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# FGF signalling restricts haematopoietic stem cell specification via modulation of the BMP pathway

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Haematopoietic stem cells (HSCs) are produced during embryogenesis from the floor of the dorsal aorta. The localization of HSCs is dependent on the presence of instructive signals on the ventral side of the vessel. The nature of the extrinsic molecular signals that control the aortic haematopoietic niche is currently poorly understood. Here we demonstrate a novel requirement for FGF signalling in the specification of aortic haemogenic endothelium. Our results demonstrate that FGF signalling normally acts to repress BMP activity in the subaortic mesenchyme through transcriptional inhibition of *bmp4*, as well as through activation of two BMP antagonists, *noggin2* and *gremlin1a*. Taken together, these findings demonstrate a key role for FGF signalling in establishment of the developmental HSC niche via its regulation of BMP activity in the subaortic mesenchyme. These results should help inform strategies to recapitulate the development of HSCs *in vitro* from pluripotent precursors.

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**H**ematopoietic stem cells (HSCs) ultimately maintain all lineages of blood and immune cells throughout the lifetime of an organism. This feature underlies the long-term efficacy of bone marrow transplantation, frequently used as therapy for blood disorders including leukaemia. Immune incompatibility between donor and host, insufficient number of donors and the rarity of HSCs within many donor tissues have led to a search for alternative approaches to traditional HSC-based therapies. Recent breakthroughs using induced pluripotent stem cells have brought hope of *in vitro* derived, patient-specific HSCs, which could circumvent these issues. Despite decades of effort, it is not currently possible to generate bona fide HSCs from pluripotent precursors. The development of novel HSC-based therapeutics may thus depend on obtaining a more precise understanding of the native molecular events that occur *in vivo* during HSC formation.

In all vertebrate animals examined, HSCs arise during embryogenesis from a specialized population of arterial cells localized in the ventral side of the dorsal aorta (DA) termed haemogenic endothelium<sup>1</sup>. This endothelial–haematopoietic transition<sup>2</sup> appears to exist only transiently, and is characterized by changes in gene expression and shape in ventral aortic endothelial cells as HSC precursors emerge and then enter circulation<sup>2–6</sup>. A prerequisite for HSC emergence appears to be the normal specification of arterial fate, most importantly proper formation of the DA. At the molecular level, arterial identity is governed by multiple extrinsic signals. In the zebrafish embryo, hedgehog signals from the notochord/floor plate regulate the expression of Vascular Endothelial Growth Factor A (VegfA) and calcitonin in the somites, which in turn regulate expression of Notch receptors in the DA<sup>7–11</sup>. Modulation of any of these signalling pathways alters arterial development and therefore HSC formation.

Recent studies have demonstrated that HSC formation is disrupted by defects in the Wnt16 (ref. 12), VegfA<sup>13</sup> and bone morphogenetic protein 4 (Bmp4) (ref. 14) pathways without concomitant loss of aortic fate. Interestingly, each pathway regulates different steps of HSC development. In zebrafish, Wnt16 controls early HSC specification through its regulation of the somitic Notch ligand genes *deltaC* and *deltaD*, whose combined action is required for the Notch-dependent specification of HSCs, but not for arterial development<sup>12</sup>. More recently, it was confirmed in *Xenopus* that arterial fate and HSC emergence can be uncoupled based on VegfA isoforms. The short isoform controls arterial fate likely through Notch4, while HSC emergence depends on the medium/long isoforms and Notch1 (ref. 13). Finally, Bmp4 that is localized to the subaortic mesenchyme is responsible for the polarization of HSC formation from the ventral side of the DA<sup>14–17</sup>. Smad1, an intracellular activator of the BMP pathway, transactivates the *runx1* promoter *in vitro*, suggesting that Bmp4 may act directly upstream of *runx1* (ref. 18), which is required for the emergence of HSCs across vertebrate species<sup>8,19–21</sup>. Just before the onset of definitive haematopoiesis in zebrafish, the aortic region switches from a BMP repressive to activated environment<sup>14</sup>. The mechanism of this sudden change remains unknown.

Interplay between BMP and fibroblast growth factor (FGF) signalling pathways has been described during organogenesis. In *Xenopus*, FGF and BMP signalling pathways intersect in the regulation of primitive erythropoiesis where FGF inhibits Bmp4-induced erythropoiesis through the control of *gata2* (ref. 22). The repressive role of FGF in primitive blood is conserved across the vertebrates. For instance, in the chicken embryo, FGF signals through Fgfr2 to control erythrocyte differentiation by repressing *gata1* expression in blood precursors<sup>23</sup>. In *Xenopus*, FGF was shown to act on the timing of primitive haematopoiesis by holding back the onset of the molecular programme that triggers primitive

blood formation<sup>24</sup>. Finally, in zebrafish, primitive erythrocyte formation depends on Fgf21, which also governs erythromyeloid precursor development, likely in concert with Fgf1 (refs 23,25,26). While several studies have established that FGF signalling represses primitive blood formation, FGF signalling acts as a positive regulator of adult HSCs. Fgf1 (ref. 27) and Fgf2 (ref. 28) can expand *ex vivo* the number of transplantable HSCs. However, this effect seems to be limited to the short-term HSC compartment *in vivo* and it is accompanied by an alteration in the terminal differentiation of erythrocytes, B cells and myeloid cells<sup>29</sup>. More recently, the role of FGF signalling in steady state conditions has been challenged and seems to be mainly required to promote mobilization and proliferation of HSCs under stress-induced conditions<sup>30,31</sup>. FGF signalling appears to have multiple roles in blood development; however, its potential role in the emergence of HSCs has not been addressed.

