thymus and bone marrow3. In zebrafish, previous studies4–6 have established that the thin space separating the dorsal aorta and axial vein in the trunk region is homologous to the mammalian aorta-gonad-mesonephros4. Recently we described how zebrafish HSCs derive directly from endothelial cells of the aorta floor through a specific transformation that we have called the endothelial hematopoietic transition7. These first HSCs then enter the bloodstream to seed a transient embryonic site of hematopoiesis, the caudal hematopoietic tissue (CHT)4,8, where they expand and differentiate before colonizing the thymus and kidney, the definitive sites of hematopoiesis in fish4,5,8.

For the study of hematopoietic stem and progenitor cells (HSPCs), their behavior and the influence of successive microenvironments on their lineage commitment, parabiosis has been a powerful tool in mouse9,10 and quail-chick chimeras11. In parabiosis experiments, two genetically marked organisms are surgically conjoined to share a common blood circulation, making it possible to analyze the interactions of the circulating cells from one partner with the potential homing sites in the other partner; this strategy provides the means to discriminate whether a mutation that perturbs these interactions acts on the HSPCs or on the homing site. However, parabiosis has not yet been applied to zebrafish. Here we describe a methodology based on the fusion of zebrafish blastulae that leads to parabiosis between embryos of two genetic backgrounds of interest. This methodology can be applied to live imaging studies at single-cell resolution for the study of, for example, the effects of diffusible signals on cell lineage specification or the cell-autonomous versus non-cell-autonomous effects of specific genes. We illustrate the value of zebrafish parabiosis by following HSPCs in vivo and studying their interactions with stromal niches.

The technique consists of fusing two differentially marked zebrafish blastulae and allowing them to develop as partially fused embryos that share a common blood circulation.

Figure 1 | Zebrafish parabiosis by blastula fusion: experimental procedure. (a–g) Images of zebrafish blastulae (a–c; e–g, top) and parabiotics (d; e–g, bottom) through a dissecting microscope under transmitted light (a–c, e–g) or red fluorescence merged with transmitted light (d). The boxed region in a is shown enlarged in b and c. Dechorionated embryos were placed in methylcellulose (a), scratched with a glass needle (b) and allowed to fuse (c). Rhodamine dextran–injected embryos were pink under transmitted light (a–c; e–g, top) and fluorescent (d). A, animal pole; M, margin; I, intermediate region (between A and M). Distinct fusion patterns resulted from different blastula fusion orientations: A-M (e), M-I (f) and I-I (g) fusions are shown. Scale bars, 250 μm. See also Supplementary Video 1.

1Institut Pasteur, Unité Macrophages et Développement de l’Immunité, Département de Biologie du Développement et Cellules Souches, Paris, France. 2Centre National de la Recherche Scientifique, Unité de Recherche Associée 2578, Paris, France. 3Unité Mixte de Recherche 5235, Dynamique des Interactions Membranaires Normales et Pathologiques, Université Montpellier 2, Montpellier, France. 4These authors contributed equally to this work. Correspondence should be addressed to K.K. (karima.kissa@univ-montp2.fr).

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First, we remove the chorions from embryos of two different backgrounds at the 128-cell blastula stage (Online Methods). Using a Pasteur pipette, we collect both embryos and transfer them together into a methylcellulose drop covered with high-calcium Ringer’s solution containing antibiotics. With a fine paintbrush, we then gently move the methylcellulose around both embryos, bringing them closer to each other in the proper orientation for fusion (Fig. 1a and Online Methods; detailed protocol in Supplementary Note). Using a pulled glass micropipette, we detach a few cells from both blastulae at their contact point (Fig. 1b and Supplementary Video 1). The methylcellulose is then moved again around the blastulae so as to press the ‘wounds’ against each other and promote fusion (Fig. 1c). The next day, we replace the high-calcium Ringer’s solution containing dissolved methylcellulose with embryo water containing antibiotics (Online Methods). Between 17% and 56% of the blastula pairs, depending on the developmental stage (Supplementary Fig. 1), developed into viable and partially fused embryos that shared a common blood circulation (‘parabiotes’) and that otherwise displayed normal overall morphology and spontaneous movement (Fig. 1d–g).

