LETTERS

Haematopoietic stem cells derive directly from aortic endothelium during development

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A major goal of regenerative medicine is to instruct formation of multipotent, tissue-specific stem cells from induced pluripotent stem cells (iPSCs) for cell replacement therapies. Generation of haematopoietic stem cells (HSCs) from iPSCs or embryonic stem cells (ESCs) is not currently possible, however, necessitating a better understanding of how HSCs normally arise during embryonic development. We previously showed that haematopoiesis occurs through four distinct waves during zebrafish development, with HSCs arising in the final wave in close association with the dorsal aorta. Recent reports have suggested that murine HSCs derive from haemogenic endothelial cells (ECs) lining the aortic floor[1,2]. Additional *in vitro* studies have similarly indicated that the haematopoietic progeny of ESCs arise through intermediates with endothelial potential[3,4]. Here we have used the unique strengths of the zebrafish embryo to image directly the generation of HSCs from the ventral wall of the dorsal aorta. Using combinations of fluorescent reporter transgenes, confocal time-lapse microscopy and flow cytometry, we have identified and isolated the stepwise intermediates as aortic haemogenic endothelium transitions to nascent HSCs. Finally, using a permanent lineage tracing strategy, we demonstrate that the HSCs generated from haemogenic endothelium are the lineal founders of the adult haematopoietic system.

Precisely how the first HSCs are generated in the vertebrate embryo has been a matter of controversy for several decades. Recent studies have strongly supported the postulate of haemogenic endothelium, ECs that transiently possess the ability to generate HSCs during vertebrate development[5]. By targeting expression of the Cre recombinase specifically to cells of the vasculature it was shown, using floxed reporter genes, that HSCs were generated from *Cdh5*$^{+}$ (also referred to as VE-Cadherin) precursors, indicating that HSCs arise from endothelium or shared endothelial precursors[1]. Furthermore, experiments using an inducible *Cdh5:Cre*^{ERT2} transgene indicated that ECs within the region flanked by the aorta, gonads and mesonephros (AGM) in the mid-gestation mouse embryo contained the majority of HSC potential[1]. In addition, conditional deletion of the *Runx1* transcription factor gene in *Cdh5*$^{+}$ cells led to loss of HSCs, indicating that Runx1 function is key in the transition from endothelium to HSC[2]. *In vitro* studies have also suggested that ESC derivatives can generate haematopoietic cells through haemogenic endothelial intermediates[3,4,6]. It remains to be determined, however, which regions of the embryo, or extra-embryonic tissues, possess endothelium with haemogenic potential.

The generation of cells having HSC characteristics has been observed in close association with arterial endothelium[5,7]. In particular, the ventral floor of the dorsal aorta (DA) has been shown by a number of investigators to be the primary birthplace of HSCs[8]. In the zebrafish embryo, we[9] and others[10] demonstrated previously that expression of a *cmyb:eGFP* transgene marks nascent HSCs along the ventral aspect of the DA between 28 and 48 hours post-fertilization (h.p.f.). To determine whether these cells arise directly from vascular precursors, we generated *cmyb:eGFP*; *kdrl:memCherry*[11] double transgenic animals and performed confocal time-lapse imaging. Between 28 and 32 h.p.f., expression of the *kdrl* transgene (also known as *flk1* and *vegfr2*) within the zebrafish equivalent of the AGM region is localized to the aorta, vein and developing intersomitic vessels; haematopoietic expression of the *cmyb* transgene initiates in cells along the DA around this time (Fig. 1a, b). Four-dimensional imaging demonstrated that *cmyb:eGFP*$^{+}$ cells arose directly from *kdrl:memCherry*$^{+}$ cells specifically along the ventral aspect of the DA (Supplementary Movies 1, 2). As shown in Fig. 1, *kdrl:memCherry*$^{+}$ ECs displaying typical flattened morphology were occasionally observed to transform into spherical shapes, forming buds that extended into the lumen of the DA. By virtue of the membrane-specific expression of mCherry, buds were observed to initiate as *kdrl*$^{+}$*cmyb*$^{-}$ cells transitioned to *kdrl*$^{+}$*cmyb*$^{+}$ cells (Fig. 1). In contrast to the proposed budding of mammalian HSCs into aortic circulation[5], we almost always observed HSCs to migrate ventrally towards the caudal vein (CV; Supplementary Movies 1, 2). This is consistent with previous observations[12], which indicated that AGM HSCs enter circulation via the dorsal wall of the CV in the zebrafish.

