SUPPLEMENTAL MATERIAL

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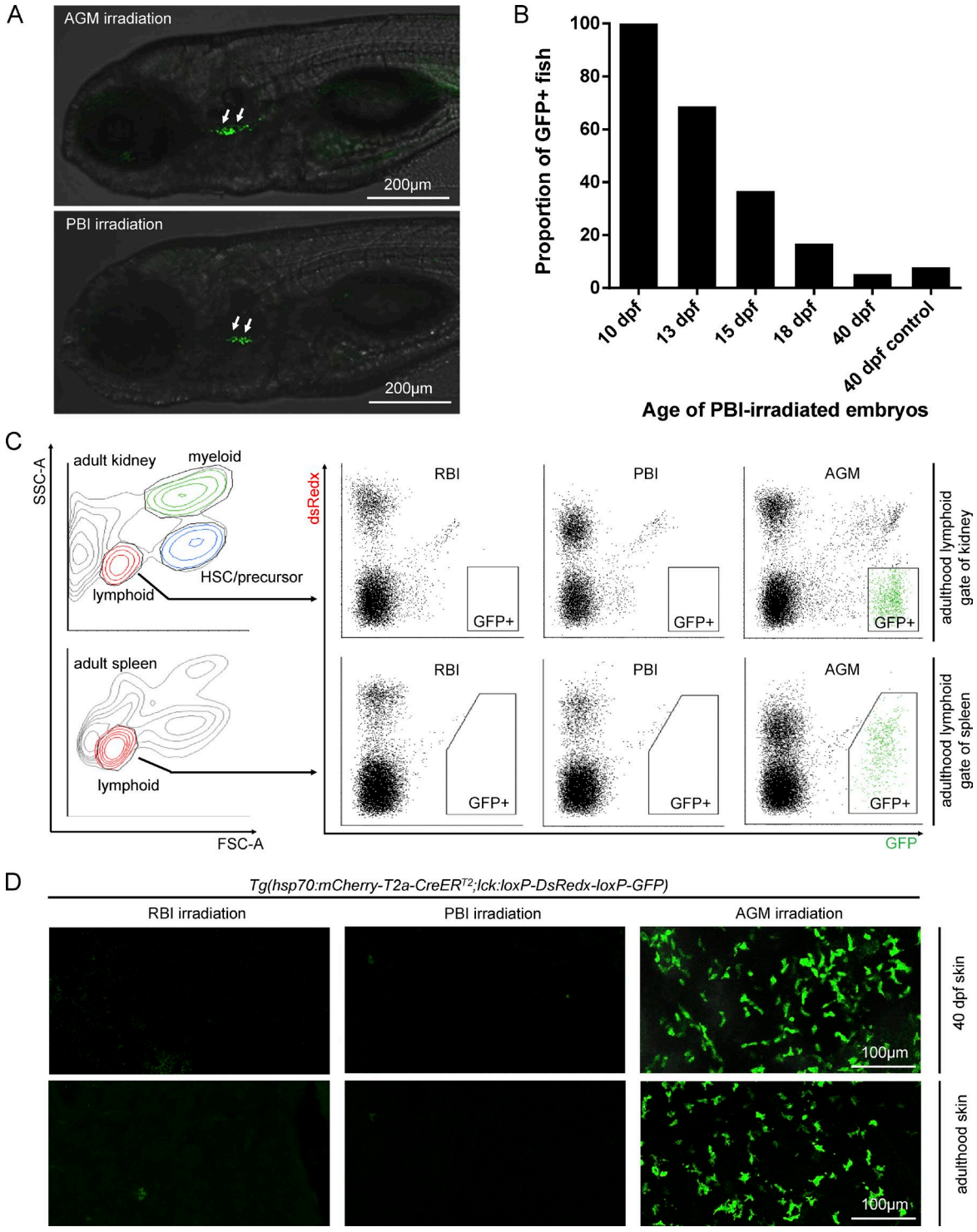


