Pdgf signalling guides neural crest contribution to the haematopoietic stem cell specification niche

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Haematopoietic stem cells (HSCs) support maintenance of the haematopoietic and immune systems throughout the life of vertebrates, and are the therapeutic component of bone marrow transplants. Understanding native specification of HSCs, to uncover key signals that might help improve in vitro directed differentiation protocols, has been a long-standing biomedical goal. The current impossibility of specifying true HSCs in vitro suggests that key signals remain unknown. We speculated that such signals might be presented by surrounding ‘niche’ cells, but no such cells have been defined. Here we demonstrate in zebrafish, that trunk neural crest (NC) physically associate with HSC precursors in the dorsal aorta (DA) just prior to initiation of the definitive haematopoietic program. Preventing association of the NC with the DA leads to loss of HSCs. Our results define NC as an important population to interrogate to find unknown HSC specification signals.

Trunk NC follow stereotypical migration routes, and give rise to a variety of adult structures including neurons, glia and endocrine cells of the parasympathetic and sympathetic nervous system (SNS), as well as pigment, depending on their location in the embryo6–8. An interplay of guidance cues and adhesive interactions controls migration and correct positioning of these cells6–8. Interestingly, trunk NC that become SNS neurons coalesce bilaterally along the length of the DA in zebrafish and mammals8,9, positioning them within signalling range of HE cells.

Here we demonstrate that trunk NC physically associate with the DA just prior to initiation of the haematopoietic program and are in close proximity to nascent runx1+ HSC precursors. We show that ventromedially directed migration of NC to the DA is dependent on platelet-derived growth factor (Pdgf) signalling, and that perturbing this path or other requirements for NC patterning leads to defective HSC specification. Interestingly, in mammals, trunk NC derivatives play important roles in the adult bone marrow HSC support niche10,11. Although the bone marrow niche is temporally, anatomically and functionally distinct from the specification niche, NC contribution to both suggests a potential point of continuity. Our results define a previously unknown cellular component of the HSC specification niche in a vertebrate.

RESULTS

Trunk NC contact the DA prior to and during HSC specification

We wanted to identify the origin of cells in contact with HE of the DA immediately prior to initiation of the definitive haematopoietic specification of normal HSCs arise from haemogenic endothelium (HE) in the ventral floor of the dorsal aorta (DA)1–3. Stromal cells residing in stem cell microenvironments often act as a ‘niche’ to contribute important cues that regulate behaviour1. It is very likely that HE cells receive specification signals from stromal cells constituting an HSC specification niche. To date, there have been no previous reports on the identity or origin of stromal cells required for HSC specification in the embryo, but such cells, if they exist, would be an important population to interrogate to find unknown specification signals. We hypothesized that cellular components of the specification niche might arise from migratory cells, providing a level of control over timing and presentation of inductive cues presented to the HE1,5.

The zebrafish represents an ideal model vertebrate system in which to address the origin of niche cells, owing to the high level of conservation in programming of haematopoietic ontogeny4 and its receptiveness to direct observation of cellular fate by time-lapse confocal microscopy using transgenic fluorescence reporter lines.

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RESULTS

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We wanted to identify the origin of cells in contact with HE of the DA immediately prior to initiation of the definitive haematopoietic

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program. To investigate the possibility that NC might migrate to the DA at the right time and in sufficient proximity to regulate HSC specification, we carefully analysed their localization over the critical time window. We tracked NC positions over 11 h of development, both by in situ hybridization for the zebrafish specific NC marker crestin12 relative to the arterial marker fnmb2a and by live time-lapse confocal imaging in double transgenic (Tg(fli1a:EGFP;sox10:mRFP)) embryos where enhanced green fluorescent protein (EGFP) is expressed in endothelial cells and membrane-bound red fluorescent protein (mRFP) in NC (Fig. 1a–h and Supplementary Fig. 1a–f and Supplementary Videos 1 and 2). The ventral migration of trunk NC initiates rostrally in the embryo and proceeds caudally6,7,12, with the first migration of cells that include SNS progenitors between somite and neural tube visible by 16 h post fertilization (hpf)13,14 and continuing over the next several hours12 with some streams reaching halfway around the notochord and others reaching the dorsal aspect of the DA by 20–21 hpf (Fig. 1b–d and Supplementary Fig. 1a,b and Supplementary Videos 1 and 2). By 23 hpf, the time when the onset of the definitive haematopoietic program is first observable by expression of the conserved HSC precursor marker runx1 in the HE13,15, trunk NC had migrated into close proximity with the DA (Fig. 1a–a′,i–i′ and Supplementary Fig. 1d and Supplementary Videos 1 and 2). A separate second stream of pigment-fated NC, which migrate ventrolaterally between the somite and overlying ectoderm as opposed to ventromedially7, had also begun their migration by this stage (Fig. 1i). By 24 hpf, ventromedially migrating NC were in contact with the DA endothelium in the sub-aortic space (Fig. 1g,j–j′ and Supplementary Fig. 1e). NC remained in close association with the vasculature through 30 hpf and very likely into adulthood, given their contribution to the SNS. Thus, trunk NC achieve close contact with the DA immediately prior to and during initiation of the definitive haematopoietic program, and are therefore present at the right time and place to instruct HSC specification.

**Pdgf signalling is required for ventromedial migration of trunk NC**

If NC association with the DA is required for initiation of definitive haematopoietic programming, preventing this association should result in loss of HSCs. Pdgf signalling regulates key cell migration events in the embryo13 including cranial NC migration7,14, although the role of Pdgf signalling in trunk NC migration has not been well defined14. We confirmed expression of pdgfra and pdgfrb in trunk pre-migratory NC15,16 (Supplementary Fig. 2a–f). Consistent with previous reports16, we did not observe expression of either receptor in endothelial cells by in situ hybridization through 24 hpf, when HSC specification initiates (Supplementary Fig. 2a–f), excluding any cell-autonomous role for Pdgf signalling in HSC specification. We were, however, able to confirm the previously reported expression of pdgfrb in the floorplate and hypochord16 as well as both receptors in the ventral somite15,16. Pdgfra interacts with Pdgf (Pdgfaa and Pdgfab paralogues in zebrafish) and Pdgfc ligands, while Pdgfrb interacts with Pdgfb ligands13. We found that pdgfab and pdgfc are expressed in the medial aspect of the somite, along the ventromedial migratory route of trunk NC, prior to and during migration (E.W.D., manuscript in preparation). These results demonstrate that Pdgf receptors and ligands are expressed in a pattern consistent with a role in controlling migration of NC to axial structures such as the DA.