To confirm the haematopoietic nature of these budding AGM cells, we performed flow cytometry on dissociated *kdrl:RFP*[13]*; cmyb:eGFP* embryos at 36 h.p.f., the time point at which we observed the peak in number of *kdrl*$^{+}$*cmyb*$^{+}$ cells. Embryos were dissected to separate anterior, head tissues from the posterior trunk/tail region that contains the AGM. In accordance with our microscopic observations, no *kdrl*$^{+}$*cmyb*$^{+}$ cells were observed in anterior regions (Fig. 2a) above background. By contrast, 0.25% of posterior cells were *kdrl*$^{+}$*cmyb*$^{+}$ cells (Fig. 2b). We thus reasoned that these double-positive cells represented the nascent HSCs observed in our imaging experiments (Fig. 2c). *kdrl*$^{+}$*cmyb*$^{+}$ cells could be subdivided on the basis of differing levels of the *cmyb:eGFP* transgene (Fig. 2b); each subset, along with single positive posterior fractions, were highly purified by FACS and queried for expression of haematopoietic and vascular genes by quantitative PCR (qPCR). As expected, expression of endothelial genes, including *kdrl*, *cdh5* and *lmo2*, were highly expressed in *kdrl*$^{+}$*cmyb*$^{-}$ cells (Fig. 2d). In general, early *kdrl*$^{+}$*cmyb*^{lo} precursors maintained similar expression levels of these vascular markers. As *cmyb:eGFP* levels increased in maturing *kdrl*$^{+}$*cmyb*$^{+}$ cells, however, expression of most endothelial genes dropped markedly. By contrast, expression of *cd41*, one of the earliest markers of mesodermal commitment to definitive haematopoiesis[14,15], initiated in *kdrl*$^{+}$*cmyb*$^{-}$ cells and increased as *kdrl*$^{+}$ cells became *cmyb*$^{+}$ (Fig. 2d). We observed little to no *cd45* expression in either *kdrl*$^{+}$*cmyb*$^{-}$ or *kdrl*$^{+}$*cmyb*^{lo} cells. As these precursors matured, however, we observed concomitant upregulation of *cd45* in *kdrl*$^{+}$*cmyb*$^{+}$ and *kdrl*$^{-}$*cmyb*$^{+}$ subsets (Fig. 2d). These results are consistent with findings in the mouse. On embryonic day 10, murine

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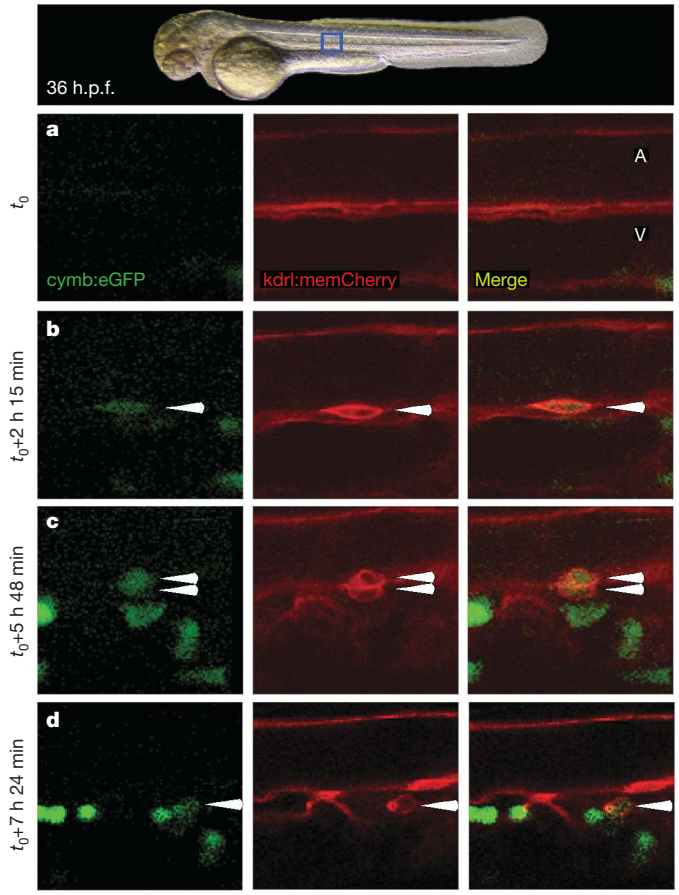

Figure 1 | Direct imaging of HSC emergence from the embryonic aortic floor. a–d, Time-lapse imaging of a double transgenic *cmyb:eGFP, kdrl:memCherry* embryo between 30 and 38 h.p.f. Four sequences from Supplementary Movie 1 are presented, documenting the stepwise emergence of HSCs from haemogenic endothelium in denoted region (blue box, upper panel). For each time point, the GFP, memCherry and merged images are shown. memCherry, GFP double-positive cells are denoted by white arrowheads. A, aorta; V, vein.