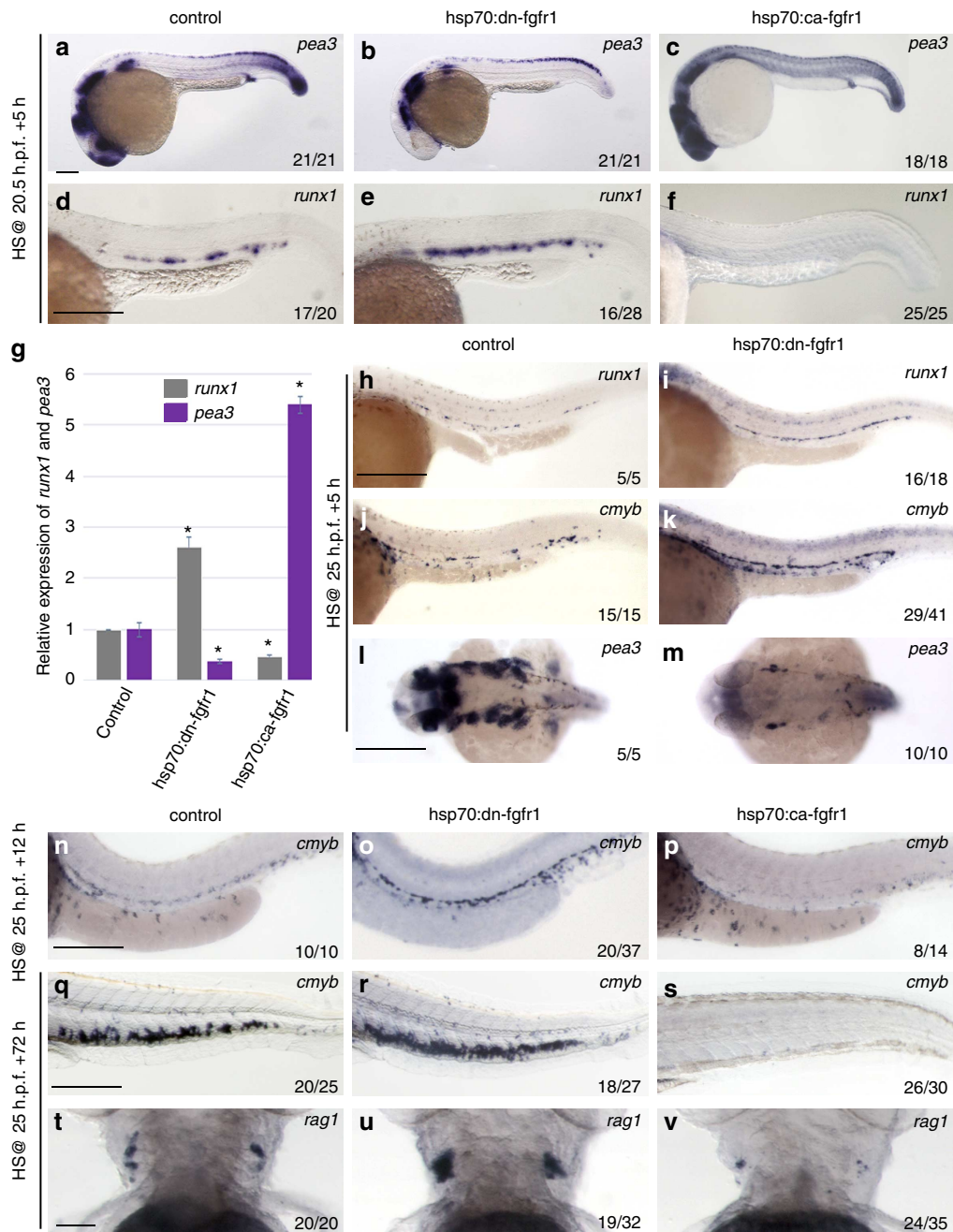
In this study, we have discovered a key repressive role for FGF signalling in HSC emergence through its regulation of the BMP pathway. Together with the data in the accompanying paper (Lee *et al.*), which reveals an earlier positive role for FGF in programming the HSC lineage, these findings suggest that precise temporal inhibition as well as activation of FGF signalling may aid *in vitro* approaches to instruct HSC fate from pluripotent precursors.

## Results

**FGF signalling is a negative regulator of HSC formation.** To functionally test whether or not FGF signalling is required for definitive blood formation, we utilized transgenic zebrafish in which FGF signalling can be inducibly abrogated or enforced by heat-shock induction of a dominant-negative Fgfr1-EGFP fusion protein (*hsp70:dn-fgfr1-EGFP*)<sup>32,33</sup> or a constitutively active Fgfr1 mutant protein (*hsp70:ca-fgfr1*)<sup>34</sup>, respectively. This time-controlled approach allowed us to avoid mesoderm patterning defects induced by early FGF misexpression and subsequently target different developmental events according to their timing<sup>35</sup>.

To identify temporal windows when FGF signalling is involved in HSC development, we initially targeted the early stage of arterial specification by inducing the *hsp:dn-fgfr1* transgene at 17 h.p.f. (hours post fertilization, 15 somite stage). At this stage, primitive blood and endothelial cells are specified and the first sign of arterial specification is detectable in the endothelial precursors that are migrating from the lateral plate mesoderm to the midline<sup>36</sup> to form the primitive vascular cord<sup>37–39</sup>. Transgenic embryos were then sorted based on the expression of green fluorescent protein (GFP), and GFP-negative embryos were used as sibling controls. Following induction of *hsp:dn-fgfr1*, definitive haematopoiesis initiated normally and there was no significant difference in *runx1* expression between GFP + and GFP – animals (Supplementary Fig. 1A,B). Interestingly, arterial and endothelial differentiations were unaffected, based on the normal expression of *deltaC* and *kdrl*, respectively (Supplementary Fig. 1C–F), suggesting that FGF signalling is not required for arterial differentiation or vascular development during the convergence of vascular precursor cells to the midline.

To investigate possible later requirements for FGF signalling in HSC development, embryos were heat shocked at 20.5 h.p.f. (23 somite stage), just before *runx1* expression along the aortic floor marks initiation of the definitive HSC programme. To verify loss of FGF signalling, we analysed the expression of *pea3*, a direct transcriptional target<sup>40</sup> of FGF signalling. In embryos heat shocked at 20.5 h.p.f., *pea3* expression decreased, and was accompanied by an increase in *runx1* expression (Fig. 1a,b,d,e). At the stage when the heat shock was performed, the aortic region contains the precursors of the HSCs that will specifically express



**Figure 1 | FGF signalling represses HSC formation and maintenance.** (a–f) Embryos were heat shocked at 20.5 h.p.f. and analysed at 26 h.p.f. (a–c) *pea3* expression is downregulated in *hsp:dn-fgfr1* (b) and upregulated in *hsp70:ca-fgfr1* embryos (c) compared with controls (a). (d–f) Aortic expression of *runx1* is enhanced in *hsp70:dn-fgfr1* (e) and depleted in *hsp70:ca-fgfr1* embryos (f) compared with controls (d). (g) Quantification of *runx1* and *pea3* mRNA expression in dissected trunks normalized to *efl1α*. Expression of each gene was set to 1 in the control (mean ± s.d.,  $n > 3$ ,  $*P < 0.001$ , Student’s *t*-test). (h–m) Embryos were heat shocked at 25 h.p.f. and analysed at 30 h.p.f. (h–i) *runx1* expression is increased in *hsp70:dn-fgfr1* embryos (i) compared with controls (h). Similar results are seen with *cmyb* expression in control (j) and *hsp70:dn-fgfr1* embryos (k). (l,m) Dorsal view of *pea3* expression in the head shows downregulation of *pea3* on depletion of FGF signalling. (n–v) Embryos were heat shocked at 25 h.p.f. and analysed 12 h and 72 hpHS. (n–p) *cmyb* expression is more intense in the *hsp70:dn-fgfr1* embryos (o) compared with control embryos (n). Conversely, embryos in which FGF signalling is increased display a drastic diminution of *cmyb* expression (p). (r,q–s) Comparison of *cmyb* expression in the CHT of heat shocked transgenic embryos and controls. The augmentation of HSC numbers detected at 26 h.p.f. on FGF modulation is maintained in the CHT of the *hsp70:dn-fgfr1* embryos while the CHT of the *hsp70:ca-fgfr1* embryos are devoid of *cmyb* cells. (t–v) Effect of FGF signal alteration on T cells. FGF ablation (u) or augmentation (v) has opposite effects on *rag1* + cells. Scale bars, 50 μm (a), 200 μm (d,h), 250 μm (l,n,q) and 50 μm (t).

*runx1* from 23 h.p.f., but also primitive blood that can be distinguished from HSC based on *gata1* expression. The effect of the modulation of FGF signalling is restricted to the HSCs as shown by the absence of alteration of *gata1* expression in transgenic embryos (Supplementary Fig. 2A). EMPs

(erythromyeloid progenitor), bipotent precursors that arise in the posterior blood island<sup>41</sup>, are not affected on FGF modulation (Supplementary Fig. 2A). In converse experiments, where FGF signalling was enforced via 20.5 h.p.f. induction of the *hsp:ca-fgfr1* transgene, embryos exhibited embryo-wide upregulation of *pea3*



expression and a substantial decrease in *runx1* expression along the aortic floor (Fig. 1c,f). Despite strong GFP expression at 5 h post heat shock (hpHS), *hsp:dn-fgfr1* embryos displayed only a slight decrease in *pea3* expression, leading to the conclusion that cellular turnover of the truncated receptor may outpace the turnover of GFP. To address this, *pea3* expression was analysed in *hsp:dn-fgfr1* embryos from 23 to 27 h.p.f. At 2 hpHS, *pea3* was nearly absent in GFP + embryos. Expression of *pea3* gradually returned to normal around 6 hpHS (Supplementary Fig. 2B). Effects on *runx1* expression followed this trend. Between 2 and 4 hpHS, a greater proportion of *hsp:dn-fgfr1* embryos showed stronger upregulation of *runx1* than when analysed between 5 and 6 hpHS (Supplementary Fig. 2C). The effects of Fgf modulation on *runx1* expression were confirmed by quantitative PCR (qPCR) using complementary DNAs from the dissected trunks of *hsp:dn-fgfr1* and *hsp:ca-fgfr1* embryos (Fig. 1g).