We found that the developmental stage at which blastula fusion is performed is critical for successful parabiosis. Blastula fusion must be performed between the 256- and the 30% epiboly stages to result in viable parabiotic embryos. Before the 256-cell stage, fusion usually led to a single embryo of both genetic backgrounds surrounding the two yolk sacs (data not shown). Blastula fusions performed at the 256- to 512-cell stages led to 40% viable parabiotic embryos by 1 dpf after fertilization (d.p.f.), 49% when performed at 1,000-cell/high stages and 56% when done at sphere/dome stage. The success rate then decreased abruptly to 17% by the 30% epiboly stage, mainly because of separation and/or death of the embryos overnight (Supplementary Fig. 1). Therefore, in all subsequent experiments, blastulae were fused between the 256- to 512-cell stage and just before 30% epiboly.

We quantified the effect of blastula orientation on fusion success and the resulting parabiosis patterns (Fig. 1e–g and Supplementary Fig. 2). Blastulae fused by their animal poles mostly gave rise to viable but malformed embryo pairs (10 of 17; Supplementary Fig. 2a,d,e). Blastulae fused by their blastoderm margins or by margin to intermediate region mostly separated during the experiment (27 of 38 pairs). Among the 11 successful fusions, 4 were malformed or died and 8 survived. Of these eight to survive to parabiosis, five gave rise to parabiotes with separate heads (Fig. 1f and Supplementary Fig. 2c–e). In contrast, fusions between the animal pole of one blastula and the intermediate or marginal region of the other led to a high rate of viable, well-formed parabiotes by 2 d.p.f. (22 of 51); of these, 19 were fused by their heads (Fig. 1e and Supplementary Figs. 2b,d,e and 3). Blastulae fused by their intermediate regions also yielded a high proportion of viable parabiotes by 2 d.p.f. (43 of 95), most of which (31 of 43) had separate heads (Fig. 1g and Supplementary Fig. 2d,e). Thus, by selecting the proper orientation upon blastula fusion, one can favor the generation of parabiotic pairs fused in patterns appropriate to the biological question to be addressed.

To assess the frequency of shared circulation of the fused embryos, we fused transgenic gata1a:dsred blastulae, in which erythroid cells and early primitive myeloid cells are labeled, with nontransgenic partners. We analyzed 24 parabiotes displaying different fusion patterns (fused heads or fused tails) at 2 d.p.f., and all of them displayed shared blood circulation as evidenced by
DsRed+ erythroid cells circulating in both embryos. This indicates that their vascular systems had anastomosed in the fused region. By 4 d.p.f., 22 of the 24 parabiotes were still alive, and in 18 of them DsRed+ cells were still circulating in both embryos.

We applied this technique to follow the behavior of primitive hematopoietic cells in parabionts. We fused two embryos harboring different reporter transgenes: pu1:gfp12, which labels primitive myeloid cells (macrophages and granulocytes), and gata1a:dsred13 (Fig. 2a–h; note: pu1 is also known as spii1b). At 18.5 h post fertilization (h.p.f.) in parabiotic embryos displaying separate trunks and tails, GFP+ primitive myeloid cells and DsRed+ primitive erythroid and myeloid cells were detected in only the embryo of origin, at their normal location for this developmental stage (Supplementary Fig. 3). By 23 h.p.f., primitive myeloid cells born in the yolk sac of either embryo had started to invade the other embryo (Fig. 2a and Supplementary Video 2). By 25 h.p.f., blood circulation started, and the DsRed+ erythroid cells born in the trunk of the gata1a:dsred embryo were then seen circulating within both embryos, demonstrating the shared circulation of the parabionts (Fig. 2a–h, Supplementary Fig. 3 and Supplementary Video 2). By 48 h.p.f., GFP+ myeloid cells and DsRed+ myeloid and erythroid cells were found in the CHT of both parabionts (100%, n = 35; Fig. 2c–h).