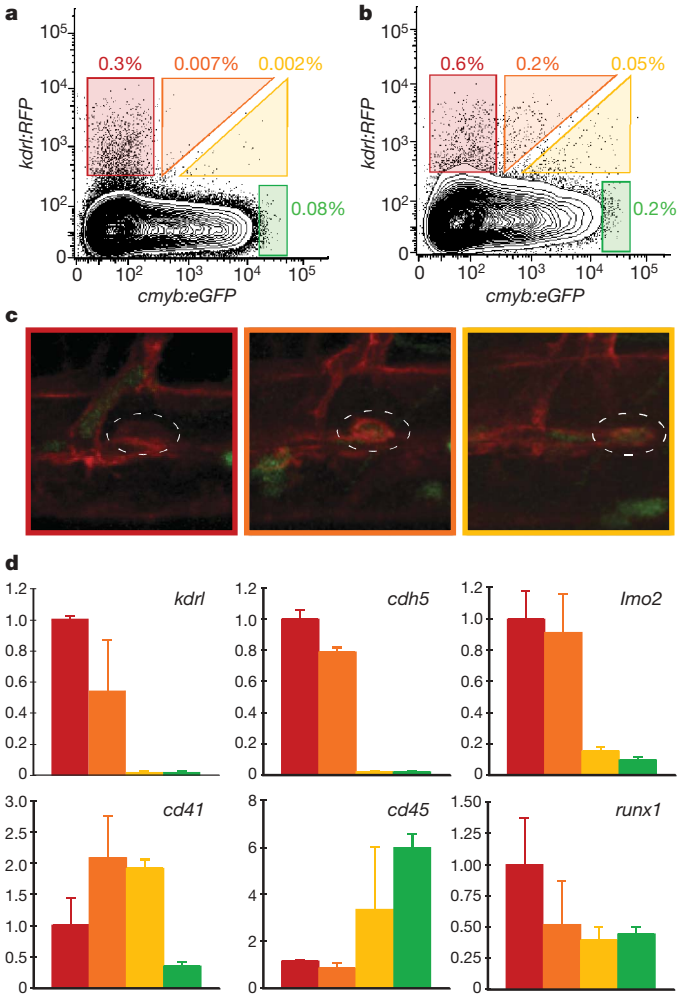

Figure 2 | Prospective isolation of aortic haemogenic endothelium and nascent HSCs. a, b, Double transgenic *cmyb:eGFP; kdrl:RFP* embryos were dissected to separate anterior (**a**) from posterior (**b**) AGM-containing tissues at 36 h.p.f. Throughout the figure, the cellular fraction including haemogenic endothelium is denoted by red boxes or bars, nascent HSCs by orange boxes or bars, maturing HSCs by yellow boxes or bars, and mature HSCs by green boxes or bars. **c**, Correlation of FACS expression profiles to stepwise HSC emergence in *kdrl:memCherry; cmyb:eGFP* embryos (left, haemogenic endothelium; middle, nascent HSC; right, maturing HSC). Images captured from Supplementary Movie 1. **d**, Quantitative PCR expression for endothelial (top panels) and haematopoietic (bottom panels) genes in purified $kdrl^+cmyb^-$ (red), $kdrl^+cmyb^{lo}$ (orange), $kdrl^{lo}cmyb^+$ (yellow) and kdr^-cmyb^+ cells (green). Units on *y*-axis represent fold changes from the $kdrl^+cmyb^-$ reference standard, which is set at 1.0. We note that the kdr^-cmyb^+ population contains some neuronal cells, effectively diluting the vascular and haematopoietic signals. Error bars, s.d.

AGM HSCs do not express CD45 (encoded by *Ptprc*)[14,15]. By day 11, however, embryonic HSCs become $CD45^+$[14,15]. Collectively, these results support the proposition that the $kdrl^+cmyb^+$ cells observed to arise from the ventral wall of the DA are definitive haematopoietic precursors.