We directly tested the requirement for Pdgf signalling in NC migration by examining trunk crest migratory behaviour in embryos where Pdgf signalling was impaired by pharmacological inhibition, in animals where Pdgf receptors were knocked down by morpholino injection, and in animals mutant for pdgfra (Fig. 2 and Supplementary Fig. 3). Although the abundance of crestin+ NC appeared unchanged in animals treated with the Pdgfr tyrosine kinase inhibitor V (inhV)17, which inhibits both receptor isoforms, the progress of ventromedial trunk NC migration was arrested at the horizontal myoseptum, approximately even with the notochord (Supplementary Fig. 3a–b′.g). To confirm a requirement for Pdgf signalling we knocked down expression of individual receptors by injection of morpholinos targeting either pdgfra18 or pdgfrb19. As with inhV treatment, crestin levels initially appeared normal, but NC migration was significantly arrested at the horizontal myoseptum (Fig. 2a–b′,e and Supplementary Fig. 3c–e′,g). Interestingly, NC migration was affected in only 48% of Pdgfrb morphants (Supplementary Fig. 3d–e′,g), whereas in Pdgfra single morphants and in Pdgfra/b double morphants, where PdgfraMO was injected at a sub-effective dose, migration defects were nearly fully penetrant and had not recovered by 36 hpf (Supplementary Fig. 3f–i). Since Pdgfra appeared to be the primary receptor required, we confirmed this requirement in mutant animals carrying a hypomorphic allele of pdgfra (pdgfra+1059, ref. 19; Fig. 2c–e). Finally, we examined NC migration relative to axial vasculature by time-lapse imaging in Pdgfra morphant Tg(fli1a:EGFP;sox10:mRFP) double transgenic embryos (Fig. 2f–m and Supplementary Videos 1–4). Trunk NC delaminated normally from the neural tube and were migrating ventromedially between 20 hpf and 21.5 hpf (Fig. 2h–j and Supplementary Fig. 4a–c and Supplementary Videos 3 and 4). At 23–24 hpf, when haematopoietic programming initiates, trunk NC in morphant animals had still not progressed beyond the limit of the ventral notochord (Fig. 2b,k and Supplementary Fig. 4a–e and Supplementary Videos 3 and 4), whereas they had contacted the DA in uninjected controls (Fig. 1 and Supplementary Fig. 1a–e and Supplementary Videos 1 and 2). We never observed migration of NC streams beyond the ventral aspect of the notochord in Pdgfra morphino-injected animals through 30 hpf by time-lapse imaging (Supplementary Figs 3k and 4 and Supplementary Videos 3 and 4), or 36 hpf by in situ hybridization (Supplementary Fig. 3h,i), and NC never contacted the DA (Fig. 2 and Supplementary Figs 1, 3h–k and 4 and Supplementary Videos 3 and 4). Trunk NC migrating along the ventrolateral migration pathway were unaffected (Supplementary Fig. 3i,k). Our results demonstrate that Pdgf signalling is required for migration of medial stream trunk NC, probably SNS progenitors, beyond the ventral aspect of the notochord to a point of contact with the DA.

**Pdgf signalling is required for HSC specification**

Since NC association with the DA was prevented in Pdgfa-deficient embryos, we examined specification of HSCs in these animals. We looked for expression of runx1, a master regulator and marker of definitive haematopoiesis13,20, at 24 hpf and cmyb+ HSC precursors20 at 33 hpf in the DA. We also examined rag1 expression in the paired thymi at 4 days post fertilization (dpf), which labels T lymphocytes1,21,22 that necessarily develop from an HSC precursor and are lost in embryos where definitive haematopoiesis fails1,21,22. HSCs and T
Figure 1 NC contact the DA prior to and during HSC specification. 
(a,a’) Progress of NC migration, marked by crestin expression relative to
the DA, marked by efnb2a expression in trunk lateral view (a; dorsal up,
 anterior left) or in a transverse section of the same embryo (a’; dorsal
up) at 23 hpf. Level of transverse section is indicated (red bar, a); yellow
arrowheads indicate ventral limit of NC progress. Scale bars, 100 µm.
(b–h) Live lateral z-stack volume projection of NC migration through the trunk
using the NC reporter sox10:mRFP in comparison with fli1a:EGFP-
labelled endothelium (b; scale bar, 100 µm) or still images of virtual transverse
trunk sections from the same embryo with dorsal up in single and merged
channels (c–h) from time-lapse confocal microscopy from 20 hpf to 27
hpf. The dorsal extent of the DA is indicated by the dotted line; the
position of the transverse section projection is indicated by the white box
in b (scale bar, 50 µm, magnification x200); yellow arrowheads indicate
ventral limit of NC progress. (i–j’) Confocal transverse mid-trunk section
dorsal up) at 23 hpf (i,i’) and 24 hpf (j,j’) showing proximity of NC to
the DA (magnification x400; scale bar, 50 µm; i,j). The white boxes in
i,j indicate the zoomed region in i’; yellow arrowheads in i,j’ indicate
NC in close proximity to the DA; white arrowheads indicate cells of the
pigment cell lineage migrating ventrolaterally (i,j) (scale bar, 50 µm, i’, j’). nt,
nose; no, notochord; da, dorsal aorta; pcv, posterior cardinal vein; isv,
intersegmental vessel.

cells were absent or dramatically reduced in Pdgfra-inhibited embryos
(Fig. 3a–n) demonstrating that Pdgfra-mediated signalling is required
for the initiation of the haematopoietic program and specification of
HSCs. Double staining for crestin confirmed defective NC migration
in HSC-deficient animals (Fig. 3a,d,g,j). The defect in T lympho-
cyte development was not due to a requirement for Pdgfr signalling