Next we investigated the migration and homing of definitive HSPCs in parabionts. In the kdr1:gfp transgenic line, GFP is expressed in all vascular endothelial cells and their HSPC progeny9. Upon fusion of a kdr1:gfp with a nontransgenic wild-type embryo, colonization of the wild-type CHT by GFP+ HSPCs was detected in 7 of 7 parabiotic pairs with separate trunks and tails examined, starting at 48 h.p.f. (Fig. 2i), and this population expanded over the next days (Fig. 2j). At 3 d.p.f., GFP+ HSPCs were found to colonize the thymic rudiment of the wild-type parabiont (Fig. 2k). Thus, HSPCs born from the aorta of the kdr1:gfp partner were able to reach and settle in the successive hematopoietic niches of both parabionts with the same timeline as in normal development.

Another useful aspect of this approach is the opportunity to track circulating or migrating cells marked by widely expressed reporter transgenes. In pu1:gfp//gata1a:dsred parabionts—that is, parabionts with each embryo harboring a separate transgene—GFP+ myeloid cells could be clearly observed in the gata1a:dsred tail (Fig. 2f) without the strong ectopic GFP expression observed in the muscles of the pu1:gfp embryo (Fig. 2e); conversely, observation of DsRed+ myeloid and erythroid cells was easier in the pu1:gfp partner tail (Fig. 2c), where DsRed was not expressed in epithelial mucus cells (Fig. 2d). In kdr1:gfp//wild-type parabionts, tracking GFP+ HSPCs in the CHT, thymus and kidney was also easier in the nontransgenic parabionte (Fig. 2i–k and Supplementary Video 3) owing to the lack of vascular GFP expression.

Last, we applied the blastula fusion technique to study the role of genes affecting hematopoiesis using the mind bomb (mib) mutant, in which Notch signaling is disrupted and definitive HSPCs do not form4. We fused mib mutant blastulae with cd41:gfp transgenic blastulae, in which HSPCs weakly express GFP9 (note: cd41 is also known as itga2b). In parabionts with separate trunks and tails that shared a common bloodstream, we observed GFPlow HSPCs colonizing the CHT and thymus of mib mutants (data not shown), whereas in parabionts in which the mib embryo had no blood circulation, we observed only thymus colonization (n = 3, Fig. 2l). These results show that Notch signaling deficiency does not affect the ability of the CHT and thymus to attract and host wild-type HSPCs. They also confirm and extend previous results showing that blood circulation is required for CHT but not thymus colonization1,2.

We here demonstrate the usefulness of the zebrafish blastula fusion technique for studying hematopoietic cells and their interactions with stromal niches. Beyond hematopoietic cells, this technique can be used for studying other migratory cells such as neural crest cells or circulating signals and their interaction with their target tissue or processes such as innervation or vascular bud formation. It can be a powerful alternative to cell transplantation experiments14 for investigating cell-autonomous versus non–cell autonomous gene function15, notably as both situations to be tested (mutant-cell migration into wild-type tissue and vice versa) occur within every parabiotic pair. Finally, reverse genetic tools such as antisense morpholinos and mRNA injection can be applied to either partner before fusion, thereby further extending the range of potentially valuable applications of this simple and powerful technique.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

K.K. designed the experiments. D.L.D., Z.R., J.-M.G., M.G. and K.K. performed the experiments. K.K. wrote the manuscript with input from D.L.D. and P.H.