Figure S1. **Transient appearance of PBI-derived T cells (related to Fig. 1).** (A) *lck*:GFP$^+$ T cells (arrows) are restricted in the thymus of the AGM- and PBI-irradiated *Tg(hsp70:mCherry-T2a-CreER^{T2};lck:loxP-DsRedx-loxP-GFP)* fish at 5 dpf. (B) The proportion of GFP$^+$ embryos in the PBI-irradiated *Tg(hsp70:mCherry-T2a-CreER^{T2};lck:loxP-DsRedx-loxP-GFP)* embryos at different stages (n = 25). The GFP$^+$ T cells are gradually reduced as fish develop and by 40 dpf, the percentage of GFP$^+$ fish in the PBI-irradiated group drops to the baseline comparable to the 4-hydroxytamoxifen–treated control group. (C) Diagram of flow cytometry analysis of adult kidney (top) and spleen (bottom). Lymphoid gate (red), myeloid gate (green), and HSC/precursor gate (blue) are shown. The same gate profiles are also used in Figs. S2 and S5. (D) Imaging of *lck*:GFP$^+$ T cells on the skin at 40 dpf and adulthood. *lck*:GFP$^+$ cells are abundantly detected in the AGM-irradiated (right lane) but completely absent in the RBI-irradiated (left lane) and PBI-irradiated (middle lane) fish.