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in thymic immigration because inhibition of Pdgfr signalling with inhV affected rag1 expression only if embryos were treated prior to the specification of HSCs (Supplementary Fig. 5l–q) and not during developmental stages when thymic colonization is occurring. Runx1+/+ cells were reduced or absent in the DA of inhV-treated embryos (Supplementary Fig. 5a,b,k) and in embryos conditionally expressing a
Figure 3. Pdgfra is required for HSC specification. (a–l) Lateral trunk views (a,b,d,e,g,j; dorsal up, anterior left) of expression of the HSC marker runx1 (blue) and the NC marker crestin (red; a,d,g,j), the HSC marker cmyb (b,e,h,k), and ventral head views of the T lymphocyte marker rag1 (c,f,i,l) in uninjected (a–c), Pdgfra morphant (d–f), wild-type and pdgfra<sup>b1059</sup> heterozygous (g–i) and pdgfra<sup>b1059</sup> homozygous mutant sibling (j–l) embryos at the times indicated. Green arrowheads, runx1<sup>+</sup>; red arrowheads, cmyb<sup>+</sup>; blue arrowheads, rag1<sup>+</sup> cells when present (a–l); blue brackets enclose the DA–NC interaction region (a,d,g,j). The numbers in the bottom right corner indicate numbers of zebrafish embryos analysed for the indicated gene, versus the total number of zebrafish analysed. Zebrafish randomly selected from three clutches are represented by the image (a–l). Scale bars, 100 µm.

(m,n) Average number of runx1<sup>+</sup> (m) or cmyb<sup>+</sup> (n) cells in each condition (for uninjected and Pdgfra morphants, 20 zebrafish embryos were randomly selected from three clutches; for wild type and pdgfra mutants, 20 zebrafish embryos exhibiting the phenotypes (inferred mutants) displayed in g–l were selected from three clutches). (o) Average number of mCherry<sup>+</sup>GFP<sup>+</sup> cells in the DA in each condition (for uninjected and Pdgfra morphants, 20 zebrafish embryos from two clutches each). Error bars indicate standard error of the mean (m–o). ***P < 0.0001 by Student’s t-test for each (m–o). See Supplementary Table 3. (p,q) Close-up lateral views of the trunk vasculature with fluorescently labelled HSCs in uninjected (p) and Pdgfra morphant (q) cmyb:GFP;kdrl:mCherry transgenic embryos at 36 hpf; HSCs are yellow cells indicated by yellow arrowheads. Scale bars, 50 µm; magnification ×200. (r–r′) Co-localization (indicated by white arrowheads) of runx1<sup>+</sup> (blue) in a lateral trunk view of the DA and crestin<sup>+</sup> NC (red) (scale bar, 100 µm (r) and 50 µm (r′)); and cross-section (scale bar, 100 µm (r″)) at 24 hpf. Magnification ×160. nt, neural tube; no, notochord; da, dorsal aorta.
heat-shock-inducible YFP-fused dominant-negative pdgfrb transgene (Tg(lacZ:Gal4;UAS:dnRag1))6, which very likely inhibits both Pdgfrs due to receptor heterodimerization13. HSC emergence from HE between 26 hpf and 36 hpf (ref. 3) can be visualized as GFP+ mCherry+ cells in Tg(cmyb:GFP;kdr1:mCherry) double transgenic embryos3. We observed a drastic reduction in the number of emerging HSCs in Pdgfr morphants compared with un-injected controls (Fig. 3o–q). Last, we confirmed a recently published contribution of Pdgfrb signalling to HSC specification25. Runx1+ cells were moderately reduced in about 41% of Pdgfrb morphants (Supplementary Fig. 5f–h,k), phenocopying a pdgfrb mutant allele25, and this reduction could be enhanced by co-injection of a sub-effective dose of Pdgfra morpholino (Supplementary Fig. 5f–k), indicating a genetic interaction between these receptors during initiation of the haematopoietic program and that Pdgfr is the dominant receptor involved.

The presence of a normal DA is a prerequisite for HSC specification1–3, so we wanted to determine the integrity of pre-haematopoietic tissues in Pdgfr-deficient embryos. The morphology of Pdgfra- and Pdgfrb-inhibited embryos was grossly normal at 24–26 hpf (Supplementary Fig. 2g–l), with beating hearts and circulating gata1+ primitive erythroid cells (which do not derive from an HSC; Fig. 4a–c,s), although occasionally embryos exhibited yolk sac oedema and a ventrally directed bend in the tail (Supplementary Fig. 2g–l). Pre-haematopoietic mesoderm marked by tal1 (also known as scl)1,5 was present (Fig. 4d–f,s) and cdh5 (also known as ve-cadherin) expression revealed normal endothelium1,5 (Fig. 4g–i,s). Importantly, efnb2a, notch1b and gata2b expression together revealed that the DA and HE were present and patterned normally1,5,24 (Fig. 4j–s). These results demonstrate that haematopoietic competent endothelium is present in Pdgfr-signalling-deficient embryos and suggest that the role of Pdgf signalling in HSC specification is to trigger initiation of the haematopoietic program. As we did not observe Pdgfr receptor expression in the endothelium itself26 (Supplementary Fig. 2a–f), the requirement for Pdgf signalling must reflect its necessity in some other tissue such as NC, probably through regulation of availability of a relay signal.