COMPETING FINANCIAL INTERESTS

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**ONLINE METHODS**

**Zebrafish stocks and embryo rearing.** Wild-type AB; *pu1:gfp*, *gata1a:dsred*, *kdr:gfpl* and *cd41:gfp* transgenic; and *mind bomb* mutant embryos were raised and staged according to Westerfield’s [16](http://zfin.org/zf_info/zfbk/zfbk.html). Embryo water was Volvic water with 280 µg/ml methylene blue and 0.003% phenylthiourea (PTU) to inhibit melanin synthesis [16].

**Rhodamine-dextran cell labeling.** 1 nl of a 10 mg/ml rhodamine-dextran (MW = 10,000 Da; Invitrogen) solution was injected to embryos at the one- to four-cell stage.

**Blastula fusion procedure.** 4% methylcellulose was prepared by dissolving 2 g of methylcellulose powder in 50 ml embryo water. Complete dissolution required several hours under agitation as well as vigorous vortexing. It was then stored at room temperature. Lower, 2–4%, methylcellulose concentrations were also used successfully; a lower concentration allows an easier reorientation of embryos, whereas a higher concentration requires better dexterity but also ensures a higher pressure between both blastulae, resulting in a higher success rate of fusion. High-calcium Ringer’s solution (200 ml) was prepared by adding 4 ml of 5 M NaCl solution (116 mM), 200 µl of 3 M KCl solution (2.9 mM), 400 µl of 5 M CaCl₂ solution (10 mM) and 1 ml of 1 M HEPES solution (5 mM) to 195 ml embryo water and was stored at 4 °C for a few weeks.

Embryos were manually dechorionated at the 256-cell stage with fine (Dumont #5) forceps [16] in two separate scratchless glass Petri dishes. Seven to nine small drops of 4% methylcellulose were deposited on a 30-mm plastic Petri dish and covered with high-calcium Ringer's containing antibiotics (50 U/ml penicillin-streptomycin, 50 U/ml ampicillin, 0.5 µg/ml kanamycin and 0.5 µg/ml gentamicin). Two embryos, one of each of the two genetic backgrounds to be fused, were collected with a glass Pasteur pipette. The tip of the pipette was used to make a small well in the methylcellulose drop, to which the embryo pair was then transferred. The methylcellulose around them was then moved with a fine paintbrush to bring them close to each other and in proper orientation for fusion. A pulled glass micropipette was then used to detach a few cells from both blastulae at their contact point, as demonstrated in Supplementary Video 1. The methylcellulose was then moved again around the blastulae so as to press the wounds against each other. Blastula fusions were performed in this way on successive blastula pairs starting at the 512-cell stage up until the 30% epiboly stage. Fusions were performed at room temperature during a 2.5-h time window (the time it takes for embryos at room temperature to go from the 512-cell stage to 30% epiboly), which allowed the generation of 30–40 blastula fusions per embryo clutch per experimenter. After blastula fusion, the dishes were left on the bench for 20–30 min to avoid any shaking that could separate the two blastulae. The dishes were then fully filled with HCR containing antibiotics and transferred to the incubator at 28 °C. The next morning, the HCR solution with dissolved methylcellulose was replaced by embryo water containing PTU and antibiotics as above.

**Figure 1 and Supplementary Video 1** were generated using a MZ16 stereomicroscope (Leica) equipped with a three–charge-coupled-device video camera (HVD-20; Hitachi), recorded on minIDV tapes and captured with BTV Pro (http://www.bensoftware.com/) and iMovie softwares; **Figure 2a–j,** was generated using a MacroFluo (Leica) equipped with a Roper camera and MetaView software.

**Time-lapse confocal fluorescence imaging of live zebrafish embryos and larvae.** Embryos were anesthetized with tricaine [16], immobilized in 1% low–melting point agarose on 35-mm glass-bottom dishes (Iwaki), covered with tricaine containing embryo medium [8] and imaged on an SPE confocal inverted microscope (Leica) (Fig. 2k and Supplementary Videos 2 and 3) as described previously [7].