Before 26–27 h.p.f., the close proximity of primitive erythrocytes within the DA and posterior cardinal vein (PCV) to the floor of the DA<sup>42</sup> makes it difficult to distinguish them from emerging HSCs, since each lineage shares expression of early haematopoietic markers. We therefore shifted the heat-shock regimen to 25 h.p.f. and fixed at 30 h.p.f. By this time, erythroid precursors would have entered circulation, which allows visualization of the haemogenic endothelial marker *cmyb* by whole-mount *in situ* hybridization (WISH). Induced *hsp70:dn-fgfr1* embryos showed elevated expression of both *runx1* and *cmyb* in the DA (Fig. 1h–k; Supplementary Fig. 2D) within the period during which the FGF transcriptional target *pea3* is still downregulated (Fig. 1l,m). In mammals, HSCs leave the DA region quickly after their emergence to seed the fetal liver and the thymus. In zebrafish, a similar shift occurs: *runx1* + /*cmyb* + cells migrate from the DA to the caudal haematopoietic tissue (CHT) and the thymus<sup>43–45</sup>. To ascertain whether the expanded pool of *runx1* + /*cmyb* + cells are HSCs, FGF signalling was modulated at 25 h.p.f. and the effect on HSCs was monitored in the DA, the CHT and the thymus at 36 h.p.f. (25 h.p.f. + 12 h), 3 (25 h.p.f. + 48 h) and 4 days post fertilization (d.p.f.) (25 h.p.f. + 72 h; Fig. 1n–v; Supplementary Fig. 2E,F). In induced *hsp70:dn-fgfr1* embryos, *cmyb* expression is still expanded in the DA 12 hpHS (Fig. 1n,o). Conversely, embryos in which FGF signalling was enforced are devoid of *cmyb* cells in the DA (Fig. 1n,p). Similarly, at 48 and 72 hpHS, *hsp70:dn-fgfr1* embryos showed a more robust expression of *runx1* and *cmyb* in the CHT, while *hsp70:ca-fgfr1* embryos showed a drastic decrease of the *runx1* + and *cmyb* + cells (Supplementary Fig. 2E; Fig. 1q–s). qPCR analysis of dissected CHT confirmed that *runx1*, *cmyb* and *CD41* levels of expression vary according to the modulation of FGF signalling (Supplementary Fig. 2F). T cells are

thought to be the first functional derivatives of HSCs. They are first detected around 3 d.p.f. and by day 4, *rag1* expression becomes robust in the thymus. The effect of FGF signalling modulation at 25 h.p.f. also affects the number of thymic *rag1* + cells (Fig. 1t–v). Importantly, the increase in the number of *rag1* + cells was observed only in *hsp70:dn-fgfr1* embryos whose blood circulation was unaffected.

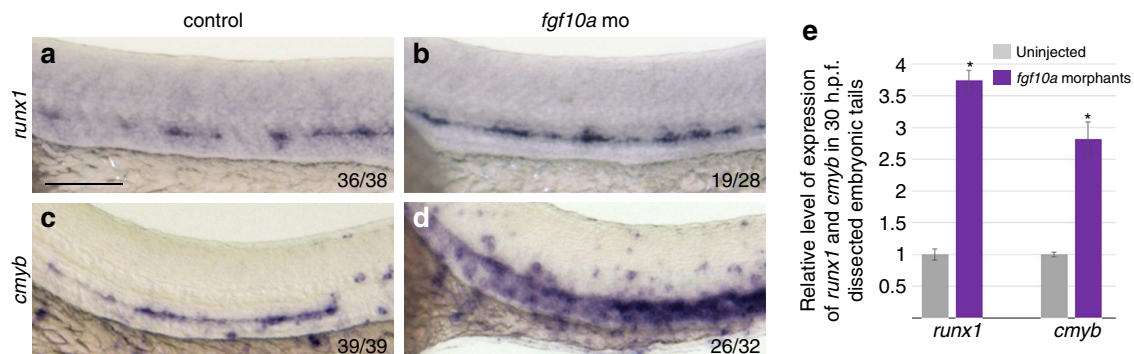
Taken together, these results demonstrate that FGF signalling is important in the establishment of haemogenic endothelium, acting to repress the specification of HSC fate from the aortic floor.

### Fgf10a represses HSC formation by acting on fgfr2 and fgfr3.

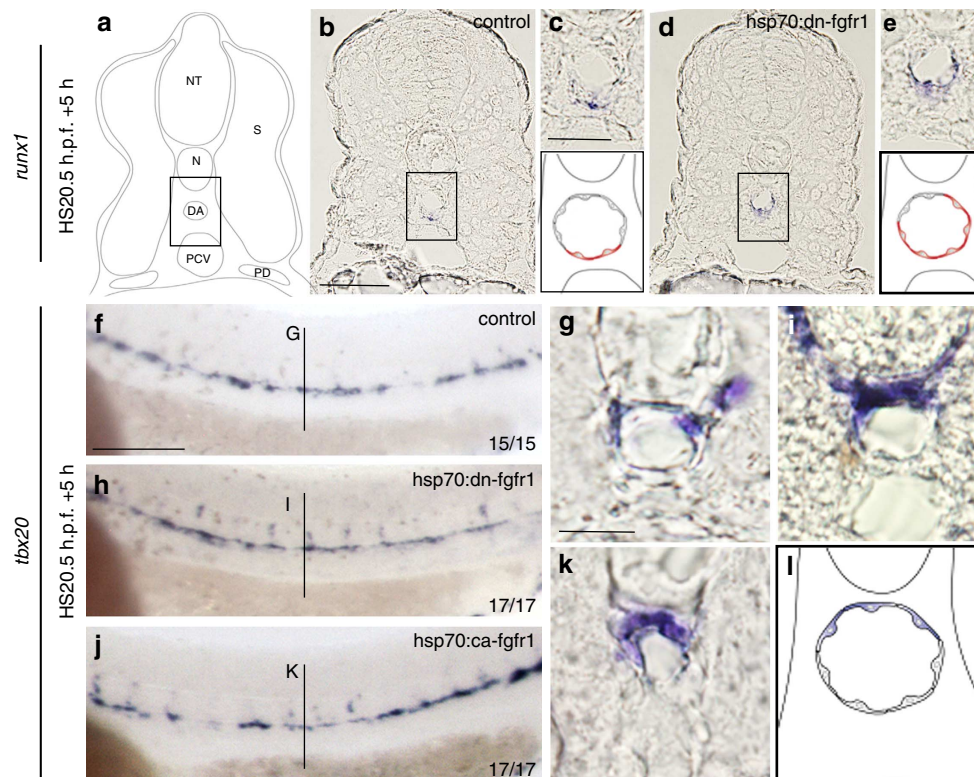
To identify the cell types that may mediate the effects of FGF signalling on HSC emergence, we examined localization of Fgf receptor expression at 20.5 h.p.f. and at 24 h.p.f. (Supplementary Fig. 3). *Fgfr1a*, *fgfr1b* and *fgfr4* were not detected in the tissues surrounding the DA at either time point (Supplementary Fig. 3A–H,Q–T). *Fgfr2* showed strong expression in the pronephric ducts, the hypochord and the neural tube at 20.5 h.p.f. (Supplementary Fig. 3I,J). In contrast to *fgfr2*, *fgfr3* transcripts were detected in the somites at 20.5 h.p.f. (Supplementary Fig. 3M,N). At 24 h.p.f., *fgfr3* is expressed throughout the trunk, whereas *fgfr2* expression is restricted to the neural tube, the pronephric ducts, the hypochord and in cells surrounding the axial vasculature (Supplementary Fig. 3K,L,O,P). The localization of each receptor suggests that the effects of FGF modulation on HSC formation may act through *fgfr2* and/or *fgfr3*. However, morpholino knockdown of these receptors failed to phenocopy the increase in *runx1* and *cmyb* expression observed in the *hsp70:dn-fgfr1* transgenic line. Loss of either receptor led to the absence of *runx1* expression in the DA at 26 h.p.f. (Supplementary Fig. 4). The discrepancy between the phenotype observed in morphants and that observed in *hsp70:dn-fgfr1* embryos suggests that *fgfr2* and *fgfr3* may be required at earlier stages of mesoderm or vascular development.