Trunk NC association with the DA is required for HSC specification

Our results are consistent with a model where trunk NC present a signal or signals, to the HE that is required for HSC specification. NC make direct contact with nascent runx1+ HSCs in the DA (Fig. 3r–t) at high frequency (76%; n = 15 zebrafish embryos). These results suggest that NC interaction with HE is required for HSC specification; however, since pdgfra and pdgfrb are expressed in tissues beyond NC, we wanted to confirm that NC are indeed required by preventing crescent specification or migration using multiple additional perturbations. Transcription factor AP-2 alpha (Tfap2a)7,25, sox1026 and syndecan4 (sdc4)7,27 are known regulators of NC specification and migration, and multiple morpholinos and mutants targeting these genes are available that allow independent means of disrupting NC association with the HE. None of these genes is expressed in the DA endothelium during HSC specification25,27. NC specification and migration was prevented and medial crest cells did not contact the DA in embryos injected with morpholinos targeting tfap2a, sox10 and sdc4, as well as in tfap2a213 and sox10241 mutants (Fig. 5a,d,g,i,o and Supplementary Figs 6a,d,g,j,m,r and 7a–h)25–27. Interestingly, migration of ventromedially migrating NC that contact the DA was less strongly affected in the sox10 mutant, colourless, despite severe defects in the development of the ventrolaterally migrating pigment precursors (Supplementary Figs 6g,t and 7e,f,l–w). As previously described, the Sox10 morpholino phenocopied the pigment phenotype of the sox10+241 mutant28 (Supplementary Fig. 7v–v’), but had a stronger effect on the ventromedially migrating population of NC (Fig. 5a,d,o). The Tlapa2aMO phenocopied the pigment development delay of the tfap2a213 mutant25 (Supplementary Fig. 7x–x’) and caused aberrant splicing of tfap2a messenger RNA (Supplementary Fig. 7a) as described previously25. Runx1+ and cmyb+ cells in the DA at 24–26 hpf and 33 hpf respectively, as well as rag1+ T lymphocytes at 4 dpf, were significantly reduced or absent in Tlapa2a, Sox10 and Sdc4 morphants and in tfap2a213 and sox10241 homozygous mutant embryos (Fig. 5a–n and Supplementary Figs 6a–q and 7i–p), indicating defective HSC specification. Strikingly, rare runx1+ cells that we were able to find in sox10+241 mutants, as well as Tlapa2a and Sdc4 morphants that had only partial defects in ventromedial NC migration, were usually at the locations where crest had reached the DA, strongly suggesting that close proximity is required for HSC specification (Supplementary Fig. 6d,j,m). We further observed significantly reduced numbers of emerging HSCs at 36 hpf in Tg(cmyb:GFP;kdr1:mCherry) embryos injected with Tlapa2aMO, Sox10MO or Sdc4MO (Fig. 5p–s and Supplementary Fig. 6s–u). Tlapa2a, Sox10 and Sdc4 morphant embryos were grossly normal, had beating hearts and circulating primitive erythroid cells further confirmed by gata1 expression (Fig. 6a–d,y and Supplementary Fig. 7q–t), although Sdc4 morphants occasionally exhibited a bend in the posterior aspect of the tail (Supplementary Fig. 7s). The expression of tal1, cdh5, efnb2a, notch1b and gata2b confirmed that, as in Pdgfr-deficient embryos, pre-haematopoietic tissues had developed normally in these animals (Fig. 6e–y). In all, ten independent means of preventing NC association with the pre-haematopoietic DA—pharmacologically, by five different morpholinos, one dominant-negative transgenic, and three mutants—result in significantly impaired specification of HSCs. Our results demonstrate a requirement for the association of trunk NC with the DA for initiation of the haematopoietic program and the specification of HSCs, very likely by presentation of one or more short-range inductive signals triggering the initiation of the haematopoietic programming.

Catecholamine neurotransmitters are not required for HSC specification

A previous report implicated SNS neurons, derivatives of early trunk NC, in the emergence of HSCs in mouse embryos through catecholamine neurotransmitter signalling29. Biosynthesis of catecholamines requires the enzymes tyrosine hydroxylase (th) and dopamine-β-hydroxylase (dbh)30–32, both of which are also markers of overt SNS neuronal differentiation31,32. Expression of th and dbh in zebrafish trunk SNS neurons is not present until at least 36 hpf (ref. 31), well after initiation of haematopoietic programming at 23–24 hpf. We confirmed that these genes were not expressed during the initiation of haematopoietic programming and that even by 5 dpf expression was present only in the superior cervical ganglion (Fig. 7a–f). To confirm that early inductive signals are not catecholamine synthetic enzymes, we disrupted their synthesis with a morpholino against th32.
Th morphants had normal numbers of *runx1*- and *cmyb*-expressing cells at 24 and 36 hpf respectively, despite strongly reduced levels of *th* mRNA in morphants (Fig. 7g–m). Our results indicate that while catecholamine signalling may be important for early HSC maintenance, pre-neuronal trunk NC must contribute a different, earlier signal required for initiation of haematopoietic programming.

**DISCUSSION**

Our results demonstrate that trunk NC migration to the DA, regulated by Pdgf signalling, is required for HSC specification. We propose that these cells contribute to a cellular HSC specification 'niche', although it is possible and very likely that additional cell types may also be required. NC very likely present pro-haematopoietic inductive
Figure 5 NC are required for HSCs. (a–l) Lateral trunk views (a,b,e,g,h,j,k; dorsal up, anterior left) of expression of the HSC marker runx1 (blue) and the NC marker crestin (red; a,d,g,j), the HSC marker cmyb (b,e,h,k) and ventral head views of the T lymphocyte marker rag1 (c,f,i,l) in uninjected (a–c), Sox10 morphant (d–f), wild-type and tfap2a<sup>ts1213</sup> heterozygous (g–i), and tfap2a<sup>ts1213</sup> homozygous mutant (j–l) embryos at the times indicated. Green arrowheads, runx1<sup>+</sup>; red arrowheads, cmyb<sup>+</sup>; blue arrowheads, rag1<sup>+</sup> cells when present; blue brackets enclose the DA–NC interaction region (a,d,g,j). The numbers in the bottom right corner of panels indicate numbers of zebrafish embryos analysed for the indicated gene, versus the total number of zebrafish analysed. Zebrafish randomly selected from three clutches are represented by the image (a–l). Scale bars, 100 µm. (m–o) Average number of runx1<sup>+</sup> (m) or cmyb<sup>+</sup> (n) cells in each condition and average number of NC streams ventral to the notochord at 24 hpf for the indicated conditions (o) (n = 20 randomly selected zebrafish embryos from three clutches each; m–o). (p–r) Close-up lateral views of the trunk vasculature with fluorescently labelled HSCs in uninjected (p), Sox10 morphant (q) and Tfap2a morphant (r) Tg(cmyb:GFP; kdrl:mCherry) transgenic embryos at 36 hpf; HSCs are yellow cells indicated by yellow arrowheads; scale bars, 50 µm; magnification x200. (s) Average number of mCherry<sup>+</sup>GFP<sup>+</sup> cells in the DA in each condition (n = 8 zebrafish embryos (p,q), and n = 7 zebrafish embryos (r) from two clutches). Error bars indicate the standard error of the mean (m–o,s). ***P < 0.0001 by Student’s t-test for m–o; ***P = 0.0004 for s. See Supplementary Table 3.
Figure 6 Arterial HE and primitive blood are normal in NC-impaired animals. (a–x) Lateral trunk views (dorsal up, anterior left) of in situ hybridizations for markers of primitive erythrocytes (gata1, a–d), pre-haematopoietic mesoderm (tal1, e–h), vascular endothelium (cdh5, i–l), arterial identity (efnb2a, m–p; notch1b, q–t) and HE gata2b (u–x) in uninjected (left column), Tfap2aMO (middle left column), Sox10MO (middle right column) or Sdc4MO (right column) embryos at 24 hpf. Scale bars, 100 µm. (y) Percentages of embryos exhibiting the specified phenotypes relative to the appearance of expression in uninjected controls with total number (n) of embryos examined. See Supplementary Table 2.