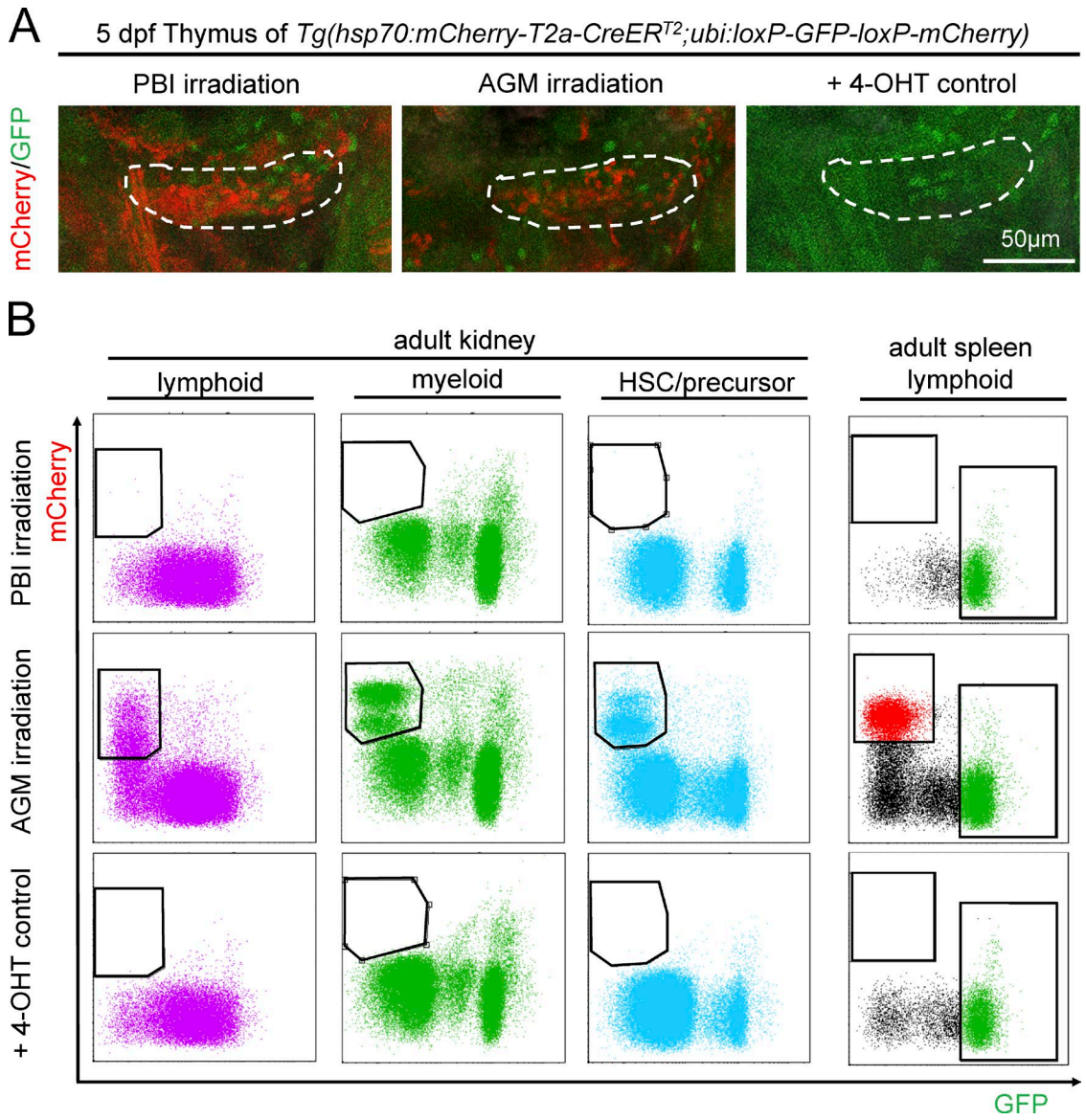


Figure S2. **The PBI is not capable of generating HSCs in situ (related to Fig. 1).** (A) Images of *ubi*:mCherry^{+} cells in the thymus of 5 dpf *Tg(hsp70:mCherry-T2a-CreER^{T2};ubi:loxP-GFP-loxP-mCherry)* embryos. Both PBI-irradiated (left) and AGM-irradiated (middle) embryos show apparent mCherry signals in the thymus, whereas control embryos treated with 4-hydroxytamoxifen only (right) have no mCherry signals. Dashed lines depict the thymus. (B) Flow cytometry analysis of hematopoietic cells in the kidney marrow and the spleen of adult *Tg(hsp70:mCherry-T2a-CreER^{T2};ubi:loxP-GFP-loxP-mCherry)* fish. The AGM-irradiated fish contain abundant mCherry^{+} cells in the lymphoid, myeloid, and HSC/precursor pools, whereas the PBI-irradiated and control fish have no mCherry^{+} cells. Gate profiles are similar to those shown in Fig. S1 C.