In zebrafish, 27 Fgf ligands have been identified<sup>46</sup>. At the stage of the heat shock, *fgf10a* is expressed throughout the trunk<sup>47</sup>, which made it a good candidate. To analyse its potential role in HSC specification, knockdown experiments were carried out using a splice-blocking morpholino (Fig. 2). Our LOF (loss of function) experiments showed that depletion of *fgf10a* gives a similar phenotype to the phenotype observed in the *hsp70:dn-fgfr1* embryos. At 30 h.p.f., in morphant embryos, *runx1* and *cmyb* expressions are extended along the entire DA (Fig. 2a–e).

Taken together, our results confirm that FGF signalling acts as a negative regulator of definitive haematopoiesis. This function is mediated by *fgf10a*, which likely signals through *fgfr2* and/or *fgfr3*.



**Figure 2 | Loss of *fgf10a* mimics the effect of FGF ablation.** (a,b) *runx1* expression is expanded along the entire DA in the morphant embryos. Similarly, loss of *Fgf10a* significantly increases *cmyb* expression in the aortic region (c,d). Comparison of the relative levels of expression of *runx1* and *cmyb* by qPCR in control and morphant embryos (e; mean  $\pm$  s.d.,  $n = 3$ ,  $P < 0.001$ , Student's *t*-test). Scale bar, 150  $\mu$ m.



**Figure 3 | Loss of FGF signalling expands *runx1* dorsally without affecting dorsal polarization of the DA.** (a) Schematic representation of a trunk section of 26 h.p.f. embryos. (b) Aortic localization of *runx1*<sup>+</sup> cells in control (b,c) and *hsp70:dn-fgfr1* (d,e) embryos. Expression of *tbx20* in control (f,g), *hsp70:dn-fgfr1* (h,i) and *hsp70:ca-fgfr1* (j,k) embryos. The black line (G,I,K) denotes where sections were made. (l) Schematic representation of *tbx20* expression in the roof of the DA. N, notochord; NT, neural tube; PD, pronephric duct; S, somite. Scale bars, 30 µm (b), 200 µm (f) and 20 µm (g).

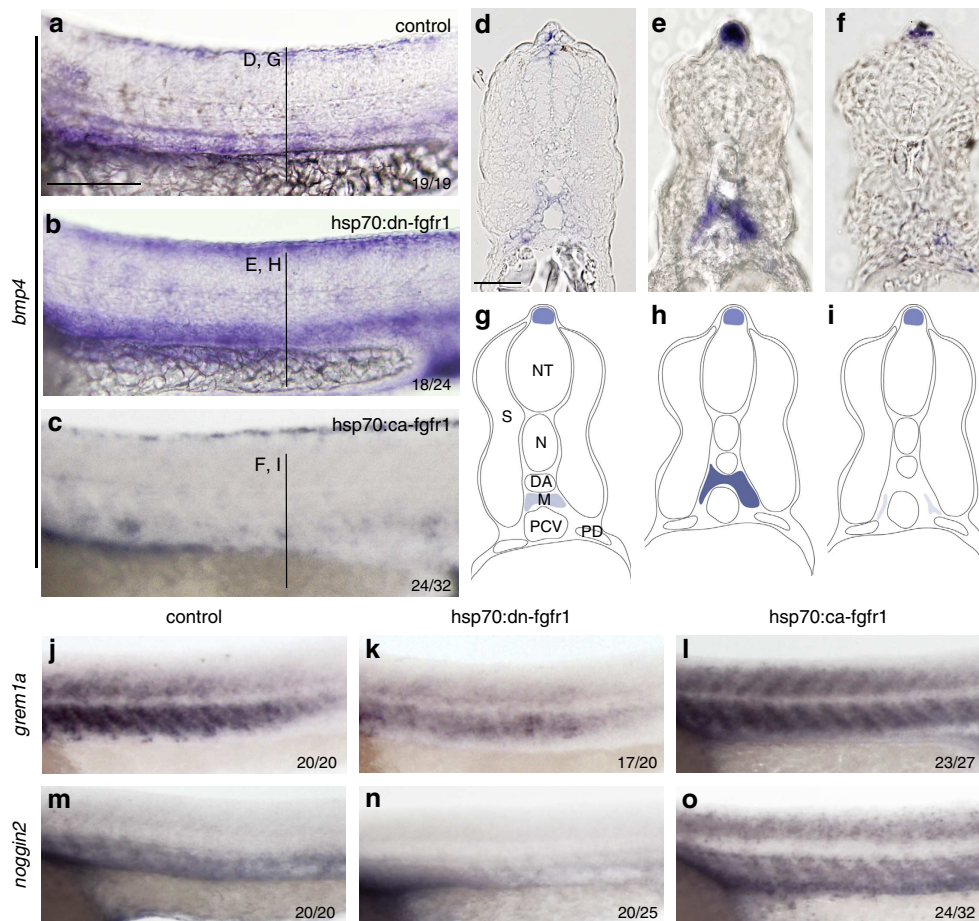
**FGF acts independently of the Notch and Vegf pathways.** In both mouse and zebrafish, Notch signalling is required for aortic and HSC specification<sup>9,21,48,49</sup>. As *fgfr2* is expressed in the aortic region and *fgfr3* in the somites, it is possible that the effects of FGF signalling on HSC emergence could be due in part to the effects on the Notch pathway. In zebrafish, enforced expression of the Notch intracellular domain (NICD) throughout the embryo is sufficient to generate an excess of HSCs<sup>21</sup>. Similarly in mice, genetic depletion of COUP-TFII, which normally represses Notch in the PCV, leads to the formation of ectopic haematopoietic clusters in the PCV<sup>50</sup>. We therefore investigated whether the modulation of HSC number observed in the DA following loss or gain of FGF signalling might be due to effects on the Notch pathway. Transgenic *hsp70:dn-fgfr1* or *hsp70:ca-fgfr1* embryos were heat shocked at 20.5 h.p.f., fixed at 25 h.p.f. and then assayed for Notch-related vascular and arterial gene markers by WISH (Supplementary Fig. 5A–O). Following either loss or gain of FGF function, the integrity of the vascular system was unaffected, as indicated by normal *kdrl* expression (Supplementary Fig. 5A–C). Aortic markers, including *gridlock* (a target of the Vegf pathway<sup>9</sup>; Supplementary Fig. 3D–F), *notch1b* (Supplementary Fig. 5G–I) *deltaC* (Supplementary Fig. 5J–L) and *ephrinb2a* (a target of the Notch pathway<sup>48</sup>; Supplementary Fig. 5M–O), were unchanged following modulation of FGF signalling. These results indicate that the effects of FGF signalling on HSC fate are not dependent on downstream Notch signalling events. To test the converse, that is, whether or not FGF signalling requirements are downstream of Notch, Notch signalling was blocked using *N*-[*N*-3,5-difluorophenacetyl]-*L*-alanyl-*S*-phenylglycine Methyl Ester (DAPM), a small chemical inhibitor of NICD released from Notch receptors. If the increase in HSC number following FGF inhibition acts downstream of Notch, blockade of Notch signalling in the same temporal window as FGF