signals to haemogenic precursors in the HE and thus recommend themselves as a population to be interrogated to identify unknown HSC specification signals. The proximity of NC to runx1+ cells, in wild-type embryos and in perturbations where there are rare, properly migrated NC, suggests that such a signal is likely to act at short range.

Our results also provide a demonstration that Pdgf signalling regulates migration of the trunk NC. Pdgfra mutant mice and zebrafish exhibit severe craniofacial defects due to defective migration and increased apoptosis of the cranial NC14,19,33, as well as aortic arch and ventricular septal defects as a result of defective cardiac NC migration33. In the trunk, we find that Pdgf signalling regulates specifically the ability of ventromedially migrating NC to pass the horizontal myoseptum, although whether the requirement is instructive or permissive is not clear. In all cases, pdgfra appears to be the key
Figure 7 Catecholamine biosynthesis enzymes are not required for HSC specification. (a–f) Lateral views (dorsal up, anterior left) of expression of the catecholamine biosynthesis enzymes tyrosine hydroxylase (th; a,c,e) and dopamine-β-hydroxylase (dbh, b,d,f) during (a,b) and after (c–f) HSC specification. Yellow arrowheads indicate expression in neurons of the head; red arrowheads indicate expression in the superior cervical ganglion. The numbers in the bottom right corner of panels indicate numbers of zebrafish embryos analysed for the indicated gene, versus the total number of zebrafish analysed. Zebrafish randomly selected from one clutch (a–f) or two clutches (g–j) are represented by the image. Scale bars, 200 µm. (g–j) Expression of the HSC markers runx1 (g,i) and cmyb (h,j) in uninjected (g,h) or Th morphant (i,j) embryos, at the indicated developmental stages; green and red arrowheads indicate runx1+ and cmyb+ cells respectively in two separate experiments. Scale bars, 100 µm. (k) RT-PCR showing a reduction in the amount of th mRNA in Th morphant compared with uninjected embryos. (l,m) Average number of DA runx1-expressing (l) and cmyb-expressing (m) cells in uninjected and Th morphants; n = 20 randomly selected zebrafish embryos; error bars indicate the standard error of the mean; NS, result not significant, P = 1.000 (l) and P = 0.0689 (m) by Student’s t-test. See Supplementary Table 3. Number of embryos displaying the depicted expression is indicated. Unprocessed original scans of blots are shown in Supplementary Fig. 8.

receptor involved. NC in the trunk that follow the ventromedial migration path give rise notably to sympathetic neurons. Pigment cell precursors, which migrate ventrolaterally, were unaffected. As sympathetic neurons remain in association with the DA to adulthood, it seems probable that NC-derived specification niche cells contribute to the SNS, although we cannot exclude the possibility that different derivatives are involved. Pdgf signalling also regulates migration of additional cell types, such as vascular smooth muscle cell precursors, so it is conceivable that the requirement for Pdgf signalling in establishing the specification niche extends beyond the direction of NC cell migration.

NC contribute both to the specification niche and the adult homeostasis niche in the bone marrow; however, the two regions are temporally, anatomically and functionally distinct. Whereas the purpose of the specification niche is to establish the haematopoietic program in endothelial cells de novo and promote the mobilization
to embryonic expansion tissues while preserving stemness, the adult niche directs diverse behaviours, such as choices between quiescence and proliferation, self-renewal and differentiation, circadian egress, and probably lineage priming. The mammalian bone marrow contains at least two kinds of trunk NC derivative: sympathetic neurons and nestin+ mesenchymal stem cells (MSCs). Nestin+ MSCs are perivascular cells that regulate HSC quiescence and are innervated by adrenergic sympathetic neurons. At least some of these MSCs are PDGFRα+ and have HSC support activity. Sympathetic neurons regulate mobilization of HSCs from the adult niche by stimulating noradrenergic signalling-dependent downregulation of CXCL12 (also known as SDF1). Thus, NC or their derivatives direct stem and mobilization behaviours both during HSC specification and in the adult niche, raising the possibility that the same or similar HSC signal transduction programs are activated in both cases. As we move forward, it will be important to better understand the molecular nature of the NC embryonic HSC specification signals, both for use in directed differentiation and possibly as a tool for regulation of adult HSCs.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of this paper.

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

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AUTHOR CONTRIBUTIONS

E.W.D. and W.K.C. designed all experiments. E.W.D. performed all experiments. E.W.D. and W.K.C. analysed all results and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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**METHODS**

**Zebrafish husbandry.** Zebrafish were maintained and crossed as previously described. All protocols were approved by the IACUC and AVMA, and all experiments were performed in accordance with all IACUC and AVMA guidelines. For details, see Table 1.

**Cloning, constructs and RT–PCR.** pdgfra (RefSeq: NM_131149) was amplified from 24 hpf cDNA; th (RefSeq: NM_131149) and dbh (RefSeq: NM_001109694) were amplified from 96 hpf cDNA. For all embryos where pdgfrb was used, strain cDNA was used in all cases. See Supplementary Table 1 for primer sequences. PCR reactions were run with an annealing temperature of 52°C for 35 cycles. pdgfra was cloned into pCRII-TOPO (Invitrogen) according to the manufacturer's instructions. cDNA was generated from purified RNA using the SuperScript III First-Strand Synthesis System for RT–PCR (Invitrogen) as indicated.