In order to trace the progeny of ECs in the zebrafish embryo, we used an indelible marking system using a floxed reporter transgene and a Cre driver that is specific to endothelium via *kdrl* upstream promoter/enhancer elements[16]. In the zebrafish, there exist two orthologues of the mammalian *flk1* gene, *kdr* and *kdrl*. The former exhibits a pan mesodermal expression pattern[17], whereas *kdrl* and transgene construct (*Tg*) *Tg(kdrl:Cre)*^{s898} (Supplementary Fig. 1) are expressed only in endothelium in a manner nearly identical to that of the murine *cdh5* gene[18,19]. Thus, the *kdrl:Cre* line used in our studies mimics the *cdh5:Cre* knock-in mouse line used in previous studies[1,2]. We crossed *kdrl:Cre* animals to animals carrying a *Tg(bactin2:loxP-STOP-loxP-DsRed-express)*^{sd5} '*switch*' reporter transgene in which 10.5 kb of upstream β-actin promoter/enhancer sequence is followed by a 5.7 kb floxed 'super-stop' cassette. Immediately downstream is a $DsRed^{express}$ gene that serves as a reporter for Cre-based removal of the super-stop cassette. As presented in Supplementary Fig. 2, reporter gene expression was never observed in the absence of Cre, and induction of Cre in 24 h.p.f. embryos led to stable reporter expression within nearly all haematopoietic cells for 1 year.

To test whether the $kdrl^+cmyb^+$ haematopoietic precursors observed in the AGM are bona fide HSCs, we generated *kdrl:Cre; switch* animals for long-term studies. Compared to whole kidney marrow (WKM) isolated from single transgenic *switch* animals that showed no expression of DsRed (Fig. 3a), double transgenic *kdrl:Cre; switch* animals showed the vast majority of leukocytes to be labelled at 6 months of age (Fig. 3b, c). Analysis of a large cohort of double transgenic animals showed that over 90% of WKM cells were marked at 3 months of age (Fig. 3b). At 6 months of age, over 96% of WKM myeloid cells expressed the $DsRed^{express}$ lineage tracer (Fig. 3c). Because this cellular subset is comprised of over 90% neutrophils, which are characterized by lifespans restricted to a few days, this result indicates that the vast majority of, if not all, HSCs were marked by the *kdrl:Cre* transgene during their embryonic formation. Finally, marked WKM was sorted by DsRed expression level and subjected to