inhibition should not prevent *runx1* upregulation in the DA. In accord with this hypothesis, *hsp70:dn-fgfr1* embryos treated with DAPM maintained strong expression of *runx1* in the DA (Supplementary Fig. 5P–U). These results demonstrate that the increase in HSC number observed in absence of FGF signalling acts in a dominant manner with respect to loss of Notch signalling. Taken together, our studies on the interaction of Notch and FGF suggest that the effects of FGF on HSC fate either occur independently or downstream of the roles of the Notch/Vegf signalling axis during arterial development and HSC formation.

#### **FGF signalling does not affect dorsal polarization of the DA.**

Since the increase in HSC marker expression in the DA is not a result of overactivation of the Vegf or Notch signalling pathways, we reasoned that it may be due to an increase in the number of *runx1*<sup>+</sup> cells in the DA or the surrounding mesenchyme. We thus examined *runx1* expression in transverse sections following induction of the *hsp70:dn-fgfr1* transgene at 20.5 h.p.f. In wild-type (WT) controls, rare *runx1*<sup>+</sup> cells were visible only in the floor of the DA (Fig. 3a–c). Following loss of FGF signalling, the expression of *runx1* in the DA was expanded beyond the floor region to the roof of the aorta (Fig. 3d,e). Cells expressing *runx1* were never detected in the surrounding mesenchyme or neighbouring PCV, suggesting that ectopic *runx1*<sup>+</sup> cells must transit through an arterial precursor. Since *runx1* is normally expressed only in the aortic floor, the ectopic appearance of *runx1*<sup>+</sup> cells in the aortic roof may indicate that FGF signalling is involved in DA polarization. In mice and zebrafish, DA polarization depends on opposing morphogen gradients; dorsal identity is established by Hedgehog secretion from the notochord, whereas ventral identity relies on BMP production from ventral domains<sup>14–16</sup>.





**Figure 4 | FGF signalling regulates *bmp4* expression as well as *noggin2* and *gremlin1a*, two BMP antagonists.** (a–c) *bmp4* expression is altered following manipulation of FGF signalling. *bmp4* levels of expression are increased in *hsp70:dn-fgfr1* embryos (b,e) and decreased in *hsp70:ca-fgfr1* embryos (c,f). (d–f) Transverse sections of embryos from WISH samples (a–c). (g–i) Schematic representing *bmp4* expression in control (g), *hsp70:dn-fgfr1* (h) and *hsp70:ca-fgfr1* (i) embryos. (j–o) *gremlin1a* (j–l) and *noggin2* (m–o) expression is reduced in *hsp70:dn-fgfr1* embryos (k,n) and enhanced in *hsp70:ca-fgfr1* embryos (l,o). M, mesenchyme. Scale bars, 200  $\mu$ m (a) and 50  $\mu$ m (d).

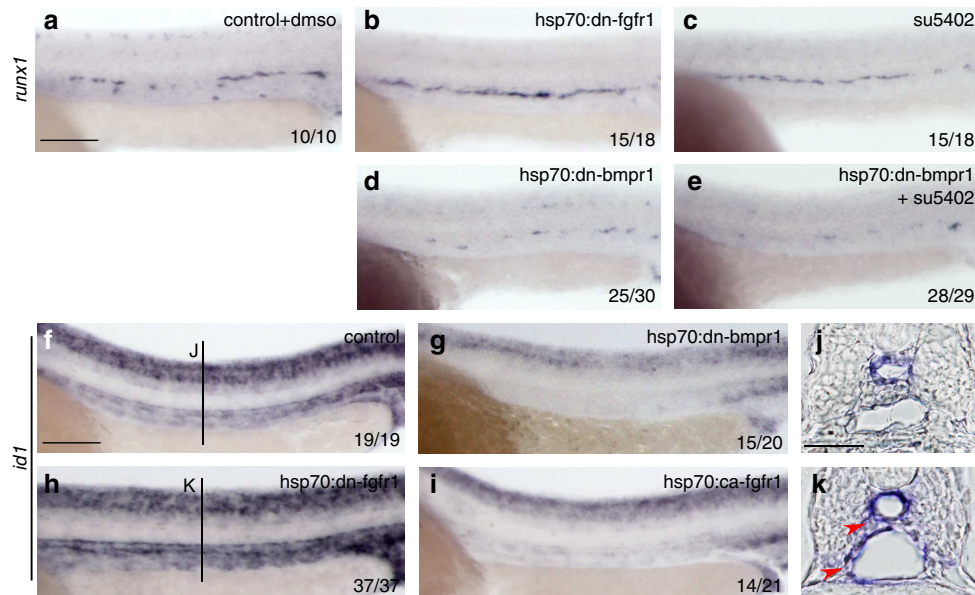
We examined the expression of *tbx20*, a transcription factor regulated by Hedgehog signalling<sup>8,14,51</sup>, which distinguishes the dorsal side of the DA. Neither activation nor inhibition of FGF signalling had any effect on *tbx20* expression (Fig. 3f,h,j). Examination of transverse sections confirmed that only cells in the roof of the DA and in the developing intersomitic vessels expressed *tbx20*, indicating that dorsal polarization is not affected by FGF modulation (Fig. 3g,i,k,l).

**FGF controls HSC formation by modulating BMP activity.** We next investigated whether FGF regulates the ventral polarization of the DA by modulating BMP activity. In zebrafish, we previously demonstrated that *bmp4* is required for the emergence and maintenance of HSCs<sup>14</sup>. Whereas *bmp4* is normally expressed in the mesenchyme underlying the DA (Fig. 4a,d,g), *bmp4* expression was upregulated in the aortic region in the absence of FGF (Fig. 4b,e,h). Conversely, in embryos with FGF overactivation, *bmp4* was absent from the aortic region (Fig. 4c,f,i), supporting the idea that FGF signalling may regulate HSC formation via its effects on the BMP pathway.

Since BMP signalling activity is tightly regulated by several antagonists<sup>52</sup>, we also examined their expression at the time of heat shock. At 20.5 h.p.f., the DA is surrounded by several BMP antagonists, including *chordin* from the pronephric ducts, as well as *noggin1*, *noggin2* (ref. 53) and *gremlin1a*<sup>54</sup>. Although *chordin* is

an important regulator of primitive haematopoiesis<sup>55</sup>, it is dispensable for HSC formation<sup>14</sup>. We therefore focused on *noggin1*, *noggin2* and *gremlin1a*. In WT embryos, *noggin1* is barely detected in the ventral side of the somite at 24 h.p.f., while *gremlin1a* and *noggin2* are strongly expressed in the sclerotome (Fig. 4j,m)<sup>53,54</sup>. In induced *hsp70:dn-fgfr1* embryos, both *gremlin1a* and *noggin2* were markedly downregulated (Fig. 4k,n; Supplementary Fig. 6). In contrast, when FGF signalling was enforced, there was a substantial upregulation of sclerotomal *gremlin1a* and *noggin2* (Fig. 4l,o; Supplementary Fig. 6). Augmentation of FGF activity was also observed to induce ectopic expression of *gremlin1a* and *noggin2* in the most dorsal compartment of the somite (Fig. 4l,o). Together, these results demonstrate that FGF signalling represses *bmp4* expression directly and concomitantly induces expression of the BMP inhibitors *noggin2* and *gremlin1a* in the neighbouring somite.