**In situ hybridization.** In situ hybridization probes were produced as previously described using either digoxigenin- or fluorescein-labelled NTPs (Roche); constructs were linearized (restriction enzymes from NEB) and transcribed (polymerase from Roche) with: pcS2+ runx1 (EcoRI, T; C. Burns), pBK-CMV cmyb (BanHI, T; L. Zon), pCRII cagl (HindIII, T; N. Trede), pBS efna2a (Xhol, T; D. Ransom), pCRII gata2b (Nhel, T; D. Traver), pCRII crestin (BanHI, T; T. M. Lardelli), pC532.2 pdgfrb (Xmal, SP6; B. Appel), pC52.2 pdgfrb (NotI, SP6), pCRII–TOPO dbh (Xbal, SP6), pCRII–TOPO dbh (NotI, SP6). For RT–PCR experiments, RNA was isolated from un.injected, th morphant, or thap2a2 morphant 24 hpf embryos using Trizol reagent (Ambion) according to the manufacturer's instructions. PCR reactions were run with an annealing temperature of 51°C for 35 cycles and products were visualized in 1% agarose gel. Product sizes were determined by comparing product bands with bands of known size from the SuperScript III First-Strand Synthesis System for RT–PCR (Invitrogen) according to the manufacturer's instructions. PCR reactions were run with an annealing temperature of 51°C for 34 cycles and products were visualized in 1% agarose gel. Product sizes were determined by comparing product bands with bands of known size from the GeneRuler RNA Ladder Mix (Invitrogen no. SM0331).

**Whole-mount in situ hybridization (WISH), microscopy and embryo sectioning.** Whole-mount in situ hybridization was performed as previously described. NBT/BCIP solution (Roche) and Fast Red solution (Sigma) were used for the colorimetric reaction to detect digoxigenin- and fluorescein-labelled probes respectively. For all embryos where in situ hybridization was to be performed after 22 hpf, except thap2a and sox10 mutants and where th or dbh expression was to be examined, melanin production was inhibited by treatment of embryos with 1-phenyl-2-thiourea (PTU) at 22 hpf. Since the development of pigmentation in thap2a and sox10 mutants was used as an indicator of the zygosity of the mutant alleles, pigmentation was either left intact or embryos were bleached post-fixation by hydrogen peroxide treatment. For embryos being examined for th and dbh expression, hydrogen peroxide treatment was used to remove pigment as PTU treatment has been shown to inhibit the development of catecholaminergic neurons. Embryos were photographed in 3% methyl cellulose using a Leica DFC310FX camera mounted on a Leica M205FA stereo. Unless otherwise indicated, at least three independent clutches of embryos were examined in each experiment. For quantification of runx1- and cmyb-expressing cells, unless otherwise indicated, twenty embryos from three independent clutches were quantified. In all cases except for experiments involving the pdgfra allele, the quantified embryos were randomly selected. For experiments involving the pdgfra allele, homozygous embryos were inferred on the basis of phenotypic ratio and phenocopy of the Pdgfra morphant phenotype. Confocal images of live embryos and fixed tissues were acquired using a Nikon C2 laser scanning confocal system using a 20× Plan-Apo (0.75 NA) or a 40× Plan-Fluor (1.3 NA) lens as indicated. For live z-stack time-lapse imaging, embryos were anaesthetized in 0.04% tricaine solution and embedded in 0.4% low-melting-point agarose; z-stack thickness ranged from 40µm to 50µm with an optical section thickness ranging from 2µm to 2.5µm; time-lapse length was 10 h, pinhole size: 1.2 AU, with 488 nm and 561 nm laser lines set at 5% power. Five uninjected and five PdgfraMO-injected embryos were filmed in total; each was from a different clutch and filmed on different days. Analysis was performed with Nikon Elements 4.30. Tg(cmyb:GFP; kdr:mCherry) embryos were fixed with 4% paraformaldehyde at the stages indicated and were embedded in 4% low-melt agarose prior to imaging. Confocal Z-stacks of Tg(cmyb:GFP; kdr:mCherry) embryos ranged between 30µm and 40µm with an optical section thickness of 2.5µm and the 488 nm and 561 nm laser lines set at 10% and 5% respectively, pinhole size: 1.2 AU. Tg(fli1a:EGFP; sox10:mRFP) embryos were fixed in 4% paraformaldehyde and embedded in 3.5% agarose. Sections (50µm) were generated on a Leica VT1200 vibratome and mounted in glass-bottom dishes (MatTek) for imaging on the confocal microscope, pinhole size: 1.2 AU, 488 nm laser power: 10%, 561 nm laser power: 5%.

**Genotyping.** Ssox10 and thap2a homozygous embryos were identified by established defects in embryo pigmentation and additionally, defective neural crest cell migration as indicated by crest expression. For pdgfra embryos, numbers of embryos indicate the fraction of total embryos in a heterozygous–incross-derived clutch—which included wild-type, heterozygous and homozygous individuals—that display the depicted phenotype. Genotype conclusions were inferred from NC migratory and haematopoietic phenotype. Observed phenotypic percentages, 21/99 embryos (21.2%) scored as decreased runx1, 18/85 (21.2%) decreased cmyb, and 19/72 (26.3%) decreased ntl, roughly conform to the expectation of 25% homozygous mutant individuals from a heterozygous incross. NC migration phenotypes, in embryos old enough, cosegregate with and confirm genotypic assignment.

**Statistics and reproducibility.** Student's t-test was used with a 95% confidence level for all experiments. Unless otherwise indicated, at least three independent clutches of embryos were examined. No statistical method was used to predetermine sample size. Individual embryos were selected from pools of embryos and assigned to uninjected or morpholino-injected categories randomly. Individual uninjected, morphant, wild-type and mutant embryos were randomly selected for examination of gene expression and time-lapse microscopy. The investigators were not blinded to allocation during experiments and outcome assessment. Total embryo numbers are indicated in the figure legends or Supplementary Table 2.