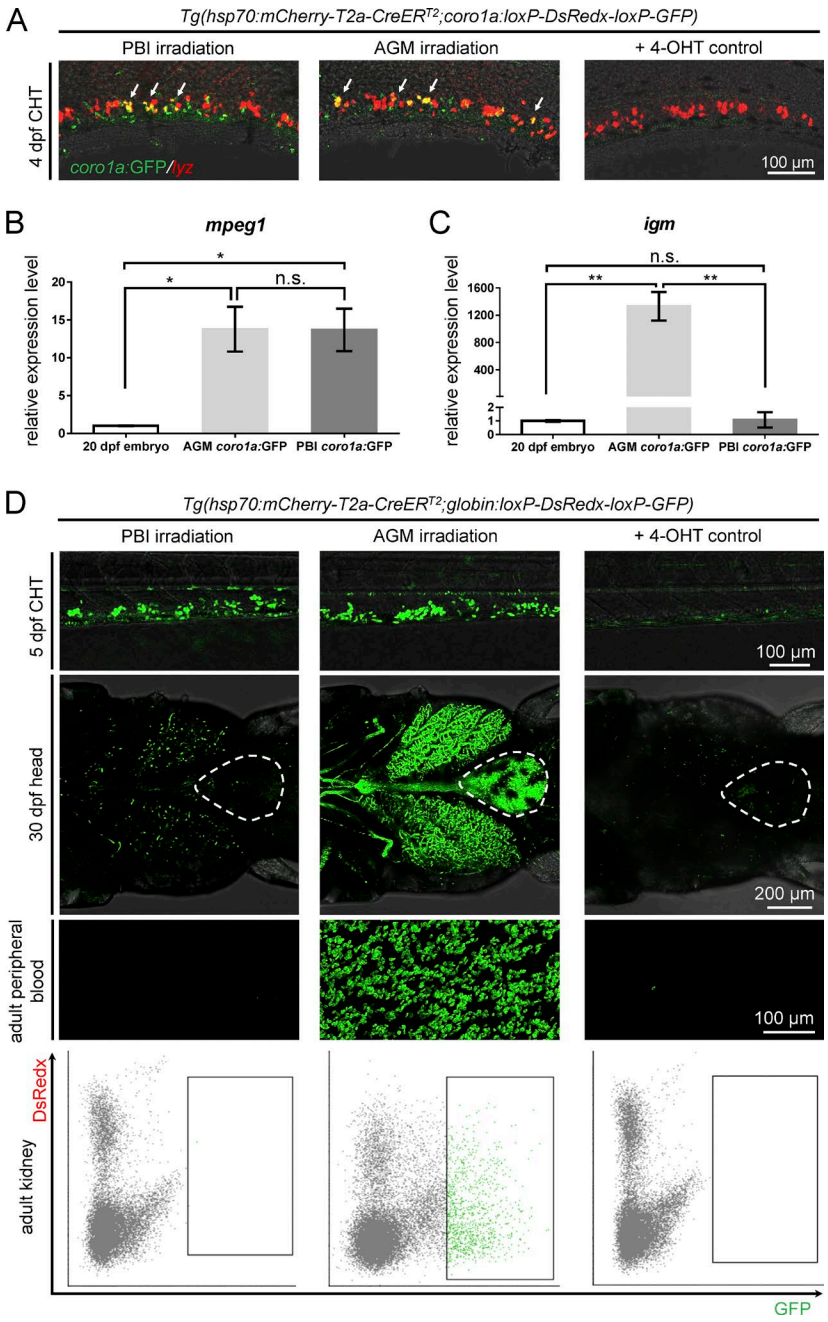


Figure S3. **The PBI in situ generates myeloid and erythroid cells but not B lymphocytes (related to Fig. 1).** (A) Images of *coro1a*:GFP$^+$/*lyz*$^+$ neutrophils (arrows) in the CHT of 4 dpf *Tg(hsp70:mCherry-T2a-CreER^{T2};coro1a:loxP-DsRedx-loxP-GFP)* fish irradiated in the PBI (left) and AGM (middle). *coro1a*:GFP$^+$ cells are detected by anti-GFP antibody, and *lyz*$^+$ cells are detected by whole-mount in situ hybridization. Result show that both the PBI and AGM in situ generate GFP$^+$ myeloid cells, whereas control group fish (right) have no GFP$^+$ cells. 4-OHT, 4-hydroxytamoxifen. (B and C) Relative expression levels of *mpeg1* (macrophage marker) and *igm* (B cell marker) in the AGM- and PBI-derived *coro1a*:GFP$^+$ cells isolated from 20-dpf embryos. The *coro1a* :GFP$^+$ cells from the AGM- and PBI-irradiated fish are sorted by FACS and subjected to RT-PCR analysis. Three independent experiments (in each group, 20 fish are pooled together, and at least 1,000 cells are collected) are conducted. cDNA prepared from 20-dpf whole embryos are used as control. Data are represented as mean ± SD. Unpaired, two-tailed *t* test was performed to determine significance. *, $P < 0.05$; **, $P < 0.01$. (D) Images of *globin*:GFP$^+$ erythroid cells at different stages in the *Tg(hsp70:mCherry-T2a-CreER^{T2};globin:loxP-DsRedx-loxP-GFP)* fish irradiated in the PBI and AGM at 28 hpf. The *globin*:GFP$^+$ erythroid cells are monitored at 5 dpf (CHT region, lateral view), 30 dpf (head region, ventral view), adult peripheral blood (by blood smear), and adult kidney marrow (by flow cytometry analysis). Dashed lines indicate the heart. The AGM-derived *globin*:GFP$^+$ erythroid cells are abundantly detected from embryonic stage to adulthood, whereas the PBI-derived *globin*:GFP$^+$ erythroid cells are present in embryonic (5 dpf) and juvenile stages (30 dpf) but not in adulthood. *globin*:GFP$^+$ erythroid cells are rarely seen in the 4-OHT control group.