To further analyse how FGF and BMP signalling interact, we tested whether inhibition of BMP signalling in FGF-depleted embryos would affect *runx1* expression. Inhibition of FGF signalling, either using the *hsp:dn-fgfr1* transgenic animals or a small chemical inhibitor su5402, increases *runx1* expression in the DA (Fig. 5a–c), while blockage of BMP signalling abrogates *runx1* expression (Fig. 5d). Inhibition of FGF signalling in a BMP-repressed environment could not rescue HSC production, supporting the idea that FGF acts upstream of the BMP pathway (Fig. 5e).



**Figure 5 | Epistatic analysis of BMP and FGF signalling interaction.** (a–c) *runx1* expression is increased in *hsp70:dn-fgfr1* embryos (b) and embryos treated with su5402 (c) compared with controls (a). Overexpression of *hsp70:dn-bmpr1* impairs emergence of HSCs (d) compared with control (a) or FGF-inhibited embryos (b,c). HSC emergence is not rescued in *hsp70:dn-bmpr1* embryos following blockade of FGF signalling using su5402 (e). (f–k) *id1* expression is reduced on inhibition of BMP signalling (g) as well as augmentation of FGF signalling (i) compared with control embryos (f). In the absence of FGF signalling, *id1* expression is increased in the vasculature and in some cells surrounding the vessels (h and k, red arrows) compared with control embryos (f and j). Scale bars, 100  $\mu$ m (a,f) and 30  $\mu$ m (j).

To further examine the epistasis between the FGF and BMP pathways, we sought a molecular marker of BMP activity. The transcriptional repressor *id1* is a known target of BMP signalling<sup>56</sup>, and its targeted deletion in the mouse embryo impairs haematopoiesis by affecting the proliferation and the self-renewal of HSCs<sup>57</sup>. In zebrafish, *id1* is expressed in developing neural tissue, somites and axial vasculature (Fig. 5f); this expression is largely ablated following inhibition of BMP signalling (Fig. 5g). Inhibition of FGF signalling leads to an increase in *id1* expression in the vasculature (Fig. 5f,h), which becomes more apparent in transverse sections (Fig. 5j,k). Conversely, stimulation of FGF significantly decreases *id1* expression (Fig. 5i). Together, these results further demonstrate that the FGF signalling pathway acts upstream of BMP signalling to regulate HSC emergence.

Finally, we performed genetic rescue experiments to determine whether enforced BMP signalling could rescue loss of HSCs in *hsp70:ca-fgfr1* animals. Enforced activity of the BMP pathway was achieved following induction of a constitutively active bmp receptor 1b (*hse:ca-bmpr1b*) construct in transient transgenic animals, as previously described<sup>58</sup>. Compared with WT siblings, *hsp70:ca-fgfr1* animals induced at 20.5 h.p.f. showed loss of HSCs accompanied with an increase in *pea3* expression (Fig. 6a,b,e). Induction of the *hse:ca-bmpr1b* transgene alone showed a robust increase in *runx1* expression without affecting *pea3* (Fig. 6c,e). As predicted by our results above, enforced activity of BMP signalling could rescue HSC development in *hsp70:ca-fgfr1* animals (Fig. 6d,e).

In this experiment, BMP signalling was enforced at the receptor level, bypassing therefore any potential effect of the Bmp antagonists *noggin2* and *gremlin1a*. In *hsp:ca-fgfr1* embryos, their expression levels are elevated suggesting that they may reinforce the BMP-repressive environment in the DA. According to this hypothesis, overexpression of *noggin2* or *gremlin1a* following inhibition of FGF signalling should prevent *runx1* increase. Overexpressions of *noggin2* and *gremlin1a* were achieved by mRNA injection into *hsp70:dn-fgfr1* embryos and

analysed for *runx1* expression. As predicted, both *noggin2* and *gremlin1a* repress HSC formation when injected in control embryos (Fig. 6f,h,j). Similar results were obtained when *noggin2* and *gremlin1a* were overexpressed in *hsp:dn-fgfr1* embryos (Fig. 6g,i,k), confirming that both antagonists are acting downstream of FGF signalling and upstream of *bmp4/bmpr1*.

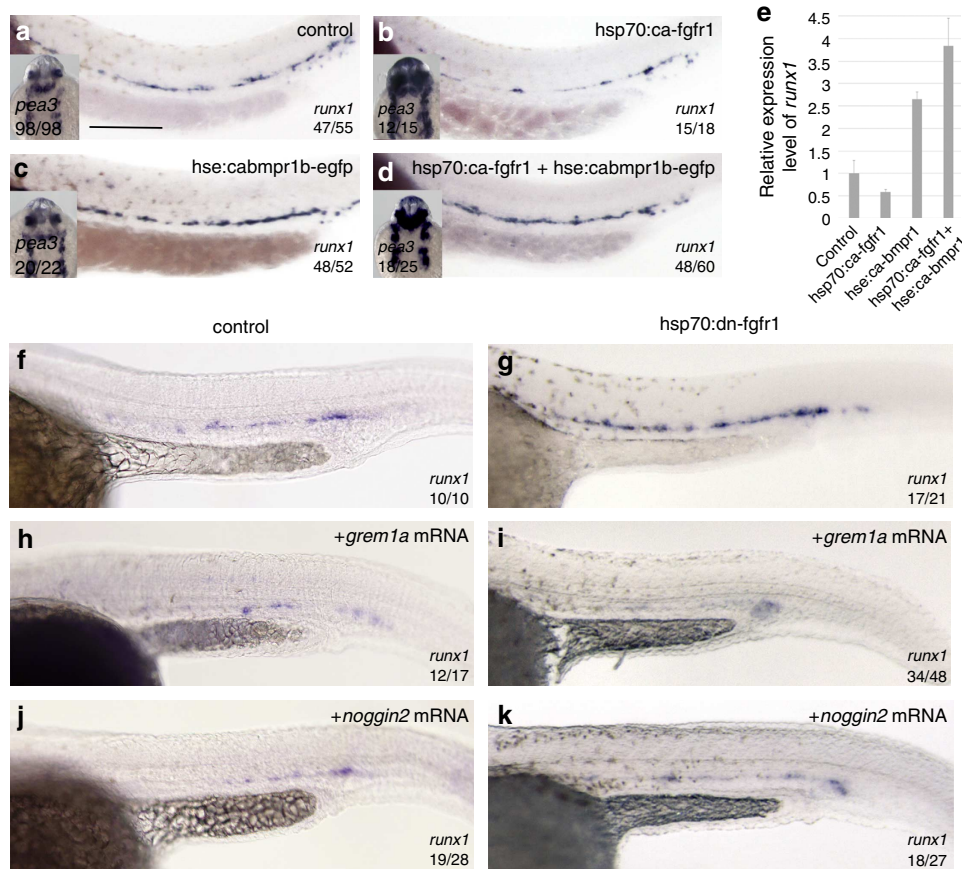
Collectively, our results indicate that FGF signalling controls the emergence of HSCs by modulating the activity of BMP signalling in the aortic region. The inhibition of BMP signalling by FGF acts at two levels, first by repressing the transcription of *bmp4* in the subaortic mesenchyme and second by increasing the expression of BMP antagonists in the neighbouring somite. These results suggest that the level of FGF signalling controls the capacity of the aortic microenvironment to support or repress the formation of HSCs (Fig. 7).