**Data availability.** Source data for Figs 2e, 3m–o, 5m–o, s, 6y and 7l,m and Supplementary Figs 3g, 5k and 6–r,t have been provided as Supplementary Table 3. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

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Supplementary Figure 1 NC contact the dorsal aorta prior to and during HSC specification. Trunk lateral views (a-f; dorsal up, anterior left) of NC migration progress using the NC reporter sox10:mRFP relative to endothelial cells labeled with EGFP in fli1a:EGFP animals. Images are still frames from a z-stack volume projection in single and merged channels (a-f) from time lapse confocal microscopy from 20 h.p.f. to 27 h.p.f. as indicated. Arrowheads indicate NC streams that are positioned ventral to (yellow arrowheads) or dorsal to (blue arrowheads) the notochord. Scale bar = 100 μm, mag. = 200X. isv – intersegmental vessel, da – dorsal aorta, pcv – posterior cardinal vein.
Supplementary Figure 2  Pdgfr expression before and after HSC specification. Expression of *pdgfra* (a–c") and *pdgfrb* (d–f") in wild-type embryo trunk in either lateral view (a–f; dorsal up, anterior left), transverse section (a'–f'; dorsal up, level indicated by red line in a–f), and dorsal trunk view (a"–f"'; anterior up) prior to (18 h.p.f., 21 h.p.f.) (a–b", d–e") and during (24 h.p.f.) (c–c", f–f") HSC specification. Developmental stages are indicated. Numbers in the bottom right corner of panels indicate numbers of zebrafish embryos analysed for the indicated gene, versus the total number of zebrafish analysed. Zebrafish randomly selected from one clutch are represented by the image (a–f"). Scale bars = 100 μm. Lateral views (dorsal up, anterior left) of whole uninjected, Pdgfra, Pdgfrb and Pdgfra/b double morphant, DMSO and inhV treated embryos at the developmental stages indicated, demonstrating overall normal morphology (g–l). Scale bars = 200 μm.
Supplementary Figure 3 Pdgfr-mediated signalling is required for NC migration to the DA. NC migration visualized by crestin expression in lateral (dorsal up, anterior left) and transverse (dorsal up) views of DMSO (a-a’) or inhV (b-b’) treated embryos, uninjected embryos (c-c’), a subset of embryos injected with 10 ng of PdgfrbMO displaying normal NC migration (d-d’) and embryos injected with both PdgfraMO and PdgfrbMO (f-f’). Numbers in the bottom right corner of panels indicate numbers of zebrafish embryos analysed for the indicated gene, versus the total number of zebrafish analysed. Zebrafish randomly selected from three clutches are represented by the image (a-f, h-i). Positions of transverse sections are indicated by the red bars (a-f). Scale bars = 100 μm (a-f, h-i). Average number of NC streams ventral to the notochord at 24 h.p.f. for the indicated conditions, error bars indicate standard error of the mean, n=20 is the number of randomly selected zebrafish embryos from three biologically independent clutches each, the triple asterisk indicates a statistically significant result between bars, from left to right, p-value was < 0.0001 by Student’s T-test in all cases (g), see Supplementary Table 3. Lateral views (dorsal up, anterior left) of crestin expression in uninjected (h) and Pdgfra morphant (i) embryos at 36 h.p.f. NC streams that have migrated ventral to the notochord are indicated by yellow arrowheads (a-f, h-i), red brackets enclose the AGM region (a-f), green arrowheads indicate the presence of melanophores (h-i). Lateral z-stack volume projections of the trunk (dorsal up, anterior left) with vasculature in green (fli1a:EGFP) and NC in red (sox10:mRFP) in uninjected (j) and Pdgfra morphant (k) embryos, blue (k) and yellow (j) arrowheads indicate NC streams which are dorsal to and ventral to the notochord respectively, scale bar = 100 μm, mag. = 200X. nt – neural tube, no – notochord, DA – dorsal aorta, PCV – posterior cardinal vein.
Supplementary Figure 4  Pdgfra-mediated signalling is required for NC migration to the DA. Lateral z-stack volume projections of the trunk (dorsal up, anterior left) with vasculature in green (fli1a:EGFP) and NC in red (sox-10:mRFP) in embryos injected with Pdgfra MO (a-f). Still frames from time lapse confocal microscopy from 20 h.p.f. to 27 h.p.f. as indicated. Blue arrowheads indicate NC streams that are dorsal to the notochord. Scale bars = 100 μm, mag. = 200X. isv – intersegmental vessel, da – dorsal aorta, pcv – posterior cardinal vein.
Supplementary Figure 5 Pdgfr signalling is required for HSC specification. Lateral trunk views (dorsal up, anterior left) of expression of the HSC marker runx1 in DMSO (a) and inhV treated embryos (b) or dnpdgfrb:YFP; hsp70::GAL4 embryos that were either heat shocked (c, d) or not heat shocked (e), wild-type uninjected embryos (f), subset of Pdgfrb morphants displaying normal HSC specification (g) a subset of Pdgfrb morphants displaying defective HSC specification (h), Pdgfra morphants (i) and low-dose Pdgfra and Pdgfrb combined morphants (j) at 24 h.p.f. Green arrowheads identify runx1 expressing HSC precursors. Average number of runx1+ cells in the DA for the indicated conditions (k), n is the number of randomly selected zebrafish embryos from three biologically independent clutches, error bars indicate standard error of the mean, triple asterisk indicate p-value < 0.0001, single asterisk p = 0.0411 by Student’s T-test. See Supplementary Table 3. T-lymphocyte expression of rag1 in 4 d.p.f. embryos (ventral head view, anterior up) treated with DMSO at 18, 24 and 48 h.p.f. (l, n, o) or inhV at 18, 24 and 48 h.p.f. (m, o, q), blue arrowheads indicate rag1+ T-lymphocytes, when present. Numbers in the bottom right corner of panels indicate numbers of zebrafish embryos analysed for the indicated gene, versus the total number of zebrafish analysed. Zebrafish randomly selected from three clutches are represented by the image (a-j, l-q). Scale bars = 100 μm (a-j, l-q).
Supplementary Figure 6 NC are required for HSCs. Lateral trunk views (dorsal up anterior left) of *in situ* for the HSC marker *runx1* (blue) and the NC marker *crestin* (red) (a, d, g, m), the HSC marker *cmyb* (b, e, h, k, n), and the T lymphocyte marker *rag1* (c, f, i, l, o, ventral head views) in uninject- ed or Tfp2a morphant (a-f), wild-type and *sox10* m241 heterozygous (g-i), *sox10* m241 homozygous mutant (j-l) and Sdc4 morphant (m-o) embryos at the developmental stages indicated. Green and red arrowheads (a, b, d, e, g, h, j, k, m, n) indicate cells expressing *runx1* and *cmyb* respectively, blue arrowheads (c, f, i, l, o) identify *rag1* expressing T-lymphocytes when present, red brackets (a, d, g, j, m) enclose the AGM region. Numbers in bottom right corner indicate number of individuals in the clutches represented by the image (a-o). Scale bars = 100 μm (a-o). Averagel number of *runx1*+ (p) or *cmyb*+ (q) DA cells, or average number of NC streams ventral to the notochord at 24 h.p.f (r) in the indicated conditions. Numbers in the bottom right corner of panels indicate numbers of zebrafish embryos analysed for the indicated gene, versus the total number of zebrafish analysed. Zebrafish randomly selected from three clutches are represented by the image (a-o). Fluorescently labelled HSCs in uninjected (s) and Sdc4 morphant (t) *cmyb-GFP;kdrl:mCherry* transgenic embryos at 36 h.p.f., HSCs are yellow cells indicated by yellow arrowheads, scale bar = 50 μm, mag. = 200X. Average number of mCherry+GFP+ cells in the DA for the indicated conditions (u), n = 8 zebrafish each. Error bars indicate the standard error of the mean (p-r, u). Triple asterisk indicates p-values < 0.0001 in (p-r) and p = 0.0007 in (u) by Student’s T-test. See Supplementary Table 3.
Supplementary Figure 7 NC are required for HSCs; NC mutant and morphant validation. Lateral trunk views (dorsal up, anterior left) of in situ for the NC marker crestin (a-h) and the HSC marker runx1 (i-p) at 24 h.p.f. in uninjected (a, i), Tfap2a morphant (b, j), Sdc4 morphant (c, k), Sox10 morphant (d, l), wild-type and sox10<sup>m241</sup> heterozygous (e, m), sox10<sup>m241</sup> homozygous mutant (f, n), wild-type and tfap2a<sup>ts213</sup> heterozygous (g, o) and sox10<sup>ts213</sup> homozygous mutant (h, p) embryos. Red brackets enclose the AGM region (a-h), green arrowheads indicate runx1 expressing cells (i-p). Numbers in the bottom right corner of panels indicate numbers of zebrafish embryos analysed for the indicated gene, versus the total number of zebrafish analysed. Zebrafish randomly selected from three clutches are represented by the image (a-p). Scale bars = 100 μm (a-p). Lateral images (dorsal up, anterior left) of whole uninjected (q), Tfap2a morphant (r), Sdc4 morphant (s) and Sox10 morphant (t) embryos at 24 h.p.f. showing embryo morphology. RT-PCR showing aberrant mRNA splicing in Tfap2a morphant (arrows) compared with uninjected embryos (u). Lateral views (dorsal up, anterior left) of uninjected controls (v), wild-type and sox10<sup>m241</sup> heterozygous (w), wild-type and tfap2a<sup>ts213</sup> heterozygous (x) embryos have increased numbers of melanophores compared with Sox10 morphant (v’), sox10<sup>m241</sup> homozygous mutant (w’) and tfap2a<sup>ts213</sup> homozygous mutant (x’) embryos (red arrowheads indicate presence of increased numbers of melanophores) at the developmental stages indicated (v-x’). Scale bars = 200 μm (q-t, v-y’).
Supplementary Figure 8 Unprocessed Blots. Unprocessed agarose gel electrophoresis image for RT-PCR experiments examining the effects of splice-blocking morpholinos targeting *th* and *tfap2a* on mRNA levels and splicing (a). Expected sizes for normal transcripts and approximate sizes for aberrantly spliced transcripts are indicated. Lanes are labelled 1 through 7 and the identity of each band and the associated embryo injection conditions are indicated in the table.
Supplementary Table Legends