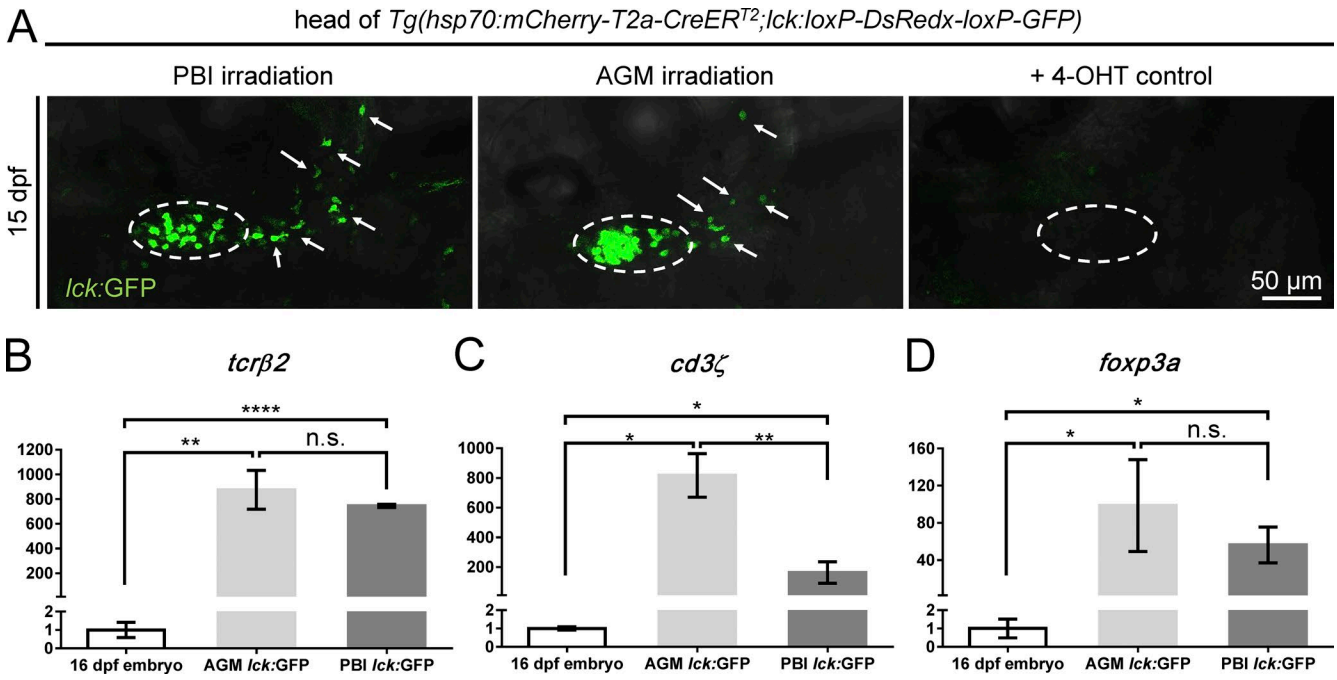


Figure S4. **The PBI–derived T lymphocytes are competent to mature (related to Fig. 2).** (A) Images of *lck*:GFP$^+$ T cells in the head region of 15 dpf *Tg(hsp70:mCherry-T2a-CreER^{T2};lck:loxP-DsRedx-loxP-GFP)* fish irradiated in the PBI and AGM at 22 and 26 hpf, respectively. Dashed lines depict the thymus region. In both PBI-irradiated (left) and AGM-irradiated (middle) fish, a substantial number of *lck*:GFP$^+$ cells egress from the thymus and reach the neighboring tissues (arrows), indicating that these *lck*:GFP$^+$ cells are mature T cells. Control fish contain very few GFP$^+$ signals. (B–D) Relative expression levels of *tcrβ2*, *cd3ζ*, and *foxp3a* in the AGM- and PBI-derived *lck*:GFP$^+$ T lymphocytes isolated from 16-dpf embryos. Three independent experiments are conducted. Data are represented as mean ± SD. Unpaired, two-tailed *t* test was performed to determine significance. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.