## Discussion

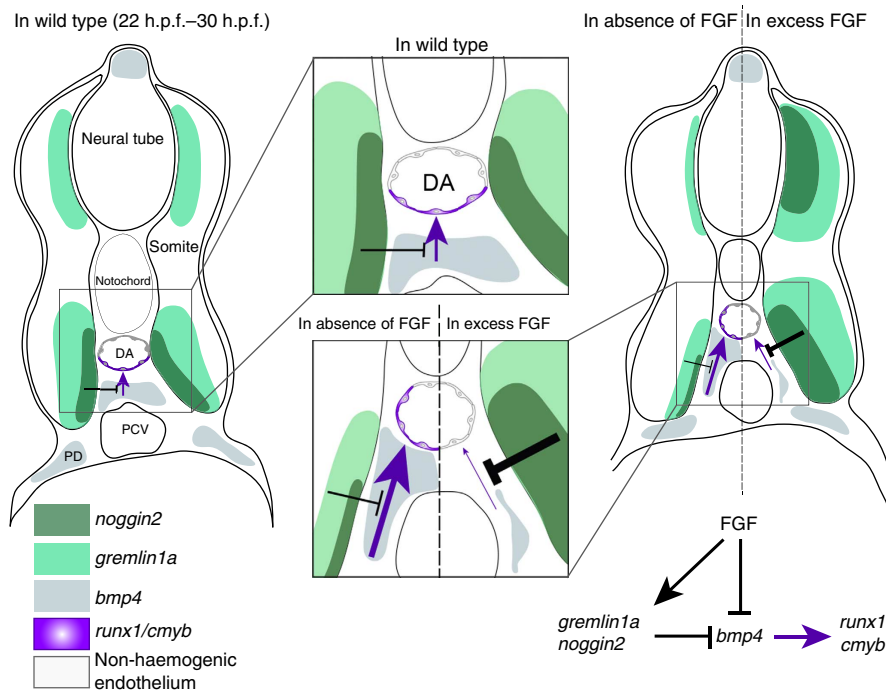
Despite FGF signalling playing key roles in the formation of mesoderm and the vascular system, no previous studies have examined potential roles for FGF in HSC development. Studies in adult mice have demonstrated that HSCs express *Fgfr1*, and that provision of *Fgf1* *ex vivo* can stimulate HSC expansion<sup>27</sup>. More recent work, however, has demonstrated that *Fgfr1* is not required for the normal homeostasis of adult HSCs, but rather in haematopoietic recovery following injury via irradiation or chemotherapy by stimulating HSC proliferation<sup>30</sup>. FGF signalling may therefore be important in regulating the number of adult HSCs.

A current bottleneck in the field of regenerative medicine is the inability to instruct HSC fate *in vitro* from pluripotent precursors, including induced pluripotent stem cells. This is due, at least in part, to an incomplete understanding of the native factors that are required to specify HSCs during embryonic development. In this study, we have demonstrated a novel requirement for FGF signalling in the generation of HSCs. We show that FGF represses the emergence and maintenance of HSCs in the DA by blocking





**Figure 6 | Ectopic activation of BMP signalling rescues *runx1* expression in an activated FGF background.** (a–d) *runx1* expression increases in the DA following activation of the BMP pathway (c) compared with controls (a). *runx1* expression in *hsp70:ca-fgfr1* (b) embryos is rescued by activation of a *hse:ca-bmpr1b* transgene (d). Quantitative analysis of *runx1* expression (e). Chart shows results obtained from one representative experiment with three biological replicates (mean ± s.d.). *pea3* expression in the head (inserts, a–d) is upregulated on FGF activation. (f–k) *runx1* expression is impaired on overexpression of either *gremlin1a* (h) or *noggin2* (j) compared with control (f). Inhibition of FGF signalling fails to rescue *runx1* expression when *gremlin1a* (i) and *noggin2* (k) are overexpressed. Scale bars, 200 µm (a).



**Figure 7 | Role of FGF signalling in the formation of HSCs.** Model for the regulation of HSC emergence by FGF signalling.



BMP signals that originate from the aortic mesenchyme. This negative role is in contrast to the role that FGF signalling has in adult haematopoiesis, where it promotes HSC amplification. Along with the results of the companion paper (Lee *et al.*), it is now apparent that the FGF pathway is required at multiple stages of development to properly specify HSC fate.

HSCs originate from arterial precursors, which depend on the Notch and Vegf signalling pathways for their specification and differentiation<sup>7,9,11,21,48,49,59</sup>. Unlike the results of Lee *et al.*, where early FGF signalling (14–17 h.p.f.) is required within the somite to bridge the Wnt16-mediated expression of the Notch ligand *deltaC*, the subsequent FGF signalling requirement (22–30 h.p.f.) for HSC emergence lies downstream of Notch function. Neither Notch- nor Vegf-dependent gene expression programmes were affected following modulation of FGF activity after 20.5 h.p.f. Moreover, the combined inhibition of Notch and FGF signalling failed to decrease *runx1* expression in the DA, indicating that FGF acts downstream of the Notch pathway in HSC emergence. Interestingly, while FGF inhibition increased the number of HSCs emerging from the DA, we did not observe ectopic *runx1* + cells outside of the DA, suggesting that FGF signalling affects only arterial precursors previously specified by Notch signalling.

Following the induced repression of FGF signalling, *runx1* expression expands dorsally within the DA without affecting dorsal identity, as defined by the normal expression of *tbx20*. This finding suggests that the expression domains of *runx1* and *tbx20* are not mutually exclusive, confirming our previous report<sup>14</sup>. Interestingly, the expanded pool of *runx1* + */cmyb* + cells behave as normal HSC. Ectopically induced HSCs have the capacity to migrate from the DA, seed the different haematopoietic organs and differentiate into T cells in the embryos showing normal blood flow 2 to 3 days after heat shock.

The proper establishment of ventral aortic identity, in contrast to the dorsal identity, appears to depend on the FGF pathway. FGF signalling controls the ventral polarization of the DA by restricting *bmp4* expression in the mesenchyme around the aortic endothelium. The timing and location of *bmp4* expression in the subaortic mesenchyme is conserved among several classes of vertebrates<sup>14,15,17,60</sup>. Interestingly, clusters of blood cells emerge from both the dorsal and ventral side of the DA in mouse embryos<sup>61</sup>. However, the adult reconstituting potential is restricted to the ventral clusters<sup>62</sup>, supporting the idea that the ventral mesenchyme provides the cues critical to conferring stem cell potential. This hypothesis is supported by previous findings, where early resection of ventral mesenchyme led to loss of aortic *runx1* expression and haematopoietic cluster formation<sup>3</sup>. Our analysis of *fgfr2* and *fgfr3* expression patterns showed that, while *fgfr3* is mainly found in the somitic tissue at the time of HSC emergence, *fgfr2* transcripts are detected in the few cells surrounding the axial vasculature corresponding to the territory of expression of *bmp4*. This tissue localization suggests that *fgfr2* may mediate the effect observed on *bmp4* expression when FGF signalling is modulated. *Fgf10a*, whose loss gives rise to a similar haematopoietic phenotype to that observed in the *hsp:dn-fgfr1*, was shown to preferentially interact with *fgfr2* (ref. 63), supporting the existence of an axis involving *fgf10a/fgfr2/bmp4* to play the role of a switch that triggers the aortic blood programme. However, we cannot rule out that other Fgf ligands may be involved in the regulation of HSC specification from the haemogenic endothelium.