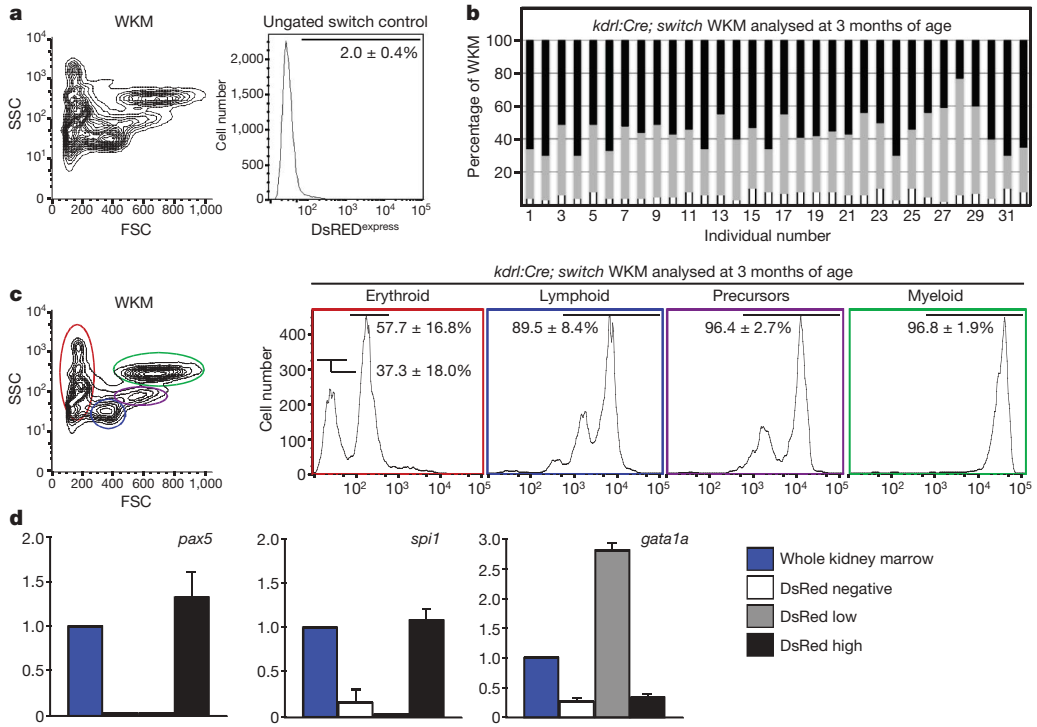

Figure 3 | Long-term lineage tracing of embryonic endothelial cells. a, Flow cytometric analysis of WKM from a transgenic *bactin:switch* adult animal. **b**, Bar graph shows the percentage of switched cells (DsRed^{hi} shaded black; DsRed^{lo} grey) in WKM of double transgenic *kdrl:Cre; bactin:switch* adult animals ($n = 32$). DsRed^{-} cells are represented in white. **c**, Histogram plots show percentages of switched haematopoietic lineages at 6 months of age in WKM (average ± s.d., $n = 5$). **d**, Quantitative PCR expression of switched (DsRed^{hi} in black; DsRed^{lo} in grey) and non-switched cells (white bars) at 3 months, for B lymphoid (*pax5*), myeloid (*spi1*) and erythroid (*gata1a*) genes. Units on *y*-axis represent fold changes from WKM, the reference standard set at 1.0. Error bars, s.d.

qPCR for lineage-affiliated genes. As presented in Fig. 3d, *pax5* (B lymphocyte-affiliated) and *spi1* (myeloid-affiliated) expressing cells were contained within DsRed^{high} cells, and *gata1a*-expressing cells within the DsRed^{low} fraction. These data are consistent with our previous demonstration that the β-actin promoter is highly expressed in leukocytes, but is silenced upon erythropoietic differentiation[20]. Thus, lineage tracing of *kdrl*$^{+}$ haemogenic endothelium demonstrates robust, multilineage, long-term population of the adult haematopoietic organ. Furthermore, because expression of *kdrl* is rapidly extinguished as HSCs arise from haemogenic endothelium (Fig. 2d), and because *kdrl*$^{+}$ *cmyb*$^{+}$ transitional intermediates were no longer detectable in larval or adult stages (Supplementary Fig. 3), our lineage tracing results support the proposition that HSCs no longer arise *de novo* following their specification in the embryo.

In summary, our imaging and lineage tracing studies demonstrate that the first HSCs generated in the zebrafish embryo arise directly from haemogenic endothelium lining the ventral wall of the DA. These results complement previous studies in the avian[21], amphibian[22] and mammalian embryo[1,2,7] and show that the cellular mechanisms of HSC generation are highly conserved across vertebrate evolution. The finding that HSC development requires transition through a haemogenic endothelial intermediate should aid efforts to instruct HSC formation *in vitro* from pluripotent precursors, a necessity for therapies designed to replace the adult blood cell lineages.

METHODS SUMMARY

Tg(kdrl:HsHRAS-mCherry)^{s896} animals (referred to as *kdrl:memCherry* for clarity) were described previously[11]. *Tg(kdrl:Cre)*^{S898} and *Tg(bactin2:loxP-STOP-loxP-DsRed-express)*^{sd5} adults were mated, and their progeny screened for the presence of DsRed^{+} vasculature at 48 h.p.f. Positive embryos were raised to adulthood; some were sacrificed to analyse WKM at several ages (7 weeks to 6 months) by flow cytometry. Flow cytometry was performed as described[9], and Sytox was used as a vital dye to exclude dead cells. Imaging was performed on an SP5 deconvolution confocal microscope (Leica). For time-lapse imaging, double transgenic *Tg(kdrl:HsHRAS-mCherry)*^{s896}; *Tg(cmyb:eGFP)* embryos were first screened for fluorescence, then anaesthetized in tricaine and embedded in agarose. Time-lapse imaging was usually performed between 22 and 36 h.p.f. in an environmental chamber maintained at 28 °C. Raw data was analysed using Volocity software (Improvision), and exported in QuickTime format.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions J.Y.B., N.C.C. and D.T. designed experiments. J.Y.B. and D.T. wrote the manuscript, with key input from N.C.C. and D.Y.R.S.; J.Y.B. performed experiments. B.S. and S.T. generated and characterized the *bactin:switch* reporter line. N.C.C. and D.Y.R.S. generated *kdrl:Cre* and *kdrl:memCherry* transgenic lines.