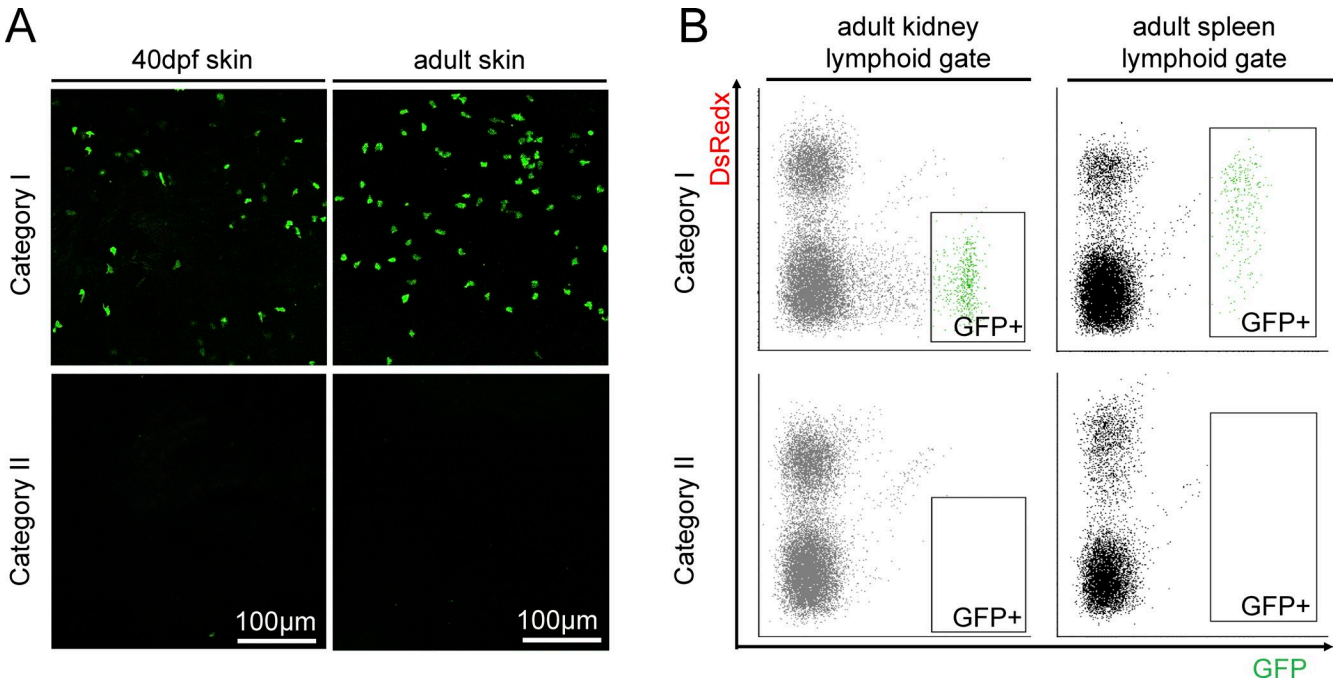
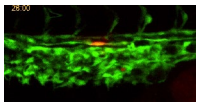


Figure S5. **AGM single spot–irradiated fish display two distinct patterns of *lck*:GFP⁺ T cells (related to Fig. 4).** (A) Images of *lck*:GFP⁺ T cells in the skin of 40-dpf and adult *Tg(hsp70:mCherry-T2a-CreER^{T2};lck:loxP-DsRedx-loxP-GFP)* fish that are irradiated in a single small spot of the AGM at 22–24 hpf. Category I fish contain abundant GFP⁺ T cells in the skin, whereas category II fish contain very few GFP signals. (B) Flow cytometry analysis of lymphoid lineage in the kidney and the spleen of adult *Tg(hsp70:mCherry-T2a-CreER^{T2};lck:loxP-DsRedx-loxP-GFP)* fish. Category I fish contain abundant GFP⁺ T cells in the kidney and spleen, whereas GFP⁺ T cells are absent in category II fish. Gate profiles are as described in Fig. S1 C.



Video 1. **Time-lapse imaging of endothelial-hematopoietic transition in the PBI of *Tg(flk1:Dendra2)* embryos (related to Fig. 3).** Time-lapse confocal imaging shows a photoconverted endothelial cell (red) in the ventral wall of caudal aorta of *Tg(flk1:Dendra2)* embryo undergoes endothelial-hematopoietic transition to form a nascent hematopoietic progenitor, which subsequently divides into two daughter cells and migrate to the CVP niche. Time on the upper-left corner indicates the developmental stage of the embryo. Each frame was collected with 10-min interval, and video is displayed at 15 frames/s.