Our attempts to mimic the *hsp70:dn-fgfr1* phenotype by knocking down *fgfr2* and *fgfr3* failed and morphant embryos showed a drastic decrease in *runx1* expression at 26 h.p.f. Knowing that Fgf receptors can heterodimerize<sup>64</sup> and that both *fgfr2* and *fgfr3* are expressed early in the forming somites<sup>65,66</sup>, it is

possible that *fgfr2* and *fgfr3* may be required in the somites to promote HSC specification in concert with *fgfr1* and *fgfr4*. To better understand how and when each receptor mediates the activity of the FGF signalling, new tools offering time control and tissue specificity have to be developed.

Our previous studies demonstrated that *bmp4* is crucial for HSC formation in zebrafish<sup>14</sup>. Attempts to locally increase *Bmp4* activity using the zebrafish mutant in *chordin*, a *Bmp* antagonist, failed to increase *runx1* expression in the DA<sup>14</sup>, suggesting that *bmp4* alone is insufficient for HSC formation or that other *Bmp* antagonists regulate HSC emergence. Our current findings indicate that the regulation of BMP signalling by FGF acts at multiple levels. First, enforced expression of a constitutively active *Bmp* receptor 1 rescued *runx1* expression in *hsp70:ca-fgfr1* embryos, indicating that BMP signalling is sufficient to trigger the definitive haematopoietic programme in the DA. This result, along with the finding that combined inhibition of FGF and BMP failed to generate HSCs, indicates that FGF signalling acts genetically upstream of BMP. In addition, inhibition of FGF function substantially increases *bmp4* expression in the subaortic mesenchyme and prevents expression of two BMP antagonists, *noggin2* and *gremlin1a*, in the surrounding somites. Here we report that overexpression of either *noggin2* or *gremlin1a* is enough to prevent *runx1* upregulation in the absence of FGF. This indicates that local increases in *bmp4* should be accompanied by inhibition of the *bmp4* antagonists, *gremlin1a* and *noggin2*, to trigger the definitive blood programme. Collectively, these results indicate that ablation of FGF signalling intensifies the effects of BMP signalling in the DA on the haematopoietic programme by both enhancing the expression of *bmp4* directly and repressing the expression of local BMP antagonists.

In conclusion, we show that the FGF signalling pathway is a negative regulator of HSC emergence through its control of *bmp4* function underlying the aortic floor. FGF signalling may thus provide a missing link in what regulates the developmental switch from a BMP repressive to supportive environment that is linked to the emergence of HSCs from ventral aortic endothelium. These findings suggest that careful modulation of the FGF/BMP signalling axis may be important in the instruction of HSC fate in regenerative medicine approaches.

## Methods

**Zebrafish strains.** WT AB\* and transgenic lines, *hsp70:dn-fgfr1* (Tg(*hsp70:dnfgfr1-EGFP*)<sup>pd1</sup>)<sup>33</sup>, *hsp70:ca-fgfr1* (Tg(*hsp70:XIa.fgfr1*, *cryaa:DsRed*)<sup>pd3</sup>)<sup>34</sup> and *hsp70:dn-bmpr1* tg(*hsp70:dn-bmpr1-EGFP*)<sup>67</sup>, were maintained and stage as previously described in ref. 68. All animal work was carried out according to UK Home Office and UCSD IACUC regulations and under the appropriate project license.

**Heat-shock conditions.** Embryos were heat shocked by transferring them into prewarm E3 medium for 30 min at either 39 °C for *hsp70:dn-fgfr1*, *hsp70:ca-fgfr1* and *hsp70:ca-fgfr1* injected with *HSE:ca-bmpr1b-EGFP* construct<sup>58</sup> or 43 °C for *hsp70:dn-bmpr1*, then transferred to 28 °C until fixation. Transgenic embryos were selected based on their reporter expression or by genotyping as previously described in ref. 69. After *in situ* hybridization, embryos were subdissected. Single heads were incubated for 60 min at 95 °C in lysis buffer (25 mM NaOH, 0.2 mM EDTA), samples were then buffered with 40 mM Tris-HCl, pH 8. Genomic DNA was used for PCR amplification to detect DsRed transgene using forward 5'-CAT CCTGTCCCAGTCC-3' and reverse 5'-CCCAGCCCATAGTCTTCTTC TGC-3' primers (255-base-pair product).

**Chemical treatments.** Small-molecule inhibitors were resuspended in dimethylsulphoxide (DMSO) and diluted in E3 medium. Su5402 (Calbiochem) was used at 5 μM and DAPM (Calbiochem) at 100 μM (ref. 70). Control embryos were treated with the corresponding volumes of DMSO added to E3 medium just after heat shock.

**WISH.** Embryos were fixed in fresh paraformaldehyde 4%, dehydrated in EtOH and assayed for WISH as described in ref. 8. RNA probes were labelled with

digoxigenin (Roche) and detected using an anti-Dig antibody (1/5,000, Roche). Embryos were stained using a solution of NBT/BCIP (nitro-blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate p-toluidine salt) (Roche).

**Wax sectioning.** Embryos were dehydrated in EtOH 100% overnight, transferred into xylene for 30 min and then embedded in wax. Blocks containing stained embryos were sectioned at 10 or 4  $\mu\text{m}$  using a microtome (Leica). Sections were transferred onto glass slides, incubated at 37 °C overnight. Wax was removed in xylene and EtOH 100, 70 and 50%, and rehydrated in PBS. Slides were mounted and imaged. For the Fgf receptors, representative embryos were selected and transversally sectioned using a razor blade. Slices of embryos were then soaked in glycerol and imaged.

**Transient transgenesis and injection experiments.** 20 pg of *HSE:ca-bmpr1b-EGFP* transgenesis construct<sup>58</sup> combined with 25 pg of transposase mRNA was injected in one-cell stage of AB\* or *hsp70:ca-fgfr1* embryos. As a negative control, the construct was injected without transposase.

One-cell stage embryos were injected with morpholino solution diluted in water. Injected and uninjected embryos were incubated at 28 °C until fixation. Sequences and working concentrations are available in Supplementary Table 1.

**Real-time PCR.** Total RNA was isolated from dissected trunk embryos 5 h after heat shock using the RNaseasy Micro Kit (Qiagen). Single heads of *hsp70:ca-fgfr1* were used for genotyping, while corresponding trunks were kept individually on dry ice. Positive trunks were then pooled and processed as other samples with Superscript III Reverse Transcriptase (Invitrogen). qPCR was performed with Sybr Green (Applied Biosystems) and analysed by the comparative method ( $\Delta\Delta C_t$ ) with *ef1a* housekeeping gene as internal control. Statistical analysis was performed using *t*-test. Primer sequences available in Supplementary Table 1.

**Statistical analysis.** All the experiments presented in this study were performed at least three times. Data were collected from independent experiments and are given as the mean  $\pm$  s.d. Student's *t*-test was used for statistical comparisons and  $P < 0.05$  was considered statistically significant.

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### Author contributions

C.P. led the study, conducted the experiments, analysed the data and wrote the paper; T.P. and F.C.S. conducted the experiments, analysed the data and edited the manuscript; Y.L. analysed the data; and D.T. and R.P. supervised the study and edited the manuscript.

### Additional information

**Supplementary Information** accompanies this paper at <http://www.nature.com/naturecommunications>

**Competing financial interests:** The authors declare no competing financial interests.

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