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METHODS

Zebrafish husbandry. Zebrafish strains AB*, *Tg(kdrl:HsHRAS-mCherry)*^{s896} (ref. 11), *Tg(kdrl:RFP)*^{la4} (ref. 13), *Tg(cmyb:eGFP)* (ref. 10), *Tg(hsp70l:Cre)*^{zf36} (ref. 23), *Tg(kdrl:Cre)*^{s898}, and *Tg(bactin2:loxP-STOP-loxP-DsRed-express)*^{sd5}, were mated, staged and raised as described[24], and maintained according to UCSD IACUC guidelines.

Generation of transgenic animals. *Tg(kdrl:Cre)*^{s898} transgenic animals were generated following cloning of a 6.8 kb fragment of *kdrl* promoter/enhancer sequences[25] upstream of a promoterless Cre construct. The construct was cloned into the pIsceI meganuclease vector. We injected 200 pg of linearized DNA into one-cell-stage transgenic reporter embryos; founders were identified by screening for fluorescent progeny. Three *Tg(kdrl:Cre)* founders were recovered with identical expression patterns but varying levels. *Tg(kdrl:Cre)*^{s898} exhibited the strongest expression; this line was used for these studies. *Tg(bactin2:loxP-STOP-loxP-DsRed-express)*^{sd5} transgenic animals were generated as follows: a loxP-flanked transcriptional STOP cassette, which contains four SV40 late polyadenylation signals in tandem, was excised from pBS.DAT-LoxStop vector[26] and ligated upstream of the fluorescent gene in the pDsRed-Express-1 vector (Clontech). A 10.5 kb fragment immediately upstream of the *bactin2* translation start site was cloned by PCR in three fragments and sequentially ligated upstream of the STOP cassette. The 5′ boundary of the fragment is 5′-GGTAGAGCCTTACATTT CTTCGTATTCTCA-3′. The transgenic construct was then excised and ligated into a Tol2 transgenesis vector[27]. The resulting construct was co-injected with Tol2 mRNA into one-cell stage embryos to generate transgenic founders.

Flow cytometry. Embryos were collected at desired stages of development and anaesthetized in E3 medium containing 0.1 mg ml^{-1} tricaine (Sigma). Disaggregation into single-cell suspension was achieved as described previously[9]. Juveniles and adults were sacrificed at 7 weeks to 6 months, and WKM was dissected and mechanically resuspended to obtain single-cell suspensions. Flow cytometric acquisitions were performed on a FACS LSRII (Becton Dickinson), and cell sorting was performed on a FACS ARIA (BD). Analyses were performed using FlowJo software (Treestar).

RNA isolation and qPCR. RNA was isolated from sorted cells using an RNeasy kit (Qiagen), and cDNA was obtained using the qscript cDNA super mix (Quanta Biosciences, Gaithersburg, MD). qPCR reactions were performed using the Mx3000P QPCR system (Stratagene) according to the manufacturer's instructions (Stratagene). Biological triplicates were compared for each subset. For each independent experiment, elongation-Factor-1-alpha (*ef1a*) expression was scored for each population. The signals detected for each transcript of interest were then normalized to *ef1a*, data were analysed by the $\Delta\Delta C_t$ method according to manufacturer's recommended protocol (Stratagene), then normalized to expression in WKM that was defined as 100% for all analyses, except for *hbae1* where 8–12 somite stage (ss) *kdrl*$^{+}$ cells were used as the reference. Primers were designed with Primer3 software[28]. Primers: *cdh5-for*, TTCAAGAATCCT GTCATTGG; *cdh5-rev*, ATGTGCTGTAACCTGGAATG; *kdrl-for*, CTCCTGT ACAGCAAGGAATG; *kdrl-rev*, ATCTTTGGGCACCTTATAGC; primers for *lmo2*, *cd41*, *cd45* and *runx1* were described previously[9].

Fluorescent microscopy and time-lapse imaging. Embryos were imaged using a Leica SP5 inverted confocal microscope (Leica). GFP, DsRed and mCherry were excited by 488, 543 and 594 nm laser lines, respectively. For time-lapse imaging, embryos were embedded in agarose (0.7% in E3 medium) containing tricaine anaesthetic at a temperature of 30 °C. *z*-stacks were taken every 3 to 5 min. Movies were created following processing with Volocity software (Improvision).

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