Supplementary Table 1 PCR primer sequences and dosages for mopholino oligonucleotide reagents.

Supplementary Table 2 Appearance of marker gene expression relative to wild type control embryos.

Supplementary Table 3 Statistics Source Data

Supplementary Video Legends

Supplementary Video 1 NC contact the dorsal aorta prior to and during HSC specification (transverse projection). Virtual transverse section (dorsal up) through the mid-trunk using confocal z-stack volume projection time lapse depicting NC migration (red, sox10:RFP) relative to axial vessel endothelial cells (green, fli1a:EGFP) over a 7 hour period from 20 to 27 h.p.f. in wild-type animals.

Supplementary Video 2 NC contact the dorsal aorta prior to and during HSC specification (lateral projection). Lateral (dorsal up, anterior left) z-stack volume projection time lapse depicting NC migration (red, sox10:RFP) relative to axial vessel endothelial cells (green, fli1a:EGFP) over a 7 hour period from 20 to 27 h.p.f. in wild-type animals.

Supplementary Video 3 Pdgfra-mediated signalling is required for NC migration to the DA (transverse projection). Virtual transverse section (dorsal up) through the mid-trunk using confocal z-stack volume projection time lapse depicting NC migration (red, sox10:RFP) relative to axial vessel endothelial cells (green, fli1a:EGFP) over a 7 hour period from 20 to 27 h.p.f. in embryos injected with 7 ng PdgfraMO.

Supplementary Video 4 Pdgfra-mediated signalling is required for NC migration to the DA (lateral projection). Lateral (dorsal up, anterior left) z-stack volume projection time lapse depicting NC migration (red, sox10:RFP) relative to axial vessel endothelial cells (green, fli1a:EGFP) over a 7 hour period from 20 to 27 h.p.f. in embryos injected with 7 ng of PdgfraMO.